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Changing Diversity in Changing Environment

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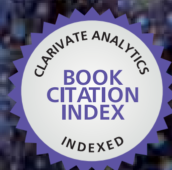

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CHANGING DIVERSITY IN CHANGING ENVIRONMENT

Edited by **Oscar Grillo** and **Gianfranco Venora**

Changing Diversity in Changing Environment
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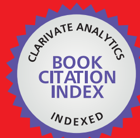

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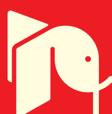
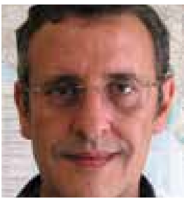


Meet the editors

Dr. Oscar Grillo was born in Catania (Sicily) in 1977, he is a food technologist with an international PhD in applied and environmental botany. Since 2003 he has been working as researcher at the Stazione Sperimentale di Granicoltura per la Sicilia, a governmental institute of agronomic research, mainly working with computer vision applied to food matrices and plant structures, above all seeds, and in particular studying wheat and the related leguminous. Currently, he is working also at the Sardinian Germplasm Bank of the Biodiversity Conservation Centre of the University of Cagliari on projects devoted to seed characterization and identification by image analysis. Results of his work have been published in many peer-reviewed journals papers and international conference papers. Referee for a few peer-reviewed journals, many times he has been invited as teacher/lecturer/speaker by some universities and research centres in Spain and Italy. He has trained many MSc and PhD students, who have made their own contributions to the agronomical and botanical research.



Dr. Gianfranco Venora is a biologist, born in Caltagirone (Sicily) in 1958, where he lives and works. He took his University degree in 1981, and since 1982 he has been working as researcher at the Stazione Sperimentale di Granicoltura per la Sicilia. His working expertise is mainly about durum wheat and leguminous breeding. At the beginning of 1990, after some years of experience on karyotyping cropped and wild species of agronomical importance, he was fascinated by computer vision applied to food matrices and plant anatomical structures, studying wheat and the related leguminous. He was recently nominated as professor to the research doctorate on Applied and Environmental Botany of the University of Cagliari. Many peer-reviewed journals published his papers and he was invited as speaker in many international conferences, and as teacher/lecturer by some universities and research centres in Germany, Netherlands, Bulgaria, Spain, Czech Republic and Italy. Gianfranco Venora is currently referee for about 15 peer-reviewed journals and tutor of many MSc and PhD students.

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Preface

The wonderful diversity of life organisms in our planet is so numerous that we haven't identified and characterized most of them yet. It is demanding work, considering the quickness of each species to react to ecological and environmental changes and the richness of existing biomes. Nevertheless, the study of the evolutive processes and spatial dynamics is in constant movement.

Mainly focused on minor and major animal species, from birds to fish, by way of amphibians, from bugs to microbes and arachnids, this volume provides several study cases about the diversity of many life forms in diverse ecosystems. It contains 17 chapters written by internationally renowned contributors, presenting meticulous research findings achieved both with traditional and innovative approaches, as well as critical reviews of the most relevant and impacting aspects of the biodiversity, covering the most ecologically interesting areas of the planet. *Changing Diversities in Changing Environment* includes systematic and phylogenetic studies, biogeographic distribution analysis and diversity richness evaluations, some of them even considering the economical effects and the future perspectives about the managing and conservation plans.

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1

Examination and Comparison of Microbial Diversity in Field-Scale Sewage Sludge Composters

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1. Introduction

While the earth's biodiversity has been studied in detail, to date, microbes have been completely omitted from meta-analytical studies of biodiversity data sets (Balmford & Bond, 2005; Díaz et al., 2006). In fact, biodiversity data sets are far from being considered a comprehensive global resource (Collen et al., 2008). Since the origin of prokaryotes on Earth over 3.5 billion years ago, the extent of evolutionary diversification within this group has been truly immense (DeLong & Pace, 2001; Payne et al., 2009). Microbial communities play important biological roles, such as the global cyclical change of materials in various environments (Díaz et al., 2006). As a result, existing biodiversity includes a vast and largely undiscovered diversity of microbes, which are probably very important for the sustainability of ecosystems (Swift et al., 2004). Hence, detailed investigation to characterize the global biodiversity of microbes is a very important task.

Microbes have always formed a major component of global biodiversity, either as producers (e.g., phototrophic blue-green algae) or decomposers (e.g., heterotrophic bacteria) (Naeem et al., 2000). Furthermore, in the future, they may serve as producers of useful alternative energy sources (Ohnishi et al., 2010). For example, phototrophic microorganisms use the energy from light for the production of biomass, which is an energy source stored in all living organisms. In fact, microbial decomposers are used in industry to convert microbial biomass and organic waste materials, such as domestic garbage, into biofuels, such as methane, ethanol, and hydrogen (Swift et al., 2004; Kayhanian et al., 2007). Microbes are also used for bioremediation, which is the cleanup of pollution caused by human activities (Jørgensen et al., 2000). In this process, various microbes have been isolated from nature, which are capable of degrading spilled oil, solvents, and other environmentally toxic pollutants. Furthermore, the breadth of microbial diversity on Earth provides genetic resources that offer solutions for environmental and energy issues, and research in this area is currently expanding. Considering the serious environmental and energy issues that humans face today, a better understanding of the ecophysiology of environmental microbes is warranted to address problems such as resource depletion and environmental pollution.

These problems could be resolved by converting anthropogenic waste into renewable resources, such as clean biofuels or fertilizers (Pimentel et al., 1994). As microbial ecologists, we are interested in understanding the mechanisms underlying the existence of an individual microbe, its involvement in a microbial community, and its special abilities.

Because microbes are invisible to the naked eyes, morphological analysis is difficult (Gest, 2003). Therefore, it is necessary to study microbial diversity from a variety of perspectives, including the physiological, genetic, and phylogenetic characteristics of species, as well as other taxonomic levels. One of the primary tasks for studying the naturally occurring microbial diversity is to perform accurate macroscopic analysis of the variety, population, and/or activity of microorganisms present in a specific habitat. In the past, the lack of appropriate methodologies has hindered this task, and thereby affected the progress in studying microbial diversity (Torsvik et al., 1998). Traditionally, microbiologists have used culture-dependent approaches for the detection and isolation of environmental microbes, and the methods currently in use are based on those developed in the late 19th century (Okabe et al., 2009). These culture-dependent approaches present one of the most serious limitations to studying microbes, as they are essentially very effort intensive and slow down data assimilation (Moter & G bel, 2000). Therefore, concerted efforts are required to develop novel techniques for elucidating the taxonomic positions and activities of as-yet unknown microbes, which might contribute towards enhancing our understanding of the microbial world.

Recently, problems related to culture-dependent approaches have been resolved through the application of methods from the discipline of molecular biology, such as culture-independent approaches (Amann et al., 1990; Muyzer, 1999). Compared to culture-dependent approaches, culture-independent approaches provide a broader view of the microbial population and/or its activity, without the necessity of isolating and culturing individual organisms (Hugenholtz et al., 1998; Ranjard et al., 2000). Thus, these approaches, such as fluorescence *in situ* hybridization (FISH) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), generate accurate results in a short time. As a result, the molecular method has been used to analyze the microbial diversity of a wide range of environments, which has generated many beneficial findings. Examples include solid waste composters (Nakasaki et al., 2009), wastewater treatment plants (Wagner et al., 2002), agricultural soils (Ranjard et al., 2000), and natural rivers (Brummer et al., 2000). Furthermore, culture-independent approaches have been used to identify many novel bacterial and archaeal lineages from different environments (Oren, 2004). As a consequence, studies using these approaches have shown that the microbial world is genetically and functionally more complex and diverse than previously predicted from culture-dependent studies.

However, culture-independent approaches also have certain disadvantages, as the strains in a sample cannot be distinguished and the unique properties of a particular strain cannot be identified (Rapp & Giovannoni, 2003). While correct data about the microbial ecology is generated using a culture-independent approach, the characteristics and activities of microbial strains cannot be studied without isolation. Therefore, it would be very difficult to use these culture-independent approaches to conduct a detailed study of an individual strain for the development of an applied technology. In other words, despite the widespread use of culture-independent approaches, cultural isolation will continue to be an important but necessary method to generate new technologies.

Our research group previously used the culture-dependent approach to provide important information about unknown microbial diversity and potential available resources in a field-scale model of a sewage sludge composter that was set up in Sapporo, Japan (Ohnishi et al., 2011). During composting, microorganisms decompose solid wastes, such as urban wastes, sewage sludge, and food garbage. This process is the first step by which organic matter is recycled and absorbed into plants or other autotrophs. However, the mechanisms underlying these activities remain unclear. In this study, we investigated the microbial diversity of culturable bacteria obtained from a field-scale sewage sludge composter that was set up in Tendo, Japan. PCR amplification of 16S rRNA gene from 32 isolates was performed using universal primers, followed by gene sequencing. The gene sequences were compared with the sequences available in the GenBank databases to identify closely related sequences. The closely related sequences were aligned to construct a phylogenetic tree for these bacteria. Then, these sequences were deposited in GenBank under different accession numbers. In addition, the detailed data sets, including ribo-patterns and carbon source utilization, were compared with those obtained in a previous study by the same research group.

2. Materials and methods

2.1 Sample collection

Samples were obtained from a field scale composter (Tendo Compost Plant; 38° 21′ N, 140° 37′ E) that was used for the treatment of sewage sludge (10 t/d) from Tendo City. This composter, which had aeration holes at the bottom, was operated as a silo-type. The composted material was sewage sludge mixed with return compost and sawdust, with a final water concentration of approximately 60%. Primary decomposition was completed after 14 d (the highest temperature was 75 °C), and secondary decomposition was completed after 60 d (at a temperature of approximately 40 °C). The sample (approximately 5 kg) was taken from the surface of the compost (at a depth of 30 cm) after the completion of primary decomposition. The sample was packed in ice for transportation to the laboratory, and maintained at 4 °C until the initiation of the experiment.

2.2 Plate counts and strain isolation

Mesophilic and thermophilic bacterial counts were performed following aerobic bacterial culture on nutrient agar plates (1.0% meat extract, 1.0% polypeptone, 0.5% NaCl, and 1.5% agar (pH 7.0)). In brief, 10 g of compost sample and 90 ml distilled water were placed in a shaking flask (volume, 500 ml). The solution was homogenized by shaking at 230 rpm for 20 min. The homogenate was then serially diluted, and 100-μl aliquots of 10^1 to 10^9 sample dilutions were plated. Incubation was performed at 37 °C or 50 °C for mesophilic bacteria and at 60 °C for thermophilic bacteria. After 7 d incubation at these temperatures, colonies on plates containing 30–300 colonies were counted. A number of colonies were randomly selected from plates that had been inoculated with the highest dilution, and in which the colonies were well separated. These colonies were then purified by repeated dilution plating. The isolates were stored at 4 °C with continuous subculturing, and stocks were deep frozen at -80 °C.

2.3 16S rRNA gene sequence determination

DNA extraction from each isolate was performed using the bead beating method (Ohnishi et al., 2010). After cells were grown for 24 h, they were suspended in 1 ml sterile distilled water

by using a sterile swab, and centrifuged for 5 min (12,000 g, 4 °C). The cell pellet (1–3 mg) was then resuspended with 1 ml of extraction reagent (0.1 M NaCl, 0.5 M Tris-HCl, and 0.5% SDS (pH 8.0)). After vortexing, the suspension was transferred into a 2-ml screw-capped tube containing 0.3 g of zirconium beads (diameter, 0.1 mm), and the cells were crushed by 10 cycles (3000 rpm, 30 s) of bead beating at 30-s intervals by using an MB-200 Multi-beads shocker (Yasui Kikai). The beads and cell debris were removed by centrifugation at 20,000 *g* for 5 min. The crude DNA was purified by phenol-chloroform extraction followed by ethanol precipitation, and dissolved in TE buffer (pH 8.0). The DNA extract was then subjected to PCR amplification.

Gene fragments that were specific to the 16S rRNA-coding regions of the isolates were amplified by PCR using the 2 primers, 20F (5´-AGTTTGATCATGGCTCA-3´, positions 10–26) and 1540R (5´-AAGGAGGTGATCCAACCGCA-3´, positions 1521–1541) (*Escherichia coli* numbering system (Brosius et al., 1978)), following the method of Yanagi and Yamasato (1993). PCR amplification was carried out in a PTC200 thermal cycler (MJ Research) using reagents from a Taq PCR kit (Takara). The amplified 16S rRNA gene was directly sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems). The following 5 primers were used: 20F, 1540R, 350F (5´-CCTACGGGAGGCAGCAGT-3´, positions 341–358), 800F (5´-GTAGTCCACGCCGTAAACGA-3´, positions 803–819), and 900R (5´-CGGCCGTACTCCCCAGGCGG-3´, positions 879–898) (Ohnishi et al., 2011).

2.4 Phylogenetic analysis

Multiple alignment was performed using Clustal X (version 1.8 (Thompson et al., 1997)). Phylogenetic distances (Knuc) for the aligned sequences were calculated using the 2-parameter method of Brummer (Kimura, 1980). The neighbor-joining method (Saitou and Nei, 1987) was used for the construction of a phylogenetic tree. The topology of the phylogenetic tree was evaluated by bootstrapping with 1000 replications (Felsenstein, 1989).

2.5 Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been submitted to the DDBJ, GenBank, and EMBL databases under the following accession numbers: AB210952–AB210984 (see Table 1).

2.6 Riboprinting

Ribotyping was performed using the DuPont (Wilmington, DE) Qualicon RiboPrinter (Bruce, 1996). Some single colonies from a 24-h culture on agar plates were suspended in sample buffer, and heated at 80 °C for 15 min. After the addition of a lytic enzyme, the samples were transferred to the RiboPrinter. Further analysis, including the use of the *Eco*RI enzyme, was carried out automatically. The ribotypes were aligned according to the position of a molecular size standard, and compared with patterns stored in the library. The ribotyping profiles were transferred and analyzed with the FPQuest software (Bio-Rad Laboratories), using the Pearson correlation and default settings for optimization (2.0%) and position tolerance (1.00%) for genetic similarity. The dendrogram was generated by the unweighted pair-group method, using arithmetic averages (UPGMA) to determine profile relatedness.

2.7 Carbon source utilization

The carbon source utilizing-ability of the isolates was investigated with a Biolog GP2 microplate (Biolog Inc.). This standard 96-well microplate contained a dried film of 95 different sole carbon sources, which are mainly carbohydrates, but also other carbons such as amino and carboxylic acids, and 1 negative control. Each well contained a redox dye (tetrazolium violet) for the colorimetric determination of respiration, due to oxidization of the carbon source by cells. This system is generally used to test the ability for sole carbon source utilization of microorganisms. Basically, when a cell metabolizes a carbon source (by chemical oxidation), a redox dye (such as a tetrazolium salt) is irreversibly reduced to a purple formazan, which is then assayed colorimetrically (Bochner & Savageau, 1977).

One colony was selected from the pre-culture by using a sterile cotton swab, subcultured on BUG medium (Biolog universal growth medium), and then incubated overnight. Cells were harvested using sterile cotton swabs, and then suspended in 20 ml of GN/GP-IF inoculating fluid.

The cell density was adjusted to 20% of transmittance with a 2% range, which was assessed using a photometer model according to the range specified by the manufacturer. Thioglycolate was added to the suspension at a final concentration of 5 mM, to inhibit the production of a bacterial capsule. An aliquot of 150 μl of the suspension was immediately dispensed into each well of the GP2 microplate by using a multichannel pipette. The microplate was then incubated for 16–24 h and analyzed using an automated Biolog microplate reader at 2 different wavelengths (590 nm and 750 nm). Reactions were interpreted as positive or negative by the Biolog MicroLog 3 software, version 4.20 (Biolog Inc.).

3. Results

3.1 Culturable count

Bacteria that were grown on media under mesophilic (37 or 50 °C) or thermophilic conditions (60 °C) in Tendo Compost were counted. The number of mesophilic bacteria that grew at 37 and 50 °C was 7.0 and 5.3 × 10^9 CFU g^{-1} dry matter, respectively, while that of thermophilic bacteria was 9.1 × 10^8 CFU g^{-1} dry matter.

3.2 Phylogenetic analysis

Thirty-two isolates from Tendo Compost, comprising 15, 9, and 8 isolates grown at 37, 50, and 60 °C, respectively, were randomly selected and purified. Comparative 16S rRNA gene sequencing analysis of the isolates was completed based on gene fragments approximately 1,500 bp in size. Table 1 and Fig. 1 show the results of phylogenetic analysis of the 16S rRNA gene sequencing for the isolates from Tendo Compost, along with 18 related prokaryote species.

Using 97% 16S rRNA gene-sequence similarity as the definition of a species (Stackebrandt & Goebel, 1994), the remaining isolates appeared to represent new species. Among the isolates, 4 could be identified only to the genus level due to their low sequence similarity. Of the unknown species, 3 groups (designated as NoID D, E, and F) belonged to the phylum Firmicutes, and 1 group (designated as NoID G) to the phylum Actinobacteria. “NoID” indicates that a taxon could not be identified to the species level based on 16S rRNA gene sequence similarity. From the isolates of the NoID groups, the 16S rRNA gene sequence similarity values for known species ranged between 93.0 and 96.7%.

A phylogenetic relationship of isolates from Tendo Compost and Sapporo Compost was evaluated. The phylogenetic trees of every phylum are shown in Fig. 1. Of the 32 isolates from Tendo Compost, 14 were classified to the genus level and 18 to the species level, including 4 unknown taxa. Of the 49 isolates from Sapporo Compost, 13 were classified to the genus level and 16 to the species level, including 5 unknown taxa. In addition, from the strains isolated from Tendo Compost and Sapporo Compost, a very high correlation was shown for the 5 common phylogenetic groups, *A. aneurinilyticus*, *B. subtilis*, *T. fusca*, No ID D group, and No ID E group.

3.3 Characterization by automated ribotyping

Isolated strains from Tendo Compost and Sapporo Compost were analyzed using ribotyping. *Eco*RI digestion was incomplete for some groups, mainly the Actinobacteria and No ID groups (without No ID C and D group). As the riboprint identification database provided by the manufacturer was not complete enough to affiliate patterns to taxon names, clustering was executed based on the similarity in band profile by FPQuest. The riboprint patterns and a dendrogram of 62 isolates are shown in Fig. 2. Clustering analysis of the ribotype patterns led to the separation of strains isolated from Tendo Compost and Sapporo Compost into clusters that corresponded with the phylogenetic group obtained from the 16S rRNA gene analysis, in most cases.

3.4 Carbon source utilization of common phylogenetic groups

Twenty isolates belonging to 5 common phylogenetic groups that were extracted from Tendo Compost and Sapporo Compost were tested for 95 carbon sources. Based on the Biolog GP2 microplate results, all strains of the same common phylogenetic group showed high similarity in their carbon sources utilization pattern; specifically, *A. aneurinilyticus*, *B. subtilis*, *T. fusca*, No ID D group, and No ID E group (Table 2).

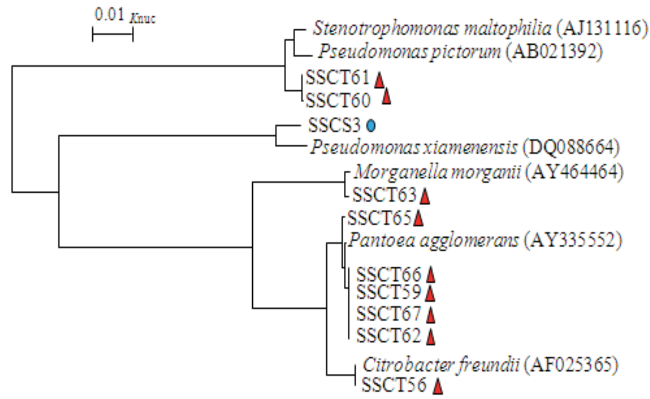
Seven of the ten utilized sole carbon sources from isolates belonging to *A. aneurinilyticus* were correlated for Tendo Compost versus Sapporo Compost (Table 2). Likewise, 23 of the 29 isolates of *B. subtilis* were correlated between the 2 compost types. For *T. fusca*, 15 of the 26 isolates were correlated. For the "No ID D" group, 17 of the 25 isolates were correlated, and for the "No ID E" group, 1 of the 7 isolates was correlated.

4. Discussion

In this study, the bacteria obtained from Tendo Compost were analyzed using the cultivation method at various temperatures, the results of which clearly showed the diversity of bacteria that could potentially be cultivated. The results indicate that various types of mesophilic and thermophilic bacteria were present in the compost at a density of around 10^9 CFU g^{-1} dry matter. Since this population size of the isolates is higher than that recorded in standard commercial composts (10^3 to 10^7 CFU g^{-1} matter), the detected isolates seem to comprise a very active group in the Tendo composting process (Pedro et al., 1999; Vaz-Moreira et al., 2008). In addition, the phylogenetic diversity of culturable bacteria was also very high. Of the 32 isolates obtained from Tendo Compost, a total of 19 species (including 4 unknown taxa) belonging to 16 genera were detected (Table 1). Hence, there was a very high diversity of bacteria at the phylogenetic level that actively contributed to the primary role of organic matter decomposition in the field-scale composting process.

Strain No.	Related species	Similarity	Accession Number	Isolation temperature
	Actinobacteria			
SSCT48	*Arthrobacter mysorens* (AJ617482)	99.7%	AB210984	37 °C
SSCT78	*Cellulosimicrobium cellulans* (X79456)	98.7%	AB210961	50 °C
SSCT54	*Cellulosimicrobium cellulans* (X79456)	99.5%	AB210980	37 °C
SSCT73	*Cellulosimicrobium cellulans* (X79456)	99.6%	AB210965	50 °C
SSCT58	*Microbacterium esteraromaticum* (AB099658)	99.1%	AB210977	37 °C
SSCT49	*Rhodococcus rhodochrous* (X79288)	99.7%	AB210983	37 °C
SSCT69	*Rhodococcus rhodochrous* (X79288)	99.8%	AB210967	50 °C
SSCT55	*Sanguibacter keddieii* (X79450)	96.7%	AB210980	37 °C
SSCT81	*Thermomonospora fusca* (AF002264)	99.9%	AB210960	50 °C
	Firmicutes			
SSCT74	*Aneurinibacillus aneurinilyticus* (AB101592)	99.9%	AB210964	50 °C
SSCT75	*Bacillus foraminis* (AJ717382)	96.7%	AB210963	50 °C
SSCT76	*Bacillus novalis* (AJ542512)	95.6%	AB210962	50 °C
SSCT84-2	*Bacillus pocheonensis* (AB245377)	93.0%	AB210954	60 °C
SSCT51	*Bacillus subtilis* (Z99104)	99.9%	AB210982	37 °C
SSCT68	*Bacillus subtilis* (Z99104)	99.9%	AB210968	50 °C
SSCT72	*Brevibacillus panacihumi* (EU383032)	99.4%	AB210966	50 °C
SSCT85	*Geobacillus thermodenitrificans* (AB190135)	99.8%	AB210952	60 °C
SSCT83	*Geobacillus thermodenitrificans* (AY608963)	99.3%	AB210956	60 °C
SSCT82-1	*Geobacillus toebii* (AY608982)	98.8%	AB210959	60 °C
SSCT82-2	*Geobacillus toebii* (AY608982)	99.6%	AB210958	60 °C
SSCT82-3	*Geobacillus toebii* (AY608982)	99.0%	AB210957	60 °C
SSCT84-1	*Geobacillus toebii* (AY608982)	99.6%	AB210955	60 °C
SSCT84-3	*Geobacillus toebii* (AY608982)	98.7%	AB210953	60 °C
	Proteobacteria			
SSCT56	*Citrobacter freundii* (AF025365)	99.5%	AB210978	37 °C
SSCT63	*Morganella morganii* (AY464464)	99.5%	AB210972	37 °C
SSCT65	*Pantoea agglomerans* (AY335552)	99.0%	AB210971	37 °C
SSCT59	*Pantoea agglomerans* (AY335552)	99.3%	AB210976	37 °C
SSCT62	*Pantoea agglomerans* (AY335552)	99.3%	AB210973	37 °C
SSCT66	*Pantoea agglomerans* (AY335552)	99.3%	AB210970	37 °C
SSCT67	*Pantoea agglomerans* (AY335552)	99.1%	AB210969	37 °C
SSCT60	*Stenotrophomonas maltophilia* (AJ131116)	98.3%	AB210975	37 °C
SSCT61	*Stenotrophomonas maltophilia* (AJ131116)	98.8%	AB210974	37 °C

Table 1. Sequence similarities of the isolated strains.



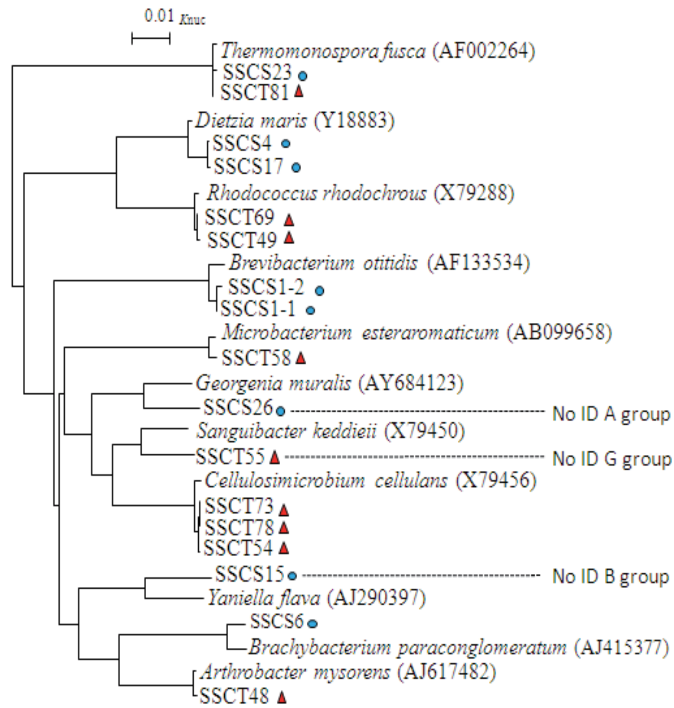


Fig. 1. Continued

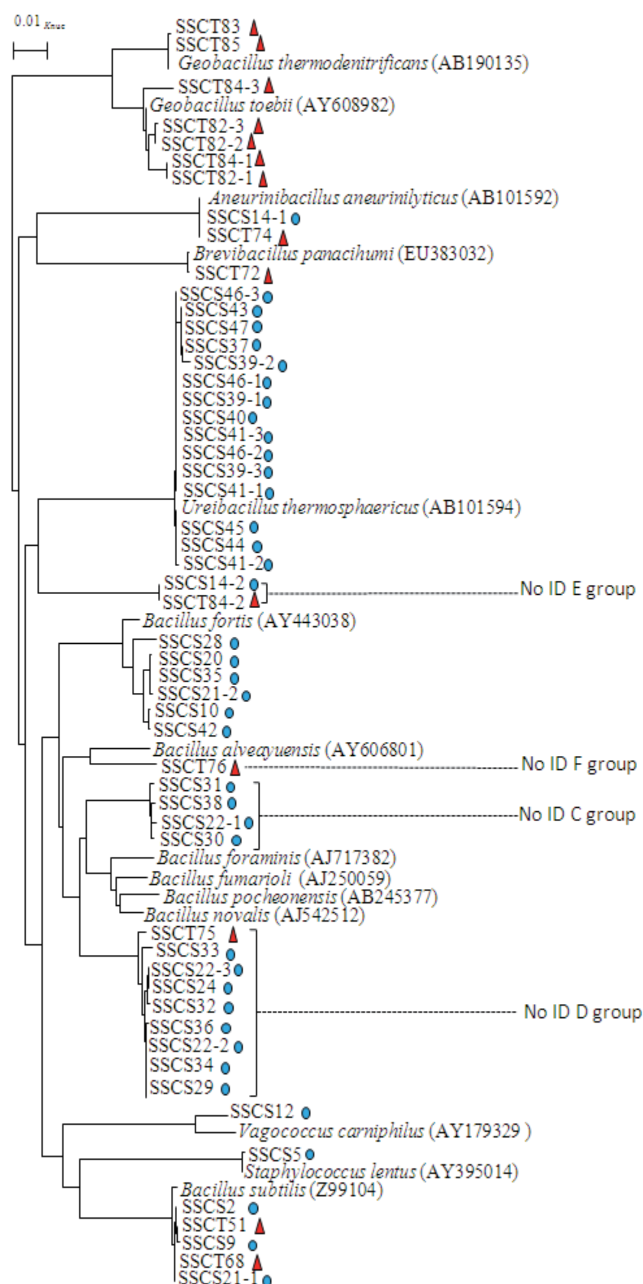


Fig. 1. Phylogenetic relationship between the isolates and other related bacteria based on 16S rRNA gene sequences. (A) Phylum Proteobacteria. (B) Phylum Actinobacteria. (C) Phylum Firmicutes. The phylogenetic tree, which was constructed using the neighbor-joining method, is based on the comparison of approximately 1,400 nucleotides of the 16S rRNA gene. Symbols are isolated: ▲, from Tendo Compost; ●, from Sapporo Compost in previous study.

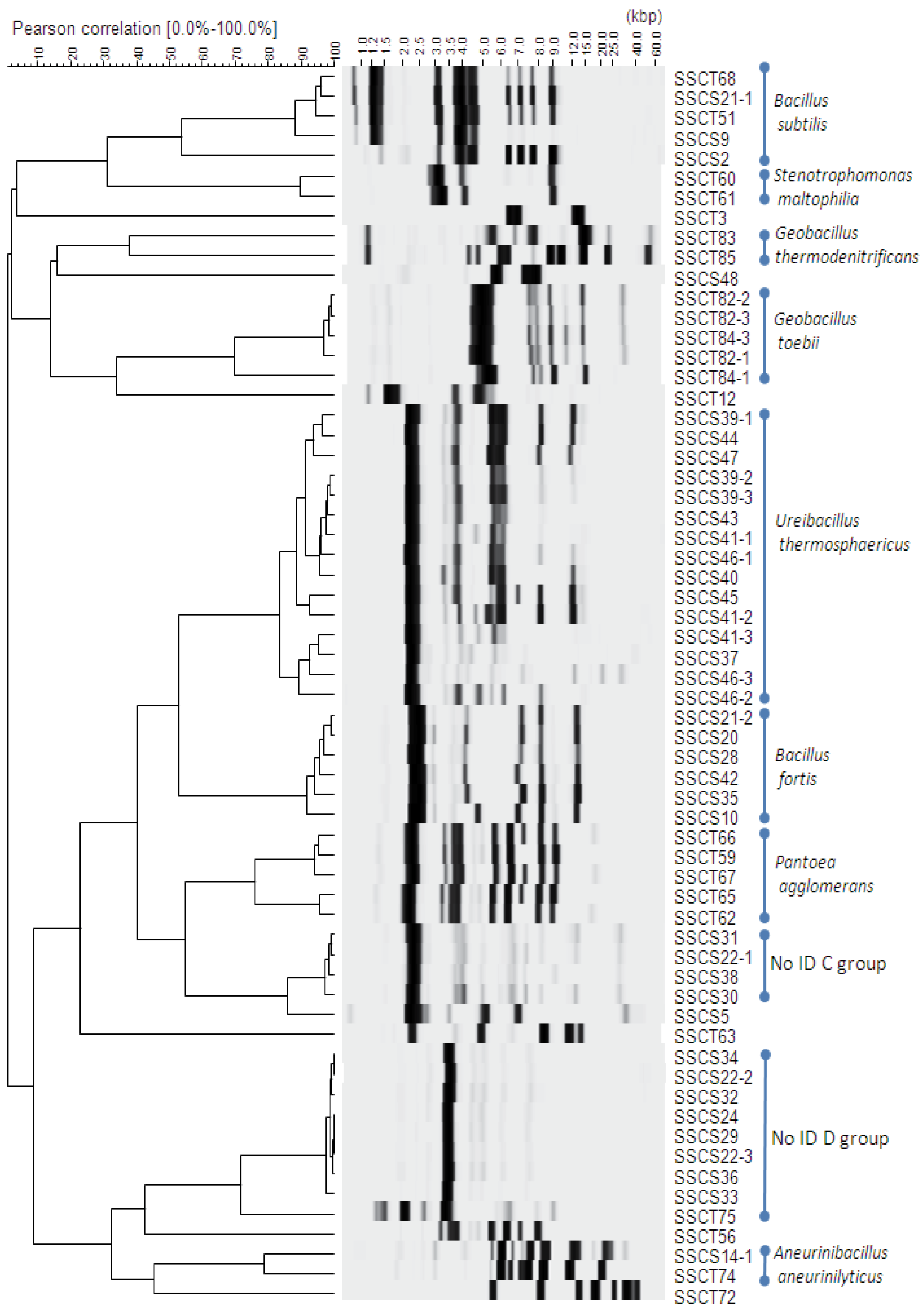


Fig. 2. Riboprint pattern obtained from isolates that were derived from Tendo Compost and Sapporo Compost. Cluster analysis was performed by the unweighted pair-group method using arithmetic averages (UPGMA) based on the Pearson correlation coefficient.

Species identification	*Aneurinibacillus aneurinilyticus*		*Bacillus subtilis*		*Thermomonospora fusca*		No ID D group		No ID E group	
	SSCT	SSCS	SSCT	SSCS	SSCT	SSCS	SSCT	SSCS	SSCT	SSCS
Dextrin			+	+	+	+			+	
Glycogen	+									
Mannan	+									
Tween 40	+	+	50	33						+
Tween 80	+	+								+
N-Acetyl-D-Glucosamine			50							
N-Acetyl-β-D-Mannosamine			+	33						
L-Arabinose					+	+				
D-Cellobiose			50	67						
D-Fructose			+	33	+	+	+			
D-Galactose			50	67						
Gentiobiose				33						
α-D-Glucose			+	+	+	+				
Maltose			+	67	+	+				
Maltotriose			+	67	+	+				
D-Mannitol						+				
D-Mannose			+	33						
3-Methyl Glucose			+	67						
α-Methyl-D-Glucoside			+	33						
β-Methyl-D-Glucoside			+	67						
Palatinose			+	67						
D-Psicose			+	+			+			
D-Ribose	+	+		+	+	+	+		+	
Sedoheptulosan			+	33						
D-Sorbitol			+	67						
Sucrose			+	+						
D-Trehalose			+	+						
Turanose			+	+						
D-Xylose						+				
Acetic Acid					+	+				
α- Hydroxybutyric Acid					+					
β- Hydroxybutyric Acid							+	88		
α- Ketoglutaric Acid	+	+				+				
α- Ketovaleric Acid	+	+			+	+	+	63	+	+
L-Malic Acid			+							
Pyruvatic Acid Methyl Ester	+	+	+	+	+		+	+		
Succinic Acid Mono-methyl Ester					+		+	63	+	
Pyruvic Acid	+	+	+		+	+	+	75	+	
Succinamic Acid	+									
Succinic Acid								75		
L-Alaninamide							+	+		
D-Alanine					+		+			
L-Alanine					+		+	38		
L-Alanyl-Glycine							+	75		
L-Glutamic Acid							+	75		
Glycyl- L-Glutamic Acid							+	75		
L-Serine							+	75		
2,3-Butanediol			+	+		+				
Glycerol			+	+		+	+	75		
Adenosine					+	+	+	75		
2'-Deoxy Adenosine					+	+	+	75		
Inosine			+		+	+	+	75		
Thymidine					+	+	+	75		
Uridine			50		+	+	+	75		
Thymidine-5'-Monophosphate						+				

+, positive reaction (100%); 1–99, percent positive reaction. SSCT, Isolates from Tendo Compost; SSCS, Isolates from Sapporo Compost. In addition to the above data, none of these isolates were able to use α–Cyclodextrin, β–Cyclodextrin, Inulin, Amygdalin, D–Arabitol, Arbutin, L–Fucose, D–Galacturonic Acid, D–Gluconic Acid, m–Inositol, α–D–Lactose, Lactulose , D–Melezitose, α–Methyl–D– Galactoside, β–Methyl–D–Glucoside, α–Methyl–D–Mannoside, D–Raffinose, L–Rhamnose, Salicin, Stachyose, D–Tagatose, Xylitol, γ– Hydroxybutyric Acid, p–Hydroxy–Phenylacetic Acid, Lactamide, D–Lactic Acid Methyl Ester, L–Lactic Acid, D–Malic Acid, Propionic Acid, N–Acetyl–L–Glutamic Acid, L–Asparagine, Putrescine, Adenosine–5'–Monophosphate, Uridine–5'–Monophosphate, D–Fructose–6–Phosphate, α–D–Glucose–1–Phosphate, D–Glucose–6–Phosphate, D–L–α–Glycerol Phosphate.

Table 2. Percentile positive results of the isolated strains using traditional biochemical tests and in BiOLOG.

However, differences in the diversity of culturable bacteria at each growth temperature were clearly demonstrated by 16S rRNA gene sequence determination. The temperature (from below 40 °C to over 60 °C) and nutritional status during the composting process was usually subject to dynamic changes. For example, the 9 isolates belonging to the Proteobacteria phylum were isolated only from mesophilic conditions at 37 °C; the 9 isolates belonging to the Actinobacteria phylum were isolated from mesophilic conditions at 37 and 50 °C; the 14 isolates belonging to the Firmicutes phylum were isolated from mesophilic and thermophilic conditions at 60 °C. Based on these observations, it appeared that in Tendo Compost, Proteobacteria and Actinobacteria are mesophiles that actively participate at temperatures below 37 °C and 50 °C, respectively. In addition, since all isolates under the thermophilic condition (60 °C) belonged to the *Geobacillus* and *Bacillus* genera, thermophiles that actively contribute at temperatures over 60 °C appear to belong to Firmicutes. Hence, the main phylogenetic groups of bacteria that actively contribute to the composting process under each temperature condition vary according to the phylum level. The same tendency was observed for Sapporo Compost (Ohnishi et al., 2011).

The commonality in culturable bacteria between Tendo Compost and Sapporo Compost was demonstrated by 16S rRNA gene sequence determination, Ribotyping, and the Biolog system (Figs. 1 and 2, and Table 2). For example, 32 isolates from Tendo Compost were classified into 16 genera comprising 19 species, including 4 unknown taxa. In comparison, 49 isolates from Sapporo Compost were classified into 13 genera comprising 16 species, including 5 unknown taxa. Five common phylogenetic groups based on 16S rRNA gene sequence were determined as *A. aneurinilyticus*, *B. subtilis*, *T. fusca*, "No ID D" group, and "No ID E" group (Fig. 1). These results show that approximately 17% of phylogenetic groups that were detected were common. Furthermore, Ribotyping showed that there was high similarity at the species level of isolates belonging to the 3 common phylogenetic groups (*A. aneurinilyticus*, *T. fusca* and "No ID D"group). In addition, carbon source utilization analysis showed that common phylogenetic groups had similar carbon source utilization abilities. Of additional significance, it was found that common phylogenetic groups existed between the 2 sewage sludge composters, despite their being located approximately 500 km apart.

The similarity in the role of isolated common phylogenetic groups between Tendo Compost and Sapporo Compost can be evaluated on the basis of carbon source utilization, in parallel to the results of past studies. For example, *A. aneurinilyticus* mainly utilized carbohydrates, polymers, and carboxylic acids, while *T.* fusca utilized a wide range of carbon sources, such as carbohydrates, polymers, carboxylic acids, and nucleosides. Furthermore, *A. aneurinilyticus* and *T. fusca* may be involved in the degradation of lignin when conditions are mesophilic during the composting process, since the bacteria of these taxa are known to degrade lignin (Chandra et al., 2007; Crawford & Crawford, 1976). Most strains of *B. subtilis* were able to utilize 23 sole carbon sources, which were mainly carbohydrates, polymers, carboxylic acids, and alcohols. The hydrolysis of starch and casein has also been observed for members of this group (Ohnishi et al., 2011). In addition, *B. subtilis* may inhibit fungal growth when compost is applied to agricultural land (Phae et al., 1990). The "NoID D" group can also hydrolyze casein (Ohnishi et al., 2011), and utilize 17 sole carbon sources, which primarily include carboxylic acid, amino acid, and nucleoside. However, this group shows weak ability to utilize carbohydrate. In addition, the "NoID D" group may be involved in the degradation of proteins when conditions are mesophilic during the

composting process (Ohnishi et al., 2011). While there is noticeable bias for carbon source utilization in 1 common phylogenetic group, this role seems to compensate for the decomposition of complicated organic matter, which occurs during the process of composting. The bias of characteristics within common phylogenetic groups seems to lead to the complementary decomposition of complex organic matter in the composting process. The "No ID E" group utilizes only a few sole carbon sources. The ability of this group to utilize a variety of sole carbon sources is very limited during the composting process. Hence, it would be of interest to identify the role of the "NoID E" group in the composting process.

5. Conclusions

The current study clarified that the diversity of cultivable bacteria is extremely high, including undiscovered phylogenetic groups that were found in Tendo Compost and their commonality to Sapporo Compost. This study found differences in the temperature required for growth in the phylogenetic groups of bacteria that were isolated. In other words, because the environmental conditions (i.e., temperature and nutritional status) of the composting process are subject to dynamic changes, the microflora that actively participates in the composting process is very protean. The growth temperature was different for the phylogenetic groups of bacteria of each phylum that were isolated from Tendo Compost. For isolates grown at 37 °C, the primary phylum was Proteobacteria; Actinobacteria also formed a large proportion of the phyla, and Firmicutes to some extent. In comparison, isolates grown at 50 °C primarily comprised Actinobacteria and Firmicutes, while those at 60 °C comprised only Firmicutes. In general, the nutritional requirement of each phylum was different, (Fierer et al. 2007), and a wide range of temperatures were recorded during the composting process (i.e., from below 40 °C to over 60 °C) within a 1-day period. Therefore, because each phylum plays different roles in decomposting, in parallel to regularly fluctuating temperatures, the early decomposition of complex organic matter may be achieved. This may be the factor that composting goes very early. Furthermore, we identified several phylogenetic groups that showed a strong correlation to a composting system that was constructed over 500 km away. These similarities may indicate that specific phylogenetic groups play a very important role in the field-scale composting process of sewage sludge.

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2

Food Microbiota Diversity

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1. Introduction

Meat fermentation and fermented sausage manufacturing are ancient processes in Europe, and typical preparations based on fermentations driven by indigenous microflora are still produced by local meat factories or artisanal producers without the use of starter cultures (Rantsiou et al., 2005a; Urso et al., 2006).
LAB (Lactic Acid Bacteria) and CNC (Coagulase Negative Cocci) are the two main groups of bacteria with technological value determining the several biochemical and physical reactions taking place during the fermentation and ripening of sausages (Rantsiou & Cocolin 2006).
Interest in preserving the biodiversity of microorganisms involved in the fermentation of food products has been reported (EC 1999; Aymerich et al., 2006). As a consequence, and also taking into consideration the technological and economic importance of these microorganisms, the availability of methodologies able to unequivocally characterise single species, potentially right up to strain level, is called for. Identification of species, particularly within the genus *Lactobacillus*, using phenotypic methods such as sugar fermentation or other biochemical traits, may produce ambiguous results and be complicated because of the presence of several LAB species with similar characteristics (Quere et al., 1997).
Molecular methods are nowadays increasingly employed to clarify the taxonomy of microbiota from sausages. The largest number of works is based on RAPD (Random Amplified Polymorphic DNA), (Rebecchi et al., 1998; Rantsiou et al., 2005a), 16S rDNA sequencing (Rantsiou et al., 2005a), PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis (Cocolin et al., 2001a; Cocolin et al., 2001b; Comi et al., 2005; Urso et al., 2006), and REA-PFGE (Restriction Endonucleases Analysis - Pulsed Field Gel Electrophoresis) analysis (Psoni et al., 2006).
The above DNA-based methodologies, with the exception of REA-PFGE, are easy, fast, and provide an accurate identification at species level, but are not as informative when it comes to characterisation of the bacterial population at strain level. Random amplification of polymorphic DNA is useful for performing identification at strain level, but the lack of reproducibility and the lack of complex band patterns, are the main limiting factors. Restriction endonucleases analysis and pulse field gel electrophoresis is a powerful method

for strain typing, but it is laborious and expensive, and only a limited number of samples can be analysed simultaneously.

AFLP (Amplified Fragment Length Polymorphisms analysis, Vos et al., 1995) is a powerful fingerprinting methodology originally developed for plants but later applied to the taxonomy of several microbiological DNA samples (Antonyshin et al., 2000; Thompson et al., 2001; Vancanneyt et al., 2005; Alter et al., 2006). The AFLP technique is particularly interesting with regard to the characterisation of the isolate to single strain level as it is more reproducible than RAPD; the complex band pattern can be modulated, thus allowing for the identification of single strains.

Among the advantages of AFLPs analysis, is that the process is amenable to full high speed automation and precise analysis, which allows for the simultaneous comparison of hundreds of colonies isolated from the same or different sources permitting an estimation of the microbial biodiversity.

The most frequently isolated LAB from fermented sausages are *Lactobacillus sakei*, *L. curvatus*, and *L. plantarum*, while among the CNC it is *Staphylococcus xylosus* that is most usually found (Cocolin et al., 2001a; Rossi et al., 2001; Aymerich et al., 2003; Cocolin et al., 2004; Rantsiou et al., 2005b).

Concerning the typology of sausages that have been analysed, several reports only consider the microbiota isolated from "Salame friulano" (Cocolin et al., 2001a; Cocolin et al., 2001b; Comi et al., 2005; Rantsiou et al., 2005a; Rantsiou et al., 2005b; Rantsiou & Cocolin 2006; Urso et al., 2006).

In the present study we have used fluorescent AFLP to analyse the genetic variability of cultured bacteria isolated from populations present in traditional North Italian fermented 'salami' in order to: (i) define the biodiversity of cultured bacteria involved in traditional meat fermentation of high quality products produced without using starter cultures; (ii) define the utility of AFLP in characterising, at strain level, the indigenous sausage microflora; (iii) collect the different isolates and identify strain specific electropherograms usable as markers for traceability of the product.

2. Material and methods

2.1 Sampling and microflora collection

Eighteen different artisanal single sausages of fermented "salami" were collected from different local manufacturers in northern Italy, in the provinces of Mantova, Cremona, Verona, Reggio Emilia and Parma, during the years 2007 and 2008. Sausage preparation, maturation time and parameters were typical of the chosen areas and differed for each considered artisanal manufacturer. Due also to the natural seasoning process taking place in specific cellars, it was difficult to ascertain these parameters precisely. In general the fermentation temperature ranged from 16 to 20 °C over a period of 90-120 days.

The isolation of bacteria was carried out as reported in Cocolin et al. (2001a). After growth on selected media (MRS - de Man, Rogosa and Sharp, and BP – Baird-Parker) for 48 h at 30 °C, twenty presumed lactobacilli and up to fifteen presumed staphylococci single colonies were selected for each sausage and two single colony isolation steps, on the same media and under the same conditions for growth, were performed. Single colonies were then inoculated into tubes containing 15 ml of liquid broth and kept at 30 °C for 48 h without (MRS) and with shaking (BP) at 125 rpm. After growth 3 ml of each culture was centrifuged

in a DNA extraction tube, and the remaining 12 ml centrifuged and re-suspended in 4 ml of sterile MRS or BP plus 25% glycerol in order to prepare four tubes for liquid N_2 storage.
All colonies were named for insertion in our general collection with the mark CCCF (Culture Collection Corrado Fogher) followed by a number starting from 4,000 to 4,499 for LAB and from 4,500 to 4,999 for CNC. In this paper, the letters before the number of the strain in the dendrograms refer to the different manufacturers and will be eliminated from the collection name after analysis.
Lactobacillus sakei DSM20100; *Lactobacillus plantarum* DSM20205; *Lactobacillus curvatus* DSM20010; *Lactobacillus casei* LC1; *Staphylococcus xylosus* ATCC35663; *Staphylococcus carnosus* ATCC51365 were used as control strains.

2.2 DNA extraction

DNA extraction was performed using the commercial kit GenElute Plant Genomic DNA miniprep kit (Sigma) following standard protocol with a few modifications limited to the preparation of the samples. Cells were grown for 48 h in 15 ml of MRS or BP medium then 3 ml was harvested by centrifugation (5 minutes (min.), 10,000 rpm). The pellet was resuspended in the provided lysis solutions, glass-pearls were added and each sample was shacked by vortexing for 1 minute to aid the disruption of cell wall, then cells were incubated at 65 °C for 20 min. The DNA was visualised and quantified by agarose gel (0.8 %) electrophoresis.

2.3 AFLP analysis

The AFLP reactions were performed as described previously (Vos et al., 1995) with minor modifications of the AFLP plant mapping protocol (Applied Biosystems). Approximately 100 ng of DNA for each sample were used for AFLP reaction. Restriction-ligation (RL) was performed in a final volume of 11 µl containing: genomic DNA (100 ng), 1X T4 *ligase* buffer, 0.05M NaCl, 0.05 µg BSA (Bovine Serum Albumine), 5 pmol *EcoRI* adapter, 50 pmol *MseI* adapter, 1 U T4 DNA *ligase* (Promega), 1 U *MseI* (New England Biolabs), 5 U *EcoRI* (New England Biolabs). The reaction was incubated for 2 h 30 min. at 37 °C. RL was diluted ten fold and 4 µl of the diluted RL reaction were considered for Preselective amplification.The pre-selective PCR (72 °C for 2 min.; 30 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min.; 60 °C for 30 min.) with *EcoRI*+A and *MseI*+C primers was performed in a 20 µl reaction volume consisting of 4 µl 10-fold diluted RL DNA, 5 pmol pre-selective primers and AFLP Core Mix (Applied Biosystems) to the final volume. The selective PCR (94 °C for 2 min.; 1 cycle of 94 °C for 20 s, 66 °C for 30 s, and 72 °C for 2 min., followed by 10 cycles of 1 °C reduced annealing temperature each cycle and 25 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min.; 60 °C for 30 min.) with *EcoRI*+AXX and *MseI*+C selective primers was carried out in a 10 µl volume consisting of 2 µl of 20-fold diluted pre-amplified DNA, 0.25 pmol of *EcoRI* primer, 1 pmol of *MseI* primer and 7 µl of AFLP Core Mix. The *EcoRI* selective primers were fluorescent dye-labeled and the following primer combinations employed: *EcoRI*+ACA / *MseI*+C, *EcoRI*+AGC / *MseI*+C, *EcoRI*+ACT / *MseI*+C. Amplified products from selective amplification were loaded and run on the ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to AFLP plant mapping protocol and analyzed, considering a threshold of 100 rfu, using GeneScan Analysis software version 3.7 (Applied Biosystems).

A similarity matrix among genotypes was constructed applying the Jaccard coefficient and the corresponding dendrogram was obtained using the UPGMA method. The closeness of fit between the original similarity matrix and the respective dendrogram was evaluated by calculating the cophenetic correlation coefficient r (Mantel 1967) between the similarity matrix and the cophenetic matrix of the UPGMA clustering (1000 permutations). A bi-dimensional visualization of the relationships between the different clusters have been obtained using the Principal Components Analysis (PCA) method. All the analyses were performed using different modules from the NTSYSpc ver 2.1.

2.4 Ribosomal DNA analysis

Two universal primers; fD1, 5'AGAGTTTGATCCTGGCTCAG3', (Weisburg et al., 1991) and 805R, 5'GACTACCAGGGTATCTAATCC3', (Tanner et al., 1998) for 16S ribosomal DNA were used for the amplifications. PCR reactions were performed in a final volume of 20 μl containing: 10 ng of genomic DNA, 1 X PCR buffer (buffer A, InCura), 2 mM MgCl2, 150 μM dNTPs, 5 pmol of each primer and 1 U *Taq* DNA polymerase (InCura). PCR amplifications were carried out with an initial denaturing step of 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 57 °C, 1 min of extension at 72 °C and a final extension step of 5 min at 72 °C. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Then fragments were direct sequenced using the BigDye v3.1 sequencing kit according to the manufacturer's instructions, and the sequences were loaded on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

3. Results

Using MRS and BP selective media 494 colonies were recovered of which 271 presumed *Lactobacillus* spp. and 223 presumed *Staphylococcus* spp.. The two groups were analysed separately and 215 out of 271 and 132 out of 223 colonies were randomly selected for molecular analyses.

Genetic relationships were determined by means of fluorescent AFLP. The *MseI* primer used for the selective amplifications was the preselective one, with an extra base (C). This was decided after preliminary trials, because the use of *MseI* selective primers with three bases gave too few peaks (usually fewer than ten, data not shown).

The use of three primer combinations was sufficient to detect several clear polymorphisms (at least two hundred for each group) and to recognise unequivocally the high number of strains considered. Only a few monomorphic bands were found and scored, and this reflects the great variability present not only between species but also within species. The use of some standard strains was useful to anchor the dendrograms and to verify the cluster-species correspondence (Table 1).

Strains isolated from all the sausage samples were considered for the analysis. The presumed LAB dendrogram (Figure 1a, 1b) and the similarity matrix were highly correlated (matrix correlation r = 0.97445; t = 52.3474, and Prob. (random Z < obs. Z) p = 1.0000). The tree was characterised by the presence of five well-defined clusters (I to V). The highest number of strains (131, 60.9 %) was clustered in group I, 15 (7 %) of the strains were in group II, 43 (20 %) in group III, 21 (9.8 %) in group IV and 5 (2.3 %) in group V. A bi-dimensional visualization of the sample set has been obtained by means of principal components analysis (Figure 2).

Genus	Species	CCCF[a] strain	Cluster
Lactobacillus	*L. sakei*	CCCF4161, CCCF4066 CCCF4000, CCCF4033 CCCF4047, CCCF4060 CCCF4044, CCCF4075 CCCF4262, CCCF4271	I
	L. coryniformis	CCCF4142, CCCF4226	II
	L. curvatus	CCCF4016, CCCF4139 CCCF4173	III
	L. plantarum	CCCF4178, CCCF4006 CCCF4032, CCCF4169	IV-1
	L. casei	CCCF4148	IV-2
Leuconostoc	*L. mesenteroides*	CCCF4059, CCCF4185	V
Staphylococcus	*S. xylosus*	CCCF4722, CCCF4716 CCCF4723, CCCF4519 CCCF4500, CCCF4707	I-1
	S. carnosus	CCCF4514, CCCF4692	I-2
	S. xylosus / saprophyticus	CCCF4520, CCCF4521 CCCF4714	I-3
	S. equorum	CCCF4556, CCCF4570 CCCF4674, CCCF4661 CCCF4671	II-1 II-2
Bacillus *Bacterium*		CCCF4560, CCCF4561 CCCF4565, CCCF4525 CCCF4530	II III
Enterococcus	*E. faecium, E. durans*	CCCF4529, CCCF4708	IV

a: Collezione Ceppi Corrado Fogher.

Table 1. Genus and Species classification of selected isolated strains based on GenBank homology search (homology greater than 98%) for a fragment at the 5′ end of the 16S ribosomal DNA gene.

Fig 1a

I

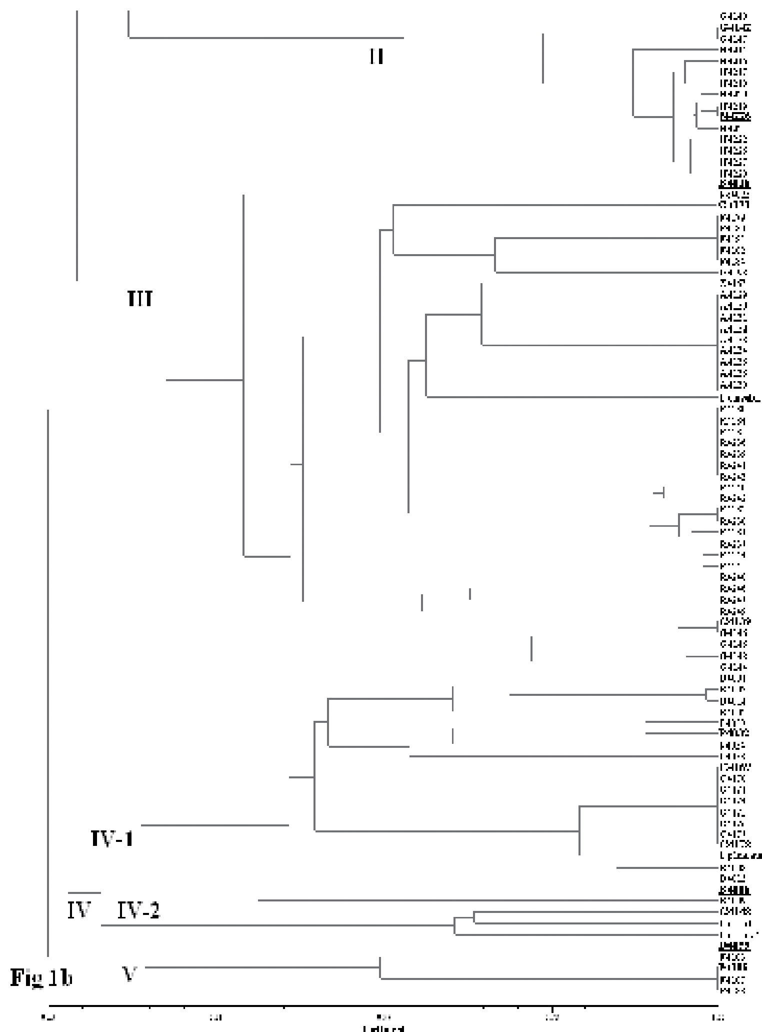

Fig. 1a, 1b. UPGMA phylogenetic tree of the Lactic Acid Bacteria population isolated from fermented sausages. The different clusters are reported. Cluster I: *L. sakei*; Cluster II: *L. coryniformis*; Cluster III: *L. curvatus*; Cluster IV-1: *L. plantarum*; Cluster IV-2: *L. casei*; Cluster V: *Leuconostoc mesenteroides*. The X axis represents the similarity coefficient obtained from the matrix constructed applying the Jaccard coefficient.

Principal component analysis (PCA) is a mathematical procedure that offers an easy visualization of the relationships between the different microbiota finding a set of synthetic variables that summarize decreasing portion of the observed variance. PCA finds those linear combinations that are maximizing the variance within the data and by using the DCENTER (Double Center) module, the Jaccard similarity matrix has been transformed to scalar product so that its eigenvalues and eigenvectors can be computed (resulting in a Principal Coordinates Analysis).

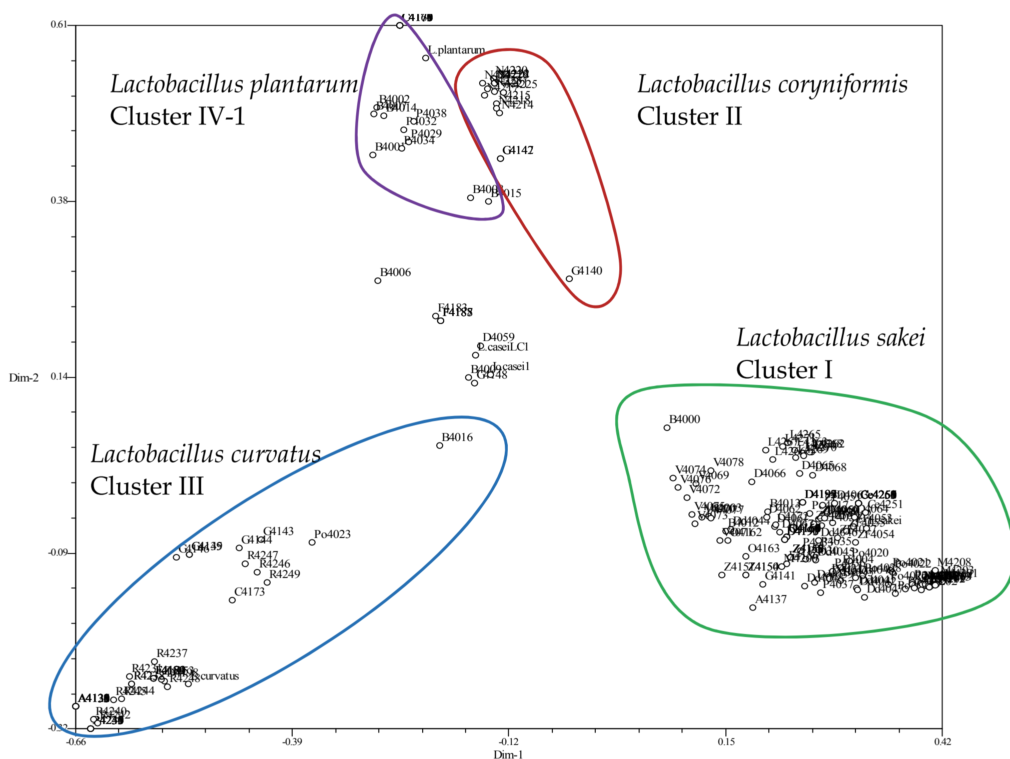

Fig. 2. Bi-dimensional representation of the presumed Lactobacilli main clusters defined by the AFLP analysis.

The first principal component (PC) is then the linear combination of the variables yielding the largest variance. The second PC explains the largest amount of the remaining variance. Thus, PCs can be sorted naturally by the explained variance and the four main clusters result to correspond to *L. sakei*, *L. coryniformis*, *L. curvatus*, and *L. plantarum*.
A more detailed comparison of the AFLP electropherograms revealed that the strains inside each cluster usually had similar banding profiles, and this made it possible to find some general banding profiles characteristic of the different clusters (Figure 3). Only one exception was present inside cluster IV, where strain CCCF4148 had a profile which was different compared with the profiles of the other strains, and therefore two sub-clusters were defined: IV-1, and IV-2.

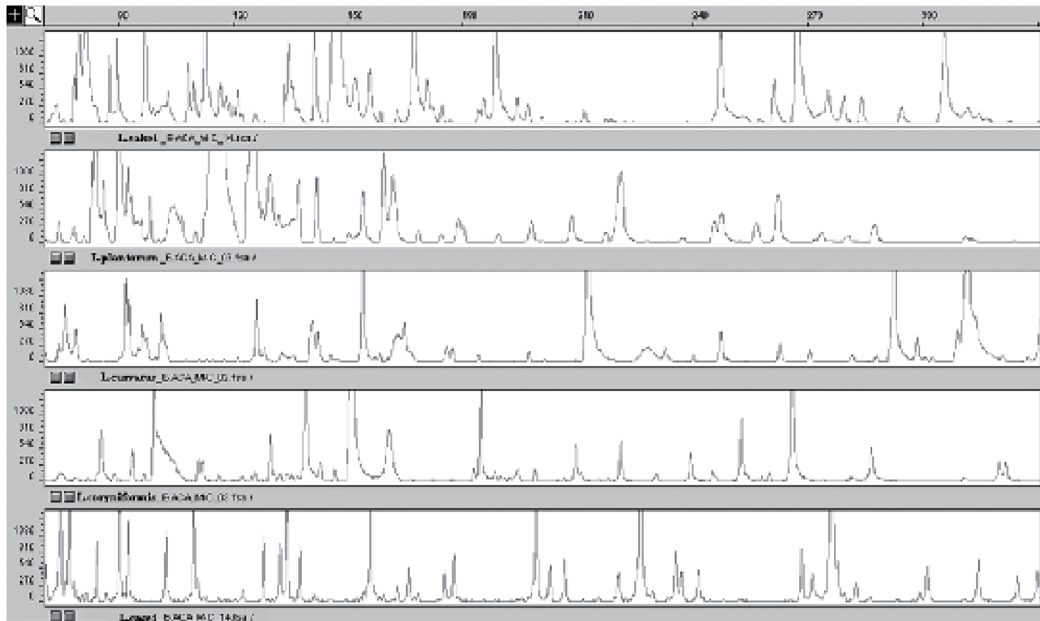

Fig. 3. AFLP electropherograms obtained using the automated genetic analyser. The five profiles are characteristic of different species of the genus *Lactobacillus*: *L. sakei* (strain 4191), *L. coryniformis* (strain 4214), *L. curvatus* (strain 4240), *L. plantarum* (Strain 4169), *L. casei* (strain 4148).

The presumed *Staphylococcus* dendrogram (Figure 4) and the similarity matrix were highly correlated (matrix correlation r = 0.94801; t = 64.2042, and Prob. (random Z < obs. Z) p = 1.0000). The tree was less defined in comparison with the LAB tree, but some main groups (four from I to IV) were still visible. The highest number of strains (66, 50 %) was clustered inside group II, 45 (34.1 %) of the strains were in group I, 7 (5.3 %) in group III, 10 (7.6 %) in group IV. Four strains (3 %) were located outside the main clusters.

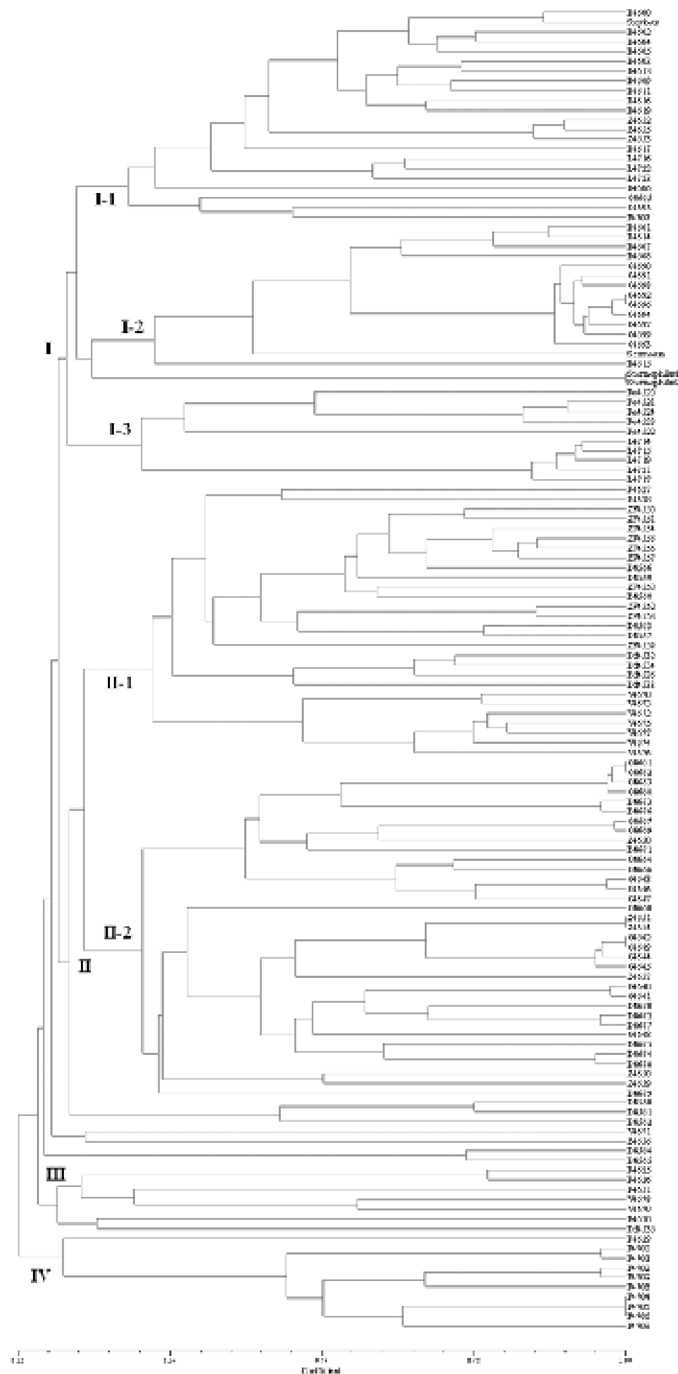

Fig. 4. UPGMA phylogenetic tree of the Coagulase Negative Cocci population isolated from fermented sausages. The different clusters are reported. Cluster I-1: *S. xylosus*; Cluster I-2: *S. carnosus*; Cluster I-3: *S. saprophyticus* / *xylosus*; Cluster II-1: *S. equorum*; Cluster II-2: *S. equorum*; Cluster III: *Bacillus* and *Bacterium* spp.; Cluster IV: *Enterococcus* spp.

As for the Lactobacilli, a PCA analysis has been obtained also for the presumed Staphylococci. The main clusters are clearly visible with the only exception of cluster I-3 that is not easily distinguished (Figure 5).

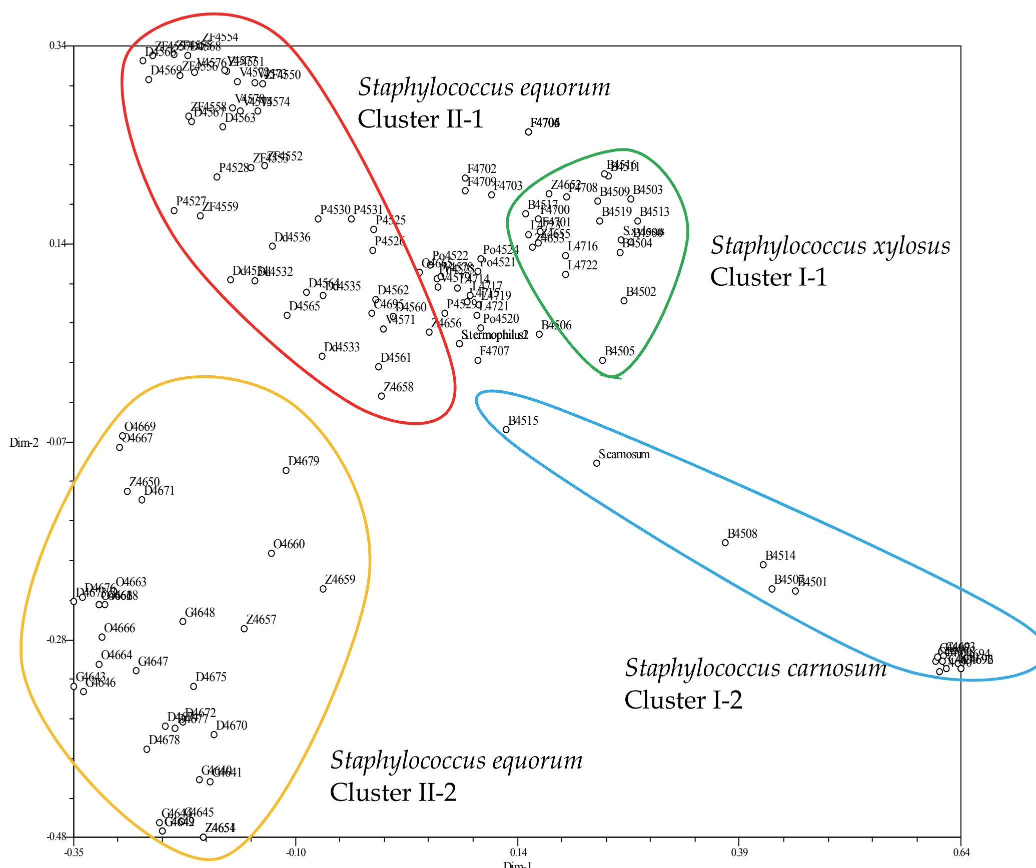

Fig. 5. Bi-dimensional representation of the presumed Streptococci main clusters defined by the AFLP analysis.

A more detailed comparison of the AFLP electropherograms of the strains inside clusters II, III, and IV revealed that they usually had similar banding profiles, while cluster I was characterised by three different AFLP profile typologies dividing the cluster into three sub-clusters: I-1, I-2, and I-3 (Figure 6).

In order to gain information about the genus or the specie corresponding to the different clusters, some strains were further analysed at ribosomal DNA level. In each group the strains were selected considering the genetic distances as reported in the similarity matrix, and those strains characterised by low similarity were selected.

The sequence of a fragment approximately 800 bp long was determined for the strains reported in Table 1, the results of a homology search is summarised in the same table.

Based on these results and on the similarities between the AFLP banding profiles, we identified the following species for *Lactobacillus*: *L. sakei* (cluster I), *L. coryniformis* (cluster II), *L. curvatus* (cluster III), *L. plantarum* (cluster IV-1), and *L. casei* (cluster IV-2). Strains inside

cluster V were recognised as belonging to the species *Leuconostoc mesenteroides*. Several strains were recognised unequivocally, while some strains showed an identical peak pattern. Indeed these could be replicates of the same strains. This situation was found mainly in groups I, III and IV-1. Inside each cluster, some sub-clusters grouping the strains isolated from the different producers were clearly visible (strains with the same letter).
The following species were identified within genus *Staphylococcus*: *S. xylosus* (cluster I-1), *S. carnosus* (cluster I-2), *S. saprophyticus / xylosus* (cluster I-3), and *S. equorum* (cluster II-1 and II-2). The strains of cluster III belong to different genera and species of *Bacillus* and *Bacterium*. Finally Strains of cluster IV belong to the genus *Enterococcus*, and the species with the greatest degree of homology were *E. durans*, and *E. faecium*. Some sub-clusters grouping strains from the same producers are visible

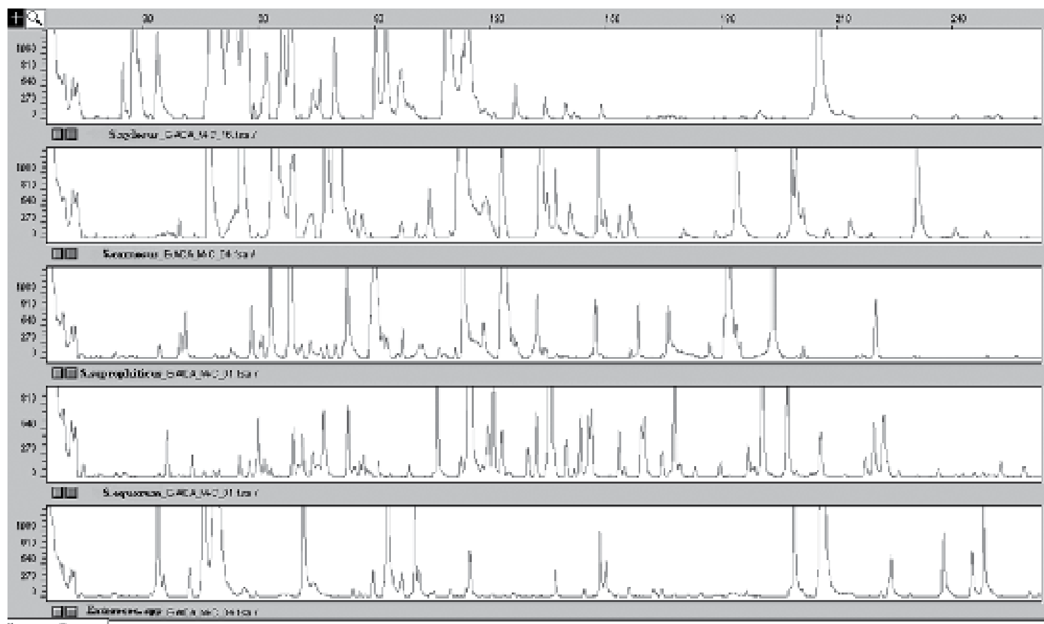

Fig. 6. AFLP electropherograms obtained using the automated genetic analyser. Panels 1 to 4 are characteristic of different species of the genus *Staphylococcus*: *S. xylosus* (strain 4722), *S. carnosus* (strain 4514), *S. saprophiticus* (strain 4714), and *S. equorum* (strain 4676). The last panel reports the profile of a strain (4704) belonging to the genus *Enterococcus*.

4. Discussion

Meat fermentation is a process with a long history in Europe, and many different countries have their own, characteristic long traditions of sausage production. Several studies have been carried out to define the biodiversity and the ecology of indigenous microflora in traditional Italian fermented products, both dry fermented sausages (Rantsiou et al., 2005a; Urso et al., 2006) and fresh sausages (Cocolin et al., 2004). The specific variety of the sausage is not always specified but, as reviewed in Rantsiou & Cocolin (2006), with the exception of a few studies concerning “Soppressata” and “Salsiccia sotto sugna”, the greatest number of these reports looking into the identification of microflora by means of molecular methods,

deal with products from northeast Italy mainly 'salame friulano' The existence of different conditions in the preparation and fermentation of sausages, both between different countries and within the same country (Rantsiou et al. 2005a; Urso et al. 2006), can determine the existence of differences in microflora dynamics (Rantsiou et al., 2005a). In this study, natural fermented sausages or 'salami' were sampled from different local manufacturers in north Italy at the end of the fermentation process. The use of reference strains both with the AFLP and the 16S ribosomal RNA gene analysis was considered in order to define the genus and, if possible, the species corresponding to the different clusters.

To assess the reproducibility of the AFLP analyses a panel of seven strains was replicated four times and the profiles consistently showed a good level of correspondence (data not shown) with a percentage of homology usually greater than 95% between the replicates. Considering this data, it is also possible that colonies showing homology greater than 95 % could be considered as replicates of the same strain.

The AFLP banding profiles of the strains grouped in the same clusters or sub-clusters showed similar patterns and the profiles of the single species can be used to rapidly classify new strains.

The potential to run AFLP on automated platforms such as genetic analysers is important since a significant number of isolates have to be characterised in order to better represent the bacterial population present in specific fermented sausages. The availability of automated platforms and the use of different dye-labelled primers provide the possibility to analyse, in a single day, several hundreds of samples. Furthermore, the use of reference strains reduces the need to perform ribosomal DNA analysis in order to define the species or the genus of the different isolates, thus further increasing the rapidity of the process.

As reported in Aymerich et al. (2006) some important points have to be considered for an optimal selection of strains and to define the microbial role in these products: i) rapid classification and identification of unknown isolates, ii) evaluation of genetic diversity among strains, iii) strain typing to assess genetic stability over time. Considering these points and based on the obtained results, fluorescent-AFLPs seem to tick all the boxes and therefore can be considered as an interesting, precise and rapid tool to identify the sausage bacterial microflora in addition to the existing and well-established methods.

Inside the genus *Lactobacillus*, we found the species *L. sakei* (61%) followed by *L. curvatus* (20 %) and *L. plantarum* (10%) to be the most represented. The same species were reported in other works (Cocolin et al., 2004; Rantsiou et al., 2005a; Rantsiou et al., 2006; Urso et al., 2006). Inside each cluster, some sub-cluster grouping strains from the same producer were defined (e.g. Strains CCCF4250 to 4261), and usually the differences between different sampling sites are greater than the differences inside single sites. This could be an important aspect to consider in the collection of microbiota biodiversity of fermented sausages.

The presence of *L. coryniformis* was also reported (Samelis et al., 1994; Rantsiou & Cocolin 2006) and, in agreement with several reports (Cocolin et al., 2004; Rantsiou et al., 2005a; Aymerich et al., 2006; Urso et al., 2006) the presence of *L. casei* is rare. Rare also are strains belonging to the species *Leuconostoc mesenteroides* which represent only 2.3 % of the LAB populations.

Inside genus *Staphylococcus, S. equorum* (48 %), followed by *S. xylosus* (21 strains, 47 % inside cluster I and 16 % of total), *S. carnosus* (14 strains, 31 % inside cluster I and 11 % of total), and *S. saprophyticus* / *xylosus* (10 strains, 22 % inside cluster I and 7.6 % of total) were found.

The same species were also found in other studies, but not in the same proportions. Rantsiou et al. (2005 c) found *S. xylosus*, *S. equorum*, and *S. saprophyticus* while they did not

find *S. carnosus*, and the percentages of the three species were respectively 48 % for *S. xylosus*, 23% for *S. equorum*, and 1.2 % for *S. sapropyiticus*. The presence of *S. carnosus* was also reported by Cocolin et al. (2001a, 2001b).
Bacteria belonging to the genus *Enterococcus* were reported by Rantsiou et al. (2005b) in similar percentages. The presence of enterococci must be evaluated carefully because they can have a role in the production of various traditional fermented foods, but at the same time certain enterococcal strains can cause human disease (Franz et al., 2003).
In this work we have analysed a significant number of typical and naturally fermented sausages produced in north Italy and provided an evaluation of the different cultured species and strains of bacteria present in these artisanal products. We have analysed all the isolates to strain level, obtaining for each one the corresponding AFLP profile demonstrating moreover that AFLP is a robust and useful technique for characterising the strain levels of cultured microbiota.

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3

Describing Parasite Biodiversity: The Case of the Helminth Fauna of Wildlife Vertebrates in Mexico

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1. Introduction

Parasites are extremely abundant and diverse in nature, representing a substantial portion of global biodiversity. At least 50% of the species living on earth are parasites of some form, considering all viruses and some bacteria, and the eukaryotic species most commonly associated with parasitology, including agents of diseases affecting not only humans, but also livestock, crops, and wildlife (Brooks & Hoberg, 2006). Interestingly, only a small fraction of the existing species are of medical or veterinary importance (Price, 1980; Poulin & Morand, 2004). There are many reasons to include parasites in any biodiversity survey, and indeed to study parasite diversity on its own. For example, parasites have been mentioned several times as elegant and sophisticated biological markers and as contemporary probes of biodiversity (Gardner & Campbell, 1992). Additionally, parasite diversity provides insights into the history and biogeography of other organisms, into the structure of ecosystems, and into the processes behind the diversification of life (Brooks & Hoberg, 2000; Poulin & Morand, 2000, 2004). In this context, parasites have, according to Brooks & Hoberg (2006), a dual and conflicting significance because they may regulate host populations, playing a central role in maintenance of genetic diversity and structuring host communities and, at the same time, they represent treats to human health, agriculture, natural systems, conservation practices, and the global economy (see Horwitz & Wilcox, 2005). For a comprehensive overview of the role that parasites play in research programs on biodiversity, the reader should refer to Brooks and Hoberg (2000) and to Poulin and Morand (2000, 2004). On the other hand, even though parasites have been proposed as indicators of ecosystem stress (e.g., Marcogliese & Cone, 1997), more recently, based on new methodological approaches, some authors have emphasized the role of parasites as indicators of environmental changes, probably as a result of a renewed interest in the impacts of climate change on earth. For instance, Vidal-Martínez et al., (2010) reviewed the usefulness of parasites as bioindicators of environmental impact, and their meta-analysis showed significant effects and interactions between parasite levels and the presence and concentration of various pollulants and/or environmental stressors. Meanwhile, Palm et al. (2011) demonstrated that fish parasites are

useful bioindicators to monitor long-term change in Indonesian grouper mariculture, and that groupers can be used to monitor environmental change in the wild.

1.1 Parasitic worms

Among eukaryotic metazoan parasites, helminths are represented by conspicuous and soft-bodied worm-shaped organisms that are commonly found living in virtually any habitat of the vertebrate host, as adults or as larval forms. In the later case, and depending on the habitat occupied by such larvae in the vertebrate´s body, the damage to the host my result in mortality, or at least, in a serious disease. According with Hugot et al. (2001) the term 'helminth' was originally used for worms living in the digestive tract of humans and animals, and thus was allied with the general concept of parasitism. Parasitism as a way of life evolved from free-living counterparts several times during the evolutionary history of life on earth. Helminths, as parasites in general, do not represent a monophyletic assemblage since under that term, members of phylogenetically not related phyla are included, i.e., Platyhelminthes ("flatworms"), Nematoda ("roundworms"), Acanthocephala ("thorny-headed worms), and Hirudinea ("leeches") (Fig. 1). Members of these groups are characterized as macroparasitic metazoans with a vermiform appearance, even though they represent independent evolutionary lineages. Some species have medical importance, e.g., *Taenia solium* and *Ascaris lumbricoides*, the first one causing diseases referred as teniosis (and cisticercosis when the larval form is the causal agent), and the second one causing ascariosis. Most helminth species possess a complex life cycle that involves one or more intermediate host, although some exhibit a direct life cycle (e.g., monogeneans).

Platyhelminths are characterized as dorsoventrally flattened acelomates with bilateral symmetry, and most of them are hermaphrodites. Among flatworms, free-living species are found, however, parasitic platyhelminths are included in three major groups, digeneans, monogeneans, and cestodes (Fig. 1) (Roberts & Janovy, 2005). Nematodes also contain free-living and parasite species; these are pseudocoelomate roundworms, with sexual dimorphism. The entire phylum Acanthocephala is represented by parasite species that infect, as adults, the digestive tract of vertebrates. Acanthocephalans are also pseudocoelomate worms diagnosed by possessing a particular attachment organ (proboscis) armed with hooks. Finally, hirudineans, commonly referred to as leeches (blood-sucking ectoparasites on a variety of hosts), belong into the phylum Annelida and are characterized as metameric celomate organisms, with some of the body segments at both extremities modified to form suckers (Bush et al., 2001).

According to estimates made by several authors, the number of known species of helminths infecting vertebrates varies between 23,670 and 52,000, with approximately 13,570 to >40,000 platyhelminths, 8,400 to >10,500 parasitic nematodes, 1,141 to >1,200 acanthocephalans, and >400 hirudineans (see Hugot et al., 2001; Poulin and Morand, 2004), however, quite possible, this biodiversity is underestimated since new helminth species are described in every volume of the major parasitological journals over the world on regular basis, and, as recently argued, the use of molecular tools is allowing a more accurate description of biodiversity by establishing a more robust species delimitation criteria, and parasites in general do not represent an exception to this trend (Nadler & Pérez-Ponce de León, 2011). Parasitologists are deeply aware that the inventory of the metazoan parasites of wildlife vertebrates on earth is far from complete. Particularly in Mexico, there is a long tradition in the taxonomic study of the helminth parasites of wildlife vertebrates, and they have been studied for more than 80 years. Due to this long tradition, a large amount of information has

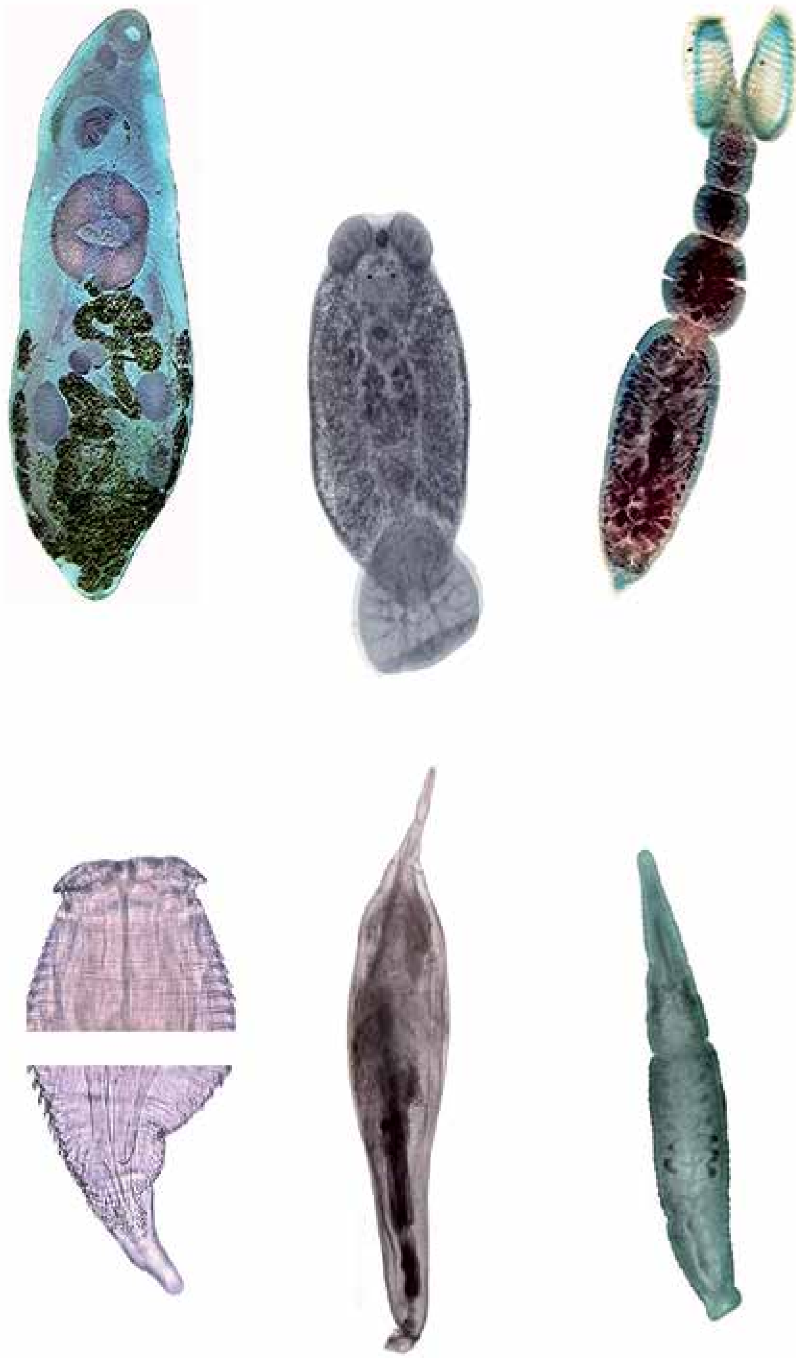

Fig. 1. Representatives of the major groups of parasitic helminths. Top line, from left to rigth, Digenean, Monogenean, Cestode. Bottom line, from left to rigth, Nematode, Acanthocephalan, Hirudinean.

been accumulated thanks to the dedicated work of national and foreign helminthologists that have contributed to the inventory of that parasite fauna. Based on the data gathered up to early 2011, in this book chapter we explore some general patterns of diversity of such parasitic group in the wildlife vertebrate fauna occurring within the Mexican territory, and we analyze these patterns in terms of host associations and geographical distribution, being aware that the inventory is not complete yet, and that unequal sampling effort may obscure some general patterns.

1.2 Mexico, a megadiverse country

The analysis we present here covers the entire country. Mexico is considered to be a Megadiverse country and, in this context, it occupies the 4th place worldwide in terms of species diversity. This is mainly the result of its position between the Nearctic and the Neotropical biogeographical zones; in addition to that, almost all the climates of the planet are found in Mexico, and it possesses a complex topography resulting from an intense geologic history (Sarukhán et al. 2009). As we recognized above, the inventory of the helminth parasite fauna is not complete yet of course, but we have gathered enough information thus far to start analyzing patterns and processes of parasite diversity and distribution, in an attempt to adjust such descriptive effort to modern taxonomic and biogeographic procedures (e.g., DNA-based taxonomy, niche modeling distribution, etc.), and to provide more accurate and realistic sampling strategies to try to complete the inventory in a timely manner, before the deterioration of some ecosystems produces the extinction of some species, or the particular habitats where they occur. Some efforts have been made to account for such patterns in the last decade; however, in this book chapter a general overview of the entire helminth fauna in wildlife vertebrates is presented for the first time, since previous analyses considered either a particular group of helminth, e.g., digeneans, or certain group of hosts, e.g. amphibians and reptiles (see Pérez-Ponce de León, 2001; Pérez-Ponce de León et al., 2002, 2007; Garrido-Olvera et al., 2006; Paredes-León et al., 2008; García-Prieto et al., 2010; Pérez-Ponce de León & Choudhury, 2010). Helminth parasites in humans as well as in domesticated (cattle, sheep, goats, swine, poultry) and companion (cats, dogs) animals are not considered in this review.

2. Gathering the data

The data on the helminth parasite fauna of wildlife vertebrates was obtained mostly from the database of the Colección Nacional de Helmintos (CNHE). The CNHE, hosted by the Biology Institute of the National Autonomous University of Mexico, is the national depository of helminth parasites in Mexico. In addition, information was also retrieved from foreign collections such as the U.S. National Parasite Collection (USNPC), Beltsville, Maryland, U.S.A., the H. W. Manter Laboratory of Parasitology, University of Nebraska-Lincoln, U.S.A. (HWML), and the British Museum of Natural History (BMNH), Great Britain, where Mexican specimens were deposited in the past. After conducting an extensive bibliographical search using databases such as CAB Abstracts, ISI Web of Knowledge, and Biological Abstracts, we retrieved all published accounts where helminth parasites in wildlife vertebrates in Mexico were reported. All the specimen data was subjected to taxonomic confirmation and the nomeclature of both, helminths and vertebrates, was updated following particular taxonomic treatments. All the data were entered into a database on Access platform and most data are available from the web site of the Unidad de

Bioinformática de la Biodiversidad (UNIBIO- Instituto de Biología, UNAM) (http://unibio.unam.mx/collections/specimens/urn/IBUNAM: CNHE). The analyses we present here considers helminth species richness in terms of two variables, the major taxa of hosts where helminths have been found, as well as the geographical distribution. The major emphasis is made on species richness, and only in some particular cases, helminth species composition is taken into account. The analysis of major taxa of hosts considers a traditional classification of the vertebrates, i.e., 5 major groups such as fish, amphibians, reptiles, birds and mammals, even though we acknowledge some of them do not represent natural groups. Geographical distribution data are presented in terms of the division of the Mexican territory into 32 states based on geopolitical boundaries, not representing biogeographical units; however, a brief discussion on the transitional role of Mexico because of its latitudinal position between the Neotropical and Nearctic biogeographical regions is presented.

3. Species richness patterns

The knowledge accumulated thus far on the Mexican helminth fauna is asymmetrical in terms of geographical distribution, host taxa analyzed, and sampling effort. All the 32 states of the Mexican Republic have been surveyed for vertebrates and their associated helminth fauna, however sampling size in terms of individual hosts as well as host species is unequal. Another feature of the asymmetrical sampling effort is that, in every state of the Mexican Republic, intensive samplings have been made in particular localities but some regions within the state still remain unexplored. The effect of sampling size in establishing an accurate parasite inventory has been widely discussed (see Poulin & Morand 2004). Inequality in sampling effort (number of studies and number of hosts examined) can influence species occurrences and richness estimates, and consequently the patterns generated from any database. Generally, parasite species are recorded from their presence in hosts and as a consequence, the effort put in sampling hosts will determine how complete the parasite inventory is. Clearly, sampling is an issue and even in detailed surveys some parasite species go undetected because an insufficient number of hosts are examined. Cumulative parasite species richness curves as a function of sample size have been proposed as an alternative method to estimate the number of living species for certain group. These curves are based on the premise that for each independent host sample, the number of known species in the parasite assemblage increases asymptotically toward the true richness value as more individual hosts are examined (Poulin & Morand, 2004). Few attempts have been made to obtain cumulative species curves for the Mexican helminth parasite fauna. For example, Pérez-Ponce de León et al. (2007) plotted the cumulative number of species of digeneans described (or recorded for the first time) against time, and this curve, for the most well-know group of helminths in Mexico, has clearly not reached the asymptote. In a previous analysis, Pérez-Ponce de León (2001) estimated that the digenean species richness in Mexico ranged from 5,300 to 8,000. Even though this number may reflect an overestimation of the size of the fauna, it indicates that the inventory of this particular group of parasitic worms is far from complete, considering we only have documented less than 650 species.

3.1 The size of the fauna

Up to January 2011, at least 1,145 vertebrates had been studied for helminth parasites, and each of these vertebrates contained at least one helminth species, although, needles to say,

most parasite surveys have been conducted only in a particular site, not covering other localities along the distribution range of the hosts; likewise, many of these studies do not report uninfected host species so the real number of analyzed hosts might be slightly higher. In addition, many vertebrate species analyzed have only once been recorded as hosts of helminth species, e.g., 52% in the case of tapeworms. In the 1,145 studied vertebrates, a total of 1,900 helminth species have been recorded, 603 of which were described as new species (referred to as holotypes). Helminths are represented by six major groups, including members of the Phylum Platyhelminthes: digeneans (634 species), aspidogastreans (5), monogeneans (331), and cestodes (271), Phylum Acanthocephala (87), Phylum Nematoda (538), and Phylum Annelida: hirudineans (34) (Fig. 2). Interestingly, a large number of species were described for the first time from some species of Mexican vertebrate. Figure 2 also shows the number of holotypes for each helminth group. It is noteworthy that the percentage of new species with respect to the total number of recorded species per helminth group varies between 14.7 (in hirudineans) and 46.2% in monogeneans. Even though two groups have received more attention in terms of the number of papers dealing with the alpha-taxonomy (digeneans and nematodes), there seems to be a correlation between the number of species recorded per helminth group and the overall diversity of each group, i.e., more species of digeneans and nematodes have been recorded, and these two as groups are the most diverse within helminths.

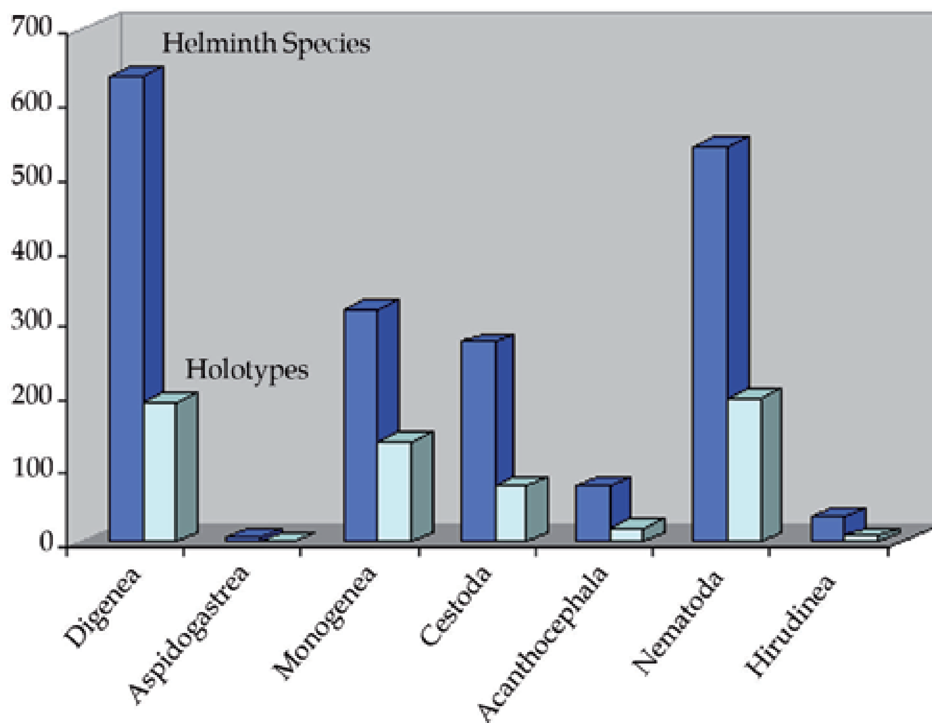

Fig. 2. Number of helminth species by parasitic group and the number of new species described for each helminth taxa.

The knowledge about the vertebrate helminth fauna in Mexico has been accumulated along eight decades, and various sampling strategies have been followed by parasitologists, both

national and foreign. During the first six decades, parasitologists mainly focused their research to describe a parasite species from a particular host species and locality, a strategy that can be referred to as "parasite species approach" (see Pérez-Ponce de León & Choudhury, 2010). The last two decades, however, witnessed a shift of the focus of survey research programs from the traditional parasite species approach to a more comprehensive survey work attending a host group, a geographical area, or both. Surveys were designed to inventory, for example, the digeneans of freshwater fishes from the sinkholes of the Yucatan Peninsula (Scholz et al., 1995), or the helminth parasites of freshwater fishes of the Nazas River, in Northern Mexico (Pérez-Ponce de León et al., 2010). At the same time, checklists where the information about certain helminth group, or certain host group, were the main focus, were also published, and these papers contributed with the update and detailed revision of the taxonomic information. For instance, Paredes-León et al. (2008) published a checklist of the metazoan parasites of amphibians and reptiles of Mexico; meanwhile, Pérez-Ponce de León et al., (2007), and García-Prieto et al. (2010) published a checklist of the digeneans and acanthocephalans of wildlife vertebrates of Mexico, respectively. As a result, a great dealt of information has been synthesized and made available for further analysis, as the one we provide in this book chapter.

3.2 Vertebrate hosts

Among vertebrates, fish are clearly the most well-known host groups. When the number of helminth species is plotted against the vertebrate group it becomes evident that fish, in general, has received more attention from parasitologists than any other vertebrate. This reflects a genuine interest of the parasitologists for that particular group, with the commercial value implicit in discovering parasite species producing diseases in economically important fish, or because some parasites are transmitted to man by consuming uncooked or raw fish, but it also shows a dual situation; on the one hand, fish are more diverse among vertebrates and, on the other hand, they represent the most easy-to-handle and easy-to-obtain host group when compared with other more charismatic, and most probably, endangered and protected vertebrates. According to recent estimates, Mexican biodiversity includes about 5,488 described species of vertebrates of which 2,692 are fish, 361 are amphibians, 804 are reptiles, 1,096 are birds, and 535 are mammals (Sarukhán et al., 2009). Of these, up to the present, 1,145 vertebrates have been studied for helminth parasites, including 674 fish, 63 amphibians, 153 reptiles, 134 birds, and 121 mammals (Fig. 3).

Overall, about 21% of the wildlife vertebrate fauna of Mexico has been studied for helminths to some extent, with parasite loads that vary from 1 to 82 helminth species per analyzed host species; however, the percentage of the hosts studied for helminths is variable among vertebrate groups. For instance, 25% of the fish fauna (including marine, brackish and freshwater), 17.5% of amphibians, 19.0% of reptiles, 12.2% of birds, and 22.6% of mammals have been studied for helminths, and at least one species has been recorded (Table 1).

Of the total number of helminth species recorded in Mexican vertebrates, 1,064 are found parasitizing fish, followed by those found in mammals (332), birds (275), reptiles (242), and amphibians (156). This total count (2,069) does not correspond with the 1,900 species given above because some of them are recorded from two or more groups of vertebrates, corresponding with the larval stage, for example in fish, and the adult in birds. As a general rule, helminths tend to keep some fidelity for the host at a higher taxonomic level such as

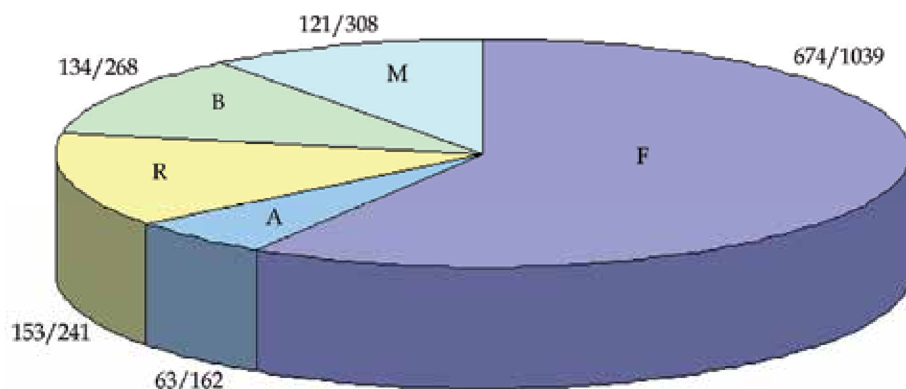

Fig. 3. Number of vertebrate species (per group) studied for helminths in Mexico, and number of helminth species reported. F = Fish, M = Mammals, B = Birds, R = Reptiles, A = Amphibians. Numbers outside the circle represent the number of studied species of hosts/ number of helminth species per group.

	Hosts			Helminths	
	Species in Mexico	Species Studied	%	No. of Species	$\overline{X}$
FISH	2692	674	25.0	1039	1.5
AMPHIBIANS	361	63	17.5	162	2.4
REPTILES	804	153	19.0	241	1.6
BIRDS	1096	134	12.2	268	2
MAMMALS	535	121	22.6	308	2.5

Table 1. Host species studied for helminths in Mexico, with respect to the total number of each vertebrate group. The number of helminth species is presented, with the mean number of species per vertabrate examined.

class or order, reflecting some sort of higher level host-specificity, i.e., parasites of mammals are usually not found in birds, and viceversa, or those found in chiropterans are not found in caviomorphs, and viceversa. Some helminth genera may contain species that infect certain host group, while other species infect a different host group. For example, rhabdiasid nematodes are typical lung parasites of amphibians and reptiles; the genus *Rhabdias* is a species-rich group and it has been demonstrated that their species are host-specific and rarely are parasites of more than one host group (Martínez-Salazar, 2006, 2008; Martínez-Salazar et al., 2009); some infect amphibians, and some infect reptiles. In most cases, the presence of a helminth species in an unusual host may be described as an accidental infection, if the worms do not reach maturity and are able to reproduce, meanwhile in some others, it is a result of an experimental infection as in the digenean *Echinostoma revolutum*, a relatively common bird digenean (duck and goose) that was intentionally used to demonstrate this parasite may infect humans (Larios, 1940). One exception to the rule might be the presence of the nematode *Spiroxys contorta* in amphibians (*Lithobates dunni, Ambystoma dumerilii*), as well as in reptiles (*Terrapene ornata*) (Lamothe-Argumedo et al., 1997).

	D	A	M	C	Ac	Ne	H	Total	Loc/States
Fish									
Cichlasoma urophthalmus	44	0	3	7	9	18	1	82	(79/7)
Petenia splendida	34	0	5	1	4	15	1	60	(42/7)
Rhamdia quelen	18	0	4	9	5	21	3	60	(61/6
Amphibians									
Rhinella marina	11	0	0	3	5	30	1	50	(39/9)
Lithobates vaillanti	15	0	0	0	3	16	0	34	(8/4)
Lithobates montezumae	22	0	0	3	0	7	0	32	(5/3)
Reptiles									
Sceloporus jarrovi	0	0	0	2	1	14	0	17	(17/16)
Kinosternon hirtipes	3	0	2	0	1	8	1	15	(16/8)
Tamnophis melanogaster	4	0	0	3	1	7	0	15	(6/3)
Birds									
Anas platyrhynchos	7	0	0	12	2	11	0	32	(8/4)
Ardea alba	18	0	0	3	2	6	0	29	(25/9)
Phalacrocorax brasilianus	12	0	0	5	1	7	0	25	(26/10)
Mammals									
Didelphis virginiana	6	0	0	2	4	16	0	28	(58/14)
*Equus caballus**	0	0	0	1	0	27	0	28	(2/1)
Philander opossum	7	0	0	1	2	6	0	16	(18/5)

Table 2. Species of vertebrates with the highest helminth species richness in Mexico. Only the top three species are listed. D = Digeneans, A = Aspidogastreans, M = Monogeneans, C = Cestodes, Ac = Acanthocephalans, N = Nematodes, H = Hirudineans, * = Wild horses.

Each host species is parasitized by a variable number of helminth species, even though this may depend upon sampling effort (considering the number of hosts analyzed and the number of localities along its distribution range). Table 2 lists the top three host species (per vertebrate group) with the highest helminth species richness. Among vertebrates, fishes (in this case three freshwater representatives) harbor a more diverse helminth fauna than any other group, however, as shown in Table 1, when data are presented as an average of the number of helminth species with respect to the number of analyzed hosts within each group, mammals and amphibians reach the highest species richness with 2.5 and 2.4, respectively. In absolute numbers, the Mayan cichlid, *Cichlasoma urophthalmus,* is the host species with the highest helminth species richness (82 species, with samples from 79 localities along seven states of the Mexican Republic) (Table 2). This fish is originally distributed in fresh and brackish waters of the Atlantic slope of Neotropical America, from the Coatzacoalcos River basin in the Gulf of Mexico southward to the Prinzapolka River, Nicaragua, including the sinkholes of the Yucatan Peninsula. Most of these 82 helminth species have been found in other species of cichlids occurring in Mexican freshwaters (about 50 species, see Miller et al., 2005), and some of these species have been

found in other fish families inhabiting Mexican freshwaters. At the vertebrate group level, helminths maintain fidelity to infect a particular host group, i.e., fish parasites are only find in fish, and not in any other vertebrate, probably with the sole exception of the Asian tapeworm (*Bothriocephalus acheilognathi*), a species that was introduced to Mexico along with common carps (*Cyprinus carpio*) from China with aquacultural purposes, and that has been found parasitizing now not only introduced but also native fish species, and even some amphibians and reptiles (Rojas-Sánchez and García-Prieto, 2008), but this is just the result of the dispersal capability of this invasive species of tapeworm. However, among the whole helminth fauna, some species exhibit a narrow host-specificity to parasitize cichlids as a group (family), a pattern that has been described as the biogeographical core helminth fauna (see Pérez-Ponce de León & Choudhury, 2005). Compared to *Cichlasoma urophthalmus,* a host species studied for helminths in 79 localities along seven states of Mexico, where 82 helminth species have been recorded, the oppossum *Didelphis virginiana*, is parasitized by 28 species of helminths, even though this mammal has been studied in 58 localities throughout a larger distributional range in Mexico that includes 14 states of the Mexican Republic, while the toad *Rhinella marina*, is parasitized by 50 worm species along its distributional range in 39 localities from 9 states, where its helminth fauna has been recorded.

3.2.1 The freshwater fish helminth fauna as a case study

Undoubtly, freshwater fish helminth parasites are the most well-known group among vertebrate parasites in Mexico. The helminth fauna consisted (up to September 2009) of 258 species in total, including 37 adult and 43 larval (metacertcariae) species of digeneans, 62 monogeneans, 15 adult and 18 larval (metacestodes) cestodes, 6 adult and 4 larval (cysthacanth) acanthocephalans, and 54 adult and 15 larval (L3) nematodes. Actually, Luque and Poulin (2007) suggested that Mexico stands out as a hotspot of parasite diversity in freshwater fishes. Based on that premise, the extent of the freshwater fish helminth parasite inventory of Mexico was evaluated using cumulative species curves by Pérez-Ponce de León and Choudhury (2010). These authors hypothesized that the inventory, as conventionally understood, is nearing completion for most helminth groups, excepting for monogeneans, where the cumulative species curve shows no tendency to reach the asymptote, indicating that further sampling and detailed alpha-taxonomy work is needed and the slope of the curve indicates more species of monogeneans will be described (Fig. 4). Interestingly, even though only 50.6% of the freshwater fish fauna in the country had been surveyed for helminths in a 80-yr period, the hypothesis is supported by empirical data and by the fact that the more species-rich groups of the native Mexican freshwater fish fauna, i.e., Cyprinidae, Cichlidae, Poeciliidae, Goodeidae, Aterinopsidae, and Ictaluridae, which overall account for 77% of the ichthyofauna, have been sampled intensively. Survey work we are conducting in areas of northern Mexico where these fish families are common, confirm this fact. In addition, in terms of geographical distribution, most of the major river drainages of Mexico such as the Lerma-Santiago and Balsas on the Pacific slope, and the Grijalva-Usumacinta, Panuco, and Papaloapan, as well as the sinkholes characteristic of the entire Yucatan Peninsula in southeastern Mexico, have been sampled to some appreciable extent. This does not mean that we will not find more species if we keep collecting data. We probably will, but it just means a slowdown in the rate of discovery of not previously recorded species of helminths.

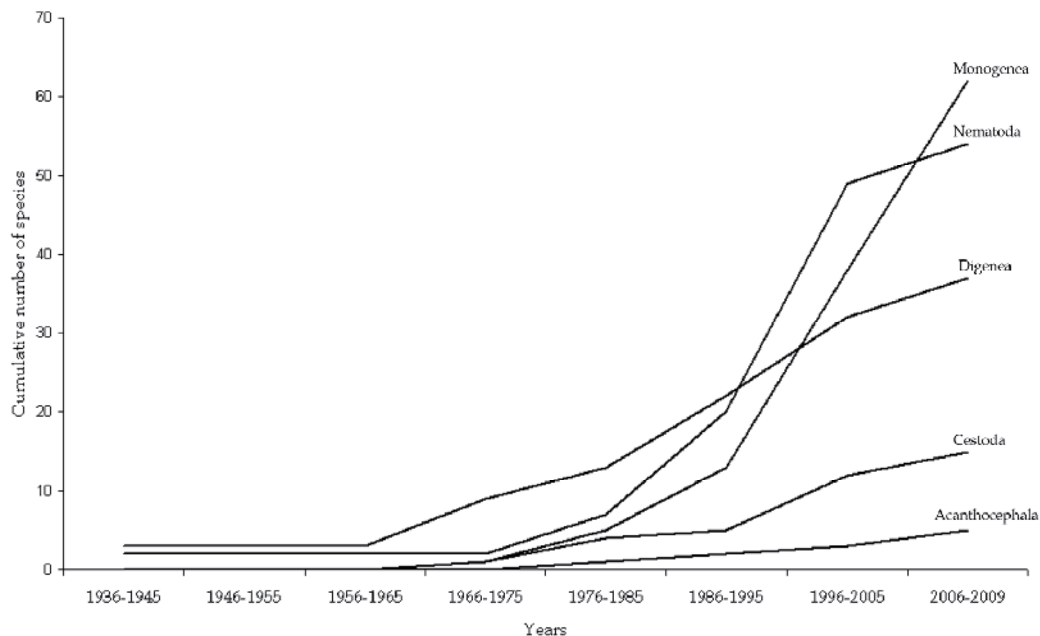

Fig. 4. Cumulative species curves for the helminth parasite fauna of freshwater fishes in Mexico. (Modified from Pérez-Ponce de León & Choudhury, 2010).

After analyzing their data, these authors contended that future survey work aimed at enhancing the biodiversity inventory of freshwater fish helminths in Mexico, should be strategic, and should combine the need to target missing components of the host spectrum with the choice of appropriate drainages based on biogeographic, faunistic, and hydrologic data. Additionally, Pérez-Ponce de León and Choudhury (2010) pointed out that the hypothesis that the inventory of this group of parasitic worms in freshwater fishes is nearing completion could be falsified if a closer look at the species delimitation criteria was made, by using molecular taxonomic methods instead of morphology-based approaches. Actually, they argue that this approach may indeed show that the helminth diversity has been seriously underestimated. At least three emblematic examples were recently published where the sequencing of various molecular markers allowed authors to demonstrate the presence of parasite cryptic species in what it was once thought to be only one species, including an acanthocephalan and a digenean infecting cichlids (Martínez-Aquino et al., 2009; Razo-Mendivil et al., 2010), and a digenean parasitizing ictalurid catfishes (Rosas-Valdez et al., 2011).

3.3 Geographic distribution

In terms of geographic distribution, sampling size is equally asymmetrical, considering the 32 states of the Mexican Republic. Figure 5 shows the number of helminth species that have been recorded in each of these 32 states, irrespective of the host group, the number of analyzed hosts, and the helminth group recorded. The states with the highest helminth species richness are Veracruz, in the Gulf of Mexico slope, with 377 species, and Jalisco, in the Pacific slope, with 285 (Fig. 5). Two states, the smallest, possess the poorer helminth fauna, Tlaxcala, with five species, followed by Aguascalientes, with 19, both in central

Fig. 5. Map of the Mexican Republic showing the number of helminth species in each of the 32 states. Dotted line represents the boundary between the Nearctic and the Neotropical biogeographical regions.

Mexico. There is no significant correlation between the size of the state and the number of helminth species reported, and clearly it also represents a biass of parasitologists to sample more intensively some localities within particular states. Veracruz and Jalisco states are the ones where the largest number of vertebrate hosts have been studied for helminths, 181 and 163, respectively (Table 3).

Figure 5 also shows a broad limit between the Nearctic and Neotropical biogeographic zones. If the two regions are considered, from a biogeographical point of view, helminth species richness is higher in the Neotropics than in the Nearctic region. The helminth species richness in vertebrates in the Neotropical part of Mexico doubles the species number in vertebrates in the Nearctic. This corresponds with general diversity patterns along a latitudinal gradient, however, due to sampling limitations, our results have to be taken with caution. As previously mentioned, this might be the result of a sampling artifact because the number of papers related with the helminth fauna of vertebrates ocurring in the neotropical part of Mexico are more than those in the Nearctic; additionally, a wider variety and number of vertebrate hosts have been studied in the entire region. In terms of helminth species composition, central Mexico represents a transitional biogeographic zone because a mixture of Nearctic and Neotropical elements are found, albeit a characteristic host association is made between the vertebrates whose origin is in the Nearctic or the Neotropics, and the helminth species that are found in them, i.e., the pattern of host fidelity is maintained. This might be established as a general pattern, and empirical data on helminth parasites of two vertebrate groups, freshwater fishes and amphibians, corroborate that observation (Pérez-Ponce de León et al., 2000; Pérez-Ponce de León & Choudhury, 2005). In these two groups of

vertebrates, it was shown that the helminth fauna possess a Nearctic or Neotropical connection, closely linked with the biogeographical origin of their corresponding hosts. For instance, helminth parasites of ictalurid, catostomid, and centrarchid freshwater fishes (typical components of the Nearctic region), harbor characteristic species of helminths that are also found in the same hosts along its distributional range extending from Canada downwards to its most southern distribution limit in central Mexico. In the case of amphibians, the same pattern is repeated and parasite biogeographic affinities coincide with host affinities, showing some degree of evolutionary association. For example, the mexican species of leopard frogs examined for parasites (*Lithobates berlandieri*, *L. brownorum*, *L. dunni*, *L. forreri*, *L. megapoda, L. magnaocularis*, *L. montezumae*, *L. neovolcanica*, and *L. spectabilis*), show a parasite fauna with 50% of the adult species (26 out of 52) having Nearctic affinities, following the origins of the host group (Hillis & Wilcox, 2005); while a minority of the parasite fauna of this group of frogs (19 %) has Neotropical affinities, particularly those found in the transitional areas (Paredes-León et al., 2008).

	Helminth Species								Analyzed Hosts
State	D	A	M	C	Ac	N	H	Total	Total
Aguascalientes	6	0	0	9	2	2	0	19	3
Baja California Norte	52	0	26	39	2	21	1	141	106
Baja California Sur	95	0	44	50	11	30	1	231	145
Campeche	53	0	44	10	10	35	1	153	60
Chiapas	35	0	7	15	5	116	3	181	115
Chihuahua	10	0	0	26	4	31	0	71	25
Coahuila	10	0	6	5	2	16	3	42	20
Colima	36	0	10	7	6	23	0	82	61
Distrito Federal	33	0	5	19	2	58	1	118	63
Durango	27	0	17	23	8	23	0	98	44
Guanajuato	13	0	10	13	1	17	1	55	26
Guerrero	58	0	33	16	9	68	1	185	91
Hidalgo	14	0	7	16	2	53	0	92	58
Jalisco	125	1	56	26	13	61	3	285	163
México	63	0	5	19	2	43	1	133	64
Michoacán	36	0	4	20	5	51	2	118	83
Morelia	10	0	7	5	3	31	1	57	38
Nayarit	28	1	20	6	6	24	0	85	71
Nuevo León	45	0	10	18	4	50	3	130	52
Oaxaca	73	0	28	23	6	62	1	193	143
Puebla	12	0	1	6	3	30	2	54	39
Querétaro	5	0	2	2	1	27	0	37	22
Quintana Roo	79	2	32	7	9	47	0	176	79
San Luis Potosi	12	0	2	4	1	24	0	43	24
Sinaloa	42	0	31	0	9	19	0	101	72
Sonora	27	0	26	22	3	40	0	118	78
Tabasco	105	1	36	13	11	32	2	200	106
Tamaulipas	32	1	21	10	6	25	4	99	47
Tlaxcala	1	0	0	2	0	2	0	5	7
Veracruz	127	3	68	35	29	114	1	377	181
Yucatán	106	2	37	26	12	83	3	269	92
Zacatecas	2	0	1	3	0	4	0	10	5

Table 3. Helminth species richness of wildlife vertebrates in each of the 32 states of the Mexican Republic. D = Digeneans, A = Aspidogastreans, M = Monogeneans, C = Cestodes, Ac = Acanthocephalans, N = Nematodes, H = Hirudineans.

3.3.1 The state of Veracruz as a case study

To illustrate the effect of sampling size, we analyzed separately the vertebrate helminth fauna of the most species-rich state in the Mexican Republic (Veracruz) in terms of the distribution of the helminth fauna. In Veracruz state, 377 helminth species have been recorded, most of them, as parasites of fishes, with 203 (53.8%). Table 3 shows the number of helminth species per helminth group and clearly, more digeneans and nematodes have been recorded in Veracruz than any other parasitic helminths, albeit this trend is also true for the other states. Interestingly, even though Veracruz is the state with the largest number of papers published, including isolated reports as well as parasite surveys, not the entire state, and of course not all the vertebrate fauna, has been equally sampled. Figure 6 shows how the distribution of the known helminth fauna is concentrated to particular areas within the state, where at least 46 helminth species have been recorded in a region of the north (in the Tamiahua lagoon and surrounding areas), and 152 and 224 species have been recorded from central Veracruz, in the regions of Alvarado lagoon, and Los Tuxtlas tropical rain forest, respectively. More species of helminths have been recorded from Los Tuxtlas, than any other region within the state of Veracruz, and that include helminth parasites of fishes (105), amphibians (54), reptiles (15), birds (13) and mammals (37). Wide areas along the state have not been sampled for vertebrates and their helminth parasites yet (Fig. 6).

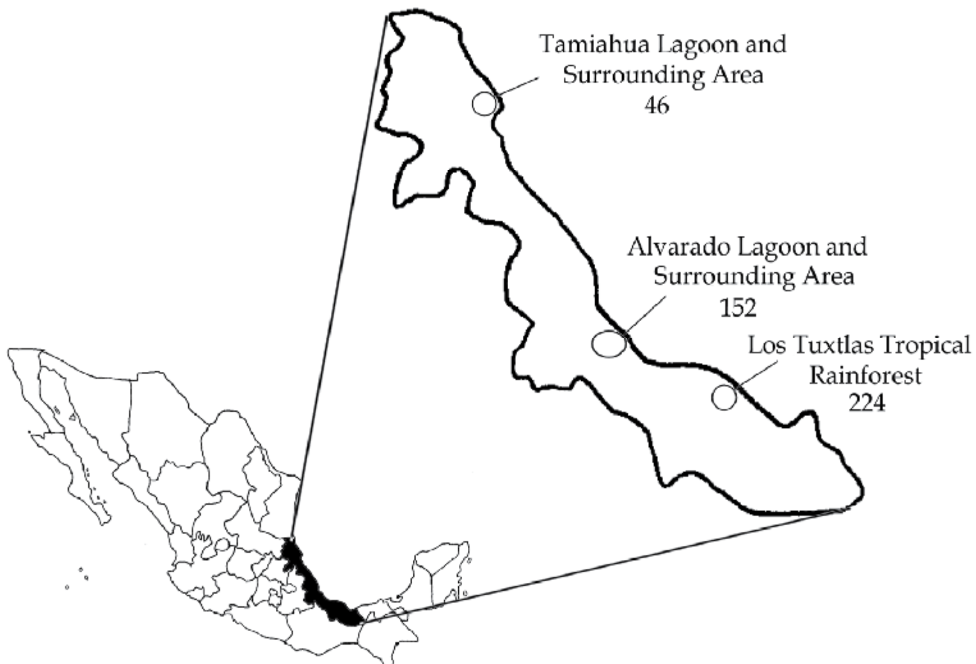

Fig. 6. Distribution of the vertebrate helminth parasite fauna in Veracruz, the state with the highest helminth species richness recorded thus far in Mexico.

4. Helminth parasite survey work: The future

After the recognition that good alpha taxonomy is central to biology, the last decade has witnessed a renaissance of the taxonomic practice. Taxonomists have recognized the

importance of using multiple data sources to establish more robust criteria for species delimitation, and to produce inventories supported by well-defined and novel protocols designed to explore and understand biodiversity. Certainly, good alpha taxonomy is crucial to overcome the biodiversity crisis, both for assisting conservation programs and documenting diversity before it is lost (Schlick-Steiner et al., 2010). Authors such as Padial et al. (2010) recognized that taxonomy as a discipline is confronted with the challenge to fully incorporate new theory, methods and data from disciplines that study the origin, limits and evolution of species. The latter authors concluded that taxonomy needs to be pluralistic to improve species discovery and description, and to develop novel protocols to produce the much-needed inventory of life in a reasonable time. Two terms have been used more frequently in the taxonomic literature, *New Taxonomy*, and *Integrative Taxonomy*, for the framework that should be used by taxonomists nowadays to bring together all the conceptual and methodological developments within the discipline (Wheeler, 2008; Padial et al., 2010). Some of these developments include virtual access to museum specimens, DNA sequencing, computer tomography, geographical information systems, multiple functions of the internet, and also that fact that taxonomic information is increasingly digitized and made available through several global initiatives (see Padial et al., 2010 and references therein).

In Parasitology, it has been recently recognized the need to follow an integrative taxonomy approach in order to obtain a more accurate description and understanding of parasite diversity, following modern taxonomic procedures that incorporate, for instance the use of molecular markers (Nadler and Pérez-Ponce de León, 2011). According to these authors, molecular tools offer an unprecedented opportunity to include new components in our discovery and description of parasite biodiversity, for example, characterization of genetic variability, population genetic structure, genetic differentiation and phylogenetic relationships. The molecular assessment of parasite biodiversity, including testing for cryptic species, is a largely unexplored opportunity for parasitologists. Deciding what species are and how to find them in nature (species delimitation) are prerequisite to characterizing this biodiversity (Adams, 1998; Nadler, 2002). For parasitic organisms, particularly those infecting humans, correct identification is crucial to understanding epidemiology, designing control programs, effective drug treatment and prophylaxis and investigating the potential for gene flow of drug resistance genes among populations (Nadler and Pérez-Ponce de León, 2011). One of the results of using an integrative taxonomy approach in parasite taxonomy is the recognition of cryptic species (those morphologically similar but genetically distinct). Recognizing cryptic parasite species from all kinds of hosts will permit a more accurate understanding of parasite biodiversity, systematics, epidemiology, evolutionary biology and biogeography. In this context, molecular data can independently corroborate that species recognized by morphological criteria are separate genetic lineages or conversely, uncover evidence that individuals appearing to be morphologically indistinguishable belong to independent evolutionary lineages. Species complexes of parasites are being revealed by molecular data where it was once thought there was either a single phenotypically variable species or a single morphologically uniform species (see Pérez-Ponce de León and Nadler, 2010 and references therein).

Characters, other than molecular markers, will be equally important in our description of helminth faunal diversity. In recent years, microscopy tools have been used to describe some traits that cannot be identified by conventional (light) microscopy. Some techniques have been of great value in helminth taxonomy such as the scanning electron microscopy (SEM) and confocal microscopy (CM). Halton (2004) argued that parasite surfaces have understandably

demanded most of the attention of microscopists, largely as a result of the pioneering studies using transmission electron microscopy. Among all techniques, SEM has become increasingly useful in describing the surface topography of helminth parasites (Fig. 7), and that is the reason we contend that future taxonomic work intended to describe helminth parasite biodiversity, should incorporate the description of body surface traits by means of SEM. Empirical studies have demonstrated the usefulness of this microscopy technique in discovering taxonomically important traits in helminths, such as sensory receptors (papillae number, shape, size and position along body surface in digeneans) (Mata-López and León-Règagnon, 2006), or the size and shape of cuticular spines in nematodes (Bertoni-Ruiz et al., 2005).

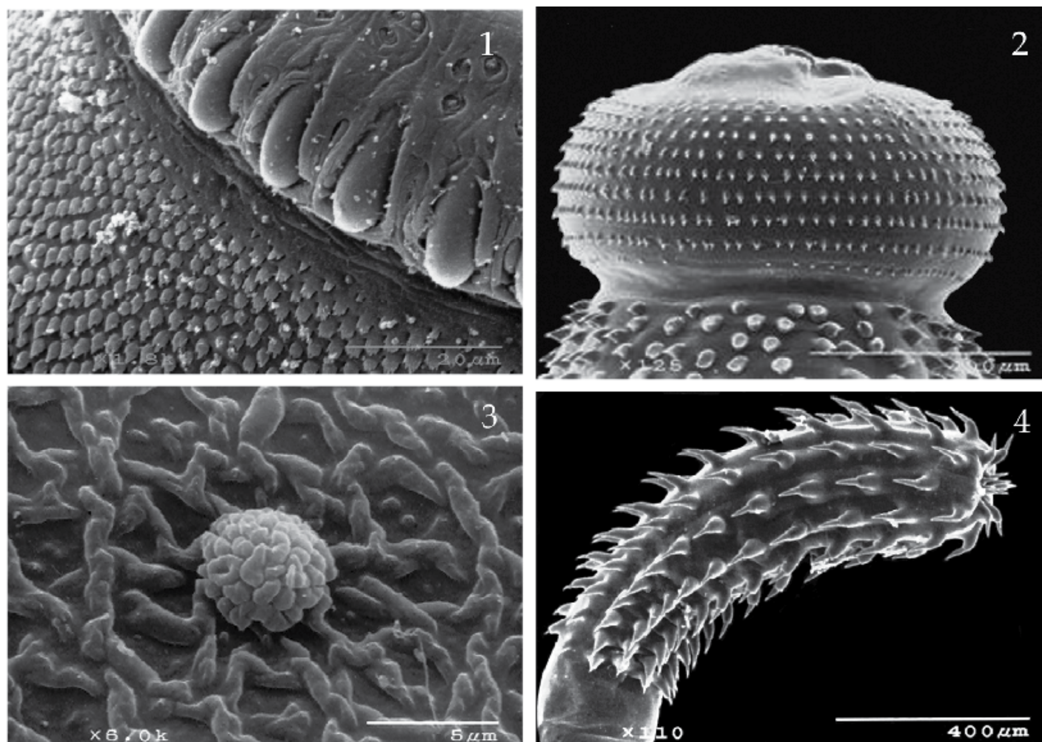

Fig. 7. SEM microphotographs of representative helminths, showing taxonomically important characters. 1 *Echinostoma* sp. (Digenea, anteriror end), 2 *Gnathostoma turgidum* (Nematoda, cephalic bulb) 3 *Phyllodistomum centropomi* (Digenea, papillae), 4 *Atactorhynchus duranguensis* (Acanthocephala, proboscis).

5. Conclusion

The main question that raises after presenting the analysis of the data we have gathered after studying helminth parasites of wildlife vertebrates in Mexico over a 80 yr period is: how far are we of completing the inventory? The short answer would be: far away of completing such inventory, considering that we have studied about 21% of the vertebrate fauna occurring within the Mexican territory. All biodiversity surveys are based on the premise that the harder you look, the most species you will find, i.e., if you spend more time searching, if you increase the size and the number of localities, and the number of collecting

trips, it is likely that you will find more species. The same argument is true for parasitic organisms but in this case we have to add the fact that the larger number of host you study, the larger number of helminth species you will find. Assuming the same rate of species discovery after studying one fifth of vertebrate hosts occurring in Mexico, this would mean that we will require at least another 320 years to complete the inventory. This kind of striking calculations parallels that made by Cribb (1998); this author estimated that to find just all trematode species in Australian vertebrates, about 160,000 hosts need to be killed and examined, taking up 30,000 days of work, not considering the time required to accomplish the species identification and description. In the case of the Mexican vertebrate helminth fauna, 1,900 helminth species have been recorded from 1,145 analyzed vertebrate hosts. Based on this data, the average number of helminths per vertebrate in Mexico is 1.66. At the same rate of species discovery, if all the vertebrates in Mexico are analyzed (5,488 species) the estimated number of helminth species to be recorded overpass 9,000, if the inventory could be completed in the next 320 years.

The result of all these species richness estimations, beyond a pesimistic view, is that survey work intended to describe biodiversity in not very well-known groups of organisms (such as helminths), lacks of a conceptual value. Part of the problem is that these calculations do not take into account some attributes of species distribution, and actually, are based on premises that are almost impossible to demonstrate. For example, we cannot assume that we will find different helminth species in each one of the vertebrate hosts we have not looked at, i.e., the assumption that the species discovery rate will be at the same pace, as more vertebrates are studied for helminths, is just wrong. The case of the aforementioned freshwater fish helminth parasite fauna illustrates this contention. No matter about 51% of the fish fauna in the freshwaters of country has been studied for helminths, we concluded that the inventory is nearing completion. Unfortunately, for the entire helminth fauna of Mexican vertebrates, based on inequality of sampling effort in terms of both, hosts, and geographic regions, cumulative species curves cannot be used. These curves, along with the use of non-parametric species richness estimators represent the best methodological approaches to estimate the number of species that would be described (Poulin and Morand, 2004). Values obtained thorough these methods allow for an strategic planning to keep working in biodiversity surveys aimed to complete the inventory of certain taxonomic group. In the case of the Mexican helminth fauna, it is unrealistic to try to complete the inventory in the near future, because clearly, a lot of work needs to be done, and some additional aspects need to be considered. One of the most important is the so-called taxonomic impediment. In recent years, the number of properly trained taxonomists has decreased dramatically, and this is not the exception for helminthology as a discipline. If the inventory work is going to be maintained, we have to be aware that more generations of well-trained taxonomists need to be produced. In addition, these new generations need to be able to use modern taxonomic methods, in addition to the expertise on the morphology of each group, and this implies the use of various molecular techniques to establish more robust species delimitation criteria, added to an appropriate knowledge of evolutionary and biogeographical methods, intended to complement molecules and morphology, to achieve a better understanding of the diversity of the helminth parasite fauna.

A second question that raises from the current analysis is: Why should be try to complete the inventory of the helminth parasite fauna of wildlife vertebrates in Mexico?. There are many reasons for such a task. Some of them are referred in the introductory section of this book chapter, but probably the most important is because parasites, in general, represent a

substantial portion of global biodiversity since at least 50% of the species living on earth are parasites of some form, considering all viruses and some bacteria, and the eukaryotic species most commonly associated with parasitology, including agents of diseases affecting not only humans, but also livestock, crops, and wildlife (Brooks & Hoberg, 2006). A second reason derives from the fact that some species of helminths that are commonly found in the wildlife, maybe become disease agents in human beings. The more we know about the diversity of this parasite fauna in the wildlife, the more we will understand about their life-cycles and the potential that some species may have in the context of emergent infectious diseases. Recently, while discussing the structure of helminth parasite faunas with respect to the invasive process in nature, Hoberg (2010) concluded that faunal baselines derived from arrays of biological specimens, integrated surveys and informatics are a permanent record of the biosphere when archived in museum collections. This author also mentioned that if we do not have comprehensive taxonomic inventories of parasites, our ability to recognize the introduction of non-indigenous parasites, and to document patterns of expansion for local faunas under a regime of environmental perturbation, would be limited.

In this book chapter we presented an overview of the general data on this parasitic group, we analyzed the information we have gathered thus far, and we presented an estimate of the number of helminth species that remain to be found if the inventory is completed. As a result, we propose here some sampling strategies in order to optimize time and resources and to contribute with valuable information on the diversity of this group of organisms. First, we contend that the inventory needs to be completed by approaching particular vertebrate groups. As for the freshwater fish parasite fauna, an approximation has to be taken with respect to other vertebrate groups. For instance, vertebrate groups have to be targeted by researchers and a sampling strategy needs to be established. After fish, mammals represent the vertebrates with a higher percentage of species studied for helminths. It is impossible to postulate that all the mammals occurring in Mexico would be studied for helminths, considering the entire distribution range for each species. Particular groups, such as caviomorph rodents, or chiropterans, or marsupials, need to be evaluated based on their diversity and geographical distribution, and then estimate the number of helminth species that would be found, based on proper sampling effort and an accurate description of the data that should include surveys that fail to find helminth parasites from a sample of hosts in a particular locality, i.e., even reporting uninfected hosts.

With no doubt, there remains much to be done and overall, the end is not yet in sight. However, a large amount of information has been produced and this analysis allow us to establish a strategic plan to address the inventory of the helminth parasite fauna of wildlife vertebrates in Mexico in the upcoming years, and more importantly, to recognize that such inventory work needs to be done under novel taxonomic procedures that guarantee the quality of the information. The inventory is not complete yet, but it is our responsability to set the better way to accomplish the task, and leave for future generations of parasitologists the task of advance in the accumulation of data with the hope that the diversity of the helminth fauna in Mexican vertebrates will be better understood, and that the generated data will be useful for other members of the scientific community.

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4

Yeasts Biodiversity and Its Significance: Case Studies in Natural and Human-Related Environments, *Ex Situ* Preservation, Applications and Challenges

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1. Introduction

Yeasts are a group of microorganisms that belongs to the Fungal Kingdom. These unicellular fungi are distributed between the Basidiomycota and Ascomycota Phyla, being a paraphyletic group. Since 1865, its study has experienced a very important advance in terms of its understanding, characterization and taxonomic accommodation. Nevertheless, it is estimated that about 99% of the potential biodiversity of this group of eukaryotic microorganisms is still unknown. That is why there is a need for increasing efforts to study yeast biodiversity, especially in mega diverse countries from the tropical regions of the planet.

To date, the majority of yeast species catalogued have been discovered in countries from the Northern hemisphere. Relatively few studies dedicated to yeast biodiversity have been done in tropical zones of the planet and in Southern hemisphere countries that embrace abundant and diverse ecosystems. A number of case studies of these approaches to yeast biodiversity are presented in this chapter, including the discovery and subsequent description of novel yeast species recently isolated in Ecuador, Brazil and Argentina. The chapter will also deal with the biodiversity of yeasts found in industry-influenced environments in Spain.

Moreover, *ex situ* preservation of yeast isolates for further characterization by physiological, morphological and molecular techniques is a fundamental issue in terms of the

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understanding and preservation of the biodiversity. Yeast culture collections play a fundamental role not only as the repositories for invaluable yeasts strains (germplasm), but also as platforms for biotechnology exploitation. Experiences and challenges that several yeasts collections are facing will also be discussed in this chapter.

2. Biodiversity of Yeast, ¿What does it mean?

The term biodiversity is an abstract expression of all aspects of the variety of life (Gaston, 1996); from bio-molecules to the variety of different species populations and communities of species. Variation is the essence of biology. Thus, biodiversity is an intrinsic feature of life.

Under this focus, biodiversity loss is one of the main global concerns. This, loss can be produced by a number of different factors related to human activities and to natural events; where competition at the intra- or inter-specific levels and even at the molecular scale, reminds us the real drama of life, where Darwin's "The Origin of Species" reaches the nerve of this fundamental issue of living organisms.

Nonetheless, an undefined number of yeast species losses can be caused due to the perturbation of habitats by humankind. As Dr. Steve James from the National Collection of Yeast Cultures in the United Kingdom points out, talking about the importance of yeast biodiversity surveys: "It's a race against time. We know that massive loss of species diversity is occurring worldwide. Our efforts are thus focused on characterizing and subsequently preserving what remains."

Global-scale conversion of tropical rainforests and agricultural intensification are major causes of biodiversity loss (Chapin et al, 2000; Hoekstra et al, 2005). Extinction is the final result of a process that starts with the vigor's weakening of certain populations. The most undesirable and irreparable effect is the complete loss of all (component) populations of a single species. This effect is uniquely evident when the fragmentation and perturbation degree of natural micro- and macro-landscapes overwhelms the "decisive threshold" (Pimm and Raven, 2000).

Some microbes do seem to be restricted to very particular environments and are endangered in as much as these environments are threatened; microbes intimately associated with other organisms share (partially) the biogeographies of their hosts. As far as they are species-specific, they could potentially become extinct along with their hosts (Weinbauer and Rassoulzadegan, 2007).

We still do not have any definitive evidence of the extinction of any yeast species: it is very hard to determine that. Nonetheless, we can presume that a number of co-evolving yeasts species have probably become extinct along with their plant or animal hosts. Studies made on bumble bees demonstrate that insects play a crucial role, not only in yeast dispersion, but also acting as a type of "wet nurse" during winter, when environmental conditions are very harsh and no flowers are present in the fields (Byrsch, 2004). In this way, extinction or weakening of insects populations can ultimately lead to the extinction of certain insect-dependent yeasts species, at least locally.

As biodiversity is not only referent to the living organism by itself, but also to the diversity of its strains and varieties. Likewise, the molecular variety of yeasts is both huge and extremely dynamic. The occurrence of a great variety of yeast strains is the result of the high mutation rate that provides these microorganisms with the ability to adapt to different environments. Mutation rate is an important parameter in evolution. It dictates the speed of adaptation in populations with beneficial mutations; in the absence of such mutations it sets

the equilibrium fitness of the population (Gregory et al, 2007). The loss of varieties in yeast strains is also a concern, but, at the same time is an issue that we cannot solve and probably we don't need to try solving.

The search for yeasts species/strains with economic potential is a way to preserve those genetic varieties that are worth being kept and used in a wide range of applications. Gregory et al in 2007 found that in *S. cerevisiae* the mutation rate per gene is in the order of 10^{-10}/base pair/generation. It is important to note that different DNA regions in living organisms have different variability. These mathematical approaches can be used to estimate the evolutionary distance in terms of time between different strains. As for *S. cerevisiae*, it has been possible to determine, based upon the nucleotide variations of several genes belonging to different strains, that this yeast species was most likely first domesticated about 11,900 years ago (Fay and Benavides, 2005). The study of ancient dormant yeast strains/species, although still in its infancy, is nevertheless a field that offers the opportunity to help better understanding of microbial biodiversity over time (Gomes *et al.*, 2010).

Dormancy in yeasts and other microorganisms plays a key role to help keep a seed bank for the future (Jones and Lennon, 2009). The biodiversity of microbial communities, of which yeasts are an integral part of has important implications for the stability and functioning of managed and natural ecosystems. Dormancy is one trait that allows species to contend with temporal variability of environmental conditions. This "bet-hedging" strategy allows dormant individuals to become members of a "seed bank", which can contribute to the diversity and dynamics of communities in future generations (Turner et al. 1998; Caceres and Tessier, 2003). The recovery of dormant yeast species from archaeological pieces as well as paleontological rests provides a means of reviving species or strains that were probably destined to become extinct (Gomes *et al.*, 2009). The techniques and the approaches already done in this field will be outlined later in this chapter.

Yeasts are also adapted to dispersion and then survival. One example of this is the cross-shaped yeast *Metchnikowia gruessii* that is dispersed by bees visiting flowers during its feeding periods in the day. This cross-formed yeast species is adapted to the glossa or tongue of the bees and so use the insect as a means of dispersal from one flower to the next. As for the studies carried out by Byrsch in 2004, this species is highly successful, very common and forms predominant populations in nectar of certain central European flowers.

The best way to get into the study of yeasts in natural environment is using ecological criteria: yeasts occupy a diverse variety of micro-ecosystems and are well adapted to a wide range of weathers, altitudes, substrates and geographical locations. It is possible to find yeasts in glaciers, high salinity lakes, water, soil, air, intestines of a variety of vertebrates and invertebrates, and even in acid waters (Russo et al, 2010) and marine deep-sea environments (Nagahama Takahiko, Biodiversity and Ecophysiology of yeast). The proper way to study yeast diversity and its function in communities is by gaining an understanding of their role in communities, so we can predict the occurrence of certain species based on the features of the micro- and macro-landscapes.

Yeast species such as *S. cerevisiae* have been used by humankind throughout history for the production of fermented foods and beverages around the world. That is why yeasts are intimately linked to our day-to-day activities related with culture, economy and nutrition. Moreover, certain yeast species are linked to human diseases, while others form part of the intestine's micro-flora in both vertebrates and invertebrates.

Nevertheless, a relatively small number of yeast species are currently being used in industry, while, a large number of species collected from natural environments and human-related micro-ecosystems are still being studied and classified. Yeast taxonomy deals with the classification and accommodation of species that are being discovered in an ever increasing number year after year.

2.1 Yeast diversity in numbers

It is believed that only 1% of all extant yeast species is currently known. From 1820 up to 2011 the number of described yeasts has increased dramatically. By 2005, more than 2500 yeast species were published. This number of species already named includes synonyms which are being taxonomically re-accommodated. Currently there are approx. 1500 recognised yeast species, which means the expected number of yeast species on Earth would be around 150,000. Large territories of Africa, Antarctica, Asia, Australia and Latin America are mainly virgin (Hawksworth, D.L, 2004). These new and hardly explored habitats represent rich sources of fungal biodiversity still awaiting discovery. To date, relatively little work has been carried out in this field in South American countries like Argentina, Brazil and Ecuador. The yeast diversity in such countries is potentially huge. For example, over 200 new species of yeasts have been found amongst 650 isolates from the guts of beetles (Suh et al. 2004, Suh & Blackwell 2005). Coleoptera species are floricolous insects and tree flux communities whose species number about 350,000. Nevertheless, not all beetle species harbour yeasts, and so its number must to be first established to predict a possible overall estimate of yeast related with them (Lachance, 2005 Yeast biodiversity and Ecophysiology).

Molecular techniques used since 2000 have greatly boosted the number of new species identified. Molecular analyses of the variable D1/D2 regions of the 26S rDNA, 18S, 5.8S and mitochondrial small subunit rDNAs gene, as well as ITS sequencing and RFLP-ITS are very useful ways to identify yeast species and invaluable tools for phylogenetic studies (Kurtzman and Fell, 2005 biodiversity and Ecophysiology). These molecular techniques, combined with microbiological and physiological tests, are being used to characterize yeast isolates and species. Most of the analyses have used rDNA sequences, however, we now know that there are no universal criteria to distinguish between genera.

Communities of yeasts are affected by natural selection which eliminates deleterious mutations and rapid fixation of adaptive alleles, just as the environment determines whether or not a species can become established within a community (Lachance, 2006). In these terms, we can understand that events of speciation and/or extinction are occurring in yeasts around us all the time at a relatively high rate due to its remarkable rate of reproduction, mutation and adaptation to changing situations.

Diversity between strains of the same species, such as *Saccharomyces cerevisiae*, has also been studied by molecular methods. It is well known that there are variations in strains and some metabolic abilities/disabilities are not necessarily linked to the species but rather to the strains (i.e. strain variable). With few exceptions, only one strain or an individual of a particular species is sequenced while hundreds of other variants, which may be important to public health, scientific research, or commercial applications, remain un-deciphered (Winzeler et al, 2002). The use of microarrays of whole genomes divided into 25mers helps to find variations between different strains of a single species, in as much as a single base substitution in these 25mers (especially those found in the center of the sequence) disrupts

hybridization (Chee et al. 1996; Gingeras et al. 1998; Troesch et al. 1999; Lockhart and Winzeler 2000). Single Feature Polymorphism SFP assessment carried out on 14 different strains of *S. cerevisiae* yielded 11,115 variations, which demonstrates the huge genotypic variation between strains of a single species and the opportunities these variations offer for the research (Winzeler et al, 2002).

Furthermore, Single Nucleotide Polymorphism (SNP) analysis is revealing relationships within strains of a single species. Moreover, the analysis of variation in gene content, nucleotide insertions and deletions, copy numbers and transposable elements are all contributing to reveal the intricate relationships between yeast species and strains (Liti, 2009). In other words, biodiversity of yeasts at intra- and inter-specific levels is a big endeavor that is still very much in its infancy taking into account the huge diversity of yeasts.

In order to ascertain and classify the yeast biodiversity in nature in an affordable way it is necessary to investigate the multiple and varied micro-ecosystems represented by substrates that may be used as a source of nutrients by yeast as well as platforms for their dispersion. From beetle guts, to flower nectar or rotten woods, there still remains a huge field to be examined in order to identify and characterise novel and known yeast species: their distribution, ecologic relationships and the understanding of the aspects involving the yeasts natural history, and feasible uses as biotechnological work horses. South America is a region that offers great potential in terms of biodiversity (macro and micro), where yeasts are being isolated from habitats that never were sampled before. Initial results from a survey run by an international consortium from Brazil, Spain and Ecuador in the Galapagos Islands at the end of 2009 (data not published) is beginning to reveal the diversity of yeasts present in various substrates such as flowers, cacti, rotten wood, turtle’s faeces, marine iguana faeces, and other substrates located in four different islands. This kind of expedition has also been done in other South American countries such as Argentina and Brazil. Several novel species have been identified in such surveys.

At this point, the identification and characterization of yeast isolates and its preservation is a task that may be accomplished by researchers. Yeast culture collections play the leading role in keeping the rich diversity of yeasts for current and future applications as well as genetic reserve. Some aspects of the biodiversity studies, yeasts preservation, novel yeast species description and its taxonomic accommodation and biotechnology applications will be developed in this chapter.

3. Ecology and biodiversity of yeasts

3.1 Yeast-insect interactions as example of biodiversity studies

In general, yeasts are suspected to engage in intimate symbiotic relationships with insects, although the nature of the interaction remains elusive in most cases (Lachance, 2006). Several examples of yeasts associated with insects have been reported in recent years (Rosa et al., 2003; Lachance et al. 2005; Starmer & Lachance, 2011). In most cases, the insects vector the yeasts and use these microorganisms as a food source. The fruit flies of the genus *Drosophila* eat yeast, digesting vegetative cells but passing spores through the gut intact and viable (Colluccio et al., 2008). Yeasts have been also described as endosymbionts in mosquito populations, lacewings, beetles and homoptera (Ganter 2006; Ricci et al. 2011). The insects rely on yeasts for various metabolic functions, including synthesis of amino acids, vitamins, lipids, sterols and pheromones, degradation of nutritional substrates, and detoxification of compounds (Suh et al. 2003; Starmer & Lachance, 2011).

Geographic gradients were identified in *Candida ipomoeae* and *Metschnikowia borealis* that are found in association with nitidulid beetles that visit short-lived flowers of morning glories and a few other plant families, indicating that historical and climatic factors play a role in shaping the populations (Lachance et al. 2001). Highly specific associations between floricolous nitidulid beetles and various yeasts, including those in the *Metschnikowia* clade, have been documented worldwide (Lachance et al. 2001b, 2005). *Metschnikowia* and related species associated with nitidulid beetles are presumed to have co-speciated with the insects (Lachance et al. 2005; Lachance, 2006). Lachance et al. (2001) suggest that *Conotelus* spp. adults feed on the nectar of ephemeral flowers and in doing so, deposit yeasts together with their fecal material in the corolla. The yeasts grow at the expense of nutrients present at the surface of the corolla. The transmission of yeasts is probably horizontal, through cross-contamination at feeding sites or possibly during copulation. One possible role of the yeasts is to assimilate low complexity carbon and nitrogen sources present in the flower and thus provide the beetle larvae with a diet that contains essential nutrients such as lipids (Nasir and Noda 2003).

More recently, a highly diverse yeast assemblage was found in the gut of various beetles families (Suh & Blackwell, 2004;) especially phytophagous Coleoptera, Homoptera, Hemiptera, Isoptera and Lepidoptera (Suh et al., 2005; Ganter, 2006; Lachance, 2006; Starmer & Lachance, 2011). In particular, it is well known that bark beetles of the weevil subfamily Scolytinae increase their host-colonizing potential by means of symbiotic relationships with fungi, which are carried within specialized structures called the mycangia, or on the body surface (Ganter, 2006). About 200 apparently undescribed species have been discovered so far from the gut of basidioma-feeding beetles, and many of those yeasts form independent clades in Saccharomycotina that have not been recognized previously (Suh et al. 2005). For example, more than 40 new beetle-associated yeast species were reported recently to form several major clades near *C. tanzawaensis*, *Meyerozyma guilliermondii*, *C. mesenterica*, and *C. membranifaciens*, and each of these clades was composed almost exclusively of insect associates (Suh & Blackwell 2004, 2005, Suh et al. 2004b, 2005).

The relationships of yeasts and insects are being discovered as studies expand: the brown planthopper (BPH), *Nilaparvata lugens*, harbors yeast-like symbiotes (YLS), especially in mycetocytes formed by fat body cells found in the abdomen. *Pichia*-like and *Cryptococcus*-like symbiotes may present a potential for biological control of this insect pest (Ganter, 2006). Also, the mutual relations of fungus-growing ants, their fungal cultivars, and antibiotic-producing bacteria suffers the interference of a black yeast counterpart that acquires nutrients from the ants' bacterial mutualist, and suppresses bacterial growth. Several yeast species were isolated from fungus garden and waste deposit of these ants, and could play an important ecological role in these substrates (Pagnocca et al. 2010).

Yeasts have also been reported associated with several species of bees, including social and solitary bees (Pimentel et al. 2005; Ganter 2006; Lachance et al. 2011). The majority of bee species, of which there are approx. 20,000 species (Michener 2000), have never been examined for the presence of yeasts (Rosa et al. 2003). The clade *Starmerella*, that includes two teleomorphic species and several asexual *Candida* species, has been isolated from honey, provisional pollen, nectar and waste deposits in hives and nests of several bee species (Rosa et al. 2003). The nature of the possible symbiosis is not known with certainty, but a role in pollen maturation is suspected (Starmer & Lachance, 2011). These yeasts were able to produce several extracellular enzymes that could metabolize the sugars and pollen stored in the nests, improving their nutritional quality (Rosa et al. 2003).

3.2 The yeasts in plant substrates: Leaves, flowers and fruits

All aerial plant surfaces, known as the phylloplane or phyllosphere are inhabited by diverse assemblages of microorganisms, and these have profound effects upon plant health and impact on ecosystem functions. The associations established on plant surfaces range from relatively inconsequential or transient to substantial or permanent (Fonseca & Inacio, 2006). The leaf surface characteristics may affect, both qualitatively and quantitatively, the immigration of yeasts to the phylloplane (Fonseca & Inacio, 2006). Leaf surfaces are colonized by members of several genera of saprophitic yeasts that provide a natural barrier against plant pathogens (Fokkema *et al*., 1979). Leaves are exposed to rapid fluctuations of temperature and relative humidity values, which may have an impact on the yeast population. Large fluxes of UV radiation are also one of the most prominent features of the leaf surface environment to which microorganisms have presumably had to adapt (Lindow & Brandl, 2003). Many plants contain a number of compounds whose adaptive significance may be a defense against invertebrates and microorganisms (Robinson, 1974). These compounds also act, in some cases, as selective agents which shape the yeast community composition (Starmer & Lachance, 2011). Some yeast species isolated from fruits have a potential use as antagonists and can serve as a biological control against post-harvest decay fruit diseases (Ippolito and Nigro, 2000; Seibold *et al*., 2004).

Flowers and other parts of plant species belonging to the Convolvulaceae, Bromeliaceae and Heliconiaceae families are rich sources of novel yeast species. Most of the novel yeast species isolated from these plants belong to the *Metschnikowia, Wickerhamiella* and *Starmerella* clades (Lachance et al. 2001; Ruivo et al. 2005; Rosa et al. 2007; Barbosa et al. 2011). In ephemeral flowers of the Convolvulaceae, the yeasts are transported by pollinating and non-pollinating flies, beetles and bees that deposit them in the corolla (Lachance et al. 2001). In the longer-lasting flowers of the Heliconiaceae, yeasts are probably introduced by a different and more diverse set of animal vectors and they may grow on the sugary compounds present in nectar (Barbosa et al. 2011).

Most yeast species isolated from flowers are supposedly nectar-inhabiting yeasts. Dense yeast communities often occur in the floral nectar of animal-pollinated plants, where they can behave as parasites of plant-pollinator mutualisms (Brysch-Herzberg 2004; Canto et al. 2008; Herrera et al. 2008, 2009 de Vega et al. 2009). Nectar yeasts, particularly at high densities, induce metabolic degradation of nectar, which can be detrimental to plant reproduction through reduced pollinator service (Herrera et al. 2008). This might originate selective pressures on plants to defend their nectars from exploiters through, e.g. the production of antimicrobial secondary compounds (Irwin et al. 2004). *Metschnikowia reukaufii, M. gruessii, C. bombi, K. dobzhanskii, Hanseniaspora* sp., *H. osmophila, Saccharomyces bayanus, Cryptococcus saitoi* and *Crypt. friedrichii* were the most frequent yeasts isolated from these substrates. The osmotic stress associated with the nectar high sugar concentrations is probably a limiting environmental factor together with the presence of secondary compounds (Nicolson et al. 2007; González- Teuber & Heil 2009). Since, the low species diversity prevailing in nectar yeast communities so far studied could reflect a generalized environmental filtering. Very low nitrogen content, another characteristic feature of floral nectars (Nicolson et al. 2007), may be yet another factor limiting the suitability of floral nectars as habitats for yeasts other than highly specialized nectarivores. A combination of osmotolerance, tolerance or resistance to secondary compounds and efficient nitrogen use possibly allows these specialists to exploit floral nectar.

Another aspect of the interaction of yeasts and nectar-producing plants is related to the fermentation of nectar sugars by yeasts. In cool environments floral warming can benefit both the plants (e.g. by faster growth of pollen tubes) and the pollinators (by providing a heat reward), and yeasts can become important floral warming agents for plants living in shady forest, which are unable to use direct sunshine to warm their flowers. Floral warming by yeasts and the attractiveness provides an example whereby yeasts in nectar could under some circumstances benefit plants, pollinators or both. Also, the abundant alcohol accumulating in the nectar of a tropical palm as a consequence of yeast metabolism may ultimately enhance the attractiveness of inflorescences to alcohol-seeking mammalian pollinators (Wiens et al. 2008).

Decaying fruits are an important microhabitat for several yeast species (Morais et al. 2006; Starmer & Lachance, 2011). These ephemeral substrates are among the most important sites of oviposition and sources of nutrition for larval and adult stages of insects, which vector the yeasts to new substrates (Ganter, 2006; Morais et al., 2006). Yeast communities on fruits of one development stage turn out to be more similar when they are located closer to each other. The similarity of neighboring groups of fruit and on neighboring trees depends cell migration and cross-contamination of fruits with yeast cells. So, distinctions in the yeast community structure in different geographical regions can be explained by differences in the conditions of their formation (Slávikova et al., 2009). Evidently, propagation through cell transfer should play an important role in formation of microbial groups on accessible substrata during a limited period of time, such as juicy fruits, flower nectar, animal excrement, etc. In works on yeast ecology, it was suggested that contamination plays the initial role in formation of the specific structure of yeast in such communities, in particular, directed phoretic transportation of yeast cells to invertebrates (Morais et al. 2006; Starmer & Lachance 2011).

3.3 Soil yeasts

Soil has been studied as a source of yeasts because of its importance in ecosystem processes (Starmer & Lachance 2011). Yeasts have been isolated from different types of soils in diverse climatatic regions (Botha 2006; Cloete et al. 2009; Vaz et al. 2011). Most studies have characterized the occurrence of yeast species, suggesting that these microorganisms are minor contributors to soil ecological processes such as carbon recycling and mineralization (Botha, 2006; Starmer & Lachance, 2011). Yeasts occur mainly in the upper surface of soil rich in organic compounds provided by the decomposition of plant materials. Typical soil yeasts include species of *Cryptococcus*, *Debaryomyces*, *Lindnera*, *Lipomyces*, *Rhodotorula* and *Schizoblastosporion* (Botha 2006, Cloete et al. 2009; Starmer & Lachance, 2011; Mestre et al. 2011, Vaz et al. 2011).

Some yeast species are associated with rhizospheric soils and can produce polyamines, such as cadaverine and spermine that could impact upon root growth (Cloete et al. 2009). The yeasts *Rhodotorula mucilaginosa, Cryptococcus laurentii* and *Saccharomyces kunashirensis* were able to produce soluble and volatile exudates that stimulated the percentage spore germination and hyphal growth of the arbuscular mycorrhizal fungus *Glomus mosseae* (Sampedro et al. 2004). Alonso et al. (2008) reported the presence of yeasts tightly associated with spores of an isolate of *G. mosseae*. These yeasts were able to solubilize low-soluble P sources (Ca and Fe phosphates) and accumulate polyphosphates. Results from inoculation experiments showed an effect of the spore-associated yeasts on the root growth of rice, suggesting potential tripartite interactions with mycorrhizal fungi and plants (Alonso et al. 2008).

Cloete et al. (2010) studied the role of rhizosphere yeasts as plant nutrient-scavenging microsymbionts in roots of a medicinal sclerophyll, *Agathosma betulina*, grown under nutrient-poor conditions, and colonized by *Cryptococcus laurentii*. The average concentrations of P, Fe and Mn were significantly higher in roots of yeast-inoculated plants, compared to control plants that received autoclaved yeast. According to the authors it was the first report describing the role of soil yeast as a plant nutrient-scavenging microsymbiont. These results suggest the potential of yeasts to improve the nutritional quality of soils for plant growth, although occurring in small numbers when compared to bacteria and filamentous fungi.

4. Case studies of biodiversity in natural ecosystems and human-related environments

4.1 Report of two novel species found in Ecuador: *Candida carvajalis* and *Saturnispora quitensis*

Ecuador is located between 1°N and 5°S on the west coast of South America. Although relatively small in size, mainland Ecuador can be subdivided nevertheless into three different and quite distinctive climatic regions: the Pacific coastal plain, the Andean highlands and the Amazon basin. In addition, Ecuador possesses a fourth region, namely the Galapagos Islands.

Climatically, the Pacific coastal plain is hot all year, with a rainy season between December and May. In the Andean highlands, the climate is markedly cooler, varying according to altitude. In contrast, the Amazon basin is hot, humid and wet all year round, while the Galapagos Islands are dry, with an annual average temperature of 25°C (77°F).

To date, very little is known about the natural yeast diversity that exists in Ecuador. In an attempt to begin addressing this scientific shortfall, and to gain a better insight into the effectsof contrasting habitats and climate variation on yeast species distribution, a survey was recently set up and initiated by the Colección de Levaduras Quito Católica (CLQCA) in Quito. The aim of the project is to catalogue, characterise and compare the indigenous yeast species found in the different ecological habitats of the four (climatic) regions of Ecuador.

Several novel species have been found since 2006, two of them are already described. In this chapter we will be referring to these two contributions to science (James et al, 2009).

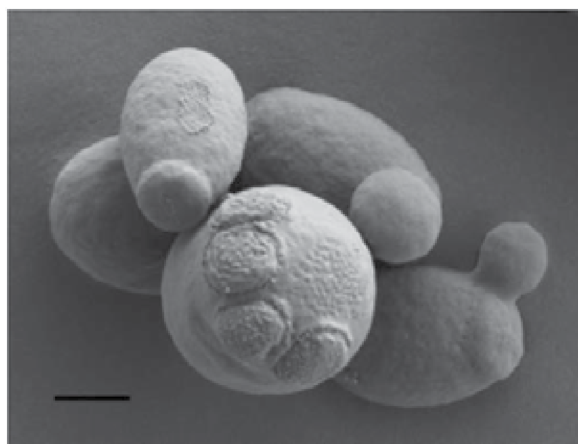
4.1.1 *Candida carvajalis* sp.nov. an ascomycetous yeast species from the Ecuadorian Amazon jungle

This yeast species was isolated from rotten wood and fallen leaf debris collected at separate sites in the central Amazonian region of Ecuador. Phylogenetically, this species belongs to the *Clavispora* clade and is closely related to *Candida asparagi*, *Candida fructus*, *Candida musae* and two as yet undescribed *Candida* species, with the six taxa collectively forming a distinct species group. The phylogenetic placement of this species, coupled with the fact that it could not be induced to sporulate in pure or mixed cultures on several media, led to the conclusion that these yeast isolates belong to a novel species of *Candida* (James et al, 2009).

4.1.1.1 Description of *Candida carvajalis* sp. nov.

Candida carvajalis – this Latin-derived epithet refers to Enrique Carvajal, father of Enrique Javier Carvajal Barriga (director at CLQCA). Although not a biologist himself, his passion for nature has nevertheless led him to become an active collaborator in the search for novel

yeast species in Ecuador. He collected these as well as other yeasts while on a number of field trips to the central Amazonian region of Ecuador (Car.va.jal.is). Figure 1.



(Image courtesy of Kathryn Cross, IFR)

Fig. 1. Scanning electron microscopic image of vegetative cells of *Candida carvajalis*strain CLQCA 20-011^{T} grown in YM broth for 1 day at 25°Cwith agitation. Scale bar = 1 μm.

On YM agar, after 2 days at 25°C, cells are spheroidal to ovoidal (3–7 to 4–8 μm), and occur singly, in pairs or in groups. Budding is multilateral. No sexual state is observed from mixed or pure cultures plated on corn-meal agar, Gorodkowa agar, potassium acetate agar, PDA and YM agar. Pseudohyphae are formed (but only in CLQCA-20-011^{T}), but true hyphae are not formed. The type strain is CLQCA 20-011^{T}, isolated from rotten wood, collected near the town of Dayuma, in the central Amazonian region of Ecuador. Cultures of the type strain and CLQCA 20-014 have been deposited with the CLQCA, Quito, Ecuador, and the National Collection of Yeast Cultures (NCYC), Norwich, UK (CLQCA 20-011^{T} as NCYC 3509^{T} and CLQCA 20-014 as NCYC 3508). The type strain has also been deposited with the CBS, Utrecht, the Netherlands, as CBS 11361^{T}.

4.1.2 *Saturnispora quitensis* sp. nov., a yeast species isolated from the Maquipucuna cloud forest reserve in Ecuador

During a pilot study to survey the yeast diversity found in the Maquipucuna cloud forest nature reserve, located 50 miles northwest of Quito, in Ecuador, CLQCA-10-042^{T} was isolated together with more than 70 other yeast strains. Sequence analysis of the D1/D2 domain of the LSU rRNA gene identified the isolates as belonging to 26 different species of the genera *Barnettozyma* (1), *Candida* (6), *Hanseniapora* (2), *Lachancea* (1), *Lodderomyces* (1), *Metschnikowia* (2), *Pichia* (3), *Rhodotorula* (1), *Saccharomyces* (1), *Saturnispora* (1), *Trichosporon* (2), *Wickerhamomyces* (3) and *Yarrowia* (1). Strain CLQCA-10-042^{T} was isolated from the fruit of an unidentified species of bramble (*Rubus* sp.), and based on its physiology and ability to produce saturn-shaped ascospores was identified as representing a *Saturnispora* species (Kurtzman, 1998). Subsequent sequence analyses of the LSU D1/D2 domain and ribosomal ITS region established that this strain belongs to a genetically distinct and hitherto un-described species closely related to *S. hagleri*. The novel species is named as *Saturnispora quitensis* sp. nov., in recognition of the location in Ecuador from where it was first found.

The yeast genus *Saturnispora* is characterised by teleomorphic species that typically produce one to four spheroidal ascospores ornamented with an equatorial ledge (i.e. saturn-shaped) and have a fairly restricted physiological profile (Kurtzman, 1998). The genus is well-supported by phylogenetic analyses based on multigene sequence analysis of the small-subunit (SSU) and large-subunit (LSU) rRNA genes, and translation elongation factor-1α (EF-1 α) gene (Kurtzman *et al*., 2008). At present, the genus comprises of seven teleomorphic species, *Saturnispora ahearnii*, *Saturnispora besseyi*, *Saturnispora dispora*, *Saturnispora hagleri*, *Saturnispora mendoncae*, *Saturnispora saitoi*, *Saturnispora zaruensis, S. serradocipensis* and *S. gonsigensis* (Morais *et al*., 2005; Kurtzman *et al*., 2008). Six anamorphic species, *Candida diversa*, *Candida sanitii*, *Candida sekii*, *Candida siamensis*, *Candida silvae* and *Candida suwanaritii*, are also accommodated within the genus (Kurtzman *et al*., 2008; Boonmak *et al*., 2009; Limtong *et al*., 2010). Collectively, these yeasts have been isolated from a wide variety of different sources and habitats including *Drosophila* flies (*D. cardinae* and *D. fascioloides*), estuarine water from mangrove forest, flowers, forest soil, insect frass, marsh water, rhizosphere of oyster grass, sauerkraut, tree bark and tree exudate (*Quercus* spp.), and wild mushroom (*Hygrophorus* sp.) (Liu & Kurtzman, 1991; Kurtzman, 1998; Morais *et al*., 2005; Boonmak *et al*., 2009; Limtong *et al*., 2010)

From an ecological perspective, *S. quitensis* is most similar to *S. hagleri*, with both species being found in neotropical regions; *S. hagleri* isolated from two different species of *Drosophila* (*D. cardinae* and *D. fascioloides*) collected in an Atlantic rainforest site in Brazil, and *S. quitensis* from a bramble fruit collected in a cloud forest site in Ecuador (0°03′09′′ N; 78°41′06′′ W; 1668 m.a.s.l). In their species description, Morais *et al*. (2005) noted that of the six identified *S. hagleri* strains, four were recovered from the crops of *D. cardinae*. This led the authors to suggest that this yeast may colonize tropical fruits and substrates regularly visited by these flies and utilised as a food source. To date, only a single strain of *S. quitensis* has been isolated. However, it seems plausible to suppose that like *S. hagleri*, additional strains of *S. quitensis* could, in future, be isolated from *Drosophila* flies and other insects which visit and feed upon tropical fruits found in neotropical regions like Maquipucuna.

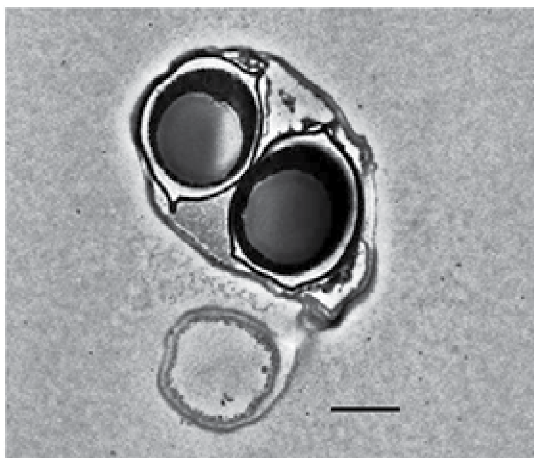
4.1.2.1 Description of *Saturnispora quitensis* sp. nov.

Saturnispora quitensis – The specific epithet *quitensis* refers to Quito, the capital of Ecuador, near where this strain was isolated (Qui.ten.sis).

Cells are spheroidal to ovoidal (4-7 x 5-8 μm) and occur singly or in groups after growth in YM broth for 2 days at 25°C. Budding is multilateral. Sediment is formed after 1 month, but no pellicle is observed. Pseudomycelia or true mycelia are not formed. After 8 days on agar media with a low nitrogen/carbon ratio (i.e. yeast carbon base with 0.01% ammonium sulphate), conjugated cells give rise to asci containing one to two spheroidal ascospores ornamented with an equatorial ledge (i.e. saturn-shaped) Figure 2. Ascospores are not liberated. Conjugation takes place between individual cells, and more commonly between cells and their buds.

4.2 Yeast species described in Argentinean environments

In recent years, numerous studies have demonstrated that Patagonian natural environments harbor a broad biodiversity of yeasts with high scientific and technological value (Brizzio & van Broock, 1998; Libkind et al., 2003, 2004a, 2004b, 2006, 2007, 2008a, 2008b, 2011a, Russo et al., 2006; de García, 2007; Brizzio et al., 2007). These studies have also shown that a large proportion of the yeast species recovered belong to undescribed taxa, in general 25 to 40



(Image courtesy of Kathryn Cross, IFR)

Fig. 2. Transmission electron micrograph of a single ascus containing two ascospores, one of which is ornamented with an equatorial ledge. Scale bar=1 µm.

percent of the species obtained from a certain substrate represent novel species. This is a clear indication of the importance of conducting bioprospection studies in microbiologically unexplored habitats of Patagonia. To date, ten novel yeast species have been formally described from Patagonian natural environments and at least 20 additional undescribed taxa have been found (Libkind et al., 2005a; 2009a; 2010a; de García et al., 2010a; 2010b; Russo et al., 2010; Wuczkowski et al., 2010). Only a few salient cases are discussed here in order to show the importance of microbial surveys in unexplored habitats.

The first formal description of Patagonian autochthonous yeasts regarded two carotenoid-accumulating yeasts (also known as red yeasts) that had the ability to produce forcefully-ejected spores (ballistoconidia). These yeasts belonged to the Sporidiobolales order of the Pucciniomycotina sub-phyllum (Basidiomycota) and were described as *Sporobolomyces patagonicus* and *Sporidiobolus longiusculus* (Libkind et al., 2005a). The sexual stage (teleomorph) of *S. longiusculus* was detected and had a particular micromorphological feature: teliospore germination gave rise to an elongated basidium, which was five to six times longer (120–275um) than those of the other member species of the genus *Sporidiobolus* (Libkind et al., 2005a). Even though all known strains of both species were collected from subsurface water of Andean lakes, their suspected primary habitat is the surrounding phylloplane, probably of *Nothofagus* spp. trees.

A similar case was that of *Cystofilobasidium lacus-mascardii*, a teleomorphic species of the Cystofilobasidiales, class Agaricomycotina (Basidiomycota) of which a single isolate was first obtained from subsurface waters of the Mascardi lake (Libkind et al., 2009a). Once it was recognized as an undescribed species, attempts to obtain additional isolates using specifically designed culture media for selective isolation were performed. Thus, new isolates were found and they happened to mate with the original isolate providing the opportunity to describe its sexual stage. Again, terrestrial environments are more likely to be the habitat of this yeast species based on its low relative occurrence in freshwater and its ability to produce a wide range of extracellular enzymes (Brizzio et al., 2007).

Another interesting case is that of *Cryptococcus agrionensis*, a novel anamorphic yeast of the Filobasidiales (Agaricomycotina, Basidiomycota) associated with acidic aquatic

environments of volcanic origin in North Patagonia. Due to the high acidity, these waterbodies also contain high concentrations of toxic metals, and thus poly-extremophile microorganisms prevail. More than seventy *Crypt. agrionensis* strains were isolated, mainly from the most acidic section of the river Agrio with a pH ranging from 1.8 to 2.7 (Russo et al., 2010). More interesting was the fact that *Crypt. agrionensis* was phylogenetically related to three *Cryptococcus* species that constitute what has been described as the Acid Rock Drainage (ARD) Ecoclade (Gadanho & Sampaio, 2009). The term 'ecoclade' refers to species that are related phylogenetically and show salient physiological adaptations associated with the physicochemical conditions present in their habitats. The ARD ecoclade (including *C. agrionensis*) have a peculiar ecology and physiology: They are only known from acidic environments and are highly resistant to heavy metals such as Cd2+, Co2+, Cu2+, Li+, Ni2+ and Zn2+. The discovery of *Crypt. agrionensis* in acidic water of volcanic origin provided evidence that the ARD ecoclade was not restricted to abandoned mines of the Iberian Pyrite region (origin of the previously known species) and demonstrated that members of this ecoclade may be found in acidic environments in general, originated both naturally and anthropically.

During our yeast biodiversity survey in the Argentinean Patagonia we came upon isolates of *Phaffia rhodozyma* (sexual form, *Xanthophyllomyces dendrorhous*), a yeast that belongs to the Cystofilobasidiales order (Class Agaricomycotina, Basidiomycota). This yeast has the ability to produce astaxanthin, a carotenoid pigment with biotechnological importance because it is used in aquaculture for fish and crustacean pigmentation (Rodríguez-Sáiz et al., 2010). Known isolates of this species had been found in exudates of trees of the genera *Betula*, *Fagus* and *Cornus* in the Northern Hemisphere, mainly at high altitudes and latitudes. We isolated *P. rhodozyma*, from the Southern Hemisphere (Patagonia, Argentina), where it was associated with fruiting bodies of *Cyttaria hariotii*, an ascomycetous parasite of *Nothofagus* trees (Libkind et al., 2007). The Patagonian population besides possessing a different habitat also showed distinct genetic features based on a detailed molecular comparison with known strains from the Northern hemisphere. However, the level of genetic divergence of the Patagonian population with respect to the remaining strains was within the intraspecific level. In addition by comparing the molecular phylogenies of *P. rhodozyma* populations with that of their tree host (Betulaceae, Corneaceae, Fagaceae, and Nothofagaceae), a good concordance was found which suggested that different yeast lineages colonize different tree species (Libkind et al., 2007). Hence, we hypothesize that the association of the Patagonian *P. rhodozyma* with *Cyttaria* derives from a previous association of the yeast with *Nothofagus*. This study provided a deeper understanding of *Phaffia* biogeography, ecology, and molecular phylogeny, knowledge essential to the study of astaxanthin production within an evolutionary and ecological framework.

The cases above, describing novel yeast species/populations, clearly illustrate the need to increase the efforts to further survey the micobiota of relatively unexplored habitats such as the emblematic Patagonia.

4.3 Yeast biodiversity in wineries, Distillerie plants and olive oil mills in La Mancha region (Spain)

Yeast ecosystems are used as raw materials in the food industry as well as in processing: the yeasts in grapes, musts and fermented musts in wineries, and in the piquettes, bagasse, grape-skins and lees used as feedstocks in the ethanol industry provide an inexhaustible supply of microorganisms.

La Mancha is the world's largest vine-growing region, with a surface area of around 600,000 hectares, i.e. roughly 50% of the country's table wine. Annual grape production, comprising entirely winemaking varieties, is around 3.6 million tonnes. This output generates roughly 600,000 tonnes of pomace or marc, produced by pressing the fermented red or white grapes which thus contain a certain amount of sugar. Pomace generally contains plant tissue residue: skin and pips from the pressing of red grapes, as well as stalks from pressed white grapes. The ratio of pomace output to grape production varies considerably, depending on the grape variety and on growing conditions. However, pomace is estimated to account for 17% of overall grape weight; within that figure, skin accounts for 8%, pips for 5% and stalks for the remaining 4%. Pomace and/or residual sugars are used as a feedstock for ethanol production.

4.3.1 Wineries

Traditional wine fermentation is a complex, heterogeneous microbiological process involving the sequential development of various yeasts and other microorganisms present in musts, such as moulds as well as lactic and acetic acid bacteria. However, it is accepted that certain strains of *Saccharomyces cerevisiae*, known as "wine yeasts", are especially well adapted to this process, and play a major role in the fermentation of grape musts; for that widely studied. Nonetheless, it is important to remember that upto 15 different genera of non-*Saccharomyces* yeasts may also be present at the start of the wine-making process, and these may contribute to the special characteristics of individual types of wine (Pretorious, 2000).

Although most wineries now use commercial starter cultures, it is usually to spend over 60 million tonnes of active dry yeasts (ADYs); nevertheless, spontaneous alcoholic fermentation, that is, fermentation carried out without the addition of commercial dry yeasts, is still typical for certain wine cellars in this wine-making area. This type of fermentation is of particular interest with a view to ascertaining the ecology of fermentation processes in respect to *Saccharomyces* and non-*Saccharomyces* yeast strains. In the case of the former yeasts, it is necessary to establish whether fermentation is carried out by one or several predominant strains or whether, in contrast, there is a succession of different strains over the course of wine-making.

Extensive ecological surveys using molecular methods of identification have been carried out with the aim of studying winery biodiversity and then selecting new yeasts better adapted to local fermentation conditions (Briones et al, 1996; Fernández-Gonzalez et al, 2001; Izquierdo et. al, 1997; Querol et. al, 1992), thus allowing the behavior of the various strains to be charted throughout fermentation.

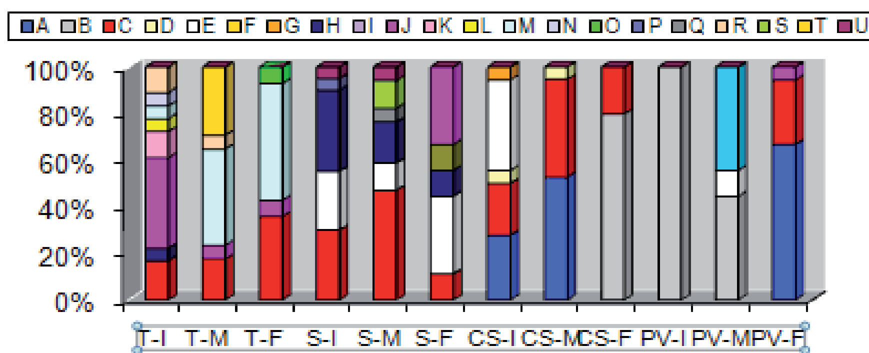
Non-*Saccharomyces* yeasts, which display low fermentative capacity and low ethanol tolerance could impart specific characteristics to the wines, and to enhance wine flavour by increasing concentrations of the volatile compounds responsible for the fruity aroma, through hydrolysis of aromatic precursors prompted by β-glucosidase enzyme activity (Arevalo-Villena et al, 2006;) or even for the production of volatile esters.

In the cellars of this area, during the early stages of wine-making there is substantial growth of non-*Saccharomyces* species. The main species found in different grapes varieties at the beginning of the process are *Candida stellata, Pichia membranaefaciens, Metschnikowia pulcherrima Hanseniaspora uvarum/guillermondii/osmophila, Kluyveromyces thermotolerans. Torulaspora delbrueckii* and *Debaryomyces hansenii* were isolated at the middle of the process, and *Lachancea fermentati (formerly Zygosaccharomyces fermentati)* were able to survive at the middle, or even until the end of fermentation.

The study of the enzymatic activities of non-*Saccharomyces* wine yeasts revealed that nearly 80% of the yeasts presented at least one enzyme of biotechnological interest. Polygalacturonase was the enzyme most commonly found and was secreted by 45% of the yeasts, whereas β-glucosidase was only observed in 14% of the yeasts. Proteolytic activity was also found in some species (Fernández-Gonzalez et al, 2000).

The analysis of restriction mitochondrial DNA is a suitable technique to study the biodiversity of *Saccharomyces* wine strains. With regard to the population dynamics of *Saccharomyces* strains, there was a greater variability of them at the start of fermentation; as fermentation progressed some initial strains were succeeded and displaced by others better adapted to environmental conditions; this succession of strains is common in spontaneous fermentation.

Saccharomyces biodiversity during vinification of the different red grape varieties is shown in Figure 3. Of the 21 genetic profiles identified, 10 contained more than four isolates. In some cases, as can be observed in the figure, the isolates are grouped in a dominant profile, i.e. 26% of isolates displayed the same profile (C), suggesting that this was a common genetic pattern in the winery; other patterns included 16% (A) or around 10% (E) of isolates, others 8% - 7%, and some of them accounted for around 2% or 3%, while the remainder contained only one or two isolates.



T: Tempranillo; S: Syrah; CS: Cabernet Sauvignon; PV: Petit Verdot. I: Beginning; M: Middle; F: End

Fig. 3. Genetic profiles of *Saccharomyces* wild strains in red grape varieties.

Some profiles were exclusive to certain varieties: profile C was isolated in abundance from all varieties; profile J was also present, though to a lesser extent, in almost all tanks; profiles A and B were characteristic of *Cabernet* and *Petit Verdot*, whilst M and T were typical of *Tempranillo,* and E and H of *Syrah*.

Other cellars showed a 65% of variability respect to a genetic patterns, found some of them repeated in some of the fermentation stages sampled. Substantial genetic differences were recorded, a customary finding for spontaneous fermentations representative for the studied region (Briones et al. 1996; Izquierdo et al. 1997).

In a study carried out in a single winery, situated around 1000 m.a.s.l. and whose wines have high quality, the sampling was done in 11 both white and red fermentations of different grape varieties (Chardonnay, Sauvignon Blanc, Cabernet sauvignon, Tempranillo, Merlot, Syrah) collecting a total of 28 samples at different stages of fermentations. The molecular analysis methods led to the determination of 23 different *Saccharomyces* mtDNA restriction patterns from 358 isolates. The degree of variability was a good parameter with

which to evaluate the number of strains actively involved in fermentation. The variability average found in this study (6.4%) was similar to those from previous studies: 8.6% (Querol *et al.*, 1994); 2.2 to 4.2% (Schütz and Gafner, 1994) and clearly lower than corresponding results from 32, 42, 38% and 23, 23, 22% in three different cellars and two consecutive years (Izquierdo et al. 1997), 22% (Torija et al. 2001) and 20.7% (Nadal et al. 1996).
The majority pattern found cluster the 56% of the isolated ones, followed of others one with a 15% and a 9%. Four restriction patterns were about 3% and the rest of the patterns whose presence was limited to one or two isolates. The main pattern was isolated in all sampled vats and in all grape varieties, both white and red; that situation is not frequently in this viticulture area, where the *Saccharomyces* biodiversity is high as recorded previously by Briones et al. (1996). In three different cellars, selected at random a large number of S. *cerevisiae* strains appeared with either the same, or a very similar, karyotype, indicating that they are strains highly characteristic of these wineries.

4.3.2 Distillery plants

Thirty-three distilleries in Spain are licensed to produce ethanol from winemaking by-products. Thirteen of these are located in the region of La Mancha.
During harvest, fermented and “fresh” pomace (from white-wine vinification) is transported to the ethanol plant, where it is mixed and stored (generally outdoors) for between 10 and 15 days; during this period, “fresh” pomaces start to ferment. After, pomaces are washed to extract ethanol. The liquid produced by this process is known as “piquette”, a mixture of alcohol (3º-4º), water and sugar; the piquette is mixed with the liquid drained off during outdoor storage-fermentation.
The piquette is fermented in stainless-steel or iron tanks for two or three days, attaining an alcohol content of between 4º and 5º (V/V). Although a few ethanol plants use active dry yeasts, fermentation is mostly spontaneous; this gives rise to a highly- varied *Saccharomyces* and non- *Saccharomyces* biota, as discussed below.
To date, little research has been carried out on the yeast biodiversity found in Spanish grape-based ethanol plants. A study of yeast populations in ethanol plants and distilleries in La Mancha sought to determine yeast biodiversity at various sites, with six different ethanol plants studied (subsequently referred to as plants A-F). *Saccharomyces* strains predominated in all ethanol plants studied; the proportion of non-*Saccharomyces* strains ranged from 14% to almost 47%. The 144 *Saccharomyces sp.* isolates matched 105 different genetic profiles; 46 profiles were from fresh piquettes, 43 from fermented piquettes and 16 from lees. In all samples and all plants, variability exceeded 50%; in five cases, variability was higher than 80%.
Fresh piquettes displayed considerable strain diversity; variability was almost 90% at plant B, and 81% at plant C. A total of 46 genetic profiles were found, 45 of which were different, while one – although infrequent (4%) – was isolated at plants A and C. Only one majority profile accounted for 22% of the yeasts isolated at plant C. Most strains were *Saccharomyces cerevisiae,* with only a small number of *S. paradoxus* and *S. bayanus* strains.
In fermented piquettes, biodiversity was greater than in fresh piquettes, and at three plants (B, D and E) different strains accounted for over 85% of the total. Plant A displayed the least genetic diversity. Piquettes at plants B and C contained negligible amounts of *S. paradoxus* and *S. bayanus,* respectively.
Lees obtained from piquette fermentation displayed less *Saccharomyces* strain diversity than either fresh or fermented piquettes. Isolates fitted 15 different genetic patterns. While a 78% strain variability was observed at plant C, lees sampled at plants A and B displayed only

50% and 57% diversity, respectively. Although patterns tended to be typical of each plant, majority profiles accounted for 57% of isolates at plant B, 33% at plant C and 30% at plant A. The greatest degree of *Saccharomyces* variability was found for fermented piquettes, although several strains co-existed in both lees and fresh piquettes. These results confirm that, whilst genetic diversity in wineries has declined considerably due to the increasingly widespread use of commercial starter cultures, *Saccharomyces* variability in ethanol plants remains considerable.

With respect to non-*Saccharomyces* yeasts, the largest percentage (49%) was found in fermented piquettes, even though the ethanol concentration varied between 4º - 5º (V/V). A total of 41% of non-*Saccharomyces* strains were isolated in fresh piquettes, and only 10% in lees. The greatest species diversity was observed in fresh and fermented piquettes, the most frequently- isolated species being *T. delbrueckii* and *C. silvae,* respectively.

Only *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*) was isolated in all three types of source. *Kluyveromyces thermotolerans* and *Wickerhamomyces anomalus* (formerly *Pichia anomala*) were isolated in both fresh and fermented piquettes, while *Candida ethanolica* was isolated in fresh piquettes and lees. The remaining species were isolated in only one type of source at the various ethanol plants.

A number of species, including *Hanseniaspora. vineae, P ichia kudriavzevii* and *Torulaspora delbrueckii,* have been reported in white-wine pomace or marc used for the production of grappa (Bovo et al., 2009) while *Zygosaccharomyces bailii* and *Saccharomycodes ludgiwii* have been identified, in smaller numbers, in agave fermentation for tequila production (Lachance, 1995).

4.3.3 Olive oil mills

Olive-fruit spontaneous microbiota comprises non-*Saccharomyces* yeasts, lactic acid bacteria (LAB) and filamentous fungi. From other research it is known that during the olives fermentation the presence of yeasts may produce compounds with suitable organoleptic attributes determining the quality and flavour of the final product (Arroyo-López et al., 2008). However the olive oil production is also important and there are few references in the literature about yeast biodiversity present in both fresh olives intended for oil production and their sub-products. Giannoutsou et al. (2004) suggested that "alpeorujo" is a good substrate for yeast growth which could be used as a feed additive, as a fertilizer in crops or as a substrate for the growth of edible mushrooms.

La Mancha is the second largest olive growing region in Spain (350,000 ha) and a major olive oil producer. No previous studies have dealt with yeast populations in local olives, nor in the by-products of olive processing, i.e. paste and pomace. Olive fruits from two varieties of *Olea europaea L.* (Arbequina and Cornicabra) were randomly picked at various olive groves; likewise, olive paste and olive pomace were also collected from different oil mills.

Fourteen different species of yeasts were identified, belonging to eight different genera (*Zygotorulaspora, Nakazawaea*, *Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida* and *Torulaspora*), thus demonstrating considerable species diversity. In fresh olive fruits, yeasts were largely outnumbered by moulds and bacteria probably due to the fact that they had not been processed (i.e. were collected straight off the tree), so the only contribution was the environmental biota. Although a similar number of isolates were obtained from paste and pomace, the latter displayed greater species diversity, with 11 different species identified. Some species were typically found in olive paste (*Nakazawaea holstii, Pichia. mississippiensis* and *Lachancea sp.*), whilst *S. cerevisiae*, *Kazachstania rosinii* (formerly *Saccharomyces rosinii*),

Candida sp. and *C. diddensiae, Zygotorulaspora florentinus* (formerly *Zygosaccharomyces florentinus*) and *Torulaspora delbrueckii* were found only in pomace.
The species most commonly isolated in the Cornicabra variety was *Pichia holstii* (39%), followed by *Lachancea fermentati* (25%), whilst the predominant species in Arbequina was *Pichia caribica* (59%) followed by *Lachancea fermentati* (23%). The remaining 11 species did not exceed 8% in either variety. *Candida diddensiae* was found in Arbequina olive variety, and similar results were obtained by Hurtado et al. (2008) who also isolated this species in Arbequina fruits. *Nakazawaea holstii* (formerly *Pichia holstii*) and *Lachancea fermentati* are yeasts associated with wastewater from continuous olive mills in Southern Italy and Spain (Barnett et al., 2000), while *Meyerozyma caribbica* (formely *Pichia caribbica*; anamorph: *Candida fermentati*) is involved in artisanal cachaca fermentation in Brazil and is found in soils in China (Barnett et al., 2000). The other species isolated are found chiefly in soils, although *C. diddensiae* has also been reported in olives in Italy.
All species isolated were fermentative to a varying degree. *S. cerevisiae* was a striking finding in this respect, and might represent a potential spoilage organism during olive oil storage. However, this should not be a problem since none of these yeasts have lipolytic activity.
Lachancea genus was isolated from olive paste of both varieties. This new genus was formed on the basis of five species; *L. thermotolerans* was chosen as type species and has been isolated from mushrooms, flowers, leaves and oil wastewaters (Naumova et al., 2007).
Biodiversity was greater in olive by-products than olive fruits, and greater in Cornicabra than in Arbequina (11 species vs. 6). Three species were common to all olive fruits and both by-products (*Lachancea fermentati, M. caribbica, Lachancea* sp.).
Candida spp. were isolated from olive paste (Torres-Vila et al., 2003), other authors have isolated yeasts in olive fruits and brines during fermentation process, including *T. delbrueckii*, *Candida boidinii*, *Cryptococcus spp*., *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* (Marquina et al., 1992; Coton et al., 2006; Hernández et al., 2007).
With regard to yeast biodiversity in oil mills, species distribution was very much dependent upon the oil mill plant. In some mills, 4 to 5 different species were identified, whereas in others (4 mills) only 2 species were isolated. *N. holstii* was isolated in all samples from Cornicabra except in one, and was not detected in the oil mills from Arbequina. Nevertheless *Lachancea fermentati* was present in the majority of mills.
Our work also shows the potential of these strains isolated from olive by-products, i.e. olive paste and pomace, suggesting that these olive wastes can also be used for industrial biotechnological purposes, for the production of enzymes, commercial preparations or fermentative processes in different industry sectors.
Characterization of these resources can also contribute to the development of a microbial bank, providing data on technological properties and enzyme characteristics for potential industrial applications. On the other hand, the quality and yield in olive oil extraction may be influenced by the presence of some yeasts with high or moderate enzymatic activities such as lipases, glucanases, cellulases, glucosidases or polygalacturonases.

5. The role of yeast culture collections: Preservation, applications and challenges

5.1 The Catholic University yeast Collection (CLQCA)

The CLQCA, or Catholic University Yeast Collection, was originally set up to carry out a survey of environmental biodiversity of yeasts in Ecuador in 2006, as a pioneer bioprospecting study. Over the last five years this collection has developed a diverse range

of techniques in order to identify and characterize yeast biodiversity. This collection has had agreements and tight collaboration with other collections such as the National Collection of Yeast Cultures (NCYC) in the United Kingdom, and the UFMG yeast collection in Brazil.

Currently, this yeast collection is the only one of its kind in Ecuador, and one of the few in South America. To date, more than 15 novel yeast species have been isolated and three of them have already been formally described and published (*Candida carvajalis*, *Saturnispora quitensis*) in collaboration with the NCYC (UK). More than 2000 yeast isolates from the 24 provinces of Ecuador have been preserved at the CLQCA. One of the most important surveys related to biodiversity was carried out in 2008 in the Galápagos Islands, where more than 800 isolates were collected from four different islands. Other Ecuadorian environments such as the high Andes, the Amazonia, and the Pacific Coast have also been sampled.

Approximately 1/3 of the isolates are already identified by ribosomal DNA (rDNA) sequencing and/or RFLP-ITS method. So far, the predominant species registered are *Candida tropicalis* (142 strains) and *Saccharomyces cerevisiae* (100 strains). However, yeasts from *Hanseniaspora*, *Pichia*, *Rhodotorula* and other genera are also well represented, as shown in Figure 4.

The CLQCA is not only a bank for the yeast biodiversity, but a biotechnology exploitation platform, where several projects are being carried out. Some of the most important ones are focused on second generation bioethanol production, biocontrol of molds, microbial archaeology and beer production.

5.2 Yeast culture collection in Patagonia

The CRUB (Centro Regional Universitario Bariloche) yeast collection is a research culture collection kept at the Applied Microbiology and Biotechnology Lab. which is held at the Biodiversity and Environmental Research Institute (INIBIOMA, CONICET-UNComahue) in Bariloche, Argentina (Northwestern Patagonia). Certainly is the most southern culture collection devoted to the preservation of native yeasts. Its collection derive from studies of yeast diversity in Patagonian natural substrates that have been mainly focused on environments with extreme conditions which impose a selective pressure towards the prevalence of adapted microorganisms with innovative physiological characteristics that can be biotechnologically relevant. Extreme environments such as glacial ice and meltwater (de Virginia et al., 2007), and acidic waterbodies of volcanic origin that have high concentrations of toxic metals (Russo et al., 2008) are being studied. Many strains have been proved to be interesting as producers of psycro-enzymes (de Virginia et al., 2007; Brizzio et al., 2007; Brandao et al., 2011), poly-unsaturated fatty acids (Libkind et al., 2008b) or because of their tolerance to heavy metals (Russo et al., 2010). However, the studies in Patagonia have been mostly concentrated in environments exposed to increased UV radiation (UVR) such as transparent mountain lakes or the phylloplane of high altitude forests. Yeasts adapted to high UVR exposure have shown to produce large quantities of photoprotective compounds (PPC) which are necessary to reduce the detrimental effect of the damaging wavelengths of UVR (Libkind et al., 2006). The synthesis of metabolites that have antioxidant and/or UV screening activities are among the strategies commonly seen in yeasts for photoprotection.

6. Biodiversity and biotechnology

Yeasts biotechnology is a growing field where novel species and its physiological abilities are potentially useful in the search of new products by means of the metabolic engineering

Fig. 4. Number of identified yeast isolates preserved at CLQCA up to 2011.

approach and the application of novel species for industrial production, not only as fermenting organisms or molecules producers, but as sources of molecules that can be purified from the structures of the yeast cell. As an example we can talk about β-glucans and mannans from cell walls that are being used as food additives for animal feed. Other examples are the partially hydrolysate yeast cells used in animal feed as well with desirable results in terms of weight and health improvements.

Biodiversity of yeasts is being studied not only to catalogue life on Earth; one of the most promising fields related to the characterization and identification of yeast diversity is related to the potential use of them in producing novel enzymes and chemicals. Psycrophilic yeasts from Antarctic substrates as well as those from high altitudes or glaciers are potential work horses in biotechnology industry to produce the breakdown of xenobiotics and pharmaceutical novel variations of molecules. Lipases from *Pseudozyma antarctica* have been extensively studied to understand their unique thermal stability at 90°C and also because of its use in the pharmaceutical, agriculture, food, cosmetics and chemical industry. Other enzymes which have been studied include extracellular alpha-amylase and glucoamylase from the yeast *Pseudozyma antarctica* (*Candida antarctica*), an extra-cellular protease from *Cryptococcus humicola*, an aspartyl proteinase from *Cryptococcus humicola*, a novel extracellular subtilase from *Leucosporidium antarcticum*, and a xylanase from *Cryptococcus adeliensis* (Shivaji and Prasad, 2009).

Other common use of the yeast biodiversity—as part of microbial communities—is in the bioremediation of oil spills. Yeasts are able to use various petroleum components as sole carbon source, showed that their biodegradability decreases from n-alkanes to high molecular weight aromatic and polar compounds. The alkanes are mainly degraded using the monoterminal oxidation pathway through cytochrome P450 system, and transformed into fatty acids with the same length of the carbon chain. Extensive studies showed that there are more than 80 genes involved in obtaining the alkane specific phenotype. Up to date, about 14 different genera of yeasts have been reported to consume hydrocarbons, exhibiting bioremediation potential uses. The abovementioned genera are: *Candida,Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus,Trichosporon* and *Yarrowia* (Mauersberger, 1996; Scheller, 1998).

Yeast can be used in foods and chemical production as they were probably one of the first organisms domesticated by humankind. Production of wine, beer and bread are three examples of the importance of yeasts in human nutrition and culture. More and more applications for yeast will arise in the next future. The diversity of yeast had been the answer to fulfill human necessities in the early times of civilization, and, undoubtedly, it is going to continue being a source of new solutions in the future.

6.1 From biodiversity to biotechnology: the case study of photoprotective compounds

Carotenoids are a group of valuable molecules for the pharmaceutical, chemical, food and feedindustries, not only because they can act as vitamin A precursors, but also for their antioxidant and possible tumour-inhibiting activity (Johnson & Schroeder, 1995). Many yeasts accumulate a variety of carotenoid pigments intracellularly are commonly known as carotenogenic or red yeast. Red yeasts were found to occur widely in aquatic environments in Patagonia, and many pigmented strains of the carotenogenic genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces*, *Sporidiobolus*, *Dioszegia*, *Cystofilobasidium* and *Xanthophyllomyces*

were isolated and are kept at the CRUB collection. These new yeast isolates from Patagonian habitats were studied for the production of biomass and carotenoids as the first step towards the selection of hyper-producing strains and the design of a process optimization approach. Patagonian yeast isolates considered as potential biomass and carotenoid sources were studied using conventional media or semi-synthetic medium employing agro-industrial byproducts (cane molasses, corn syrup, raw malt extract) as carbon sources (Libkind et al., 2004a; Libkind & van Broock, 2006). Maximum pigment production (400 ug g^{-1} cell dry weight) was achieved after optimization through a factorial design with the yeast *Cystofilobasidium lacus-mascardii*. β-carotene, torulene and torularhodin were the major carotenoids found in most yeasts (Buzzini et al., 2006; Libkind & van Broock, 2006). The exceptions were *Phaffia rhodozyma* strains which produced the biotechnologically relevant pigment astaxanthin (Libkind et al., 2008a). Moreover, photobiological studies were performed that demonstrated the photoprotective role of these carotenoid pigments in yeasts (Moliné et al., 2009), in particular torularhodin in the ubiquitous yeast *Rhodotorula mucilaginosa* (Moliné et al., 2010). Thus, the CRUB collection represents an interesting source of carotenogenic yeast strains already characterized regarding their biomass and carotenoid production performance at Lab scale and providing a variety of pigments for diverse applications.

In contrast to carotenoid pigments, Mycosporines, are water soluble UV-absorbing (310–320 nm) and very less known. They are compounds containing an aminocyclohexenone unit bound to an amino acid or amino alcohol group (Bandaranayake, 1998). Although mycosporines were initially discovered in fungal sporulating mycelia (Leach et al., 1965), it was not until recently that their synthesis was reported in yeasts by us (Libkind et al., 2004b). A number of basidiomycetous carotenogenic yeasts were found to synthesize a UV-absorbing compound (peak absorption at 309–310 nm) when grown under photosynthetically active radiation (PAR, 400–750 nm). The compound was afterwards identified as mycosporine-glutaminol-glucoside (MGG) (Sommaruga et al., 2004). More recently the MGG was confirmed as a photoprotective agent in yeasts (Moline et al., 2011) and its possible use in human sunscreens has been tested (Libkind et al., 2009c). To date, many yeast species have been detected as MGG producers (Libkind et al., 2005b; 2011b) and the level of synthesis appear to be related to the solar exposition history in the habitat of origin (Libkind et al., 2006). Thus, the diversity surveys in highly UV exposed habitats have rendered valuable isolates able to accumulate large quantities of MGG with concentrations above 5% of the dry weight (Libkind et al., 2005b, 2011a; Brandao et al., 2011). These MGG producing strains are conserved in the CRUB collection and are used in studies related to the elucidation of the genetic bases of MGG synthesis in yeasts and fungi in general.

6.2 Innovative biotechnological method to resuscitate ancient yeasts from fermenters useful in microbial archaeology

Fermenters from ancient cultures are suitable substrates to keep dormant yeasts within its pores. Both in Ecuador and Spain, yeasts isolates were recovered from archaeological pieces belonging to fermenters used by ancient cultures. The most remarkable ones were vessels from about 2500 b.C. belonging to the Iberos culture (Spain), and Sierra Norte (Ecuador) from about 200 a.C. Other remarkable yeast recovered from ancient fermenters belonged to the first brewery founded in America in 1566 (Ecuador), where wooden vessels were sampled as well.

The method developed to recover these yeast strains is based upon the so-called "resuscitation triangle", where cell walls restoring and membranes fluidization is carried out firstable; after that, a hydration step is performed; and, finally, a metabolic activation step is accomplished to resuscitate ancient and valuable yeast strains. Underlying these techniques is the nascent Microbial Archaeology that pursues an understanding of the ancient microflora and its implications for human beings by using archaeological rests as sources of ancient microorganisms. This method, allowed retrieving and isolating dozens of different yeasts, most of them belonging to species such as *Saccharomyces cerevisiae, Clavispora luisitaniae, Cryptococcus saitoi, Rhodotorula mucilaginosa*, *Meyerozyma guillermondi, Cr. diffluens, Candida parapsilosis,* and *C. tropicalis* and other undescribed species.
The method used to recover these ancient yeasts is a trade secret belonging to the Pontificia Universidad Católica del Ecuador, where the CLQCA is placed.

7. Conclusion

The yeast biodiversity study is currently being boosted by more and more groups that show interest in discover novel yeasts species and understand the ecology, physiology, and evolutionary aspects of yeasts.
As pointed out previously, there is still much to be knonwn about yeast biodiversity in vast zones of the planet. In our understanding, this gap will take a long time to be closed taking into account the still unexplored habitats occupied by these organism.
Moreover, taxonomy of yeasts faces challenges in terms of the accomodation of species inasmuch as frequently yeasts are being re-clasified. The genus *Candida* is the more extended in yeasts, nevertheless, this genus is only created to accommodate those yeasts that haven't shown teleomorphic (sexual) phase. But this characteristic can be reverted if an appropriate medium allows the yeast sporulation.
On the other hand, molecular techniques developed during the last 30 years are very valuable tools for research. Molecular approaches are fundamental in current studies of yeast for its identification and classification. Concomitantly, chemotaxonomic methods are complementary to characterize yeast strains. These methods allow the researchers find out the metabolic abilities of yeast strains whose understanding is the first step to potential use of in biotechnology and industry.
Yeast collections play a fundamental role in preservation, identification and characterization of these microorganisms; represent safe repositories where biodiversity is preserved for the future. The *ex situ* preservation of yeasts is a big effort not only in economical, but also in technical terms. Qualified personnel are needed as well as economic sources to carry out the Contamination and loss of viability are two main concerns of curators in yeast collections, that is why the ex situ preservation of yeasts is a big endeavour, even though yeasts themselves are quite small.

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5

Polychaeta Diversity in the Continental Shelf Off the Orinoco River Delta, Venezuela

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1. Introduction

Coastal waters are mainly influenced by two large spatial scale processes: upwellings and river inputs (Mann & Lazier, 2006). Rivers are the main conduits of water, dissolved and particulate organic matter, salt, and other organic materials from the continents to the sea (Alongi, 1998). In Venezuela, the largest watercourse is the Orinoco River, covering a total basin of 10^6 km^2 and discharging an average of 1,080 km^3/yr of water, and 150x 10^6 tons^3/yr of sediment to the Atlantic Ocean, representing the fourth largest river of the world in terms of discharge (Alongi, 1998). Several authors have shown that the Orinoco's river plume can extend up to 100 km from the coast line, during the rainy season, influencing the salinity patterns, coastal currents, suspended materials and nutrient concentrations in the Venezuelan Atlantic coast and the Caribbean Sea (Muller-Karger et al., 1989; Penchaszadeh et al., 2000). The dispersion of the riverine front follows a northwest direction due to the influence of the northeasterly trade winds and the Guayana current flow. The surface plume is well-mixed inshore but it is stratified on the outer shelf, creating unique environmental conditions that greatly modify the marine waters and sediments.

Very scarce information was available for this Atlantic area until recent years, as the Venezuelan government has undergone offshore gas exploration activities in the continental shelf off the Orinoco River delta (Gomez et al., 2005, Martín & Bone, 2007). This effort has allowed the scientific community to conduct large multidisciplinary base line studies for this Atlantic region, characterized by a large continental shelf, partly influenced by the Orinoco's continental waters, with salinity values ranging from 0.25 to 36.92‰ (Martín & Bone, 2007), and a steep slope, reaching more than 2,500 m deep. These studies have included the characterization of the environmental and biological settings of the area, including the benthic component. The benthic community has been recently reported in terms of the main groups inhabiting these large soft-bottom areas (Bone et al., 2007), where polychaetes represented the most important one, achieving more than 64% of the total macrofaunal abundance.

The biodiversity knowledge of the polychaeta fauna in Venezuela has been traditionally focused on shallow water areas. Previous studies have revealed a total of 40 families, 138 genera and 206 species for the Caribbean coast (Bone & Liñero, 2003), but there is no previous information for the Atlantic region or deep waters. In the course of this study we

report for the first time the biodiversity of the polychaete community inhabiting the Venezuelan Atlantic front. The area is described in terms of its environmental settings, and the role of the abiotic conditions in shaping the polychaete community is assesed.

2. Methodology

This study was conducted in the Venezuelan Atlantic coast, south-east from Trinidad & Tobago, and north-east offshore the Orinoco's River delta, between 10º16' - 8º55' N and 61º05' - 58º49'W (Figure 1). The study area was subdivided into three depth zones: a shallow continental shelf zone (from 10 - 60 m), with vertically mixed waters under the Orinoco's direct influence (1), a continental shelf zone (from 60-200 m), with no vertical mixing (2), and a deep slope zone, > 200 m (3). A total of 82 stations were sampled during November-December 2005 (rainy season): 17 in zone 1, 50 in zone 2, and 13 in zone 3 (Figure 1). Baseline studies (Gomez et al., 2005; Martín & Bone, 2007) provided sea bottom environmental data -depth, salinity, water temperature, sediment texture and organic content of the sediments- for these and other sampling stations. Samples of the benthic fauna were taken using a Van Veen dredge of 0.20 m^2 surface area. Sediment samples were hand-sieved by means of 0.5 mm sieve. All fauna was removed and polychaetes were identified down to species level when possible.

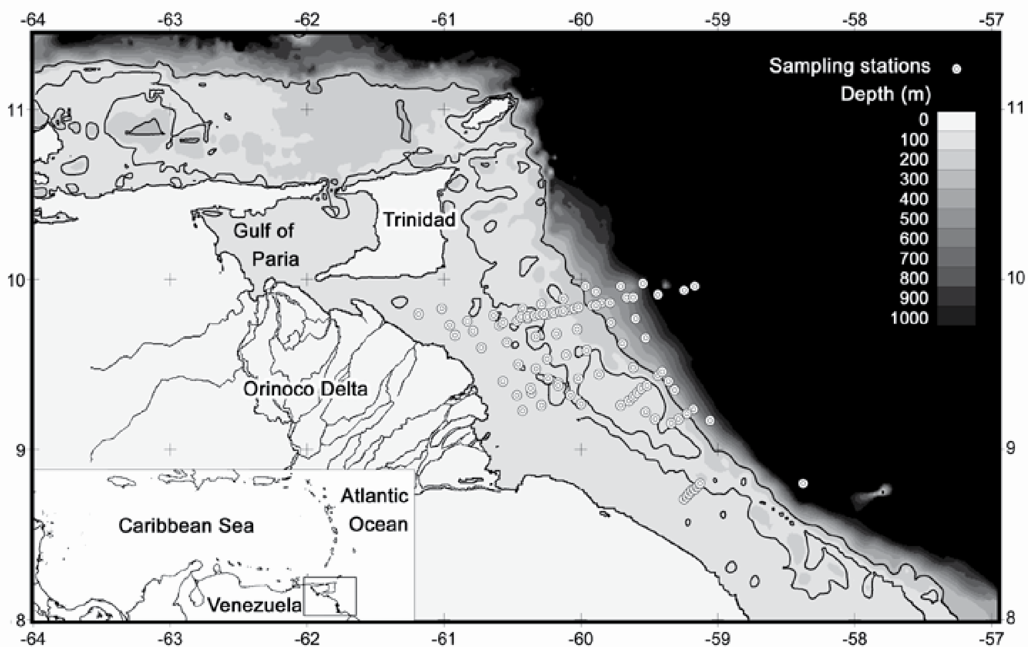

Fig. 1. Map of the Venezuelan Atlantic coast showing the sampling stations. The study area was divided in three bathymetric zones, delimited in the map by thick lines at the 60 m and 200 m isobaths.

Principal Component Analysis was applied to the environmental data in order to explore the structure of the dataset and detect any possible difference between depth zones. Univariate differences in the environmental variables according to the bathymetric zones

were tested using parametric ANOVA, followed by a Fisher LSD test. Cluster analysis was used to study species distribution patterns along the depth gradient. Non-parametric ANOVA's (Kruskal-Wallis) were used to determine statistical differences in density and richness values between depth zones, due to the high variability between the benthic samples. The relationship between polychaete density and the environmental variables (depth zone, salinity, sand, clay, and organic content of the sediment) was explored within a piecewise regression approach to account for the non-linear relationship between the response and explanatory variables. As samples were grouped according to depth in shallow (0-60 m), intermediate (60-200 m) and deep areas (>200 m), depth was treated then as a nominal variable with three levels. After a preliminary data exploration, the redundant response variable family richness (related to density, R=0.9) and the explanatory variables temperature (explained by depth, R=-0.8) and lime (related to clay, R=-0.9 and organic content, R=0.6) were excluded from the analysis. Also, two outliers with density values higher than average+3sd (86.7 ind/m^2) were excluded from the analysis.

3. Results

3.1 Environmental variables

The characteristics of the depth zones in terms of their main environmental parameters are presented in Table 1. As expected, temperature responds to the depth gradient (R=-0.87), with the deepest zone showing the lowest values (ANOVA test, $p < 0.001$, Fisher LSD test, $p < 0.001$). The behavior of the other environmental variables seems to respond to the influence of the Orinoco River, such as salinity, where the shallowest zone, near the Orinoco delta, has the lowest salinity and highest variability (ANOVA test, $p < 0.001$, Fisher LSD test, $p < 0.001$). Sediment textural analyses show that sand and silt have an opposite relationship (R=-0.92), but silt and organic content are positively correlated (R=0.65). Closest to the river influence, the shallowest zone has highest silt and organic content. The drop-off of the continental shelf is characterized by a sudden decrease of these fine components and an increase of sand, whereas in the deep waters these variables have intermediate values.

	Zone 1 (0-60m)	Zone 2 (60-200m)	Zone 3 (>200m)
Temperature (°C)	**26.86** ± 1.29 (37)	22.97 ± 3.36(37)	**6.24** ± 3.86 (40)
Salinity (‰)	**26.12** ± 13.82 (28)	**36.57** ± 0.35 (24)	35.02 ± 0.42 (35)
%Sand	**14.55** ± 21.62(38)	**63.55** ± 23.42 (60)	30.78 ± 21.20 (17)
%Silt	**73.30** ± 24.66 (38)	**25.15** ± 16.92 (60)	49.58 ± 19.80 (17)
%Clay	12.16 ± 11.67 (38)	**11.27** ± 9.52 (60)	**19.64** ± 12.67 (17)
%Organic content	**0.96** ± 0.28 (38)	**0.51** ± 0.38(60)	0.61 ± 0.35 (17)

Table 1. Average and standard deviation (number of samples) for sea bottom environmental variables measured in each bathymetric zone. Highest values in bold letters, lowest values in gray. The differences between the zones are significant for all variables (ANOVA test, $p<0.05$). Groups in different colors according to post hoc comparisons (Fisher LSD test, $p<0.05$).

The Principal Component Analysis of the environmental variables restates the clear separation between the three bathymetric zones (Figure 2). Factor 1 accumulates 41% of the

total variance. This factor includes information concerning the sediment variables: salinity (-0.57), percentage of silt (0.83), organic content (0.83) and percentage of sand (-0.72). Factor 2 (28% of the explained variance) is represented by the variables depth (-0.75), temperature (0.69) and percentage of sand (0.63).

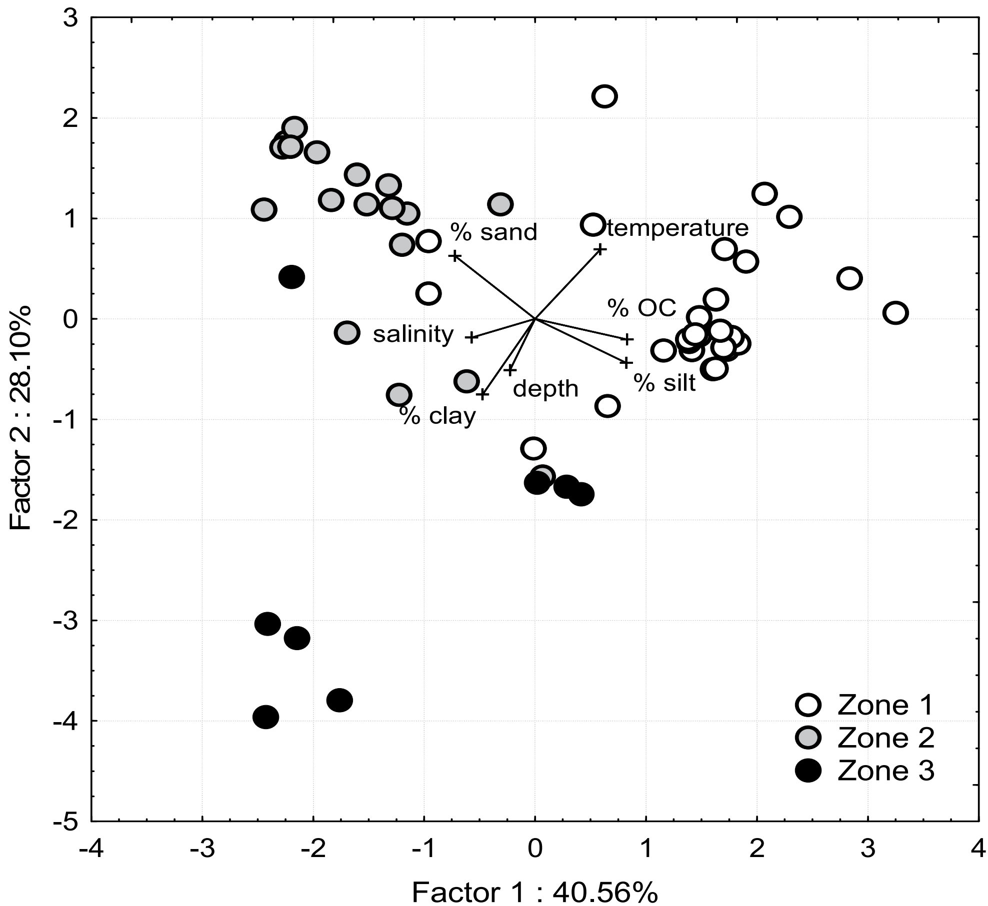

Fig. 2. Principal Component Analysis plot of site scores on the first two principal components derived from the environmental variables for sampling sites at the Venezuelan Atlantic coast according to the bathymetric zones.

3.2 Polychaete fauna

A total of 2,452 ind were collected, representing 43 families, showing a very high biodiversity for this region. None of the families were dominant, but Spionidae (13.1%), Pilargidae (11.5%) and Paraonidae (10.72%) were the most abundant ones; the rest represented less than 7% of the total abundance. At a species level, we identified 81 species for the area: 19 species in zone 1, 68 in zone 2, and 23 in zone 3 (Table 2).

We identified 19 families in the first zone, with Spionidae (19.81%), Paraonidae (14.32%), Magelonidae (11.79%) and Capitellidae (10.98%) as the most abundant. From the 19 species recorded here, 4 of them represented 53.38% of the total number of individuals: *Dipolydora*

Species	Zone 1 (0-60m)	Zone 2 (60-200m)	Zone 3 (>200m)
Paramphinome besnardi		X	X
Isolda pulchella		X	
Lysippe annectens		X	X
Melinna cristata		X	
Mediomastus californiensis	X	X	
Scyphoproctus platyproctus	X	X	
Notomastus hemipodus	X		
Notomastus lineatus		X	
Notomastus americanus		X	
Notomastus angelicae		X	
Notomastus tenuis		X	
Chaetozone cf. setosa		X	
Monticellina dorsobranchialis		X	X
Tharyx cf. setigera	X	X	X
Cossura delta	X	X	
Schistomeringos pectinata		X	
Protodorvillea kefersteini		X	
Eunice filamentosa		X	
Eunice websteri		X	
Eunice vittata			X
Marphysa sanguinea		X	
Diplocirrus capensis			X
Goniada maculata		X	
Glycinde normanni		X	
Glycera papillosa			X
Gyptis brevipalpa		X	
Heterospio cf. longissima	X		
Scolotema verrilli	X	X	
Lumbrinereis jonesi		X	
Magelona cf. berkeleyi		X	
Asychis elongata		X	
Aglaophamus verrilli	X	X	X
Nephtys squamosa		X	
Nephtys incisa			X
Nereis falsa		X	
Ceratocephala oculata		X	X
Kimbergonuphis ceproensis		X	
Kimbergonuphis tenuis		X	
Diopatra cuprea		X	
Diopatra tridentata		X	
Armandia agilis	X	X	
Ophelina cylindriculata		X	
Ophelina acuminata		X	X
Scoloplos rubra	X	X	
Scoloplos texana		X	
Cirrophorus lyra		X	X
Aricidea (acmira) cf. finitima		X	
Aricidea (acmira) simplex		X	X
Aricidea (allia) suecica		X	
Aricidea (aricidea) fragilis		X	
Cirrophorus branchiatus		X	
Levinsenia gracilis		X	X
Paraonella nordica			X
Sigambra tentaculata	X	X	X
Ancistrosyllis jonesi	X		
Sigambra wassi	X		
Pilargis berkeleyae		X	
Poecillochaetus johnsoni		X	
Eteone lactea		X	
Lygdamis indicus			X
Prionospio multibranchiata		X	
Prionospio delta	X	X	X
Dipolydora socialis	X		
Paraprionospio cf. tamaii	X	X	
Aurospio dibranchiata	X	X	
Spiophanes duplex	X	X	X
Laonice cirrata		X	X
Spio pettiboneae		X	
Spiophanes wigleyi		X	X
Sternapsis scutata	X		X
Sphaerosyllis parabulbosa		X	
Exogone (Exogone) naidina		X	
Exogone (Exogone) lourei		X	
Exogone (Parexogone) campoyi			X
Exogone (Parexogone) caribensis		X	
Haplosyllis spongicola		X	
Pionosyllis weissmanni		X	
Sphaerosyllis magnidentata		X	
Syllis (Typosyllis) ortizi		X	
Streblosoma hartmanae		X	
Terebellides stroemi		X	

Table 2. Spatial distribution of polychaete species along the bathymetric gradient.

socialis (16.76%), *Mediomastus californiensis* (13.77%), *Cossura delta* (12.19%) and *Prionospio delta* (10.66%). In zone 2 only Spionidae (10.92%) and Pilargidae (10.03%) were important, with *P. delta* (9.86%) and *Aglaophamus verilli* (9.2%) as the dominant species. Zone 3 exhibited 4 main families: Pilargidae (19.41%), Spionidae (15.16%), Paraonidae (14.84%) and Cirratulidae (9.13%), with *P. delta* (13.54%), *Levinsenia gracilis* and *Tharyx setigera* (11.28% each), and *Cirrophorus lyra* (9.03%) as the most abundant species. We found that only 5 species (6.17%) were common to all zones along the depth gradient: *T. setigera, A. verrilli, Sigambra tentaculata, P. delta* and *Spiophanes duplex*, suggesting a certain degree of specificity in the spatial distribution of most species. Eight species were common to zones 1 and 2, one species was common between 1 and 3, and 10 were common to 2 and 3. The cluster analysis indicates that the shallowest, river influenced area, has a particular polychaete assemblage and that deeper areas are more similar (Figure 3).

3.3 Patterns of association between variables

Polychaete density was related to the environmental variables in a nonlinear fashion (piecewise regression, r= 0.94 percentage of variance explained: 87.82%, Figure 4).

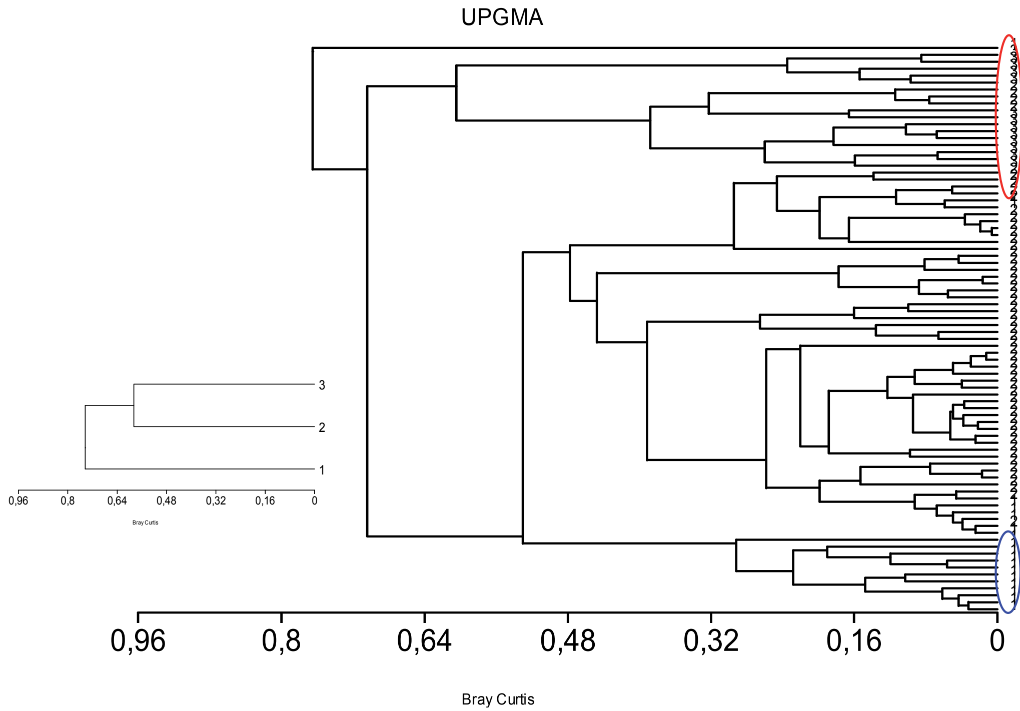

Fig. 3. Dendrogram comparing the species composition in the three depth zones.

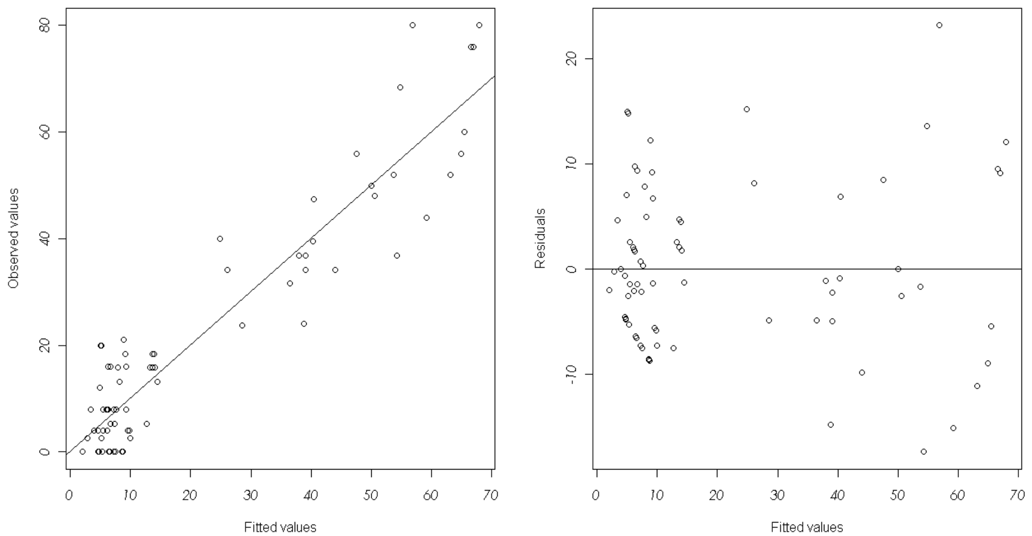

Fig. 4. Fitted values vs observed values (left) and residuals (right) for the piecewise regression applied to polychaete density data.

The river influence is determinant of the abundance patterns when densities are low (<21.19 ind/m²). However, when density is higher, depth zone, clay and organic content are the best variables explaining these patterns (Table 3).

	Intercept	Zone	Salinity	Sand	Clay	OC
M1 (density<breakpoint)	7.65	-2.97	0.22	-0.06	-0.02	1.77
p value		0.19	0.06 *	0.44	0.94	0.54
M2 (density> breakpoint)	-73.05	13.16	3.52	-0.05	-0.90	-27.32
p value		0.06*	0.12	0.36	0.03**	0.01**

Table 3. Parameter estimates and significance values of the two regression lines fitted using piecewise regression, estimated breakpoint=21.19 ind/m².

Canonical Correspondance Analysis (CCA) relating the environmental data and density of the polychaete species showed a strong association between *C. delta, M. californiensis, D. socialis, S. verrilli, S. platypatus* and salinity values (Figure 5). This result is reinforced by a significant positive correlation between salinity and total density along the depth gradient (Spearman, r: 0.36, p= 0.04), suggesting that this variable is one of the main ecological variables determining their distribution. Other environmental variables were also important in defining the spatial distribution patterns, like sediment silt content and deep water temperature, contributing to mark clear differences between zones.

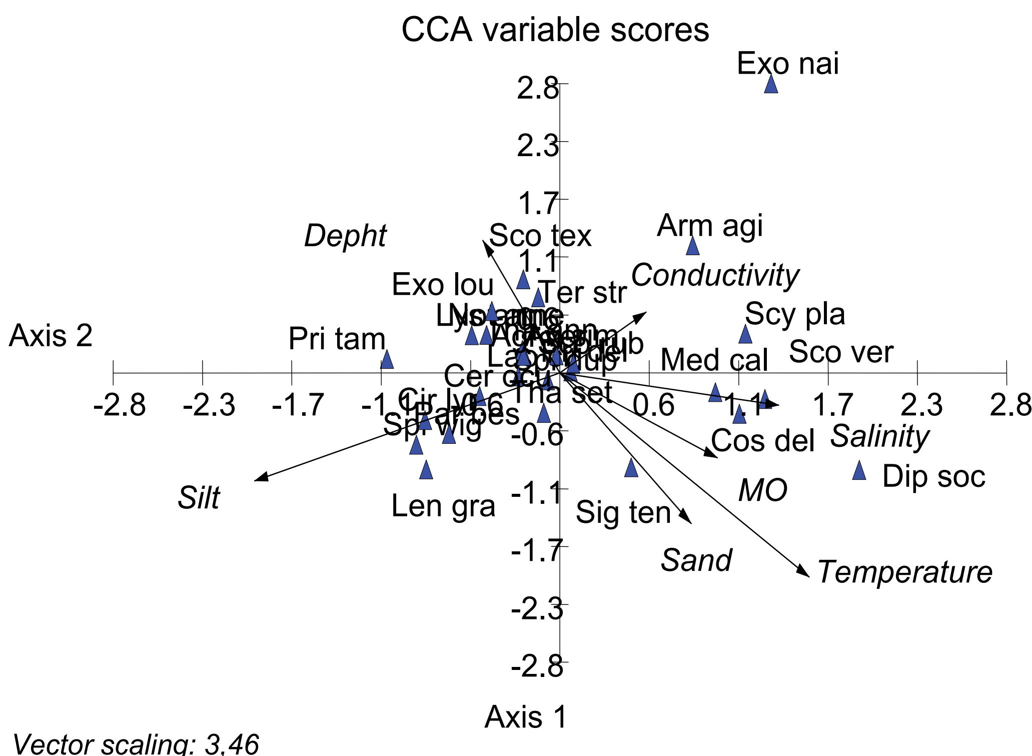

Fig. 5. Canonical Correspondance Analysis relating the environmental data and density values for the dominant polychaete species.

4. Discussion

This study represents the first contribution to the knowledge of polychaetes biodiversity from deep-sea waters in the south Caribbean, particularly for Venezuela, where such studies are very scarce. Previous research carried out at the Orinoco's river delta and its continental shelf (Bone et al., 2004, 2007) have established that polychaete communities associated to soft bottoms of this region, are characterized by high species richness but low organisms density in relation to coastal marine environments. These are expected characteristics in deep water benthic communities (Grassle & Macioleck, 1992; Nybakken, 1997; Sanders & Hessler, 1969). The presence of 43 families and 81 species of polychaetes demonstrate this high diversity. Nonetheless, the richness is lower than what has been reported for the Venezuelan coastal waters (Bone & Liñero, 2003), where over 200 species have been found. The most important polychaete families in the study site were Spionidae, Pilargidae and Paraonidae, while the rest of them presented relative abundances lower than 7%. Glover et al. (2001) and Pérez et al. (2003) pointed out that Spionidae, Cirratulidae and Paraonidae families represent between 50-60% of the total abundance in deep water areas, in the Atlantic and Pacific, independently of the sediment organic content. The authors explained that this can relate to a very similar functional structure between these communities, dominated by detritivorous polychaetes; being also present predator habits families, mainly syllids and lumbrinerids, but in a lower proportion. Probert et al. (2001) report the importance of Spionidae family for the west coast of New Zeland (48,7%), especially in shallow waters (depths < 100m), where there is a vast fresh water discharge and terrestrial sediment drag towards the marine receptor waters.
The polychaete species composition, richness and abundance found show the presence of a very marked spatial pattern. The highest average density and species number were observed at depths greater than 60 m, at zones called 2 and 3, the ones that are farther from the coastline, the Orinoco river influence and the Guayana current. At the shallowest zone (1), there is lower richness, and only some polychaete species increased their densities as in the particular case of the individuals from Spionidae (*Dipolydora. socialis*) and Capitellidae (*Mediomastus californiensis* and *Scyphoproctus platyproctus*), whose densities decreased towards the zones 2 and 3 (Balthis et al., 2006). In general, these species are opportunistic, have short reproductive and recruitment cycles (MacCord & Amaral, 2007); and are deposited organic matter consumers, taking advantage of the productivity peaks; which lets them inhabit highly disturbed systems (Méndez, 2002; Santos, 1994). Likewise, in this zone, a higher estuarine species relative abundance was observed with broad range of saline tolerance, like the case of species from Cossuridae (*Cossura delta*) and Spionidae (*Prionospio delta*) families.
The response of polychaetes to steep salinity variations was reported in Venezuela for a few spionids (*Streblospio gynobranchiata* and the polydorids group) at coastal zones affected by intense rain events that cause salinity to decrease down to 5PSU (Chollet & Bone, 2007). Bone & Rodríguez (2004) also report the presence of only 9 polychaete families and 14 species, at the littoral zone of the Orinoco's river delta, where salinity values are close to 0PSU, and higher density and biodiversity at areas with greater marine influence during the dry season, when the river discharges are lower. The result showed no significant correlation with depth or other environmental variables, except salinity (Spearman, $p<0.04$), suggesting a clear influence of the Orinoco river in the spatial distribution pattern of polychaetes found in the shallow continental shelf zone.

Similar results were found by Yáñez-Arancibia & Day (1988), who report the presence of some species from Cossuridae and Spionidae families at Términos lagoon west area, Gulf of Mexico, where the higher fluvial discharge and lower salinity values are observed, indicating that distribution of organisms at the lagoon is determined by salinity. Gaston & Nasci (1988) report the capitellid *Mediomastus californiensis* dominance all year long, at the lower Calcasieu estuary, Louisiana, USA, with salinity fluctuations between 0-23PSU, showing its euryhaline condition. On the other hand, restricted spatial distribution was observed for some families like Eunicidae, Syllidae, Onuphidae, Nereididae, Amphinomidae, Ampharetidae, Glyceridae, Fauveliopsidae, Questidae and Sabellidae, limited to zones 2 and 3, with a marked richness reduction towards zone 3 (deepest one), suggesting that they can be less tolerant to marked salinity decreases.

Our results show that this low polychaete density at shallow bottoms off the northeast Venezuelan region can be related to the Orinoco's river discharge, and that this influence determines the existence of two zones in the region, not three. One shallow, conditioned by the river discharge, with low organic matter content in sediments (< 1%), high water turbidity and extreme salinity variations (0.25-23PSU); and a deeper, an oceanic influenced and oligotrophic zone, characterized by a steep depth gradient, low temperature and marine current influences (Martín & Bone, 2007). These environmental conditions contribute to define the polychaete community spatial distribution pattern, with low densities for the first one, and higher for the second one (Martín & Bone, 2007; Pérez et al., 2003). At the shallowest zone (< 60 m depth), there is active sedimentation, with high silt and organic content. This area is replaced by another one dominated by a higher sandy fraction content, where sedimentation is scarce, which reaches about 200 m depth. Thereafter, the behavior of the data is very variable towards deeper zones, and could be related to differential sedimentation and transportation processes, as it has been suggested by Martín & Bone (2007). Muller-Karger et al. (1989) have shown that this influence may extent over 100 km offshore, especially during the rainy season, when the water volume discharged into the marine environment is higher. Orinoco's river plume effect combines with the one from the Amazon river that skirts the east coast from the south, and together they extend to 18ºN, specifically to La Mona island, between Puerto Rico and Dominican Republic, extending 300km over the Caribbean Sea (Cherubin & Robertson, 2007).

Conlan et al. (2008) found a positive correlation between macrofauna density and distance from the coast, at Beaufort, Canada, due to the Mackenzie's river influence, and a negative correlation with depth (between 10-400 m), where there is a salinity gradient from 16 to 30PSU at 75km from the coast, and a higher carbon and nitrogen concentration in sediment at the shallow area. Our results showed a different spatial pattern, where polychaete densities are related to environmental variables in a nonlinear fashion. The river influence is determinant of the abundance patterns where densities are low (<15 ind/m^2). However, where density is higher, depth, clay and organic content are the best variables explaining these patterns. In this deeper zone, the highest biodiversity by station is also reported, particularly between 60-200 m deep. The polychaete average density at zones 2 and 3 was very similar (34 and 36ind/m^2 respectively), but with only 23% of family composition resemblance; with a clear depth effect. This oceanic zone, away from the coastline, corresponds to oligotrophic waters and low primary productivity, due to the influence of the Orinoco river and Guayana current, in consequence species richness is again reduced (Alongi, 1998).

In the literature, many diverse results exist for depth effects in benthic communities spatial patterns, like is pointed out by Allen et al. (2006) at a revision of 120 investigations within the Gulf of Mexico continental shelf and the Atlantic coast of the EEUU. Aller et al. (2002) explains that despite the fact of having a well-defined depth gradient at Cape Hatteras platform, in North Carolina, they found a very similar macrofauna density from the shallow area to 800 m deep, with a reduction towards 2000 m. These density variations in the depth gradient for the Tropical Atlantic, has been attributed to nutrient entry and flux reduction at greater distances from the coast (Cosson-Sarradin et al., 1998). Aller et al. (2002) and Juul-Pedersen et al. (2008) explain that organic matter entry to marine water masses, as phytoplankton, feces or particulate rests, contribute to the existence of a extremely active benthic community, with high abundance and biomass that inhabits these deep bottoms. But benthic communities from shallow areas receive a greater organic matter input and other sources of carbon from the nearby coastal platform, showing greatest organisms' density. At the Orinoco's delta region this does not seem to be the case, where we observed differences at the shallow area due to salinity reduction as a consequence of the river discharge. Aller et al. (2002), showed that this organic matter entry to the system does not have direct relation with depth patterns, due to the zone's topography, which can alter particles deposition. As well, Glover et al. (2001) pointed out after evaluating this relationship at abyssal bottoms, that species diversity and polychaete density do not depend in a linear way on the water column productivity, but can be affected by local factors like depth, fluvial discharges and hydrodynamic patterns, suggesting that the oligotrophic condition does not limit diversity in a given zone.
The response to variation in organism density and species richness in the case of the Orinoco's delta could be adjusted to the model proposed by Alongi (1998) to explain infauna spatial distribution patterns associated to soft-bottoms areas affected by river and delta discharges. Following this model, the most influenced area by an important river discharge is the shallow one, affected by a great nutrient and sediments deposition, but does not allow the establishment of a high diverse community, like the case of our zone 1. At the shallowest zone, salinity can be reduced, and only a few polychaete species, considered as opportunistic, can increase their density with these environmental changes, being this condition adverse to many other species. This response can be favored by organic matter dragged by runoffs from the coast and nearby rivers, increasing the organic matter input, which can be a direct or indirect source of food to these organisms (Probert et al., 2001). As the distance progresses from this point of high discharge of the delta towards open sea, Alongi's model predicts reduced solid suspended amounts in the water column, which permits development of a higher primary productivity (more light penetration), this last one also favored by a higher water column mix and oxygenation, because depth is greater, which leads towards the development of a denser and more bio diverse benthic community. This situation could be present in our zone 2, which is farther away from the Orinoco's river discharges and the Guayana current effect, characterized by stable and higher salinity values and species richness. Our zone 3, the farthest one from coastline and Orinoco river influence, corresponds to an oligotrophic zone, with low primary productivity due to the lower nutrient input, so the species richness is again reduced. This deep zone is dominated by species from the Pilargidae, Spionidae, Paraonidae and Cirratulidae families, similar to Pérez et al. (2003) results from the Gulf of Mexico, who report the presence of these families at zones between 200-3800m depth. Therefore, in this work we stress the importance of studying large-scale biodiversity spatial and temporal patterns in marine environments

subjected to heavy continental influence, in order to contribute to reduce the existent gap of information, especially in tropical regions, where riverine inputs are determinant.

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6

Butterfly Diversity in a Changing Scenario

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1. Introduction

For many historical and ecological reasons, Italy is characterized by extremely high biodiversity level, which we can observe in virtually all animal and plant groups. This occurs in concomitance with relatively low levels of anthropic disturbance, at least in comparison to most areas of central Europe. The elongate shape and mountainous nature of Italy, together with its vast littoral areas, contribute to generate very diverse ecological conditions, all along a series of latitudinal and altitudinal habitat gradients. The Mediterranean basin, where Italy has centre-stage position, is among the 25 world biodiversity hotspots identified in Myers' seminal paper (Myers et al., 2000). Whatever will happen to, or affect the, Italian biodiversity will be reflected on a world-wide scale. This chapter describes animal diversity as a dynamic functional system, now increasingly threatened by various kinds of anthropogenic threats and pressures. We describe how and to what extent biodiversity is changing, taking as an example a particularly well-known animal group, such as butterflies, within an area of focal importance, such as the Mediterranean basin. We mainly discuss data that we, and our research group, have personally obtained in the course of the past several years, and even though we mainly worked in Italy, we discuss them in the more general framework of a pan-European scientific scenario.

2. The Italian butterfly biodiversity

The Italian biodiversity is among the richest in Europe. In particular, the Italian butterfly fauna includes 283 species, assigned to 79 genera and 9 families or subfamilies. It represents 37% of the total euro-mediterranean fauna. At a national level, butterfly biodiversity is higher in northern Italy, particularly in the Alps and the pre-Alps, than in the Apennines and in the main Italian islands: this is a consequence of the well-known peninsular effect (Tontini et al., 2003), which develops along a latitudinal and longitudinal gradient, were the alpine arc plays a central role. Species richness notably differs also according to an altitudinal gradient. Forty-seven species are alpine elements present exclusively or primarily over the tree line boundaries, and ninety-seven are typical of the high mountain horizon. A smaller number of species (*Colias hyale, Maculinea alcon, M. teleius, Euphydryas aurinia,*

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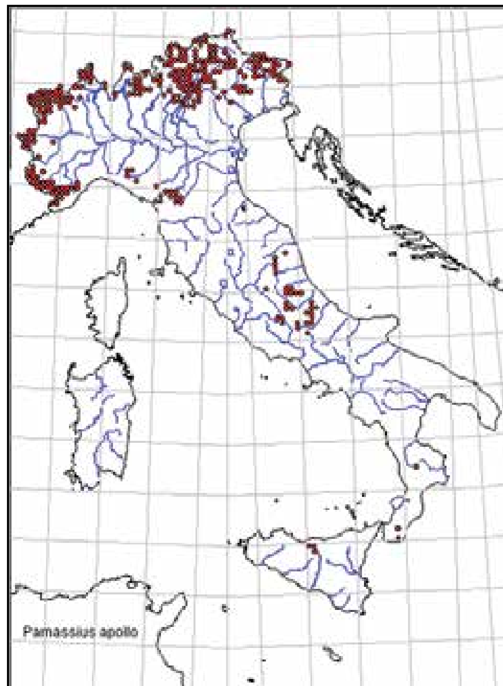
Coenonympha oedippus) live exclusively in the Padano-Venetian plains, whilst twelve species are elements connected with the Mediterranean woodlands (macchia).
The Italian endemic species *sensu stricto* are eighteen, i.e. 7.5% of the total, but this number would increase to 48 (17,1% of total), if we consider endemic species ranging across a small area of politically non-Italian territory, such as *Erebia christi* or *E. flavofasciata*.
The highest concentration of strictly Italian endemics is found in the Apennine mountains (10 species), in the small islands (4), Sardinia (3), Sicily (2) and in the xerothermic "oases" of the western Alps. During the last decade, two species newly became part of the Italian fauna: *Danaus chrysippus* and *Cacyreus marshalli*. *Danaus chrysippus* is a well-known migrant and became naturalised in several Italian regions (Apulia, Sardinia, Sicily etc.), after having already marked some brief presence in previous times. *C. marshalli* was accidentally introduced and was observed in Italy (Rome) for the first time in 1997, but its expansion throughout the whole Country was very fast.
Another species, *Lycena helle*, probably became extinct in historical times. Three more species, *Araschnia levana, Euphydryas maturna* and *Polyommatus exuberans* once supposed to be extinct, have been rediscovered in recent times (Balletto et al., 2007).
The Italian Ministry for the Environment has published in 2007 a preliminary distribution atlas for 10,000 animal species, the CKmap Project, mapped on a 10x10 Km UTM grid (e.g. Fig. 1). Data included in this database derive from the literature as well as from museum collections. As concerns butterflies, this data set currently includes well over 160 000 non-duplicated individual records. New data represents over 50% of the total data and are continuously updated (Balletto et al., 2007).

2.1 Population extinctions

In a recent paper (Bonelli et al., 2011), we focused on a database of well-documented population extinctions having occurred among Italian day-flying Lepidoptera.
Depth studies of patterns of extinction are fundamental to understand species vulnerability, in particular when population extinctions are not driven by habitat loss, but related to subtle changes in habitat quality and are due to 'unknown causes'. We used a dataset containing over 160,000 non duplicate individual records of occurrence referred to 280 butterflies and 43 zygaenid moths, and their relative extinction data, to carry out a twofold analysis. The earliest published data that we could use to evaluate extinctions are due to Hübner (1790) and de Prunner (1798). We identified ecological preferences that influence extinction probability, and we analysed if all species were equally vulnerable to the same factors. We investigated Italian population extinctions at two levels, i.e. i) Population level: data of extinctions were pooled across species, separately for their ecological features, because we hypothesized that populations sharing similar ecological traits would react to changes in a similar way, acting like '*functional types*' (Henle, 2004; Shreeve et al., 2001; Thompson et al., 1996). We tried to understand, in this way, if different ecological requirements influenced the species' probability of becoming extinct; ii) Species level: Italian species were subdivided into two groups comprising a) species that lost at least one population, and b) species that did not lose any populations during our selected time frame. We analysed differences between groups, taking into account the ecological characteristics of individual species and trying to understand which features made them more prone to extinction. Although only one species (i.e. *Lycaena helle*) may have become extinct in Italy after or around 1925, a relatively high number of populations of day-flying Lepidoptera have disappeared. At



(a)

(b)

Fig. 1. Italian distribution of *Parnassius apollo* (a) (from Balletto et al., 2007); (b) *Parnassius apollo* courtesy of C. Bertino.

least 727 populations (653 of butterflies and 74 of burnet moths), formerly occurring in 268 UTM grid squares of the 3537 included in the Italian political territory have been lost. Population extinction affected 164 species, i.e. 142 butterflies (50.5% of the Italian fauna) and 22 zygaenids (51.2% of the Italian fauna). Our analyses revealed that extinctions were non-randomly distributed in space and time, as well as across species. Most of the extinctions were recorded in 1901-1950 and, as expected, populations at their range edges were more prone to become extinct for non-habitat related causes. Ecological traits were not only unequally distributed between extinction and non-extinction events, but also not all ecological features had the same importance in driving population vulnerability. Hygrophilous and nemoral species were the most likely to experience population losses and the most prone to disappear even when their habitats remained apparently unchanged.

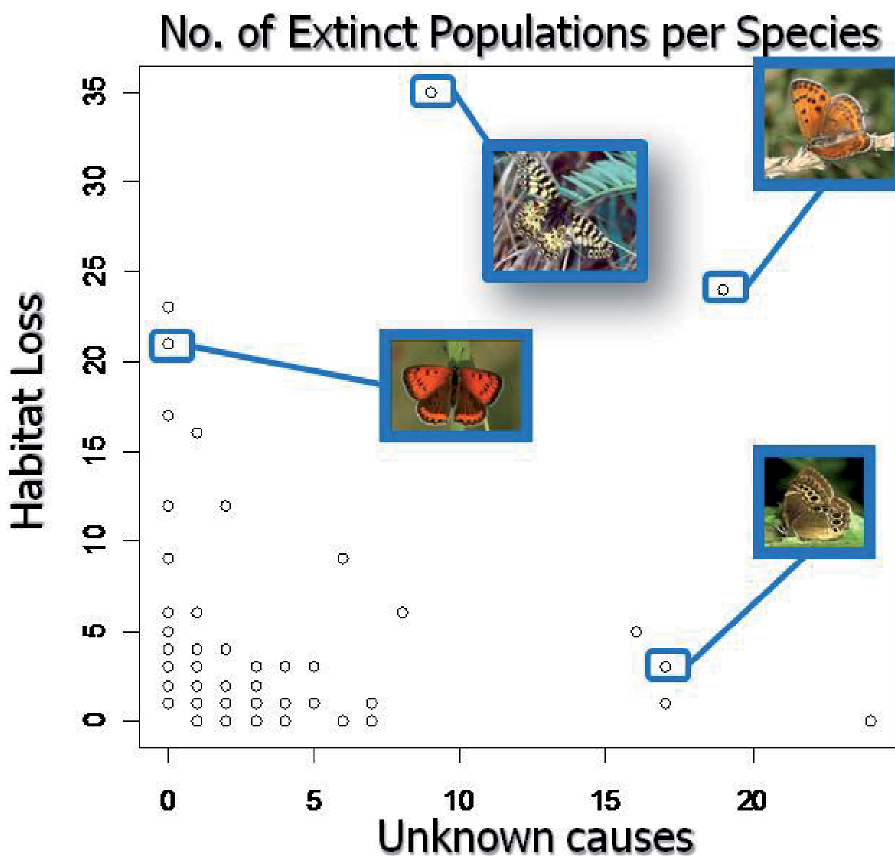


Fig. 2. Species showed a distinct pattern of vulnerability, depending on threats. Some species are more affected by unknown causes than by habitat loss (Unk. *vs.* Hab.) e.g. *Araschnia levana* (17 vs. 1), *Argynnis pandora* (16 vs. 5), *Melitaea britomartis* (24 vs. 0), *Lasiommata achine* (17 vs. 3, Fig. 2), whereas others are more affected by habitat loss, e.g. *Zerynthia polyxena* (9 vs. 35, Fig. 2), *Lycaena dispar* (0 vs. 21, Fig. 2), *Apatura ilia* (0 vs. 23), *Maculinea arion* (0 vs. 17), *Melanargia arge* (0 vs. 21). The only species that lost a high number of populations for both causes is *Lycaena thersamon* (19 vs. 24, Fig. 2).

As concern vulnerability to extinction, we tried to understand if different ecological traits, generating different ecological needs in ground water, light, temperature and general habitat preferences, influence extinction probability and vulnerability to different causes. It is, in fact, reasonable to postulate a non-random distribution of species' vulnerability (Isaac & Cowlishaw, 2004; McKinney, 1997; Parmesan, 2006). Species vulnerability depends on both ecological requirements and threat type: in fact, each species showed a distinct pattern of vulnerability, depending on threats (Fig. 2).

Habitat destruction is the main cause of extinction throughout the Italian territory, especially in the plains of the North, while hygrophilous and nemoral species are the most vulnerable. As already pointed out for many countries (e.g., van Swaay et. al., 2010) a correct conservation policy should begin by stopping urbanization and intensive agriculture and revitalising traditional agro pastoral activities. Our data, however, show that these measures may be insufficient. One-third of the Italian butterfly population extinctions, in fact, were not clearly related to habitat destruction, but linked to some more subtle degradation of environmental quality. Many, often still unknown, causes affect small, isolated populations. Van Swaay et al. (2006) indicated that butterflies are strongly declining all across Europe (-11% in the last 25 years) and that other threats, in particular global warming, will soon represent a serious matter of concern, as also indicated by Settele et al. (2008).

2.2 The extinction of Alpine species: The role of climate change

Climate studies strongly suggest that atmospheric alteration is already occurring and that changes in atmospheric composition are altering weather and climate processes. Warming of the climate system is unequivocal, as is now evident from observations of increases in global average air temperatures (Ipcc, 2007). Temperature, however, is not the only climate variable likely to change. Widespread changes in precipitation amounts, wind patterns and aspects of extreme weather have already been observed (Ipcc, 2007). Closer investigation reveals that climatic change in the alpine region during the 20th century has been characterized by increases in minimum temperatures of up to 2°C, a more modest increase in maximum temperatures, little or no trend in the precipitation data, and a general decrease of sunshine duration, all through to about the mid-1980s (Beniston, 2000).

Given the central role of climate in governing the natural environment of mountain areas and the intensity of most biological processes, it is easy to suppose a high vulnerability of such systems to the impacts of a rapidly changing climate (Beniston, 2005). Mountain ecosystems provide interesting and useful models for the early detection and study of the signals of climatic change and its impacts on ecological systems (e.g., Haslett, 1997; Wilson et al., 2005).

Yet, any assessment of climatic change and of its related impacts in the mountains has been shown to be particularly difficult, because of the complexity of interrelated factors. In these regions topography is a dominant feature of the environment (Beniston, 2005) and determines rapid successions in various environmental conditions, even along narrow altitudinal gradients. Such difficulties are stronger in the Alps, which are characterised by diverse meso-climates even showing some Mediterranean influence, and for which it is almost impossible to draw up a comprehensive scenario of past and future changes valid for the entire alpine chain (Theurillat & Guisan, 2001). Moreover, the shape of landscapes has been modelled, during millennia, by human pressure and it is now difficult to understand the relative roles of land use changes and of global warming, or to hypothesize how these

factors will interact in the near future (e.g. Motta & Nola, 2001; Körner & Ohsawa, 2005; Vittoz et al., 2008).
To explore the relative role of climate and land use changes in shaping butterfly communities in mountain ecosystems, in 2009 we investigated a valley in the Maritime Alps (Italy, Valdieri, Valasco Valley), where we sampled 7 butterfly communities, already investigated in 1978. Sampling was made by semi-quantitative linear transects. Our 7 study sites had been precisely identified (spatially) in 1978, permitting the exact repetition of the monitoring programme in 2009. They range along an altitudinal gradient (1300–1900 m) and cover different kind of habitat (broadleaved forest, subalpine heathlands, hygrophilous meadows, alpine pastures, rocky slopes and screes). In 1978, the sampling period was limited to the end of July and the beginning of August, the optimal period to study butterfly communities in mountain ecosystems. In 2009 the sampling period was extended from the beginning of June until the end of August, to cover almost all the potential flight season, in order to be sure that '*no-more-found species*' really had disappeared during our time frame and had not just suffered some phenological shift.
Data from nearby meteorological stations, both rough and elaborated trough interpolation techniques (Loglisci, unpublished data), have been used to quantify climatic changes in the study area as a whole. Visual inspection of aerial photographs of each transect, taken in 1978 (source: IPLA archives, Piedmont) and repeated in 2006 (source: Italian Ministry for the Environment), have been used to describe changes in the vegetation cover during the selected time frames and to identify the most important habitat alterations.
The comparison of butterfly communities observed in 1987 and in 2009 did not evidence any clear pattern, in terms of species richness, between the two sampling periods, but the analysis of some individual cases provided interesting results, and showed several changes in the ecological composition patterns of butterfly communities.
We observed some '*species substitution*' in our sampling sites, indicating a general loss of specialised and narrow-range species and a general gain in ecologically tolerant elements. In 2009, we recorded neither *Coenonympha darwiniana*, an alpine endemic, nor *Coenonympha glycerion*, a hygrophilous species, but we found *Coenonympha arcania* in 6 of 7 sites, where it had not been recorded in 1978. The latter is a widespread species, characteristic of the lowland ecotonal habitats and much stronger generalist than the previous two. A similar case is that of *Colias phicomone*, a xerophilous species, linked to open herbaceous habitats occurring above the tree line (subalpine and alpine belts). It was repeatedly found in 1978, but was '*substituted*' in 2009 by the massive ingression of *Colias crocea*, an ecotonal, altitudinally generalist species, generally thermophilous and characterised by very high vagility.
Still at species level, thermal preferences demonstrated importance in determining patterns and directions of change: a strong increase in the number of thermophilous species was observed in 5 on 7 transects (Fig. 3) and was accompanied by the parallel reduction in microthermic elements. For instance, *Pieris callidice*, a microthermic, xerophilous species of the open herbaceous environments of the alpine belt, disappeared completely.
We also observed a strong general ingression of the widespread and common species (e.g. Vanessines), all characterised by high vagility and high ecological tolerance, while little mobile species came in reduced frequencies. Species linked to the wooded habitats (nemoral species) strongly increased in frequency, apparently to the expenses of species linked to the open herbaceous environments occurring above the tree-line (subalpine and alpine belts).

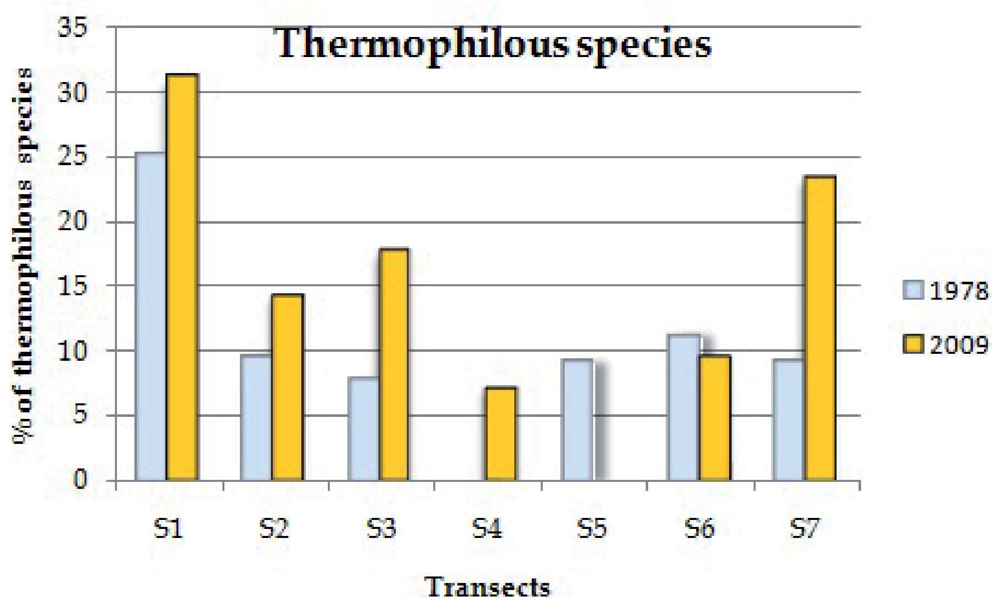


Fig. 3. Percentage of thermophilous species in each transect (classified as S1-S7) through the analysed time frame. Different colours refer to different sampling years (blue=1978; yellow=2009).

The sampling design adopted for the 2009 field season allowed us to demonstrate a phenological shift in the flight period of some species, which in 1978 occurred between the end of July and the beginning of August, but which we only recorded at the beginning of the sampling season, in 2009. The case of *Anthocharis cardamines* is somewhat peculiar, since in 2009 it reached the peak of its flight period in the middle of June and was never observed after the beginning of July, while in 1978 it was observed in 5 of 7 transects at the beginning of August.

Changes observed in community compositions and phenologies apparently went in the direction of the predicted impacts of climate changes. We did not observe any decrease in species richness, but rather a number of changes in the composition of species assemblages. Indeed, species responded individually to the changing environmental conditions (see Hengeveld, 1990). In particular, we wish to stress the loss or strong decline in a number of species whose characteristics made them potentially sensitive to climate change, e.g. the geographically localized, the poor dispersers, or the ecologically highly specialized (see McNeely, 1990).

The analysis of habitat changes identified by photo-interpretation revealed strong variation in land cover between 1978 and 2006 (most recent pictures available), with a generalised spread of forested areas (trees and/or shrubs) and a reduction in the size of grasslands. This is fully in agreement with changes observed in the composition of butterfly communities as regards individual species and their environmental preferences. The observed increase in vagile and tolerant species perfectly fits to the same pattern. The reduction of open surfaces, in fact, will not be a problem for the highly mobile species, which can easily reach another suitable site, while the less mobile species will remain 'locked' in their continuously

shrinking micro-environment (Boggs & Murphy, 1997; Shreeve, 1995). In the same way, broadly tolerant species will take advantage of a '*changing environment*', because of their strong adaptability, to the detriment of the stenotopic and more specialized species (Krauss et al., 2003; Shreeve, 1995).

In contrast, a first analysis of our climatic dataset did not provide any strongly supporting information. The analysis of temperature data did not show any clear pattern. Within the time frame between the beginning of the 1980s and 2010, we remarked only a slight increase in maximum temperature. A similarly unclear pattern was recorded by the analysis of precipitations, both in term of rainfall and in number of rainy days. Even though climatic data will have to be analysed more in detail, it is difficult, at the moment, to understand which factor(s) may be responsible for the observed vegetational shifts, or may have determined the observed changes in butterfly communities.

As we have already pointed out, the influence of climate change in the European Alps is regionally confused by human activities. Cattle grazing in the alpine pastures has been decreasing throughout the last century, allowing a fast recolonization by trees and shrubs, everywhere the treeline had been artificially lowered (Vittoz et al., 2008). Most of the observed changes in butterfly communities, however, cannot be explained only in term of changes in land use: the general increase of thermophilous species and the reduction or disappearance of some alpine and microthermic species can be a signal of a changing climate.

The same is to be said for the observed phenological shifts, even though the latter might be a consequence of weather variability during a single season, rather than of a more general pattern of warming climate.

The importance of this work derives from the fact that many previous studies aimed at understanding the effects of climate changes on community composition, relied on the comparison of contemporary data with historical data sets (atlases, collection specimens), which were often collected in a non-standardized way and referred to a much coarser spatial grain. This makes difficult to exactly detect altitudinal changes in species distribution, as well as to analyse the respective roles of single environmental factors. For these reasons, transects set in well-specified areas are more appropriate for investigating the altitudinal range shifts occurring in response to contemporary climate warming (Archaux, 2004). We suggest, however, that to gain full understanding of the underlying patterns and the relative roles of different causes, it will be fundamental that transects are repeated both in space (i.e. along altitudinal gradients) and in time, over a suitable number of years. Our data from the W Italian Alps, taken on exactly the same sites at a 30 years distance fulfil, in principle, all these needs.

2.3 The ingression of alien species

Climate change and global warming have promoted, in the last decades, the arrival and expansion of several alien species in Europe. Over the past 60 years it is estimated than about 130 exotic pests have become acclimatized in Italy and 7% of them are Lepidoptera. Most aliens species came from America, Asia, Africa and Australia (Pellizzari et. al., 2005).

The main risk factor for alien species entry is the accidental introduction owing to intensive commercial exchanges of plants and goods and an ever-increasing tourist traffic. For millennia, the natural barriers of oceans, mountains, rivers and deserts provided the isolation essential for unique species and ecosystems to evolve. In just a few hundred years

these barriers have been rendered ineffective by major global forces that combined to help alien species travel vast distances to new habitats and become invasive species.

The impacts of alien invasive species are immense, insidious, and usually irreversible. They may be as damaging to native species and ecosystems on a global scale as the loss and degradation of habitats. The scope and cost of biological alien invasions is global and enormous, in both ecological and economic terms, indeed the direct economic costs of alien invasive species run into many billions of dollars, annually.

Cameraria ohridella is a tiny moth of the family Gracillariidae and a very well known leaf-miner of the common Horse Chestnut. It was first observed, and immediately described as a new species, in the northern Greek region of Macedonia, where its food-plant is native, during the early 1980s, but it quickly invaded most of Europe (Austria, Italy, France etc) over the following two decades. The rapid geographical expansion of this parasite was mainly correlated to the passive transportation of infested plant leaves or adult moths, probably in cars or in other vehicles. As a consequence, it soon reached most of the big cities of Central and South Europe, where the Horse Chestnut is abundantly cultivated as an ornamental tree (Ferracini & Alma, 2008). Larvae develop almost exclusively on the white-flowered (not on the red flowered) species (*Aesculus hippocastanum*) to which they cause significant damage by digging their way into the leaves and stunting growth. Infected leaves are covered in small brown patches, which rapidly spread across the entire tree. They give trees an autumnal appearance and cause important aesthetic impact. The invasive success of this parasite relates both to its high rate of population growth and high dispersal capability. Once established in a site, its populations reach outbreak densities within a few years. Several generations of this moth develop per year whilst the impact of natural enemies is still limited (Settele et al., 2010). The low incidence of parasitism of the horse chestnut leaf-miner by European parasitoids, in fact, is at least partially a matter of poor synchronization between the life cycles of the native antagonists (parasitoids) with that of the introduced host moth. Therefore, the lack of suitable host when the parasitoids are ovipositing will inhibit their adaptation process (Grabenweger, 2004).

Another moth, *Paysandisia archon*, of the Castniids family, is native to Central America (Argentina and Uruguay) and was accidentally introduced into Europe, where it is spreading rapidly. In its natural range, the species is not considered a pest, but in Europe it is causing increasing concern because of the sometimes fatal damage produced to both native and exotic palm trees. The main symptoms of this infestation are in the deformation of leaves, some of which become haggard, yellow in color and bored. Larvae dig tunnels through the stem or the young leaves, causing characteristic damage (Vassiliou et. al., 2009).

Yet another, slightly different, example is provided by *Danaus chrysippus*, a widespread species in the paleotropics, which was observed for the first time in Italy (Campania) by Ochsenheimer in 1806. It soon became extinct in that area, only to reappear in Apulia in 1983. While there are doubts on whether or not its first arrival was due to passive introduction (contemporary authors blamed ships of the British Navy), its reappearance was quite natural, and probably a consequence of global warming. During the last 20 or 30 years the species has spread to the whole Mediterranean basin. Its larvae grow on Asclepiadaceae originally imported for the production of 'wild cotton'. Strictly speaking, therefore, we cannot consider this a really alien invasive species. The apparently recent arrivals of *Zizeeria knysna* (see Romano & Romano, 1995) to the tiny Italian island of Lampedusa, the recent burst of invasiveness shown by *Thaumatopoea pityocampa*, or even the fluctuating invasiveness of *Lymantria dispar* are to be viewed in a similar framework.

Some studies have highlighted that only a tiny proportion of non-indigenous species become invasive and most invasions occur in man-dominated rather than in pristine ecosystems. Moreover, indigenous and non-indigenous species are sufficiently similar that their impacts may not necessarily be different and there is evidence that introduced species will sometimes augment, rather than reduce, species diversity (see Hulme, 2003 for a detailed analysis).

However, we believe that invasive alien species are a major threat to biodiversity, as well as, sometimes, livelihoods. Many countries have limited resources to prevent the introduction and spread of invasive species, or to prioritise and implement surveillance, eradication and control. A key constraint is in the lack of direct access to suitable data and information. On a global scale, data on invasive species are sparse, geographically biased, of variable quality, and expensive to obtain. Yet, the mobilisation and improved accessibility of existing global information would represent a significant resource at national and regional levels.

Different kinds of information are needed for assessing risks of establishment, spread, negative impact and difficulties in management. For each species, information requirements for spatial modelling include native and introduced range, point occurrence/observation data as well as climate layers at appropriate resolutions. Another key piece of information is 'invasiveness elsewhere', since "only one factor has a consistently high correlation with invasiveness: whether or not the species is invasive elsewhere" (Wittenberg & Cock, 2001).

Generally speaking, hundreds of extinctions may have been caused by alien invasives. The ecological cost is in the irretrievable loss of native species and ecosystems, while the direct economic costs of alien invasive species may annually run into the billions of dollars. It is in this framework that the relevant IUCN SSG has identified the problem of alien invasive species as one of its major initiatives at a global level (see http://www.issg.org/database/welcome/).

2.3.1 Study case: The Geranium Bronze

The butterfly *Cacyreus marshalli* (Lepidoptera: Lycaenidae), commonly known as Geranium Bronze (Fig. 4, a-c), is an invasive species native of South Africa currently occurring in many parts of Europe and the Mediterranean area.

During our studies we have investigated the *C. marshalli*'s ability to spread to native *Geranium* spp. and evaluated the conservation risks that such a shift would pose for both native geraniums and cohabitant butterflies. In Europe, *C. marshalli* larvae normally feed on pelargoniums causing damage to flowers, stems and leaves (Fig. 4, b). Seriously affected plants may die as a result of the infestation. As a consequence of the fast spread and dangerousness of this pest, pelargonium were included among the species requiring quarantine and *C. marshalli* was listed as a A2 quarantine pest by the European and Mediterranean Plant Protection Organization. The key factor of its invasiveness is the relationship with the host food plant: in particular its potential to spread to native *Geranium* spp. Recently the host plant preferences of the Geranium Bronze were investigated under controlled conditions (Quacchia et al., 2008). Studies included 9 Italian native *Geranium* spp., which commonly occur in many mountainous and hilly habitats of north-western Italy, showed that under no choice conditions, at least one egg was recorded on each tested plant, except for *G. phaeum* and all the plants on which oviposition occurred were fully suitable for larval development. No statistical differences were detected in the wingspan between adults emerged from *Geranium* (Fig. 4, d) and *Pelargonium*. *Cacyreus marshalli* represents a

potential threat for both native geraniums and for geranium-consuming lycaenids, such as *Aricia nicias* and *Eumedonia eumedon*. Since the Geranium Bronze is multivoltine and accepts to lay on leaves and stems and not only on flowers, it would probably out-compete the other two, both monovoltine, in areas of sympatry. As concerns the probability that a shift onto the native geranium species can occur in natural conditions, the voltinism-suitability hypothesis suggests that the diet breadth is restricted to hosts supporting the most rapid larval development where populations are near the thermal limits. In contrast, when the same number of generation can be achieved without difficulty, relaxed selection will permit females to oviposit on hosts on which larval growth is lower (Scriber & Lederhouse, 1992). Variation in oviposition preference behaviour among different host species is heritable and responsive to selection (Renwick & Chew, 1994; Thompson & Pellmyr, 1991). The evolutionary mechanisms allowing or favouring the shift to a "new" larval host plant are rather obscure. Some authors have argued that species invasions can increase, rather than reduce, species diversity (see Hulme, 2003 for a detailed analysis). This may be true also for *C. marshalli*, but only in man-dominated, urban ecosystems. Should this species be able to spread to natural environments it would probably cause considerable ecosystem impact, by affecting both native geraniums and geranium eating lycaenids. Even apart from this, as suggested by Trematerra & Parenzan (2003), the adaptation of this lycaenid to autochthonous plants may favour its spread and establishment not only in Italy and in Mediterranean areas, but also deep into mainland continental Europe, causing serious economic and environmental losses.

Fig. 4. *Cacyreus marshalli:* a) adult; b) larva; c) pupa; d) *Geranium sanguineum*. Courtesy of S. Canterino and D.S. Ossino.

Cacyreus marshalli has never been reported as a pest species in its area of origin, probably because some autochthonous parasitoids and predators are able to keep its population under the damage threshold. In our study we have not observed any parasitoid or predator. Among parasitoids, only Sarto i Monteys (1992) reports one egg of *C. marshalli* parasitized by *Trichogramma evanescens* Westwood (Favilli & Manganelli, 2006). The introduction into Europe of the Geranium Bronze is having great impact on the plant-nursery sector, with a consequent decrease in the demand of *Pelargonium*, which are ever more often replaced by customers with other ornamental plants. Even though in nurseries the control of the pest may be carried out through the aid of common insecticides, this is not feasible elsewhere, particularly in the mountainous and hilly habitats where wild Geranium species (Fig. 4, d) commonly occur. For the regions of Piedmont and Aosta Valley, Pignatti (2002) reports the occurrence of 18 Geranium species. Should an adaptation to these native species ever occur, *C. marshalli* would become a real threat for the native flora, as well as for local biodiversity.

3. Conservation

Biological conservation is, by definition, a work in progress. Biodiversity is under ever increasing or sometimes shifting threats, invariably, although sometimes indirectly, generated by human intervention. The task of preserving biodiversity is increasingly difficult and none of our results, no matter how apparently positive in the short or intermediate term, is to be taken as granted forever. Humans increase in numbers, and even more so is increasing their use of land and energy; climate, at least partially as a consequence, changes to unprecedented rates; vegetation structure becomes disrupted and landscapes change, becoming increasingly uniform and banal. A conservationist’s work is never done.

This does not necessarily mean, however, that biological conservation is a doomed initiative, which will never see any lasting success. Many new instruments of intervention have become available to conservationists in the past several years, and many new ones may be expected to be developed in the near future.

We will discuss some of these new instruments in the following pages.

3.1 The Italian biodiversity hotspots

As it has been mentioned already, Italy is a highly species-rich region, in comparison to central and northern European countries, and the Italian fauna is generally well known, at least taxonomically and geographically. A minimum of 59, 302 native Italian animal species are currently known to exist, not less than 5845 of which are endemic to the Italian political territory.

For N European scientists, travelling in such a biodiversity-rich region may certainly be a dazzling experience, but having to deal with such a complex fauna makes a conservationist’s work extraordinarily difficult. Species interact in many ways, they form very complex food-webs and they co-operate or compete with each other in unexpectedly intricate ways. Most of these interactions are still unknown, many are only suspected or insufficiently substantiated. The embarrassing truth is that for most of our extraordinarily rich fauna we only have descriptions of the external morphology, sometimes, but not always, accompanied by some detailed information on their geographical distribution over the Italian territory. Even this, however, may be important from a conservationist’s point of view.

Identifying regions having extraordinarily high species richness has been recognised as an important conservation tool. The notion that some areas have highest biological diversity (biodiversity hotspots) has been long established. Originally Myers et al. (2000) defined biodiversity hotspots as areas "where exceptional concentrations of [endemic] species are undergoing exceptional loss of habitat", but this concept has become increasingly generalised, in more recent times, to cover all aspects of biological diversity. Hotspots of species richness are generally large areas, where conservationists will be able to create one or more reserves covering a broad range of species. A shift of emphasis from the macro-geographical to the local scale, however, has generated several difficulties. At the regional level, hotspots may harbour large numbers of common and widespread species, rather than endemic, rare, or threatened species. In other cases, they may represent areas where field studies, rather than species, have been concentrated and species distribution reflects the distribution of taxonomist, or of their sampling efforts.

In a recent work (Balletto et al., 2010) we analysed species composition across the whole Italian political territory and over a relatively broad sample of taxa, each characterized by a range of often idiosyncratic ecological requirements.

The presence or absence of individual species was sampled over a 10x10 km UTM grid and across the Italian territory, considering 471 species of zygaenids, butterflies, carabine coleoptera, amphibians and reptiles. We analysed their distributions in terms of hotspots of species richness, of rarity and of complementarity and compared the efficiency of these methods in evaluating local animal diversity. Our aim was to compare methods to assess biological diversity, and we were not dealing, as a consequence, with functional biodiversity.

We carried out this work both at national and at regional level, trying to understand if high diversity areas extracted at national level could be taken as representative of regional situations. At national level, out of 3218 10x10km UTM quadrats sampled, 161 (5% of total) had highest species richness. Islands included only 1 hotspot (Sicily). Sixty-eight species (14.4%) were not represented. They were mainly endemic (65%), insular (73.5%), or rare (25%). Working taxon by taxon, hotspots increased to 433. Only 85 (19.6%) were hotspots contemporaneously for two taxa and only 9 were hotspots for 3 taxa. Missing species were fundamentally insular species. The regional-level approach generated 467 hotspots. Eleven species were not represented (2.3%). They had marginal distributions, or were insular endemites. Hotspots of rarity numbered 235 and 10 species were not included. Results demonstrated that hotspots of high species richness are poor predictors of overall biodiversity and that they rarely coincide among animal groups.

The complementarity method identified 67 quadrats (Fig. 5). By definition, complementarity quadrats accounted for all species investigated. They failed, however, to predict the occurrence of three zygaenids, which were previously not included in the analysis. As expected, complementarity provided better results than hotspots analysis and one can assume that combining the two methods will assure that areas having the highest biodiversity values are identified. Regional or rarity hotspots should generally be preferred to hotspots of species richness and among the latter those calculated at regional level should generally be preferred, as well as hotspots of rarity. At the moment, the perfect method for assessing regional biodiversity probably does not exist. Whereas hotspots analysis aims at providing an absolute measure of local biodiversity, complementarity does not and takes a more pragmatic approach to intermediate scale conservation practice. Combining the two

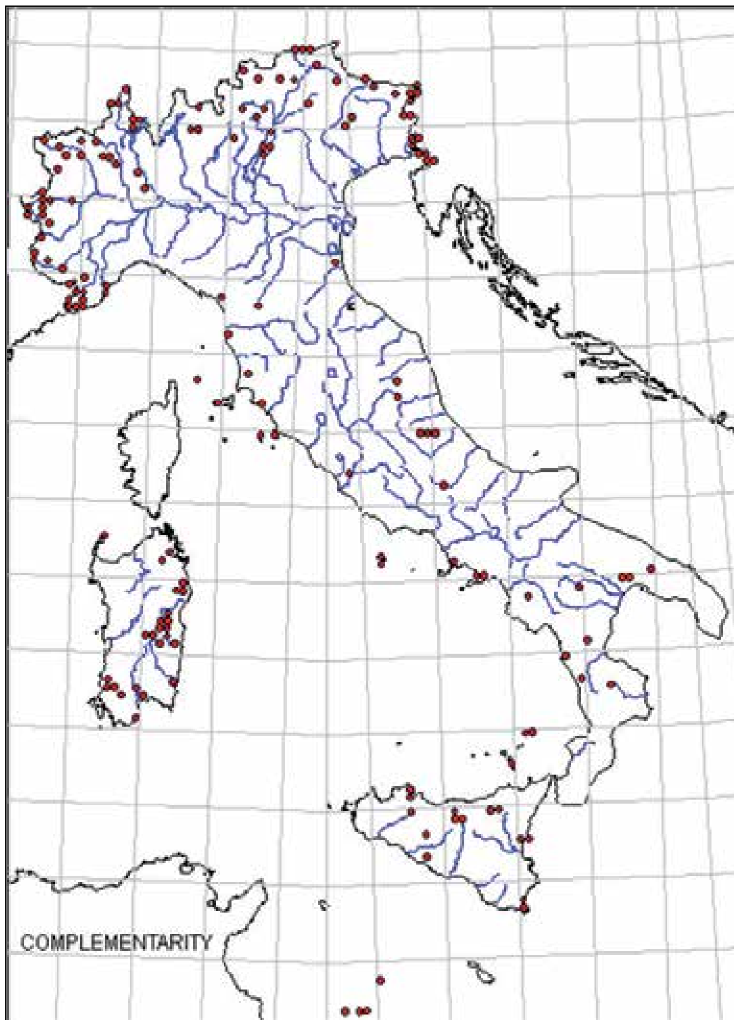


Fig. 5. Quadrates identified by complementarity analysis.

methods will assure that areas having the regionally highest biodiversity values are identified, even while working with necessarily incomplete or skewed databases. In these areas, conservationists will be able to create one or more reserves covering the broadest possible range of species, for that particular region.

3.2 The NATURA-2000 network

Partially shifting the emphasis from the community to the species level, the European legislation and in particular the "Birds Directive" (79/409/EEC) and the "Habitats Directive" (338/97/EEC) respectively require Member States to designate "special protection areas" and "special areas of conservation" that will eventually become integrated into the NATURA-2000 network of Sites of Communitarian Importance (SCIs) and finally coalesce into Special Areas of Conservation. Sites to be included within this network are designated either to ensure the appropriate conservation of some [bird or otherwise] species

deemed to be threatened within the European territory, or of a combination of other threatened animal or plant species, and of their habitats.

From a conservationist's perspective, once the designation phase is completed, this approach is expected to ensure that all species and habitats of Communitarian Interest may be afforded suitable protection. As an indirect consequence, however, it is possible that many other species, either not threatened or unknown to be threatened, are also potentially protected, in a way that is in many respects similar to the "umbrella" concept of some Conservationists.

The overall effectiveness of this process in generating a coherent network of globally important conservation areas, however, has been tested rarely and effects of its implementation on a local basis have been questioned (Troumbis & Dimitrakopoulos, 1998; Dimitrakopoulos et al., 2004).

As concerns the Italian NATURA-2000 network in particular, Maiorano et al. (2007) have investigated its potential effectiveness in affording terrestrial vertebrate species a favourable conservation status. For their analysis, these authors used available distribution models (DMs). These models, however, are generally not available for invertebrates, which represent a conspicuous percentage of threatened species.

Recently (Bonelli et al., [submitted]) we tried to assess, by actual distribution data, the proportion of the Italian terrestrial invertebrate and small vertebrate species that will be included in protected territory once the NATURA-2000 network is set in place.

For each of 429 species investigated, we calculated the ratio between the number of data of presence within the SCIs/SPAs protected sites and in non-NATURA-2000 areas ("observed value"), and we compared this value with the ratio between the areas of protected and non-protected sites ("expected value"). For individual species, the "expected value" represented the proportion of presence data that are expected to fall within a NATURA-2000 area, in case presence data were scattered at random.

Most species (427 of 429 analysed) were represented at the sites proposed for the Italian NATURA-2000 network. Only 2 species were missing, namely one butterfly (*Boloria eunomia*) and one carabid species (*Carabus* (*Hygrocarabus*) *nodulosus*). They are both rare species, which marginally enter the Italian territory, where they occupy respectively 1 and 2 UTM quadrats. Neither of them is considered threatened in Europe, at least by the Habitat Directive.

The Italian NATURA-2000 network (Fig. 6a), once fully implemented, will represent a suitable instrument to foster the conservation of many invertebrate and smaller vertebrate species. As we said, since a vast number of these sites are coincident with areas encapsulating very high biodiversity (Balletto et al., 2010), their general importance may partially transcend the currently prevailing species-by-species approach to biological conservation. The geographical distribution of SCIs on the Italian territory ensures that they include a significantly larger number of species than random. Most of the Italian NATURA-2000 sites, however, are small and this, of course, does not make them very suitable for some larger vertebrates (Fig. 6b). As it is the case in most of Europe (Gaston et al. 2008), in fact, the geography of the Italian territory, combined with high human-population densities makes identifying more numerous large-sized NATURA-2000 areas impossible. Large NATURA-2000 sites are often located within the "historical" Parks, which were created well before European Directives were even conceived, and at a time when the "ecosystem level" conservation philosophy was prevalent, at least in Italy.

In this work we showed that in most cases the main problem is the lack of type of management on which species' survival depends.
As we have already shown, a further threat to Italian biodiversity will be represented by climate change. Range shifts will pose major problems to conservation, as species that are already protected might actually shift their distribution out of the currently protected areas which were designated to preserve them. Networks of designated sites, including the NATURA-2000 network, should be managed flexibly.

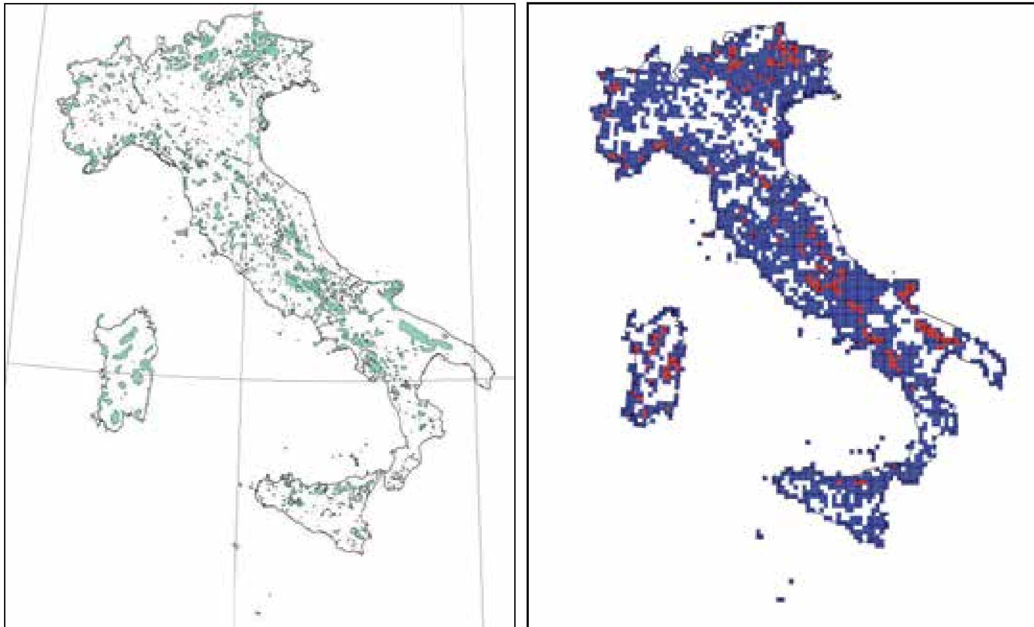


Fig. 6. a) The Italian NATURA-2000 network of protected sites (terrestrial sites only); b) Italian UTM-MGRS quadrats including over 10 ha (blue) or over 5000 ha (red) of NATURA-2000 protected sites.

3.3 The European "Red list"

The European Red List is a review of the conservation status of about 6,000 European species of dragonflies, butterflies, freshwater fishes, reptiles, amphibians and mammals, as well as of some selected groups of beetles, molluscs, and vascular plants. Ranks, ranging from Extinct (EX) down to Near Threatened (NT), or Data Deficient (DD), Not Evaluated (NE), etc. have been given to individual species according to the IUCN (International Union for Conservation of Nature) regional Red Listing guidelines (IUCN 2001). In 2010, the IUCN, together with Butterfly Conservation Europe (BCE) and in collaboration with the European Union published the Butterfly Red Data Book (van Swaay et al., 2010). The conservation status of a total of 482 European species was assessed for the purposes of this list. Almost a third of these species (142) are endemic to Europe. Species classified as either threatened (i.e. Vulnerable (VU), Endangered (EN), or Critically Endangered (CE)), or Near Threatened (NT) represent 19% of the total and are thus deemed to be of high conservation priority. A high proportion of threatened and Near Threatened butterfly species are endemic to either

Europe or the EU. This is particularly true for France, Italy, Spain, Greece and Bulgaria and, more in general, most of the threatened species are confined to one or more parts of southern Europe. The main current threat to European butterflies was recognised to be in the loss of their habitat. In many cases habitat connectivity is also important, being challenged by changes in agricultural practices, either through intensification or abandonment. Other important threats are climate change, increased frequency and intensity of fires and development of tourism and related infrastructures. As BCE partners, we provided all the necessary data on the Italian butterflies. Based on this list, in Italy we have three Endangered (EN) butterflies (*Maculinea arion, Polyommatus humedase* and *Coenonympha oedippus*), as well as seven Vulnerable (VU) species, while another 21 species are deemed Near Threatened (NT).

3.4 The "Butterflies Prime Areas"

The principal aim of the "Prime Butterfly Areas" European project was to identify the most important areas where conservation efforts should be focused as a matter of urgency. The objective of this project was of implementing the Red Data Book of European Butterflies and to suggest management measures for these areas. A site was classified as a Prime Butterfly Area if it contained a substantial resident population of at least one species which is threatened according to the Red Data Book of European Butterflies (van Swaay & Warren, 1999), or to the IUCN Red List of threatened animals. These species are called target-species. As with the Red Data Book, all data were provided by national compilers who were asked to select the 'best', in this respect, areas for every country, and to accompany their lists with all necessary information on location, key butterfly species, land uses, threats, and needed or already implemented conservation measures.

Following the criteria set by van Swaay & Warren, 32 Prime Butterfly Areas (PBA) have been identified in Italy as a first selection of the most important areas for 16 species deemed to need protection (Balletto et al. 2003 [in van Swaay et al. 2003]).

The main purpose here is therefore to inform about this selection and to provide definitions for the sites forming the Italian section of the NATURA 2000 Network. Italian Prime Butterfly Areas were identified by using 16 target species. In total, they cover 365, 209 ha of Italian territory.

The 16 target species used to identify the Italian PBA are: *Argynnis elisa, Coenonynpha oedippus, Erebia calcaria, Erebia christi, Euphydryas aurinia, Lopinga achine, Maculinea arion, Maculinea rebeli, Maculinea teleius, Melanargia arge, Papilio hospiton, Parnassius apollo, Plebejus trappi, Polyommatus galloi, Polyommatus humedase, Pyrgus cirsii*.

Two of them, *Polyommatus galloi* and *Polyommatus humedase* (IUCN global threat status: Endangered, based on the Italian Red List) are restricted to as many small areas and represent 100% of the global (and of course European) population of those species.

Rare and threatened butterflies are not widely distributed species and often occupy very small sites. In the 32 Italian PBAs the most common types of land-use are nature reserves, touristic and agricultural areas.

As concerns these areas, a very important threat is in their isolation, since the distance between two areas is sometimes too large to allow adults' interchange. Also management types (or lack of management) and hectic natural events represent a common problem. All sites are in strong need that active management practices are planned and implemented as soon as possible. Providing a correct management of Prime Butterfly Areas will not only

support the conservation of a few target-species, but will allow to reach a number of other far-reaching objectives fostering the conservation of many butterflies and insect species in general.
Preserving biological diversity for the future generations is the single most important objective of conservation biology.

3.5 The role of the Alps in butterfly diversity conservation

Many European butterflies species are restricted to the alpine, sub-alpine and mountain habitats with 25 species confined in the Alps and other 66 with a wider distribution in mountain habitats. The Alps are among the most important hotspots of endemism in Europe (Essl et al., 2009). As concerns their distributions, narrow-range species were strongly influenced by Pleistocene glaciations, both in the case of plants (Tribsch, 2004) and of some invertebrates, including butterflies (Hüemer, 1998). Since the Alps are the largest and highest mountain system in Europe, it is not surprising that a high number of endemic species, typical of the mountain environments, is found here, as observed in many Lepidoptera and especially in micro-moths (Varga & Schmitt, 2008). Some of these endemic species are distributed throughout the Alps, while others are local endemism found only in parts of them. Two types of local hotspots of endemism have been identified. The first group includes species whose ranges are restricted to peripheral regions of the Alps and mainly confined to the intermediate and low altitudes, as observed in some species of the genus *Erebia* and in some Lyceanids (Varga & Schmitt, 2008). The highest concentrations of these local endemics are found in the southwestern and southeastern regions of the Alps, which include large areas at low altitude, not covered by ice during glaciations. These areas were probably used as centers for glacial survival, where these species experienced shifts in altitude but not expansion of their range on a large scale (Schmitt, 2009). Shifts in elevation, in response to Pleistocene climatic fluctuations, may have repeatedly promoted evolutionary processes and speciation. The second group is represented by species confined to the inner parts of the Alps, such as a large number of micro-moths (Hüemer, 1998) and some butterfly species (i.e. *Erebia nivalis*) (Varga & Schmitt, 2008). These species are generally restricted to high mountain habitats and might have survived glaciations in small ice-free areas, on the slopes of the inner Alpine mountains (Schmitt, 2009).
The Italian fauna includes 283 species of native butterflies (Balletto et al., 2007), 106 of which are located in the Italian Alps (25 are strictly alpine) and 64 in other mountain habitats. A recent study has identified the Alps as one of the most important biodiversity hot-spots of the Italian peninsula combining the use of the niche- modeling with a complementarity-based method called zonation (Giradello et al., 2009). In the Italian Alps, butterfly species form loose associations, characterized by low inter-specific competition for space and other resources (Tontini et al., 2003). Density-independent processes (Den Boer, 1998) generally determine the population sizes. Butterfly communities are composed by various combinations of stenotopic species, occurring together with some erychorous or, sometimes, migratory species. Most of the butterfly associations are inseparably associated with vegetational types, along a successional gradient. This seral connection, however, breaks down above the treeline, dominated by grasslands where the ecological conditions are characterized by a unique set of physical and biotic parameters (Nagy, 1998; Ozenda, 1985). These grasslands are well represented in the Italian Alps, and are generally less affected by human activities than at lower elevations (Tontini et al., 2003).

Perhaps the single most important factor that will affect the mountains system in the future is global warming, which will also have severe effects on animal communities. In the last two decades, climate changes were associated with increases in northern latitudinal limits (Hill et al., 2002; Parmesan et al. 1999), elevational shifts (Descimon et al., 2006), contraction of range size (Parmesan et al., 1999) and phenological modifications (Roy & Sparks, 2000; Stefanescu et al., 2003). At high altitudes, continuous changes in temperature and other climatic parameters are expected to have a strong effect on plants and the associated animal assemblages (Beniston et al., 1996; Walther, 2003). A study on *Boloria titania* and its foodplant *Polygonium bistorta* strongly suggests that climate change has a potential to disrupt trophic interactions because co-occurring species do not react in a similar manner to global change. *B. titania* might considerably expand its future range in case its host plant (*Polygonum bistorta*) has unlimited dispersal, but it may lose from 50 to 75% of the its current distribution if the host plant will be unable to fill its projected ecological niche space, or from 79 to 88% if the butterfly is assumed to have highly limited dispersal (Schweiger et al., 2008).
Climate change is already impacting many populations across Europe (in particular the tundra species, such as *Colias hecla* and *Euphydryas iduna*) and is likely to affect additional species even more significantly, in the future (Settele et al., 2008). In the recent Atlas of Climate Risk, Settele et al. (2008) examined the current distributions of 294 European butterflies and evaluated their future ranges at the years 2050 and 2080, on the basis of variations projected on 22 climatic variables. Their results predict important losses in climatically suitable areas, particularly in the South of Europe and the Italian peninsula. The Alps, in contrast, where many SCIs and Bird areas are located, will further increase their importance for biological conservation, since many stenotopic species will become concentrated in this area. Strict stenotopy is generally a consequence of adult behavior, rather than the larval biology and the genus *Maculinea* offers a rare example in Europe.

3.5.1 Habitat preferences of *Maculinea arion* in the Alps

Projections of climate change scenarios predict that many of *Maculinea arion* populations will disappear from Europe in the next 50 years and those close to the southern limits of the species' range will be the most threatened (Casacci et al., 2011). While the vast majority of the Italian populations of *M. arion* are expected to become extinct, some will survive in mountain areas, especially in the Alps (Settele et al., 2008 – Fig. 7).
M. arion is a lycaenid parasite of ants of the genus *Myrmica* and its survival depends on the presence and abundance of two resources, a specific foodplant, *Thymus pulegioides* and one or more *Myrmica* host ants. We collected data on the distribution of thyme and on the abundance of *Myrmica* ants. We measured sward height close to the each nest, as an indicator of the microclimatic niche, the distance between the ant colonies and the butterfly foodplant, as well as the structure of vegetation patches in 14 in the Western Italian Alps (Val Ferret: Aosta) (Casacci et al., 2011).
Results from our study showed that colonies of different *Myrmica* species occur at different distances from *T. pulegioides* plants, probably as a consequence of their different microhabitat requirements. *M. sulcinodis* (one of the host-ant species recorded together with *M. lonae*) colonies occur in the nearest range with respect to *M. arion* foodplants, which can suggests that they have similar habitat demands as those of Thymus foodplant. Therefore, larger overlapping and smaller distances can results in higher probability of adoption for *M. arion* larvae, since a higher number of foraging workers can be found in the near

Fig. 7. Alpine habitat of *Maculinea arion.*

surrounding of Thymus plants. *M. sulcinodis* is, moreover, also the most abundant *Myrmica* species on pastures with a high number of *M. arion* adults, whereas it is a scarce species on patches with lower butterfly abundance. It seems, therefore, that *M. arion* mostly inhabits patches where these two resources fully overlap each other.

Thomas and his colleagues (1998b) have pointed out that, for conservational purposes, it is necessary to have precise information about the sward height and the niche preferences of the host ant species. In England, a strong correlation between the turf height and the *Myrmica* community was found (Thomas et al., 1998b, 2009). In particular, M. *sabuleti* (the unique host-ant of English *M. arion* populations) has a very narrow niche, corresponding to a grass height ranging between 0 and 3 cm. The ant's density decreases with increasing grass height, until disappearing over 7 cm (Thomas et al., 2009). Thus, inconspicuous changes in grazing regime and vegetation structure have caused the host ants to be replaced by similar but unsuitable congeners, explaining the extinction of *Maculinea arion* populations in 1979 (Thomas et al., 2009).

In Val Ferret, the host specificity of *M. arion* is apparently not limited to one single species of the genus *Myrmica*. Moreover, it appears that each *Myrmica* species requires relatively broader niches in relation to the grass height. In the Italian Alps, the greater tolerance of the *Myrmica* species with respect to increasing grass height, may be the result of much more pronounced daily and monthly temperature fluctuations. Data obtained in Val Ferret during July 2009 show that the average daily maximum temperature was 27 °C,

while the minimum temperature was about 11 °C. In contrast, climatic data collected in England at *M. arion* sites show that the average daily maximum temperature is 19.5 °C while minimum temperature is 13.5 °C. Thus, the much more pronounced daily and monthly fluctuations occurring in Val Ferret might have pushed *Myrmica* species to become much more adapted to broader niches and tolerant with respect to marked changes in the sward height.

3.5.2 Larval ecology of *Colias palaeno*

Colias palaeno (Lepidoptera: Pieridae) is classified as vulnerable to climate change by the authors of the '*Climatic Risk Atlas of European Butterflies*' (Settele et al., 2008). In recent years it has undergone a great decline in many parts of Europe, in particular in the low altitudes of South Germany, where it is present only on some peat bog margins, while it disappeared in the last ten years from many, apparently unchanged, habitats (Dolek et al., 2007). One possible explanation could be linked to larval survival, before and/or after overwintering. Peat bogs are environments characterized by high humidity levels on oligotrophic soils, and are potentially vulnerable to changes in air temperature, rainfalls regime and general atmospheric composition. These changes can determine micro-habitat alterations, including micro-climate and larval host plant characteristics, and consequently the performance and feeding behaviour of the small larvae. *Colias palaeno*'s single larval host plant is *Vaccinium uliginosum.* In the lowlands of South Germany this plant is only present in peat bogs. In the Alps, in contrast, it grows in many different ecological conditions, even in much drier places, such as the *Vaccinium* and *Rhododendron* mountain heaths. In the Alps, *C. palaeno* populations are probably not (yet) threatened, but quantitative data on their distribution and life-history traits in the mountain ecosystems are not available. In recent years (2008-2009), we started on a coordinate project between Germany and Italy, in order to compare larval performances and survival rates in different habitats and altitudes, and thereby trying to understand the main drivers of decline. In fact, in many butterfly species, the larval stages have more specific requirements than those of the adults, because they are less mobile. They are, therefore, strongly linked to the micro-environment occurring at the oviposition site and have longer 'life' than the adults (Albanese et al., 2008; Bergmann, 1999). Understanding larval requirements is essential for identifying what '*habitat quality*' means for the majority of butterflies, while understanding the larval needs of target species may be crucial to design adequate management practices (Ellis, 2003).

Colias palaeno is a spring-developing, but heliophilous, organism and hibernates as a small larva (2nd or 3rd instar). Therefore, this species may be particularly sensitive to the cooling of microclimates in spring, which can be determined by the seasonal advance of plant growth because of climatic warming and nitrogen deposition (Wallisdevries & van Swaay, 2006). Moreover, a specialized association of a monophagous larva with its host species may create an opportunity for climate change to impact on larval feeding (Dennis & Shreeve, 1991). Changes in soil moisture, soil temperature, precipitation, air temperature etc. can affect the nutrient content or palatability of the host plant. Any change in food quality may be harmful to specialised herbivores, since it may force them to increase foraging time, and thereby expose them to predators or increase the potential for food limitation (Ayres, 1993). Dury et al. (1998) have found changes in the digestibility of leaf materials, due to reduced leaf nutritional quality, as a result of reduction in foliar nitrogen concentration and parallel increase in condensed tannin content. In fact, plant secondary chemicals can constrain

herbivore growth by deterring consumption and by interfering with digestive efficiency (Ayres, 1993). Therefore, two separate factors may be potentially responsible for the decline of *C. palaeno* : i) changes in micro-environment structures, in particular in features influencing local micro-climate; ii) alterations in the general leaf quality of the larval host plant.
Some preliminary results obtained on Alpine populations suggest a dependence between polyphenols content and larval survival rate. Higher levels of secondary metabolites could have detrimental effects on larval performance, while lower amounts of phenolic compounds have been observed in the heath lining the timberline, a kind of environment not present in the lowlands area of great declined of South of Germany (*unpublished data*).

3.6 The functional hotspots of biodiversity - The myrmecophilous insect

Pre-adult myrmecophilous butterflies spend a variable amount of time living in association with ants. Most of them are commensal or mutualist organisms, either left undisturbed by ants, or becoming actively protected by them. In nature, we observe many different levels of myrmecophily, ranging from facultative (butterfly larvae are occasionally tended by ants while feeding on their food-plant, more rarely on aphids etc.), to obligate (butterfly larvae cannot survive unless they are taken into the ants' nests where they fulfil their trophic need and spend the climatically worst parts of the year; see Fiedler, 1991). Butterfly larvae, in fact, may be tended within their phytophagous (more rarely carnivorous) foraging areas, or inside the ants' colonies, depending on cases (Hölldobler & Wilson, 1990; Thomas et al., 2005). In Europe, *Plebejus argus* is among the relatively few obligate mutualistic myrmecophilous Lycaenids, and is associated to *Lasius niger* ants. Other myrmecophilous butterflies have evolved as ants' social parasites and most of them are rare in comparison to the abundance and distributions of their ant hosts (Hölldobler & Wilson, 1990; Thomas et al., 2005). Butterflies of the genus *Maculinea* are among the best documented examples of myrmecophilous insects. They are obligate parasites, strictly and only depending on the ants classified in the *Myrmica* genus. Larvae of these butterflies, after spending a short period while feeding on a specific foodplant, penetrate into the *Myrmica* ant-colonies, where they spend 11 to 22 months (Barbero et al., 2009a,b; Thomas et al., 1998a; Witek et al., 2006). Such a close and obligate interaction shelters myrmecophilous butterflies from seasonal climate variations, but leaves them vulnerable to large scale environmental changes, since their survival depends on the persistence of multiple factors, such as a strictly specific larval host plant (LHP) and a similarly species-specific ant. Most of them are rare and protected in Europe (e.g. Habitat Directive Annex II).

3.6.1 Host ant specificity

Regional populations of specialist insect species, such as myrmecophiles, may be adapted to their local environment more strongly, and at smaller scales (Schönrogge et al., 2006), than is recognized in current paradigms of host specificity. Across Europe investigations of the host-ant specificity of *Maculinea* butterflies have demonstrated that patterns are very complicated. Both single- and multiple-host populations are currently known to exist for each of *Maculinea* species, with the only exception of *M. nausithous*, which is associated to one *Myrmica* species (*M. rubra*), both at local and at broad geographical scale (Casacci et al., 2011; Patricelli et al., 2010; Pech et al., 2007; Tartally et al., 2008; Witek et al., 2008). Moreover, on the base of host specificity data obtained for *M. alcon* and *M. rebeli*, Pech et al.

(2007) postulated that single-host specialization tends to develop near the butterfly range edges. Thus, populations such as those of *M. rebeli* in Spain or in France, or of *M. arion* (Fig. 8) in England, may prove even more vulnerable to climate or habitat changes than other European populations.

Fig. 8. *Maculinea arion* on its alternative LHP – *Origanum vulgare.*

The survival of myrmecophilous insects depends on the interactions among several species, so that a precise understanding of host specificity patterns is required before we can set up any appropriate habitat management. This may be even more essential for single host butterfly populations, since they are adopted to an ant-host possessing narrow niche preferences, such as in the case of the *M. arion* populations living in England (Thomas et al., 2009).

3.6.2 Habitat fragmentation and dispersal ability

The myrmecophilous life style and its associated high host-specificity tend to select for a limited dispersal. In fact, *Maculinea* butterflies show very low mobility, compared to most other butterflies (Nowicki et al., 2005a) and distances covered by *Maculinea* individuals typically vary between 50 and 400 m, even though movements of over 5 km were sometimes observed in *M. arion* (Nowicki et al., 2005b). Many insect species are currently forced to live in fragmented populations, and occupy habitats which in the course of time became disrupted into small, isolated patches. Their low dispersal ability makes myrmecophilous butterflies more vulnerable to the effects of low genetic variability, generating high extinction probabilities for some particular subpopulations. Moreover, since they depend on host ants as well as on foodplant availability they are also more sensitive to habitat fragmentation, since their survival depends on the abundance and density of both resources on each particular habitat patch (Nowicki et al., 2007, 2009). Metapopulation studies carried out on *M. arion* in the Alps have shown that only sites with high density of Thymus (foodplant) and host-ant

(*Myrmica sulcinodis*) support high numbers of butterfly adults (Casacci et al., 2011). Similar effects of habitat quality were observed for *Plebejus argus*, whose persistence was determined by the presence and density of mutualistic ants (Seymour et al., 2003).

3.6.3 Land use and management regime

Apart from habitat fragmentation, another main cause for the decline of butterfly populations is in changes in land use. Most myrmecophilous butterflies are associated to some particular type of grassland, either hygrophilous or xerophilous depending on the species. The persistence in time of such habitats is necessarily man-dependent, since they will become invaded by trees and shrubs as soon as they will be abandoned. Many of them, as a consequence, were transferred to other types of land use, while some management regimes such as mowing or grazing were suspended. All across Europe, this resulted in strongly declining biodiversity, during the last decades (van Swaay, 2002). A classical example showing how changes in habitat management may lead to local extinctions relates to the native English populations of *M. arion*, which became extinct in 1979 (Thomas, 1980). In England, the survival of *M. arion* larvae depended on the presence and abundance of only one host ant - *M. sabuleti*. In N Europe, this ant species is associated with short-turf grasslands, and prefers warm and sunny places. Changes in habitat management related to the loss or strong decrease in grazing rabbits (disease), as well as the cessation of grazing by cattle and sheep, caused the short-turf grass to be replaced by longer turf, and resulted in a cooler soil microclimate. This, in turn, favoured other *Myrmica* species like *M. scabrinodis*, which displaced the main host ant *M. sabuleti*. After all these changes the survival of *M. arion* larvae decreased dramatically and finally led to population extinction (Thomas, 1980). Nowadays, thanks to a highly-organized management programme, *M. arion* was successfully reintroduced to its native area, starting from populations from S Sweden (Thomas et al., 2009).
Johst et al., (2006) presented a model showing how mowing regimes influence population persistence in two great blue butterfly species, i.e. *Maculinea teleius* and *M. nausithous.* Factors responsible for density-independent and density-dependent (myrmecophily) mortalities of larval stages were taken into account. Results indicated that mowing once per year, or even every second or third year, was the most suitable type of management for both *Maculinea* species.

3.6.4 Climate changes

It is well know that butterflies react to temperature rises by adapting their phenology to that of their larval host plant and by searching for suitably cooler places by shifting in latitude or altitude (Roy & Sparks, 2000; Stefanescu et al., 2003; Wilson et al., 2007; Settele et al., 2008). Changes in latitude have been observed in some species (e.g. *Polygonia c-album* see Thomas, 2005) but no changes have been demonstrated to occur in many others. Species such as *M. alcon*, *M. nausithous*, *M. teleius* are all strictly linked to the hygrophilous grasslands and, as mentioned above, they have low dispersal capability (Nowicki et al., 2009). Since they typically occupy lowland meadows, these *Maculinea* species will be probably scarcely prone to shift in altitude. It is interesting to notice that even though each *Maculinea* butterfly exploits a species-specific larval host plant at least in most of its range, some species can exploit a second host plant. We can speculate that, while a shift onto a congeneric food-plant might occur in connection with natural dispersion events, or the colonization of new habitats, a shift to a more taxonomically distant LHP might occur as a response to changes

in the phenology of the primary host plant. This could be the case for *M. arion* in Italy, where it ranges from the 200 m of some peninsular sites, to over 2000 m in the Alps, and occurs in a wide variety of habitats, from sub-Mediterranean to Alpine.
We can also speculate that, during the last glaciations, in the north of Italy Thymus-eating *M. arion* may have found refuge in the lowlands at the base of the glaciated Alps (Schmitt, 2009), while it may have survived at higher altitudes, in the hotter latitudes of central and southern Italy. After de-glaciation, following the natural spread of its host plants and ants, the Alps may have been re-colonized (Schmitt, 2009). At the same time, the rising temperatures on the already warm grasslands occupied during glaciations might have resulted in changes in LHP phenology. This may have led *M. arion* to either of two possible responses: i) in the places where, by chance, a taxonomically distant but phenologically similar plant, became successfully exploited, there was a change in LHP (*Origanum vulgare*), or ii) there where these changes were impossible, butterflies were forced to adapt their phenology to that of the Thymus plants (see Fig. 9). To partially support our supposition, we can observe that in each Italian site where *Maculinea arion* exploits *Origanum vulgare* (lowland populations) also Thymus is present, but has anticipated phenology. In these communities, the flight period of *M. arion* occurs at the same time as for the *Thymus*-eating populations occurring in the Alps. On the contrary, in those *M. arion* populations where no host plant shift was possible, butterflies adapted their phenology to their *Thymus* host plants, so that their flight period occurs much earlier than in the alpine, as well as the lowland population of the North of Italy.

Fig. 9. *Thymus* spp. *Maculinea arion* main LHP.

The current global climate warming may be expected to cause similar effects as those of past de-glaciation times. Were this the case, the conservation of the alpine populations of *M. arion,* where an altitudinal shift cannot occur, will be assured, in some areas, by the coexistence of the two possible larval host plants, or in other areas by the capacity of this butterfly to adapt its phenology to that of thyme. On the other hand, the future of those populations which became adapted to a second LHP (*O. vulgare*) will be assured by the butterflies' capacity to adapt their phenology, or otherwise by shifting onto a third host plant, as yet unknown. Before we can evaluate the butterfly's ability to adapt its phenology to that of the host plants, it will be fundamental to gain information on the possible effects of temperature on the phenology of the host-ants' nest. It is well know, in fact, that *Maculinea* larvae acquire most of their body mass after their winter diapause (Thomas et al., 1998a; Witek et al., 2011), i.e. at a time when the nest is active and food (ants' brood) is widely available. In the lack of a full understanding of temperature effects on nest activity, it will impossible to identify the break point between the butterfly's need to adapt to the LHP phenology, on the one hand, and to spend enough time inside the *Myrmica* nest to acquire sufficient body mass to conclude its larval cycle, on the other. Any failure in balancing between these needs will result in a major threat for all those population in which phenological shifts are already observable. An early emergence will be favoured only on those cases when larvae will have eaten enough to pupate early.
Butterflies of genus *Maculinea* have become 'flagships' of European biodiversity conservation (Thomas & Settele, 2004) and are perceived as umbrella species covering many grassland communities (Casacci et al., 2011; Randle et al., 2005; Spitzer et al., 2009). Special conservation efforts should be focused on those habitats where myrmecophilous insects are abundant, since protecting these sites will also allow us to protect many rare plant and invertebrate communities.
In a broader time frame, it will be important for us to gain precise knowledge on the ecological requirements of myrmecophiles under current climate and habitat change scenarios, in order to try and reverse current declines, by restoring optimum ecological conditions (Thomas et al., 2009). Management activities suitable for creating cooler micro-topographies and cooler successional stages will mitigate the impact of climate warming both in the short- and in the intermediate- term.

4. Conclusions

Species' extinctions are rapidly increasing everywhere in the world, as well as in Europe (Hobbs & Mooney, 1998). Species loss proceeds by increasing the extinction rates of individual populations, rather than by sudden and complete species losses (Ehrlich, 1994). Species, in fact, may lose a lot of their local components (i.e. populations) before they become threatened or even scarce in nature and population decline is a reversible event, while species' extinctions are not. So that detecting, understanding and halting this trend must be central in national and regional conservation planning.
Butterflies have attracted wide public attention owing to their endangered status, their beauty as adults and their extraordinary life histories, thereby becoming a flagship group. Their short life-cycle is sometimes very complex e.g. in the case of myrmecophiles. Butterflies have well-known ecological preferences and quickly respond to the action of drivers of change even more and faster than other well-known taxa like birds and vascular plants (Thomas et al., 2004; Warren et al., 2001). In addition they are a good indicator group

for other insects taxa (Thomas, 2005; van Swaay et al., 2010), but at least in Europe they are increasingly restricted to a small and fragmented portion of landscape.
For centuries most of Europe's land has been used by humans to produce food, timber and fuel and provide living space, so that currently in western Europe more than 80% of the land is under some form of direct management (European Environment Agency, 2010). Consequently, European species are to a large extent dependent upon semi-natural habitats created and maintained by human activity, particularly on traditional, non-intensive forms of land management. These habitats are under pressure from agricultural intensification, urban sprawl, infrastructure development, land abandonment, or desertification. Many species are directly affected by overexploitation, persecution and impacts of alien invasive species, as well as by climate change being set to become an increasingly serious threat in the future. Although considerable efforts have been made to protect and conserve European habitats and species diversity (e.g. Butterfly Prime Areas, Red Data Book, NATURA-2000 Network etc.) decline continues to be a major concern in the region.
Because of its geographical position and latitudinal and altitudinal gradients, Italy plays a central role in biodiversity conservation in this changing scenario.

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7

Bee Diversity in Thailand and the Applications of Bee Products

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1. Introduction

This chapter provides information on honey bees (genus *Apis*) and their reasonably close relative group, the stingless bees within the Meliponini Tribe. Their taxonomic position, common morphology and behaviour are defined and explained. Also, a species identification of the four native Thai honey bee species, including the comb and nest structure, worker morphology, species distribution and description of each species behaviour, is summarized. Beyond their role as pollinators, honey bees and stingless bees have important economic, ecological and social values for many rural people in Asia. Especially, wild honey bees are hunted for their products (honey, brood and wax), providing many people with a useful component of household income. Therefore, the applications of bee products, which are important for many rural people in Asia including Thailand, are briefly outlined.

2. The genus *Apis*

Honey bees are classified in the Apini tribe within the subfamily Apinae and family Apidae (Ruttner, 1988). They are part of the large insect order Hymenoptera that includes bees, wasps, ants and sawflies (Gullan & Cranston, 2000). Apis is the only genus of true honey bees and is comprised of the ten Asian species and one Western species (Oldroyd & Wongsiri, 2006).

Some of the most discriminate morphological criteria for worker bees of the genus *Apis* are: the compound eyes covered with erect long hairs, a strongly convex scutellum, the pollen press on the hind leg, the greatly elongated marginal and submarginal cells of the forewing and the jugal lobe in the hind wing (Oldroyd & Wongsiri, 2006).

All honey bee species are highly social insects. Oldroyd & Wongsiri (2006) revealed at least three criteria for defining the eusociality form in honey bees that correspond with that of Wilson (1971). First, an individual larva is reared and cared for by a multitude of workers, and no one larva receives special attention compared to the others (of the same caste), except those going to be queens. Second, they have a pronounced reproductive division of labour, which is that one individual monopolizes reproduction (queen) while others are sterile (workers) for most or all of the time. Third, the form of eusociality in honey bees has overlapping generations. Therefore, during the short life span of workers they are surrounded by their sisters and brothers.

Usually, the social structure of a honey bee colony is composed of a single fertile female queen, several thousand sterile female workers, and, at certain times, a few hundred males (drones) (Fig. 1). The queen and workers both develop from fertilized eggs (diploid, 2n = 32) that are heterozygous at the sex locus. Their different and irreversible development trajectorie are thus not directly genetically predetermined but rather are determined solely epigenetically (environmentally) by their feeding and other treatments that they recieve as larvae. Unlike the queen and workers, drones or functional males are hemizygotes (haploid, n = 16) and develop from unfertilized eggs under the arrhenotous sex determination system. Note that fertilised eggs that are homozygous at the sex locus will develop as diploid males, but their functionality and fertility is limited.

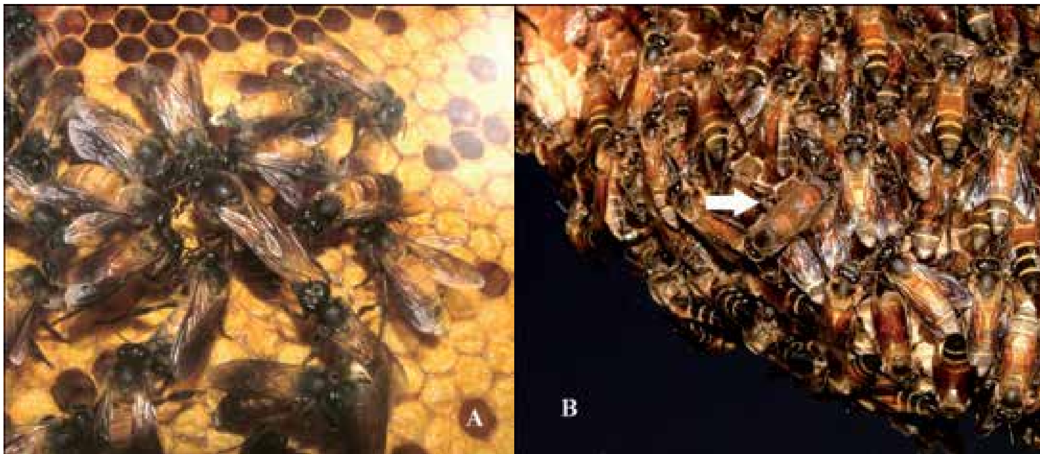

Fig. 1. The size dimorphism between castes of the giant honey bee, *Apis dorsata* F. is less pronounced than other *Apis*. (A) A queen is surrounded by her workers. Her thorax is slightly longer and broader than workers'. (B) Drones have larger eyes (white arrow) but are slightly shorter than workers. Photo by S. Wongvirat.

Within a hive, the queen is the only fertile female so she is the mother of all diploid (queen and worker) members (Crane, 1990), whilst she is typically (under normal circumstances) the mother of all unfertilized eggs (functional drones) as well. Interestingly, a virgin queen can mate with many drones, and so limits the chances of a matched mating (homozygous at the sex locus) and diploid male production (Gould & Gould, 1988). Such high level of polyandry is especially the case for *A. dorsata* queens that have mating frequencies of up to 88.5 (Wattanachaiyingcharoen et al., 2003). Such polyandry, given sperm mixing, leads to asymmetrical levels of the genetic relatedness between workers within colonies and has a profound effect on the bee biology and on the evolution of sociality in bees (Oldroyd & Wongsiri, 2006). A queen can release twenty or more pheromones from her mandibular gland (Crane, 1990). These queen pheromones are volatile compounds, which are important in ensuring colony cohesion within the nest and the dominance of the single queen that heads the colony. The queen's mandibular gland pheromenes induce retinue physiology and behavior in workers (Slessor et al., 1988). For instance, they inhibit the worker's ovary development leading to non reproductive females, and stimulate workers to release pheromones (e.g. Nasonov pheromone) attracting other workers. They can stimulate workers to forage and regulate worker coherence in a swarm or abscorn (Crane, 1990).

Although workers are typically sterile, (in some circumstances some workers can lay unfertilized eggs which if left unattacked by other workers will develop as drones), they have many activities in a colony. For example, a very young adult worker cleans vacated brood cells. Then, at about five-days old, it can feed young larvae and a queen since its hypopharyngeal glands locating in its head are fully active to synthesize royal jelly. Later, these glands start to degenerate at 10-days-old (Crane, 1990). Next, the glands change to produce wax for comb building and to clean the colony. At about two weeks of age, the venom sac is full (Crane, 1990), and some worker bees become active as guards of the colony. As the workers develop from two to four weeks of age, their hypopharyngeal glands secrete increasing amounts of invertase and glucose oxidase, enzymes used in making honey from nectar (Gould & Gould, 1988). At the final stage, the workers will go out of a hive to forage food.

Drones are normally fertile (haploid) males. In all *Apis* spp. except *A. dorsata* and *A. laboriosa*, drones are reared in drone cells on the periphery of the brood nest (Oldroyd & Wongsiri, 2006). These cells are similar to the worker cells in shape and orientation, but the hexagous cells are about three times larger than those of workers (Gould & Gould, 1988). Drones do nothing except leave the colony and mate with a virgin queen. Then, they die. The morphology of the honey bee penis (genitalia) is unique to the genus (Michener, 2007) so it is one of the most useful species identification characters (Radloff et al., 2011).

3. Diversity and distribution of honey bees

From Oldroyd & Wongsiri (2006), three subgenera of honey bees are currently recognized (Table 1), and these differ in the location and structure of building their hive. The two dwarf honey bee species from the subgenera *Micrapis*, *A. florea* and *A. andreniformis*, build a single comb surrounding a twig, while the giant honey bees (subgenera *Megapis*), *A. dorsata* and *A. laboriosa* build a single massive comb under a branch or cliff overhanging or under the eves or roof of a building. Cavity-nesting honey bees (*Apis*), *A. mellifera, A. cerana, A. koschevnikovi, A. nuluensis* and *A. nigrocincta*, build multiple comb nest in cavities.

A recent molecular phylogeny (Lo et al., 2010) added two new taxa to the existing genus *Apis*, one each in the subgenera Megapis and Apis. Based on Bayesian and maximum parsimony phylogenetic trees, their analysis support recognition of *A. indica* (the plains honey bee of south India) as a separate group from the more broadly distributed *A. cerana.* In addition, it also supported classification of the giant Philippines honey bee, *A. breviligula*, as a separated species from the more broadly distributed lowland *A. dorsata*. Thus, three subgenera and 11 species of honey bee of genus *Apis* have been recognized. The distribution of these species is highly uneven (Fig. 2). Interestingly, nine of these 11 species of honey bee can be found in the South-east Asia region, and combined with molecular phylogentic estimates of diveregnce times within the genus, supports that Asia is the most likely birthplace of the *Apis* genus.

In Thailand, there are five *Apis* species which are *A. andreniformis*, *A. florea*, *A. dorsata*, *A. cerana* and *A. mellifera* (Rattanawannee et al., 2007). The first four species are native to Thailand but *A. mellifera* has been introduced by man (anthropogenic) into the country for the apicultural industry (Wongsiri et al., 1996). To recognize these four native species, Rattanawannee et al. (2010) revealed that geometric morphometric analysis of the single wing alone could be used to identify four Asian honeybee species in Thailand and that the sex of the individual does not impede identification. A description of each of the four native species in Thailand are provided below.

Main group	Subgenus	Species	Author	Common name	Thai name
Dwarf honey bee	*Micrapis*	*A. andreniformis*	Smith (1858)	Small dwarf honey bee / Black dwarf honey bee	Pung mim sidam/Pung mim lek/ Pung marn
		A. florea	Fabricius (1787)	Dwarf honey bee / Red dwarf honey bee	Pung mim/ Pung vee
Giant honey bee	*Megapis*	*A. laboriosa*	Smith (1871)	Giant mountain honey bee	-
		A. dorsata	Fabricius (1793)	Giant honey bee / Common giant honey bee	Pung luang
		A. breviligula	Maa (1953)	giant Philippines honey bee	-
Cavity nesting honey bee	*Apis*	*A. cerana*	Fabricius (1793)	Eastern hive honey bee	Pung prong
		A. koschevnikovi	Enderlein (1906)	Red honey bee	-
		A. nigrocincta	Smith (1861)	Sulawesian honey bee	-
		A. nuluensis	Tingek, Koeniger and Koeniger (1996)	Mountain honey bee	-
		A. indica	Fabricius (1798)	Plains Honey Bee	-
		A. mellifera	Linnaeus (1758)	Western honey bee	Pung pun

Table 1. Three subgenera of the genus *Apis* Linnaeus.

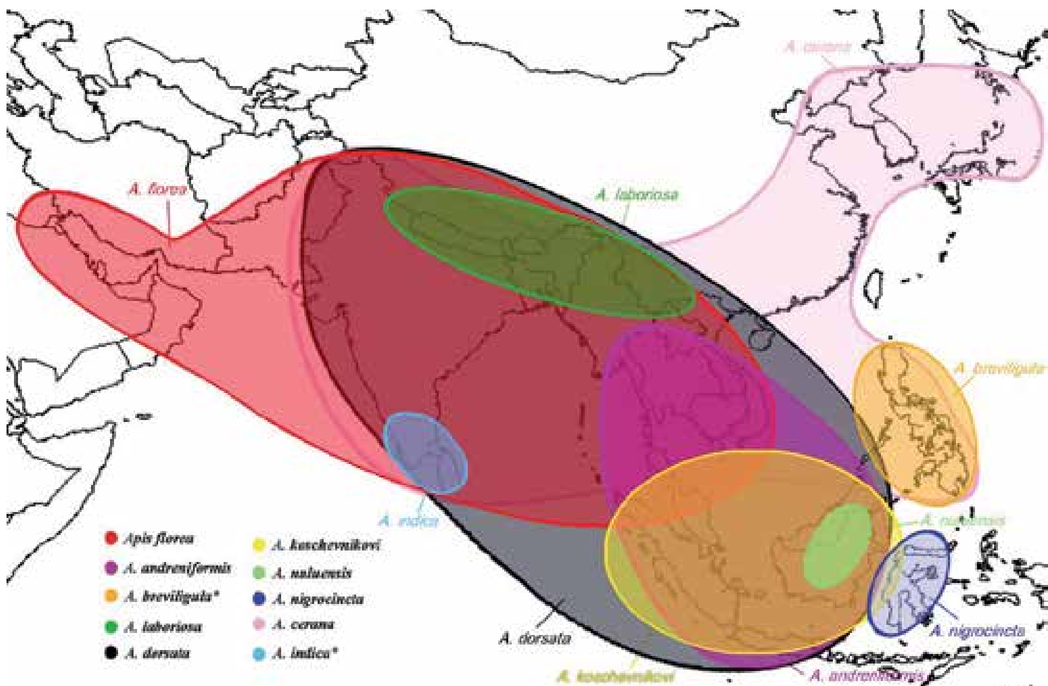

Fig. 2. Approximate distribution of 10 Asian honey bee species of genus *Apis* (amended in accordance with Ruttner, 1988; Oldroyd & Wongsiri, 2006; Lo et al., 2010).

3.1 Dwarf honey bees, subgenus *Micrapis*

The existence of two dwarf honey bee species (*A. andreniformis* and *A. florea*) as valid biological species is well revealed (Radloff et al., 2011), although they are mostly similar in worker and nest sizes. Both build an exposed single comb colony and may utilize similar resources in similar habitats (Wongsiri et al., 1996). Considering species-specific mophological characters, *A. andreniformis* workers have black hairs on their hind tibia and the dorsolateral surface of the hind basitarsus whilst *A. florea* workers have white hairs instead (Wu & Kuang, 1987). In addition *A. andreniformis* workers also have black pigment all over which makes the bees look the darkest, while *A. florea* workers have less black pigment and so are mostly yellow bees (like red dwarf honey bees). The exception is the pigmentation of the scutellum, where in *A. andreniformis* it is yellowish, while that for *A. florea* tends to be black (Wongsiri et al., 1996). Furthermore, the abdominal segments of *A. andreniformis* queens and drones are all black, whilst in *A. florea,* queens present all orange-yellow abdominal segments while drones have grey abdominal segments with white hairs (Rinderer et al., 1995).

Although the endophalli of both species have a pair of bursal cornua, the morphology of the drone's endophallus is different in the two species. In *A. florea* the fimbriate has three protrusions with a strongly curved terminal whilst for *A. andreniformis* the fimbriate has six protrusions with thick and straight terminal. In addition, the thumb-like bifurcate basitasus of the hind leg of drones (Fig. 3) is comparatively longer in *A. florea* (Wu & Kuang, 1987), being just under a half and 2/3 that of the tibia length in *A. andreniformis* and *A. florea,* respectively (Wu & Kuang, 1987).

Fig. 3. Right hind leg of two dwarf honey bee drones showing the thumb-like bifurcate basitasus. Photo by A. Rattanawannee.

Although the nests of both species are very much alike (Figs. 4 and 5), some clear differences in the nest architecture are still observed. When viewed from the edge, cells in the honey storage area of *A. florea* nests are orientated inwards towards a supporting branch (Wongsiri et al., 1996). Considering a cross section of the crown of an *A. florea* nest, there are three levels of inter organization. The first level from the edge contains very long cells that extend to a supporting branch. The second level contains cells coming from the opposite side that have their base at the sides of cells coming from the other side. The third level contains cells

Fig. 4. A nest of *Apis andreniformis* in Thailand, showing the sticky resin around the supporting branches. Photo by S. Wongvirat.

Fig. 5. An *Apis florea* nest in Thailand, showing that the comb is built around a small branch. Photo by S. Wongvirat.

coming from the top of the honey storage area that have the same pattern as cells from the second level. However, some cells open to the top surface have their base well away from

the supporting cell's base (Rinderer et al., 1996). As a consequence of the comb building process, the crown of *A. florea* nests do not contain a midrib (Oldroyd & Wongsiri, 2006). These features contrast with the honey storage area in *A. andreniformis*'s nests, where a characteristic crest appearance is evident when viewed from the outside. A cross section of the honey storage area of an *A. andreniformis* nest reveals a clear midrib structure where the bases of opposing cells come into contact as found in the brood area (Rinderer et al., 1996).

3.1.1 *Apis andreniformis* Smith, 1858

The black dwarf honey bee or small dwarf honey bee, *A. andreniformis*, is the smallest species in the genus *Apis*. It is widely distributed in the tropical and sub tropical regions of Asia, especially in the southern part of China, India, Burma, Laos, Vietnam, Malaysia, Indonesia and the Philippines (Fig. 2). It is always found at coastal flats and near foothill areas (1 - 100 m above sea level) to high mountain and forest areas at about 1600 m attitude (Wongsiri et al., 1996). The economic value of *A. andreniformis* has not been documented. However, the importance of the naturally occurring flora in the range of *A. andreniformis* probably depends on this bee species for pollination (Rinderer et al., 1995).

Since *A. andreniformis* is a rare and patchily distributed species, very little work has been reported. For example, intraspecific variation of *A. andreniformis* was reported by Rattanawannee et al. (2007). They sampled from 27 colonies (for morphometric analysis) and 32 colonies (for genetic analysis) of *A. andreniformis* throughout Thailand. In addition, three colonies for morphometric analysis and five colonies for DNA polymorphism were taken from Tenom in Sabah, Malaysia. For morphometry, 20 informative morphometric characters were used to assess the variation. Principle component analysis (PCA) yielded four factor scores. Within PCA plots, *A. andreniformis* from across Thailand and Tenom (Malaysia) formed a single group, a notion further supported by a hierarchical cluster analysis generated dendrogram. However, linear regression analysis showed clinal patterns of morphometric characters, where the body size of bees increased from the South to the North, associated with increasing altitude, but decreased from the West to the East, associated with decreasing altitude. For genetic variation, based on the sequence analysis of the mitochondrial *cytochrome oxidase subunit b* (*Cyt-b*) gene fragment, two groups of *A. andreniformis* populations from Thailand were found. However, these results are tentative, pending more extensive analyses of samples across the distribution areas of *A. andreniformis*.

3.1.2 *Apis florea* Fabricius, 1787

The red dwarf honeybee, *A. florea,* is extremly widespread in Asia, extending from Vietnam and southeastern China, across mainland Asia along and below the southern Himalayas, westwards to the Plateau of Iran and southern into Oman (Fig. 2) (Hepburn & Hepburn, 2005). However, the main habitat of this bee species is Pakistan, India, Sri Lanka, Thailand, Indochina, Malaysia, part of Indonesia and Palawan at altitudes below 1000 m (Ruttner, 1988).

Multivariate morphometric analysis of *A. florea* using 20 morphometric characters to investigate the intraspecific morphometric variation of 18 samples of *A. florea* (360 bees) from Sri Lanka, Thailand, Pakistan, Iran and Oman revealed three morphocluster groups of *A. florea* (Ruttner, 1988); (i) Sri Lanka and south India, (ii) Thailand and Oman and (iii) Pakistan and Iran. In addition, the body size of *A. florea* was observed to increase across the study range from the South to the North. Subsequent analysis using 12 morphometric

characters of *A. florea* from 26 localities in southern Iran revealed two morphoclusters of *A. florea*; a larger bee group at high latitudes (29° - 34°) and a smaller bee group at lower latitudes (<29°) (Tahmasebi et al., 2002). After combining their data with that of Ruttner (1988) and Mogga & Ruttner (1988), they also identified three morphoclusters for all *A. florea* samples. However, information on the geographical contiguity of this honey bee species was still missing (Radloff et al., 2011).

To fill the geographical contiguity of *A. florea*, Hepburn et al. (2005) performed multivariate morphometric analysis of 184 colonies (2,923 individual workers) of *A. florea* collected from 103 localities across the full distributional area from Vietnam and southeastern China to Iran and Oman. They concluded that *A. florea* was a panmictic species comprised of three morphoclusters; northwestern, southeastern and an intermediate form. They suggested that the seasonality of reproductive swarming was temporary continuous allowing gene flow throughout this panmictic species.

In Thailand, Chaiyawong et al. (2004) performed multivariate morphometric analysis of 50 *A. florea* colonies (750 worker bees) from different locations throughout Thailand. From a PCA and cluster analysis of 22 morphometric characters, they revealed only a single group of *A. florea* in Thailand. Then, after reducing the number of characters, a degree of isolation from the mainland group was obtained for Samui Island and Pha-ngan Island, but the bees from these locations were correctly regarded simply as variants. This single morphocluster for Thailand of *A. florea* was in close agreement to the report of Nanork (2001), who found no variation among sympatric *A. florea,* in Thailand using PCR-RFLP analysis of the *Cyt-bI-tRNA* coding gene region of the mtDNA.

3.2 *Apis dorsata* Fabricius, 1793

The common giant honeybee, *Apis dorsata,* is one of three species of the subgenus *Megapis*. Neither Ruttner (1988) nor Engels (1999) separated *A. dorsata* from the closely related species, *A. laboriosa*. However, various evidences have demonstrated the difference between the two giant honey bee species. For example, Underwood (1990) reported the mating flight of Nepalese *A. laboriosa* drones was during 12:30 - 14:30 h whereas *A. dorsata* drone mating flight occured just after dusk, during 18:15 - 18:50 h (Koeniger et al., 1988), suggesting a prezygotic reproductive barrier. Also, the vocal communication dance performed by *A. dorsata* workers is different from that of the silent *A. laboriosa* workers (Oldroyd & Wongsiri, 2006; Kirchner et al., 1996). Furthermore, Arias & Sheppard (2005) revealed that the ND2 and EF-1a gene nucleotide DNA sequence divergence between *A. dorsata* and *A. laboriosa* is 10.6 - 11.5%, which strongly supports seperate species status. Indeed, Raffiudian & Crozier (2007) showed that 100% of Bayesian consensus trees support the grouping of *A. dorsata* distinct from *A. laboriosa*, and supporting recognition of *A. laboriosa* as a valid species.

Other than *A. dorsata* and *A. laboriosa*, another species of giant honey bees has been reported by Lo et al. (2010). Based on Bayesian and maximum parsimony phylogenetic trees, their analysis supports recognition of the giant Philippines honey bee, *A. breviligula* Maa, 1953, as a separate species from the more broadly distributed lowland *A. dorsata*. *A. breviligula* is found northwest of the Merrill line in Luzon in the Philippines (Oldroyd & Wongsiri, 2006). This giant honey bee is strikingly distinguished from *A. dorsata* owing to black rather than yellow coloration of the abdomen and that it never forms colony aggregations as *A. laboriosa* and *A. dorsata* do (Lo et al., 2010). Therefore, three species of giant honey bee in the subgenus *Megapis* of the genus *Apis* have now been recognized.

The distribution of *A. dorsata* is over a vast geographic area in the South and Southeast Asia (Fig. 2). To the West, *A. dorsata* occurs not farther than the Indus river, and to the East, *A. dorsata* are throughout the Philippines and even cross the Wallace line. The giant honey bee is reported to present at altitudes up to 1000 - 1700 m, or even up to 2000 m during migration (Ruttner, 1988).

In Thailand, *A. dorsata* is the only species of the subgenus *Megapis* that can be found. Among honey bee spp., individual workers of *A. dorsata* are relatively large, being about 17 mm long. Thus, the giant honey bees are distinguished from the other four honey bee species in Thailand by their much larger body size and that their wings that are fuscous, and quite hairy (Oldroyd & Wongsiri, 2006). The fore and hind wings of *A. dorsata* workers are 12.96 and 8.91 mm long, respectively (Tan, 2007). The body color of *A. dorsata* workers is yellow, with tergites 2 and 3 being reddish-brown (Crane, 1990). Unlike the comb of the dwarf honey bees (*A. florea* and *A. andreniformis*), in which the crown of the comb always encircles the support, the massive single comb colony of *A. dorsata* is always attached under the surface of a stout tree branch or an overhang of a rock face, and nowadays also sometimes to the eves of buildings or other urban structures (Fig. 6) (Paar et al., 2004).

Fig. 6. A masive single comb nest of *Apis dorsata* attached under the eves of buildings at Mae Fah Luang University, Chiang Rai, Thailand. Photo by A. Rattanawannee.

Where *A. dorsata* nests are found in trees, the diameter of the supporting branches varies from 12 - 30 cm (Morse & Laigo, 1969) or much larger (Oldroyd & Wongsiri, 2006). A

slightly sloping branch is preferred (Tan et al., 1997). The width of *A. dorsata* combs varies from 43 – 162 cm, and the height from 23 – 90 cm (Tan, 2007). Honey is stored in one corner of the comb nearest the uppermost section of the comb in an area about 10 - 20 cm in a large nest (Oldroyd & Wongsiri, 2006). In the large colonies, the number of individual workers can be over 50,000 (Morse & Laigo, 1969). About 3 - 4 weeks after nesting, a colony of *A. dorsata* typically has about 4 kg of stored honey in the comb, but the highest recorded amount is 15.7 kg (Tan, 2007).

Fig. 7. An aggregation of *Apis dorsata* colonies under the roof of a temple in Chaing Rai, Thailand. Photo by A. Rattanawanee.

Three further typical characters of *A. dorsata* are as follows. First, colonies are unusual in terms of that nests often occur in dense aggregations of up to 100 or even 200 colonies on a single tree or building (Koeniger & Koeniger, 1980), and these colonies are often separated by only a few centimeters (Figs. 7 and 8). Secondly, the nest sites are occupied seasonally year after year. Interestingly, queens often return to the same nest site even after an absence of up to 18 months (Paar et al., 2000). In Thailand, aggregations of nests are formed by swarms that arrive at the onset of the dry season. Finally, colonies usually display seasonal migration between alternate nesting sites. Nest sites of these bee populations tend to be occupied for 3 - 4 months (Paar et al., 2004). Towards the end of this period, colonies abscond, leaving an empty comb (Fig. 8). The swarms leave the nest site to a new site up to 200 km away (Koeniger & Koeniger, 1980), and most like spending the wet season as combless swarms in mountainous regions (Ruttner, 1988). The proximate cause of migration may be related to available flowers. Abscornding *A. dorsata* have been observed to travel among habitats with different blooming seasons (Crane et al., 1993). The migration of

colonies may also contribute to control infections with the parasitic mite *Tropilaelaps clareae*, since it needs bee brood in order to reproduce (Paar et al., 2004). Therefore, colonies may reduce infestation levels by this parasitic mite with a period of broodless migration (Rinderer et al., 1994).

Fig. 8. Absconding nests within a colony aggregation of *A. dorsata* on a single tree in Sakonnakorn, Thailand. Photo by A. Rattanawannee.

3.3 *Apis cerana* Fabricius, 1793

A. cerana can be found throughout Asia, including in the great mountain ranges and deserts (Ruttner, 1988), except that there is no evidence of *A. cerana* occurrence in the northern Japanese island of Hokkaido. In contrast, it is widely distributed over the other islands in Japan. In Southeast Asia, *A. cerana* is restricted to the Malayan region, the West of the Wallace line (Ruttner, 1988, as shown in Fig. 2).

A. cerana is a medium sized bee (in body length) with a fore wing length of 7 - 10 mm (Oldroyd & Wongsiri, 2006). Feral colonies of *A. cerana* are found in a similar location as *A. mellifera* colonies, such as tree hollows, clefts in rocks and walls (Fig. 9) (Ruttner, 1988). They usually build three or more parallel combs attached to the roof of tree hollows (Fig. 10). Among the native Thai honey bee species, only *A. cerana* can be maintained in hives like *A. mellifera* (Wongsiri et al., 1986). However, traditional hives for *A. cerana* are substantially smaller than those constructed for *A. mellifera* (Ruttner, 1988).

Fig. 9. A feral colony of *Apis cerana* in a coconut tree hollow in Samut Songkham, Thailand. Photo by J. Kaewmuangmoon.

Fig. 10. Multiple combs withinan *A. cerana* nest. Photo by P. Nanork.

The first morphometric analysis of *A. cerana* was reported in Ruttner (1988), where the results of a PCA using 40 morphometric characters on 93 samples (18 Asian countries) revealed four main groups. Group I consisted of *A. cerana* collected from South India, Sri Lanka, Bangladesh, Burma, Malaysia, Thailand, Indonesia and the Philippines, whereas *A. cerana* from Afghanistan, Pakistan, North India, China and Vietnam were classified in Group II. *A. cerana* samples from central and east Himalaya belonged to Group III whilst Group IV contained *A. cerana* from Japan.

In Thailand, Limbipichai (1990) successfully used standard morphometrics to verify a geographic subpopulation of *A. cerana* split by the Isthmus of Kra, a biogeographic transition area (12° N latitude). This morphometric result was supported by Deowanish et al. (1996) who used PCR-RFLP analysis of the $tRNA^{leu}$ – COII region mitochondrial DNA sequence based analysis and found variation in the PCR-RFLP banding patterns among Thai samples when using *Bgl*II, *Eco*RV, *Hae*III, *Hin*fI and *Nde*I. In addition, *A. cerana* from the South of Thailand (Hatyai and Samui) could be clearly separated from the mainland population when the $tRNA^{leu}$ – COII region containing amplicon was digested by *Eco*RV and *Hin*dIII. In some support of this, Sihanuntavong et al. (1999) also reported that the *A. cerana* population from the Samui islands (South of Thailand) was distinct from the mainland populations, as determined using PCR-RFLP anlaysis using *Dra*I restriction of the PCR amplicons of the *srRNA* and *lrRNA* gene and *COI-COII* coding regions. Likewise, Songram et al. (2006) revealed eight distinct RFLP patterns of the *ATPase6-ATPase8* gene region when the DNA was digested by *Vsp*I. Overall, a strong biogeographic pattern between the northern and southern latitude bee populations in Thailand was revealed.

4. Stingless bees

Meliponini is one of the 19 tribes in the subfamily Apinae, including Apini, Euglossini and Bombini (Michener 1974, 2000). Apini and Meliponini are the two tribes that contain members that display a high level of social behavior (Arias et al., 2006). Meliponines are groups of stingless bees whose size, body color and appearance vary greatly. For example, stingless bees in some species have a slender body while those in other species have a wide body. Some appear shiny and others as hairy somewhat like small bumble bees. Also, stingless bees in some species look metallic (Crane, 1990). The number of stingless bee species of Meliponini is still controversial, but it is estimated to be about 50 times more species than *Apis* spp. (Roubik, 2006). Currently, over 600 species in 56 named genera have been recorded in the tropical and subtropical regions of the world. Of these, 400 known species exist in the Neotropical regions and at least 45 species were described in Southeast Asia (Cortopassi-Laurino et al., 2006).

Stingless bees and honey bees are both classified as highly eusocial insects (Michener, 2000), with large perennial colonies, morphologically distinct worker and queen castes and an intricate division of labour and recruitment to food sources (Peter et al., 1999). They normally have a single egg-laying queen and reproduce by division of a colony between the mother queen and a daughter, which is called reproductive swarming (Roubik, 1989).

Meliponines differ from honey bees of the genus *Apis* in many biologically significant ways. For example, they generally have no sting, do not use water to cool their nest and pure wax to build it, and the males feed at flowers while the gravid queens cannot fly (Roubik, 2006). Moreover, Peter et al. (1999) showed that single mating is the rule in stingless bees; in contrast to the well-known multiple mating of honeybee queens (Oldroyd et al., 1997), since

diploid males (from sex allele matched matings) are not tolerated and lead to the queen bee being usurped.

Stingless bees nest in cavities, which differ in locality between species and may be underground, in tree or other enclosed spaces, such as buildings and termite nests (Crane, 1990). Stingless bee species are recognizable from the characteristic nest entrances and often their particular site (Roubik, 2006). Nests are made of wax secreted from the metasomal terga mixed with resins and gums collected by stingless bee workers. Some species add mud, feces or other materials to certain parts of the construct. In all Meliponine species, the composition and texture differ in different parts of the nest (Michener, 2000). Unlike honey bees, they produce brood in the manner of a solitary bee with an egg placed on top of a food mass in a sealed cell (Michener, 2000). Inside the nest of stingless bees, there are different shapes and arrangements of brood cells and food storages (Fig. 11). Brood cells in many stingless bee species are spherical to ovoid, while food storage containers are small to large spheres, or are egg-shaped, or even conical or cylindrical (Roubik, 2006). Honey and pollen are usually stored in separated containers called "storage pots". Usually, pots are constructed together in conglomerates, as are the brood cells. Interestingly, the horizontal brood cells of stingless bees open upwards and are closed after an egg is laid. The egg is positioned on the semi-liquid mix of honey, hypopharyngeal-gland secretion and pollen. All brood cells are destroyed by workers after use and cannot be reused as they are in honey bees (Michener, 2007).

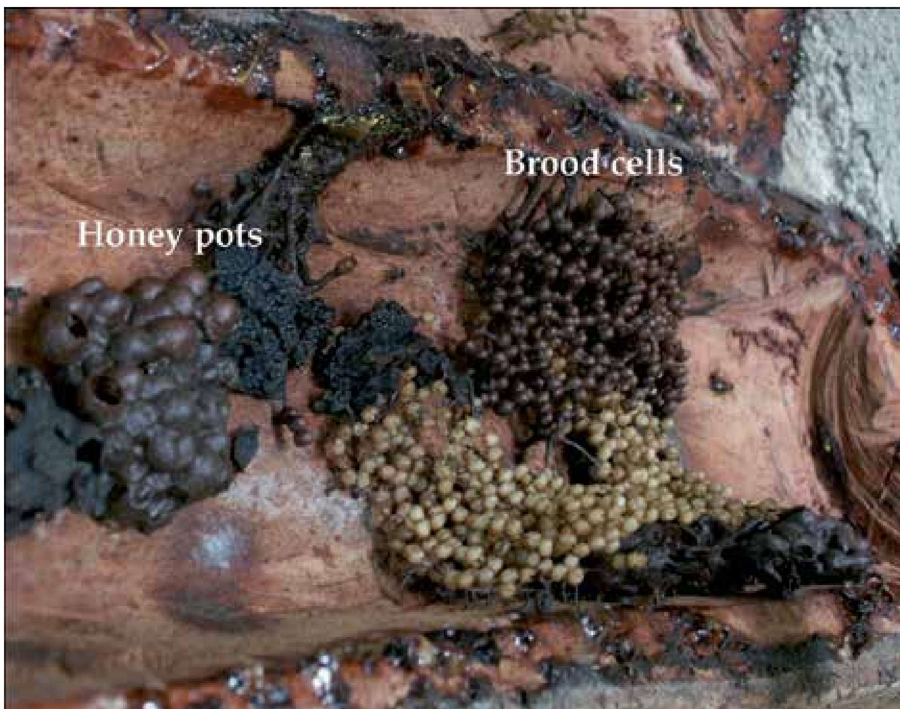

Fig. 11. Ovoid brood cells and honey pots within a *Trigona laeviceps* nest are separated.

More than 50 genera of Meliponines have been reported (Arias et al., 2006). In Thailand, only one genus, *Trigona*, is recognized as endemic with 32 species currently reported

(Klakasikorn et al., 2005). However, this genus is found extensively in tropical regions. In the Neotropics it ranges from Mexico to Argentina, in the Indo-Australian region it extends from India and Sri Lanka to Taiwan, the Solomon islands, South Indonesia, New Guinea and Australia (Michener, 2000). The Thai name for stingless bees varies across the regions, and are Channa Rong (Central), Kheetung Nee (North), Khee Suit (Northeast), and Oong (South).

5. Value of bees

5.1 Pollination value

Up to a third of the food we eat is derived from plants that are either dependent on or benefit from insect pollination (Oldroyd & Nanork, 2009), especially by honey bees (Richards, 2001). The European honey bee, *Apis mellifera*, is the most economically valuable pollinator of agricultural crops worldwide (Conte & Navajas, 2008). However, in most areas of Southeast Asia, there is no significant pollination industry. Insect pollinated crops are, therefore, completely reliant on wild bees, particularly honey bees and stingless bees, for their pollination (Rahman & Rahman, 2000).

Because of their dance language and broad foraging length, honey bees can rapidly identify and exploit the available flowers for nectar and or pollen or plant sap for propolis over a wide range (Dornhaus et al., 2006). Therefore, honey bees are better at long-distance dispersal of pollen than solitary arthropods (Oldroyd & Wongsiri, 2006). Circumstantially, honey bees may partially compensate for fragmentation by bridging the gaps between isolated plant communities (Johnson & Steiner, 2000). Corlett (2001) reported that 86% of plant species in an extremely disturbed area in Hong Kong were visited by *A. cerana*. Thus, although *A. cerena* is probably not a pollinator of all these plants, it does appears to maintain Hong Kong's diverse flora.

Lychees, *Litchi chinensis* Sonn., is one of the important commercially grown economic fruit plants in Thailand. Field trials suggested that the reduction of fruit yield by as much as 11.2% occurs in the absence of pollinators (Oldroyd & Wongsiri, 2006, as cited in Sihag, 1995), and that the majority of pollinators are honey bees and stingless bees. Wongsiri et al. (1996) reported that *A. florea* and *A. andreniformis* are excellent orchard and field crop pollinators, including for longan (*Dimocarpus longan* Lour.) and mango (*Mangifera indica* L.). Since *A. florea* is easy to maintain in orchards and is abundant throughout Thailand, this dwarf honey bee is an excellent pollinator for economic crops and wild plants (Ruttner, 1988).

The lowland forests of Asia are dominated by trees in the family Dipterocarpaceae. Since an individual tree of each species tends to be over the long distances required for efficient effective fertilization and gene flow (Itioka et al., 2001). This requires an animal vector that has species fidelity while foraging, a large foraging range, and the tendency to visit multiple trees, either as individual foragers, or via transfer of pollen among foragers in the nest. The giant honeybee has all these characteristics (Oldroyd & Nanork, 2009). In addition, Momose et al. (1998) reported that *A. dorsata* is one of the major pollinators of several dominant components of the forest canopy in Southeast Asian lowland Dipterocarp forests, one of the richest terrestrial ecosystems in the world. It was reported that *A. dorsata* pollinated at least 15 species of emergent and canopy trees at Lambir (Momose et al., 1998) and was the dominant pollinator of the upper strata in rainforests in peninsular Malaysia (Appanah, 1993), and for canopy dipterocarps in Sri Lanka (Dayanandan et al., 1990).

5.2 Products value

Bee products (honey, royal jelly, propolis, bee pollen, wax and bee venom) are of increasing economic importance. Honey is always consumed as food while royal jelly, propolis and bee pollen are useful in nutrient supplements and applied in cosmetics and traditional medicine. Furthermore, bee venom has long been used in Apitherapy. Among the different bee products in Thailand during 2008 - 2010, honey seems to be the only world trade product according to the statistical record of Thai Custom Department, Ministry of Finance of Thailand (Tables 2 and 3).

It is obvious that China's market is the biggest, both in terms of importation and exportation. Interestingly, the US exports the highest quantity of honey while relatively only a small quantity is consumed in the country. Although China exports a high quantity of honey, a much higher quantity of imported honey is observed. In contrast, Germany is the leading exporter of honey overseas. In addition, although there are many bee farms in countries such as Thailand, Myanmar and Australia, large quantities of honey still have to be imported. This suggests that a promotion program in bee apiary should be arranged and supported.

Country	2008 A.D.		2009 A.D.		2010 A.D.	
	Quantity (ton)	CIF value (million baht)	Quantity (ton)	CIF value (million baht)	Quantity (ton)	CIF value (million baht)
Australia	150.26	17.39	112.44	14.48	119.64	18.75
China	35.96	0.61	431.38	14.00	1,222.11	57.11
France	14.58	3.83	11.24	3.17	14.99	3.54
Germany	23.68	6.16	23.96	6.89	24.04	6.12
Japan	14.03	2.12	14.12	2.67	9.7	1.92
Laos	0.03	0.00	ND	ND	18.60	1.36
Malaysia	ND	ND	0.28	0.01	0.86	0.18
Myanmar	0.2	0.00	0.08	0.00	1,094.68	31.13
Switzerland	3.29	1.08	1.00	0.36	3.00	1.04
Thailand	1.79	0.41	1.79	0.31	251.40	8.57
UK	3.15	0.94	2.53	0.72	8.16	2.10
USA	0.00	0.00	0.00	0.00	0.14	0.02

Table 2. Honey imports (2008 - 2010). The data was obtained from Thai Custom Department, Finance Ministry, Thailand. ND represents no data.

Country	2008 A.D.		2009 A.D.		2010 A.D.	
	Quantity (ton)	FOB value (million baht)	Quantity (ton)	FOB value (million baht)	Quantity (ton)	FOB value (million baht)
Australia	ND	ND	ND	ND	0.00	0.00
China	400.53	38.58	174.16	12.33	447.32	32.02
France	4.00	0.83	8.54	0.96	637.93	49.82
Germany	140.10	10.29	169.22	11.96	658.20	44.48
Japan	2.68	0.29	5.76	0.60	2.56	0.33
Laos	ND	ND	0.11	0.02	0.04	0.00
Malaysia	272.34	14.69	252.90	16.20	269.11	32.22
Myanmar	ND	ND	ND	ND	ND	ND
Switzerland	ND	ND	ND	ND	0.00	0.00
Thailand	ND	ND	ND	ND	ND	ND
UK	1.79	0.35	1.79	0.26	79.03	4.67
USA	186.09	10.18	1,954.86	95.51	1,815.79	92.75

Table 3. Honey exports (2008 - 2010). The data was obtained from Thai Custom Department, Finance Ministry, Thailand. ND represents no data.

6. Application of bee products

Not only are bee products consumed as food, as mentioned earlier, but they also have long been used in medical aspects, especially in traditional medicine. Bee products are derived from plants. For example, honey is the modified form of plant nectar by alpha-glucosidase (Kubo et al., 1996). Propolis is collected from plant buds and barks (Castaldo & Capasso, 2002). Since it is hard to control the consistency of bioactivities from natural products, both in their original form and crude extract, it is important to obtain a chemical structure of the active compounds for subsequent chemical synthesis or (bio) assay of the active contents. Many purification steps were used in order to obtain a pure compound. To this end, spectroscopic techniques, such as Infrared spectroscopy (IR) and Nuclear Magnetic Resonance (NMR), have been broadly applied. Once the structures of the bioactive compounds are known, it can lead into their chemical synthesis and or serve as templates for modifications for subsequent drug development. Currently, the bioactive chemical compounds found in propolis and honey, which mainly belong to the groups of flavonoids and phenolic compounds, seem to be similar to those found in the pollen or sap of the foraged plants (Katircioglu & Mercan, 2006), as expected. Some of the bioactivities from bee products are briefly outlined below.

6.1 Antimicrobial activity

Anti-bacterial activity has been reported against pathogenic bacteria for bee products, and especially propolis and honey (Boorn et al., 2010). Overall, Gram positive bacteria are more

sensitive to the bee products than Gram negative bacteria (Marcucci et al., 2001). Active compounds may act on the inhibition of bacterial RNA polymerase (Takaisi & Schilcher, 1994), degrade the cytoplasmic membrane of bacteria (Cushnie & Lamb, 2005) or cause bacteria to lose their capacity to synthesize ATP, membrane transport and mobility (Mirzoeva et al., 1997). For example, the proliferation of *Staphylococcus aureus* (Gram^{+ve} bacteria) and *Escherichia coli* (Gram^{-ve} bacteria) is inhibited by the propolis of *Melipona compressipes* (Kujumgiev et al., 1999). Furthermore, propolis collected from the same bee species but in different regions, or different bee species in the same region show marked differences in bioactivity levels as well as susceptible microbes, as expected given the different flora available or utilized by the different bee species in different regions. For example, propolis collected from Spain yielded a higher antimicrobial activity than that collected from Mongolia (Kujumgiev et al., 1999).

However, such geographical and likely seasonal variations in the bioactivity of bee products necessitates some form of standardization of their bioactivity. There has been some progress in the improvement of the standard and acceptance in using bee products in medicine, especially medical-grade honey (Kwakman et al., 2011). Manuka honey, is one such medical-grade honey with antibacterial bioactivity (Lin et al., 2011). Given the severe problem of bacterial resistance to antibiotics, such as methicillin-resistant *S. aureus*, there is a growing need to find new antimicrobial agents. Interestingly, honey from *A. mellifera* in Ireland (Maeda et al., 2008) and from *T. laeviceps* in Thailand (Jirakanwisal, 2010) can inhibit the growth of methicillin-resistant *S. aureus in vitro* better than the currently used antibiotics. In addition, other antibiotic-resistant bacteria, such as gentamicin-resistant *E. coli*, methicillin-resistant *S. epidermidis*, vancomycin-resistant *Enterococcus faecium*, could be killed by medical-grade honey (Kwakman et al., 2008).

Other than pathogenic bacteria, antifungal activity has been reported for bee products, such as the *in vitro* inhibition of *Candida albicans* growth by propolis from Brazil (Kujumgiev et al., 1999). Interestingly the antifungal activity of the propolis extract from *A. mellifera* against *Phomopsis* spp., *Fusarium* spp. *Trichoderma* spp. and *Penicillium notatum* was greater than that seen with ketoconazole, an antifungal drug (Quiroga et al, 2006).

In addition to bacterial and fungal pathogens are severe human diseases caused by viruses. Due to their high rate of mutation, the development of new antiviral agents is always required. With respect to bee products, in 1999, Kujumgiev et al. presented that the aromatic acids and flavonoid aglycone compounds in the propolis from *M. compressipes* in Brazil could inhibit the growth of avian influenza virus *in vitro*. Furthermore, the *in vitro* replication of herpes simplex virus was also inhibited by propolis (Erukhimovitch et al., 2006) and honey (Banerjee, 2006).

6.2 Anti-inflammatory activity

Inflammation is part of the immune and general tissue damage defense response of the vascular tissues, such as for aiding removal of invading pathogens, intruders, which can be microbes, wounds, allergenic proteins, auto-immune, some chemicals, and removing damaged or necrosing tissues Although required for the healing process and part of the immune response, as outlined above, inappropriate or chronic inflammation is deleterious and can lead to, for example, asthma, atherosclerosis and rheumatoid arthritis as well as pain and poor healing. Each individual is differently susceptible to the anti-inflammatory agents or drug. It is still necessary to find out a new anti-inflammatory agent. However, this response can be inappropriate or too extreme and detrimental, driving the requirement for

topical, specific and systemic agents to control the anti-inflammatory responses. With respect to bee products, Paulino et al. (2003) reported an anti-inflammatory activity in the ethanolic extract of propolis from *A. mellifera* in Bulgaria, which had a similar anti-inflammatory activity level to that provided by indomethacin, a recent anti-inflammatory drug. Subsequentially, Hu et al. (2005) reported that the water and ethanolic extracts of propolis from *A. mellifera* in China could significantly decrease the swollen symptoms within two hours of treatment.

Other than propolis, an anti-inflammatory activity can be provided by bee pollen, such as that reported in the ethanol extract of pollen from *A. mellifera* in Brazil (Medeiros et al., 2008). The main active compounds were found to be phenolic compounds and furthermore these were similar to those found in various plants, such as berry ,vegetables, fruits and tea leaves. Moreover, it was reported that the flavanol derivatives from propolis could reduce the allergenic symptom of paw edema, inhibit the synthesis of immunoglobulin E (IgE) and immunoglobulin G_1, reduce the activity of eosinophil peroxidase and reduce the mobility of pulmonary cells. Thus, it is promising that we may be successful in finding new anti-inflammatory agent in propolis.

6.3 Free radical scavenging activity

Free radicals are oxygen-centered molecules that contain a single electron at the outermost orbit. Although they play an important role in biological processes such as in immunity (intracellular killing of bacteria) and certain redox signaling pathways, their inappropriate expression in terms of level or cellular location can lead to serious cell damage as they can bind to low-density lipoprotein (LDL) and some other compounds including proteins and DNA causing irreversible changes. The bound or modified compounds can be toxic to cells leading to premature or inappropriate cell death, and can cause mutations in the genetic materials transforming normal cells to cancer cells (Campos et al., 2003). Other than cancer, excess free radicals are linked to a diverse array of disorders, such as atherosclerosis, cerebral ischemia, cardiac ischemia, Parkinson’s disease, gastrointestinal disturbance and aging (Ames et al., 1993). It has long been challenging to find new free radical scavenging agents. However, with respect to bee products, Choi et al. (2006) reported that *A. mellifera* propolis from different regions in Korea (Yangpyeong, Boryung, Cheorwon and Yeosu) contained free-radical scavenging activity, but that they differed in their ED_{50} values between regions. Indeed, propolis from the same bee species collected in Portugal showed the same free radical-scavenging affect (Moreira et al., 2008). Both works also support the idea that natural products from different regions provide an interesting bioactivity at different efficiencies.

Other than propolis, Silva et al. (2005) reported the presence of a free radical scavenging activity in bee pollen from the stingless bee, *Melipona* subnitida, in Brazil. Analysis of the bee pollen revealed that they were from *Mimosa gemmulata*, a plant in the *Mimosaceae* family, and from plants in the *Fabaceae* family. The efficiency of the free radical scavenging activity obtained mainly depended on the organic solvents used in the extraction process. Ethyl acetate was the most efficient extraction solvent for recovery of this bioactivity, followed by ethanol and hexane, respectively. Active compounds were analyzed to be naringenin, isorhamnetin, D-mannitol, β-sitosterol, tricetin, selagin and 8-methoxinerbacetin.

6.4 Antiproliferative activity

Although cancer research has long been established, cancer is still the leading cause of death and sickness to people worldwide. Due to the high cost of cancer treatment and the

limitation of recent therapy, including the evolution and spread of resistance to current chemotherapy agents, alternative and complementary medicines are becoming of increasing interest and potential importance, especially those with a different mechanism of action. Indeed, a significant proportion of cancer research has been focused upon finding new anti-cancer agents. With respect to bee products, Awale et al. (2008) reported that the methanolic extract of red propolis in Brazil contained an antiproliferation activity against human pancreatic cancer cells (PANC-1) in tissue culture (*in vitro*). From this extract, forty-three active chemical compounds were analyzed. Among those, three new compounds, 6)a*S*,11 a*S*)-6a-ethoxymedicarpan, 2)-2′,4 ′-dihydroxyphenyl)--3methyl--6 methoxybenzofuran and 2, 6-dihydroxy-2-[(4-hydroxy-phenyl) methyl]-3-benzo-furanone, were found. In addition, Umthong et al. (2009) reported that the propolis from *T. laeviceps* in Thailand had an antiproliferative activity against the colon cancer (SW620) cell line in tissue culture. The concentration of the methanolic extract of this propolis showed a linear correlation to the anti-proliferative affect, whereas the water extract revealed a biphasic effect.

Bee pollen has also been reported to have an antiproliferative activity upon cancer cell lines in tissue culture, and this has been linked to the flavonoid composition (Rice-Evans et al., 1997). The antiproliferative activity from *A. mellifera* bee pollens collected from *Cystus incanus* L. in Croatia were found to be mediated by phenolic compounds, such as flavonol (pinocembrin), flavanol (quercetin, kaempferol, galangin and isohamnetin), flavones (chrysin) and phenylpropanoid (caffeic acid).

Overall, it is evident that the active chemical compounds and bioactivities depend mainly on the bee species, collecting sites, biogeography and other external factors.

7. Threats to and conservation of wild bees in Southeast Asia

7.1 Deforestation and destruction of nesting sites

Flint (1994) reported that between 1880 and 1980 Southeast Asia showed an average loss of forest cover area of 0.3%, which was primarily caused from agricultural expansion and commercial logging. Subsequent to 1985, deforestation has remained particulary severe in Southeast Asia (Achard et al., 2002).

Little is known about how deforestation will affect honey bees, especially the giant honey bee. Liow et al. (2001) revealed that the proportion of stingless bees and honey bees (Hymenoptera: Apidae) was very low in oil palm plantation areas and very high in undisturbed areas, which implies that oil palm plantations are not suitable in terms of either fulfilling the preferences of honey bees or the ability to support them. Palm trees do not produce nectar and their dense leaves render them unsuitable for nest building by *A. dorsata* (Oldroyd & Nanork, 2009).

The removal of nesting trees of *A. dorsata* is of great concern in their conservation (Oldroyd & Nanork, 2009). Giant honey bees tend to build their nests in aggregations, sometimes with more than 100 colonies on a single tree (Oldroyd et al., 2000). In addition, *A. dorsata* colonies often migrate long distances, but return to their previous nesting site every year (Koeniger & Koeniger, 1980). Thus, the felling of major bee trees may cause a significant decline in the *A. dorsata* populations. Although the effects of agricultural landscapes and industrialization have significantly increased in Thailand, deforestation could represent as a main threat to wild honey bee and stingless bee populations and their nesting sites should be protected (Dietemann et al., 2009).

7.2 Brood and honey hunting

Honey hunting is the general term given to the collection of honey from wild honey bee colonies. Traditional honey hunting is an important role in the life of Asian people. They have been hunting wild honey bees for more than 40,000 years (Crane, 1999) and honey bee hunting remains a widespread practice throughout the region (Oldroyd & Nanork, 2009). The existing method of honey hunting in giant honey bees is the same across Asia. Hunting *A. dorsata* and *A. laboliosa* is more ruthless, and often burning the bees with a smoldering torch of tightly-bound brush (Lahjie & Seibert, 1990). In traditional honey hunting, night time is preferred by many hunters. The smoking is considered crucial to disorientate the bees and so to reduce the number of stings received. After smoking off the bees from the comb, most honey hunters cut down the whole combs destroying all the brood and food stores. A large number of larva and young bees, some hundreds of adult bees and drones are also killed while hunting honey (Tsing, 2003). Many queens must be lost during these harvest methods, and their colonies perish along with them (Oldroyd & Nanork, 2009). Therefore, these methods of hunting may kill many colonies of *A. dorasata* within colony aggregations in one night.

7.3 Honey bee diseases and parasites

Honey bee colonies can be infected by numerous pathogens (viruses, bacteria, fungi and protozoa), and can be infested by various parasitic insects and mites (Morse & Nowogrodzki, 1990). Normally, feral honey bee populations are not threatened by the parasites and pathogens with which they have co-evolved (Oldroyd and Nanork, 2009). However, Allen et al. (1990) reported that *A. laboriosa* populations in Nepal were infected by European foulbrood (*Mellisococcus pluton*), which they attributed to environmental stress by deforestation. Moreover, *A. mellifera* colonies have been introduced into many countries in Southeast Asia. Thus, the anthropogenic movement of honey bee populations between countries increasingly exposes wild populations to novel pathogens and parasites that they have no or reduced resistance to the pathogen alone or after subsequent stress (Oldroyd & Nanork, 2009).

The *Tropilaelaps* mite is a serious external parasite of the honey bee. Its primary host was subsequently recognized as the giant honeybee, *A. dorsata* (Laigo & Morse, 1968) and it has now been reported throughout the entire distribution range of *A. dorsata* (Matheson, 1996). It is also associated with other Asian honey bees, including *A. laboriosa*, *A. cerana* and *A. florea* (Delfinado-Baker et al., 1985).

The greater wax moth, *Galleria mellonella*, is the most serious pest in honey bee colonies worldwide. Its larvae cause considerable damage to bee colonies by feeding on the wax combs and cells containing broods, honey and pollens. The wax moth larvae also destroy the comb structure by forming tunnels inside the comb (Jyothi et al., 1990). Furthermore, Tingek et al. (2004) reported that a Conopid fly, *Physocephala parralleliventris* Kröber (Diptera: Conopidae) parasitizes *A. dorsata*, *A. cerana*, and *A. koschevnikovi* in Borneo. This fly grasps foraging bees in flight and deposits a larva on the integument. Then, the larva penetrates the bee cuticle and consumes the bee from the inside.

7.4 Pesticides

Some commercial fruit orchards, particularly longan (*Dimocarpus longan*), litchi (*Litchi chinensis*) and citrus are major nectar producers which are highly attractive to honey bees

(Crane et al., 1984). Commercial sun flower (*Helianthus annuus*) plantations are one of the most important sources of pollen and nectar for bees in Thailand. However, these commercial crops are regularly sprayed with insecticides, especially in the flowering period. Oil palm (*Elaeis* spp.) orchards are also regularly exposed to insecticides, and this may contribute to the observed low number of honey bees within oil palm crops (Oldroyd & Nanork, 2009). Colonies of all bee species may lose field bees when foraging on crops that are exposed to insecticides. The regulation of pesticide use is lax in some Southeast Asia countries, and can increase the possibility of honey bees exposure to pesticides (Oldroyd & Wongsiri, 2006).

7.5 Impact of climate change

Climate influences flower development and nectar and pollen production, which are directly linked with the colonies' foraging activity and development (Winston, 1987). A major effect of climate change on honey bees stems from changes in the distribution of the flower species (Thuiller et al., 2005) on which the bees depend for food. Rain can impact on honey harvesting. For example, when acacia (*Acacia* spp.) flowers are washed by rain, they are no longer attractive to honey bees as it dilutes their nectar too much (Conte & Navajas, 2008). Likewise, an overly dry climate can reduce the production of flower nectar for honey bees to harvest, since many plant flowers produce no nectar when the weather is too dry, which makes harvesting by bees a largely hypothetical matter. In these situations, honey bees can die of starvation.

8. Conclusion

In Southeast Asia, the bee diversity is very high, especially for honey bees (*Apis* spp.). In Thailand, there are four native honey bees; *A. cerana, A. florea, A. dorsata* and *A. andreniformis*, plus the anthropogenically imported *A. mellifera*. Other than *Apis* spp., stingless bees can produce honey as well. In Thailand, there are more than 50 species of stingless bees, of which the most common is *T. laeviceps*. Besides the biology, diversity and ecology of the bees, variation, both morphometric and genetic variations have been evaluated. In addition, although the gender of bees can be distinguished easily by their morphology, geometric morphometric analysis of their wings alone could successfully distinguish the genders. Bees are classified as eusocial insects since there are three distinct castes within a hive; that is the queen, drones and workers. Not only are bees are very useful as pollinators, but their bee products, especially honey, are economically important. Other than being consumed as food, bee products, especially honey, propolis and bee pollens, have long been used in traditional medicine. They provide many bioactivities, such as antimicrobial, anti-inflammatory, free radical scavenging and antiproliferation activities amongst others. Although they are important in agriculture, at present, it is obvious that there is a decrease in the number of hives of these bees. This may be due to a combination of deforestation, hunting, diseases, pesticide and other factors. Thus, it is very important to consider the conservation of bees and promote the bee management in each country.

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8

South African Spider Diversity: African Perspectives on the Conservation of a Mega-Diverse Group

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1. Introduction

Any field of endeavour requires retrospection after a period of substantial activity. This process provides a measure of what has been achieved and identifies future directions. Studies of spider diversity in South Africa have gone through an intense growth phase over the past ten years and reached a stage in its development where reflections on patterns and processes observed could provide meaningful input into the identification of further work. This chapter establishes the background and framework for such a discussion on the path to a more holistic conservation planning that includes invertebrates.

Invertebrate conservation and diversity pose a significant challenge to planners and managers (Engelbrecht, 2010), and in spite of the central role that insects and arachnids play in terrestrial biodiversity, they still remain peripheral to decision-making processes. The reality is that, for Africa in particular, there are very few conservation areas that have both the resources and expertise to include invertebrates as part of their monitoring and management initiatives (however, see South African River Health Programme[1]).

The advent of adaptive management, with a strong emphasis on experimental implementation of alternative management options (Johnson, 1999), has informed much of recent thinking and has cast a dim light on classical inventory studies that generate species lists. However, records of the numbers of species and their distribution provides a fundamental starting point for the conservation of biodiversity (Pullin, 2002). This view also ignores the contribution that basic inventories and alpha taxonomy make to the initial development of a field.

This chapter will show that South African spider systematics and ecology are in an exploratory phase, and that traditional approaches to mapping diversity has enabled spider ecology in the country to generate species lists that are often resolved up to species level. Very few other studies on mega-diverse invertebrate groups in Africa can match this taxonomic resolution (see e.g. Formicinae). This descriptive phase will provide the

[1] www.dwa.gov.za/iwqs/rhp

foundations for more integrative work in future, and any attempts to ignore the importance of providing baseline biodiversity and taxonomic data will hamper subsequent attempts to develop a deeper understanding and appreciation of this unique heritage.
The real significance of this heritage is highlighted by recent work throughout Africa that point to the global relevance of Southern African spider diversity (Jocqué, pers. com.). Contrary to the general trend of decreasing diversity at higher latitudes (Jenkins et al. 2011), spider richness increases towards the middle and southern latitudes of Africa, i.e. a hump-shaped distribution, peaking in Southern Africa (Platnick, 1991). This region is also characterized by ancient landscapes that have remained relatively stable for millions of years, with several relictual taxa that were once more widely distributed (Jocqué, 2008).
As signatories to the Convention on Biodiversity[2], South Africa is obliged to develop a strategic plan for the conservation and sustainable utilization of this heritage. The convention also has two key objectives, which are the "identification and monitoring" of biological diversity and "public education and awareness" (articles 7 and 13). The South African National Survey of Arachnida (SANSA) was initiated to address this by discovering, describing and inventorying the South African arachnid fauna (Dippenaar-Schoeman & Craemer, 2000). In addition to this, the project also had a strong element of public involvement.
The second phase of SANSA that was initiated in 2006 saw the integration of a series of *ad hoc* projects into targeted surveys in degree squares throughout South Africa (Dippenaar-Schoeman, 2007). Annual accessions of specimens in the South African National Collection of Arachnida (Pretoria) increased three-fold from the start of this second phase (Fig. 1).
Despite the extensive sampling carried out during this phase, several gaps and areas in South Africa that are underrepresented in the database still remain (Fig. 2). This can largely be attributed to logistical challenges, restricted manpower and time necessary to sample these areas properly, particularly in the western parts of South Africa. However, there are certain patterns that are evident, several lessons were also learnt, and we detail these based on the ca. 50 000 records accessioned as part of SANSA. This chapter also provides a general overview of spider biodiversity at the biome scale and a vision of future directions of spider diversity studies in South Africa.

2. Information needs of a developing country

Based on current estimates there are a total of 2010 species in 70 families recorded from South Africa. This represents 61% of the world's family fauna. Of these species, 1220 (> 60%) are endemic to the region, including two families, Chummidae (Jocqué, 2001) and Penestomidae (Miller et al., 2010). This high level of endemicity could partly result from the low inventory levels beyond South Africa's borders.
As our database is plagued by undersampling, we provide estimates of spider diversity in some of the studies, and although species richness is a crude measure of diversity, it could provide some indication of inequalities between areas and their significance to conservation. A summary table of spider species richness found in each of the biomes suggests that the Savanna Biome is the most diverse but sampling is very uneven (Table 1). Limited sampling in the Fynbos Biome precludes any definitive statements, but it is evident that Fynbos

[2] www.cbd.int

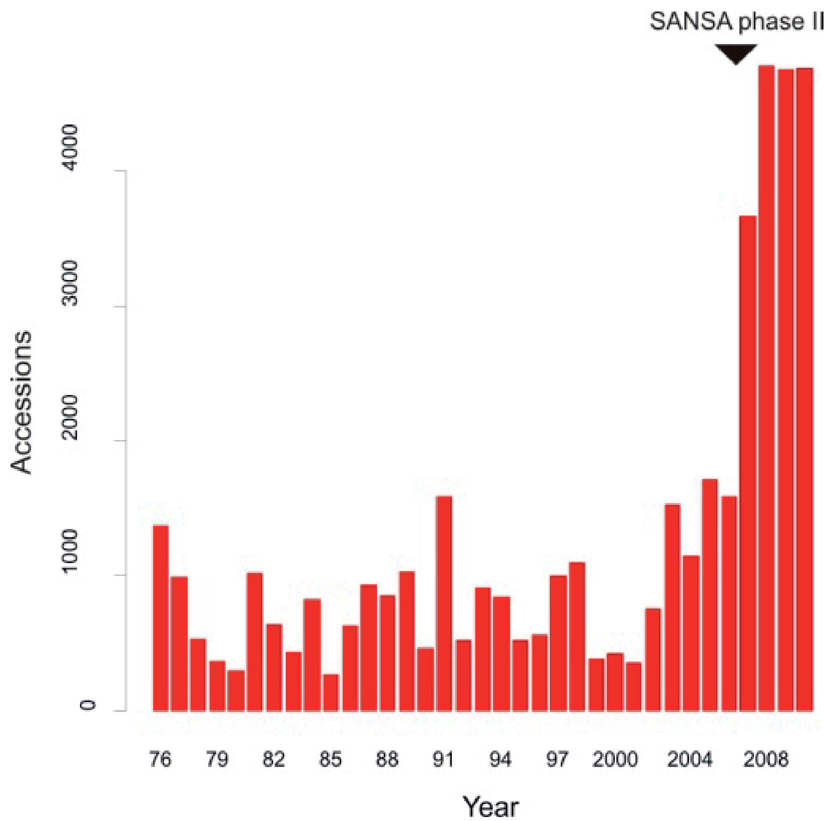

Fig. 1. Spider accessions per year in the South African National Collection of Arachnida, the largest in Africa.

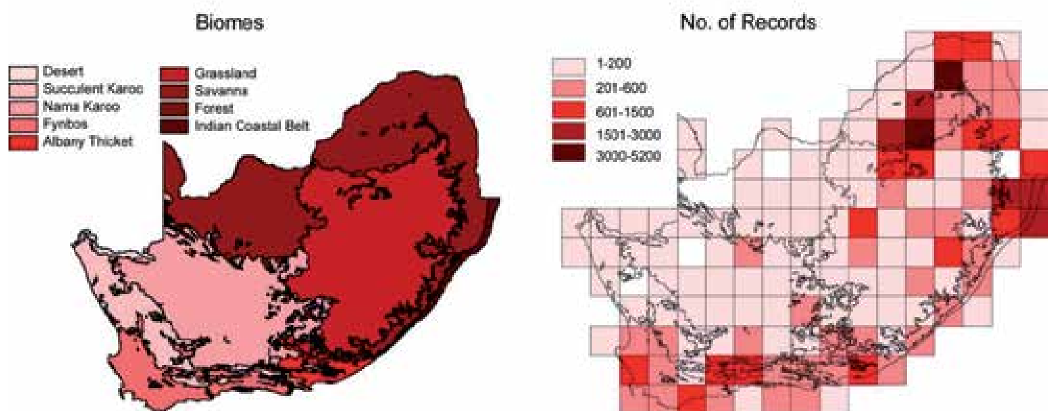

Fig. 2. Biomes of South Africa (Mucina & Rutherford, 2006) and the number of spider records for each degree square throughout South Africa.

diversity matches that of degree squares in savanna subjected to the same sampling intensity (Fig. 3). The Grassland Biome seems to be the next most diverse biome, followed by forests. In contrast, Thicket, Nama Karoo and Succulent Karoo have not been sampled sufficiently to suggest any pattern.

Biome	No. of sites	No. species
Savanna	46	1201
Grassland	27	655
Forest	5	508
Fynbos	13	636
Nama Karoo	8	464
Succulent karoo	1	219
Thicket	3	464

Table 1. Number of sites for each biome that have more than 100 accessions, together with total number of species recorded for each biome.

2.1 The Savanna, largest and most diverse biome, issues of scale and heterogeneity

Savanna ecosystems cover approximately 60% of the land surface of sub-Saharan Africa. The biome is recognized by a discontinuous overstory of woody plants and a herbaceous layer dominated by grasses. Rainy seasons are short with long dry seasons and the temperature is warm almost all year round. Rainfall is relatively high (500 – 2000 mm) but usually concentrated over short periods of time. Currently there are 1201 spp. (60% of the South African fauna) in 370 genera and 61 families known from this biome in South Africa. Of the 1201 spp., 327 spp. are endemic to the savanna and 322 are near endemics. Salticidae is the most diverse family (157 species) followed by Thomisidae (116) and Gnaphosidae (106). Characteristic taxa include species of the genus *Hersilia*, particularly those within the *Hersilia sericea* species-group (Foord & Dippenaar-Schoeman, 2006), orb-weavers such as *Araneus apricus*, *Cyclosa insulana* and *Isoxya tabulata*, and crab spiders, *viz. Diaea puncta*, *Thomisops scrupeus*, *Simorcus cotti* and *Runcinia flavida*. All these species are foliage dwellers, and the absence of diagnostic ground dwelling species are probably the result of the localised distribution of epigeal species within the Savanna Biome.

A total of 10 published surveys of the Savanna Biome have been undertaken over the last 20 years (Dippenaar-Schoeman et al., 1989; Foord et al., 2002; Whitmore et al., 2002; Dippenaar-Schoeman & Leroy, 2003; Modiba et al., 2005; Haddad et al., 2006; Dippenaar et al., 2008; Foord et al., 2008; Dippenaar-Schoeman et al., 2009; Muelelwa et al., 2010). The savanna has been better sampled than the other biomes and predictably has the most records (Fig. 2). Unfortunately, even this biome still suffers from severe undersampling, and species richness is largely a function of sampling effort, although it does seem to asymptote at approximately 350 species per degree square (Fig. 3). Some of the degree squares in this biome are the most diverse in South Africa, e.g. the degree squares that contain Ndumo Game Reserve in northern KwaZulu-Natal and the Western Soutpansberg in Limpopo Province.

Undersampling is, however, the rule rather than the exception, even in sites that have been intensively sampled (Coddington et al., 2009). For example, after 150 one-person hours worth of effort and 100 pitfalls left open for 20 days in 16 ha of a savanna vegetation type in

the Limpopo Province, Muelelwa et al. (2010) sampled less than 50% of the species present. Their data was fitted to a lognormal distribution and species richness estimates varied between 370 and 450 for the two 16 ha sites sampled (Muelelwa et al., 2010). With the default hypothesis of undersampling, the 431 species recorded from Ndumo Game Reserve (Haddad et al., 2006; Wesołowska & Haddad, 2009) is exceptional. Subsequent collecting in this reserve by canopy fogging, pitfall trapping and hand collecting has expanded our knowledge of the fauna, which now exceeds 500 species.

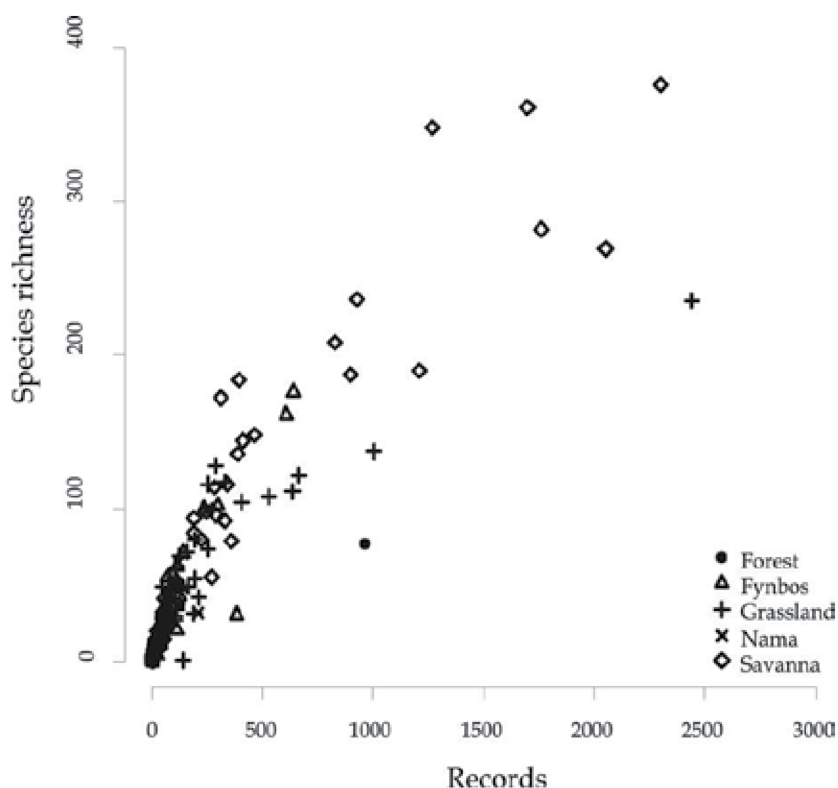

Fig. 3. Number of species vs. the number of sampling records in each of the 143 degree squares categorized according to biomes in South Africa, Succulent Karoo and the Thicket Biomes were excluded due to insufficient sampling.

Savannas are one of the most heterogeneous ecosystems, both spatially and temporally, in the world (Lomolino et al., 2006). Therefore, in addition to alpha diversity at a site, understanding the role of beta diversity and the scale at which assemblages respond to the environment are particularly important considerations for managing a particular taxon's diversity in this biome. The results from three concurrent semi-quantitative studies between May 2004 and March 2006 in Limpopo Province (Dippenaar et al., 2008; Foord et al., 2008; Muelelwa et al., 2010) provides a basis for evaluating the role of alpha vs. beta diversity in generating spider diversity at various scales. The degree of turnover between the spider assemblages of the 18 plant communities distributed over 4 sites was summarized by computing Bray-Curtis similarities and the Chao estimator of the Sørensen similarity index (Chao et al., 2005); the latter index includes the effect of undetected species.

The results from these three surveys, *viz*. Dippenaar et al. (2008), Foord et al. (2008) and Muelelwa et al. (2010), yielded a total of 642 species. The regional richness of these four sites is comparable to the national check list of Portugal, with 757 species (Cardoso et al., 2008).

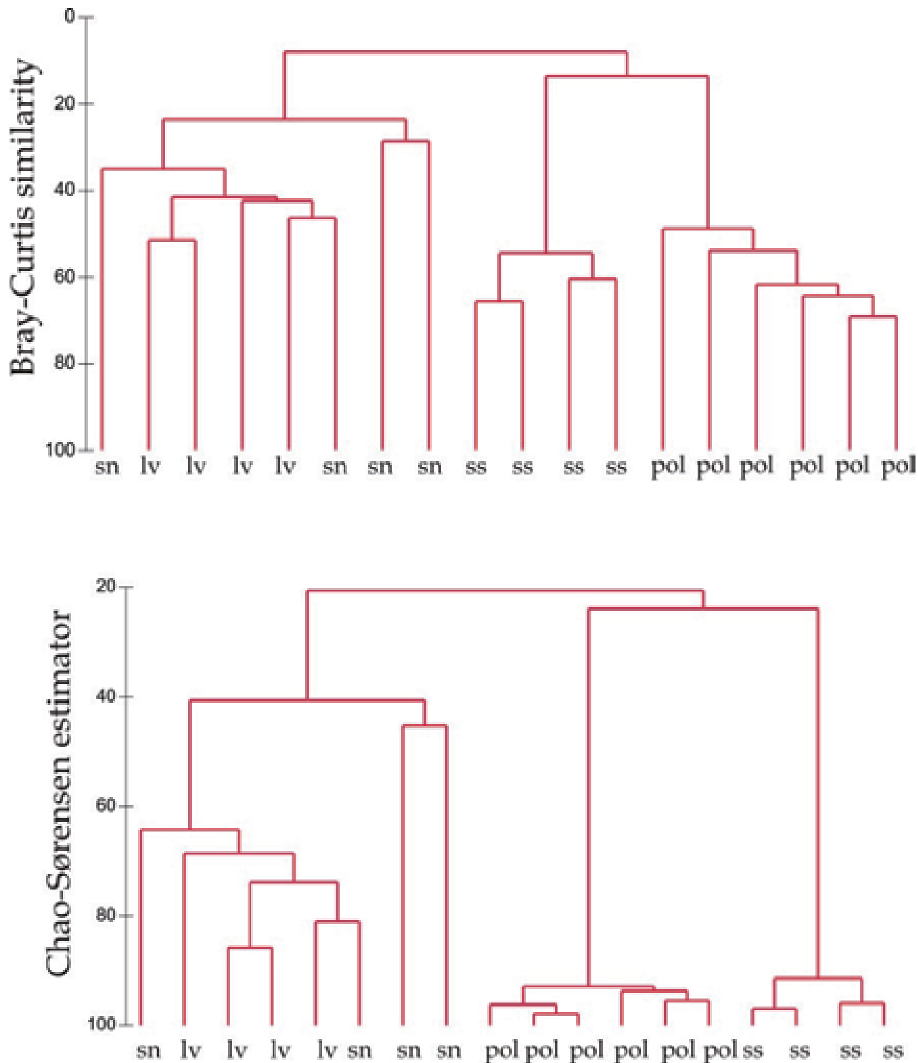

Fig. 4. Cluster diagrams of Bray-Curtis similarities and Chao-Sørensen estimators for the spider assemblages of the Limpopo valley (lv), Soutpansberg northern aspect (sn), Soutpansberg southern aspect (ss) and Polokwane Nature Reserve (pol) for semi-quantitative surveys conducted between May 2004 and March 2006.

The Chao estimator of the Sorensen index suggests that turnover between plant communities in a site is very low, particularly for the southern aspect of the Soutpansberg and Polokwane, with estimated similarities exceeding 90 for plots within these sites. Turnover between the three surveys was very high, with similarities as low as 20 and never higher than 30 (Fig. 4). Even though the plant communities within each of these three sites

differed substantially in structure, e.g. the grassland vs. woodland, estimates of similarities suggest that their spider assemblages are very similar within a site. As expected, estimates of similarities between plant communities within the three surveys increased with the amount of time spent surveying, e.g. Polokwane Nature Reserve had the highest similarity estimates (> 90) and sampling was done over a twelve month period, followed by the southern aspect of the Soutpansberg (three months), and then the Limpopo valley and Soutpansberg northern aspect (two weeks). Limited turnover between plant communities might result if identifications are not consistent between surveys, particularly when morphospecies comprise > 30% of the spiders collected. However, two of the authors (Dippenaar-Schoeman and Haddad) were responsible for species as well as morphospecies determinations in all three studies, resulting in fully reconciled species lists.

The conservation implication of these patterns is that several small reserves that are geographically widely separated would maximize the spider diversity conserved, as long as a diversity of habitats with varied structure is maintained within each of these. This large-scale regional comparison will have to be complimented with studies at more intermediate scales to establish the scale at which beta-diversity is generated. Studies in the next biome, grasslands, touch on the role of this turnover at smaller scales in generating spider diversity.

2.2 Grasslands – Discovering new biotopes and species

The Grassland Biome is found on the high central plateau of South Africa, including large parts of Gauteng, Mpumalanga, Free State, parts of North West, and the inland regions of KwaZulu-Natal and the Eastern Cape (Fig. 2). This biome is dominated by a single layer of grass and absence of trees, except in a few localised areas. There are many unique grass-dwelling spiders found on the vegetation, with special adaptations in body form, colour, and web and retreat construction. A total of 57 families represented by 299 genera and 655 species have so far been recorded from this biome; 70 spp. are endemic to the biome and 156 are near endemic. The Salticidae with 77 species, followed by the Gnaphosidae (75) and Thomisidae (64) are the most species rich families. Grassland survey results have focused on the Free State (Lotz et al., 1991; Haddad & Dippenaar-Schoeman, 2002, 2006a; Butler & Haddad, In press), Gauteng (Van den Berg & Dippenaar-Schoeman, 1991a), Mpumalanga (Makaka et al., In review) and KwaZulu-Natal provinces (Van der Merwe & Dippenaar-Schoeman, 1996).

The species richness in degree squares found in the Grassland Biome diverges from that of the Savanna Biome, and the most diverse degree square has considerably fewer species than the most diverse degree squares in the savanna (Fig. 3). Even the outlier of the grassland is probably not a true reflection of real grassland diversity as it incorporates the city of Pretoria that occurs on the edge of the Savanna Biome.

Sampling in the majority of long- and short-term studies, as well as *ad hoc* collecting, relies heavily on the use of general techniques for collecting spiders, including pitfall trapping, sweep-netting and beating foliage. While these methods are efficient for collecting large numbers and estimating species richness of spiders, they say little about the microhabitat preferences of spiders. In the case of pitfall traps in grasslands, for example, the majority of species collected can be regarded as "typical" ground-dwelling species, regularly sampled at many sites within the Grassland Biome, while singleton and doubleton species are often represented by grass- and foliage-dwelling species that have fallen into pitfalls accidentally.

As a result of these biases, supplementary methods such as hand collecting can often provide considerable information on individual species' microhabitat preferences, especially

when microhabitat structural differences can be identified and considered. For example, hand collecting at the base of grass tussocks of different grass genera (e.g. *Themeda*, *Eragrostis* and *Cymbopogon*) and litter sifting under contrasting trees (e.g. *Olea*, *Searsia* and *Cussonia*) within the same habitat in central South Africa often yield very different faunas (Butler & Haddad, In press, Haddad pers. obs.). To illustrate this point, three examples will be presented here.

The recently described tracheline sac spider *Poachelas striatus* Haddad & Lyle, 2008 was initially reported from three localities in central Free State grasslands with a range of approximately 100km. All of the material had been sampled in the two years prior to its description, with most of the specimens collected at the base of grass tussocks, where this species is often very common (Haddad & Lyle, 2008). This apparent restricted distribution was the result of two scenarios, i.e. distinct endemism in central South African grassland, or undersampling. To assess the distribution of the species in the Grassland Biome and beyond, several sites in the Free State, Northern Cape and Eastern Cape were sampled, with up to two hours of active searching at the base of grass tussocks carried out at each site. At all of the sites sampled *P. striatus* was collected, extending its range to approximately 900km and its occurrence to the Savanna and Nama Karoo Biomes (Haddad, 2010).

The purse-web spider genus *Calommata* (Atypidae) was recently revised by Fourie et al. (2011), with two South African species recognized, namely *C. transvaalica* Hewitt, 1916 and *C. meridionalis* Fourie, Haddad & Jocqué, 2011, known from savanna and grassland habitats, respectively. The initial discovery of *C. meridionalis* in the Erfenis Dam Nature Reserve in the Free State provided the first records of the genus from the Grassland Biome in South Africa. To assess its distribution, pitfalls were set out at six localities in the Free State. The species was only collected at two of these, where the soil structure could be considered as dark vertic clay or loamy-clay soils, which are usually associated with lower-lying areas near water bodies (Fourie et al., 2011). This was consistent with the soil types at Erfenis Dam where the species was first discovered. Although only known from three localities, this species is likely to be quite widespread in the Grassland Biome, and future pitfall studies aiming to collect this species should take into account the particular soil type apparently preferred by this species, as well as the restricted phenology of this species (male activity from the end of September to December). These considerations will allow for a much more accurate presentation of the distribution of the species and likelihood of success in sampling this species.

Long-necked or assassin spiders (Archaeidae) are a family of morphologically unique araneophagous spiders occurring in South Africa, Madagascar and Australia, with some species of *Archaea* recorded from Baltic amber (Wood, 2008). South African archaeids have typically been regarded as forest-dwelling specialists, primarily because most of the species were collected by leaf litter sifting in forests. Two of the South African species, *Afrarchaea harveyi* and *A. royalensis*, were described by Lotz (2003, 2006) from the type localities only, i.e. Champagne Castle and Royal Natal National Park in KwaZulu-Natal, respectively. During recent field work in January 2011, additional records of *A. harveyi* were collected in open grassland at Champagne Castle and Cathedral Peak by searching at the base of grass tussocks, under rocks and under *Berkheya* leaves close to the ground, while additional records of *A. royalensis* were collected at Royal Natal in forest leaf litter and at Platberg Nature Reserve in the eastern Free State at the base of grass tussocks in open grassland (H. Wood, pers. comm.). Therefore, the presumption that *Afrarchaea* only occur in forests is false; some of the species at least frequent moist grasslands where they can be readily collected

once their preferred microhabitats have been identified. Thus, only by sampling particular microhabitats thoroughly can the resident spiders be identified, and hypotheses alternative to "popular thinking" be proposed regarding their broad habitat/biome preferences as well as specific microhabitat preferences.

2.3 Forests – Islands of endemism?

The Forest Biome in South Africa is composed of three vegetation types, namely Afromontane (Afrotemperate) forests, sand forest and coastal forest. Together, these vegetation types only cover approximately 0.3% of South Africa's land surface (Mucina & Rutherford, 2006). To date 51 families represented by 223 genera and 508 species have been recorded from forests. The Thomisidae with 71 species are the most species rich, followed by the Salticidae (70) and Araneidae (43).

Afromontane forests are particularly under threat, with nearly 30% of this vegetation type transformed or degraded. In contrast, nearly half of the sand forest habitat is protected in formal conservation areas (Eeley et al., 2001). The South African Afromontane forests occur as small fragments within a fynbos, grassland or savanna matrix, and are predominantly found along the slopes of mountain ranges in the south, east and north-east of the country. Sand forest is very localised and only occurs on the Maputaland coastal plain of Northern KwaZulu-Natal, extending into southern Mozambique where it is known as Licuati forest. Coastal forest forms a relatively continuous belt from the Eastern Cape northwards, extending into East Africa. While the Forest Biome has been extensively sampled in South Africa, few quantitative published studies exist on the spider fauna (Van der Merwe & Dippenaar-Schoeman, 1996; Dippenaar-Schoeman & Wassenaar, 2002, 2006; Haddad et al., 2010).

Amongst invertebrates, high levels of diversity and endemism have been recorded for harvestmen (De Bivort & Giribet, 2010), snails (Bursey & Herbert, 2004; Herbert, 2006; Govender, 2007; Cole & Herbert, 2009), millipedes (Hamer & Slotow, 2002, 2009) and velvet worms (Daniels et al., 2009) in South African forests. While considerable speciation has taken place in the aforementioned groups, spiders have generally not followed suit to the same degree, with only 33 species being endemic to forest and 141 near endemics, although the origin of these forests biota is complex and ancient. Griswold (1991a) pointed to the inability of dispersal and Pleistocene vicariance to explain the biogeographical patterns of four Afromontane spider taxa, *Microstigmata* (Microstigmatidae), *Moggridgea quercina* group (Migidae) and the tribes Vidoleini and Phyxelidini (Phyxelididae), instead proposing Mesozoic origins for some of the observed patterns. A contemporary review of his findings could provide a test of this assertion, particularly since many of the species that were originally recorded from the Forest Biome are now widespread within forests and/or occupy habitats in several different biomes.

There are, however, several notable exceptions. For example, the six South African species of *Microstigmata* (Microstigmatidae) are considered to be restricted to forest habitats in the eastern half of the country (KwaZulu-Natal and Eastern Cape Provinces), with the exception of *M. zuluense* and *M. longipes* that also occasionally occur in savanna habitats in northern KwaZulu-Natal (Griswold, 1985, 1991b; Dippenaar-Schoeman et al., 2006). Using the endemism classes of Hamer & Slotow (2002) and the published data of Griswold (1985), two species could be considered site endemics (only one locality), one species a local endemic (distribution range of 11–70km), and three species as South African endemics, with a distribution range of >150km. As a result of collecting since Griswold's revision, including

SANSA sampling, one of the site endemics (*M. ukhahlamba*) can now be considered a South African endemic, occurring widely in forest patches in the Drakensberg Mountains. The first specimens of *M. longipes* from Table Mountain in the Western Cape Province were also recorded, thereby disputing Griswold's (1985) proposal that the genus is absent from the western Cape Fold Mountains. This makes the absence of the genus from the more continuous Afromontane forests of the Outeniqua Mountains of the southern Eastern and Western Cape Provinces, where extensive leaf litter collecting in forests has been conducted, even more puzzling.

Griswold (1990) recognised 54 species in his global revision of the family Phyxelididae (then a subfamily of Amaurobiidae), of which 30 species are known from South Africa. Ten of these species (33%) were suggested to be endemic to the Forest Biome or occur in closed canopy bush, while the other 20 species (67%) are troglobitic or occur in grassland, savanna, fynbos or desert habitats, occasionally also occurring in forests (Griswold, 1990). Thus, at least a few of the species of most of the South African genera seem largely specialised to occur in forest habitats.

Within the family Zoropsidae, two endemic South African genera are presently recognised, namely *Griswoldia* (formerly *Machadonia*) and *Phanotea* (Griswold, 1991b, 1994). Both Griswold and Lawrence (1953) regarded these two genera as important components of the cryptic fauna of moist South African forests. Regarding *Griswoldia*, 11 of the 12 described species were recorded from Afromontane or coastal forests, with only *G. urbense* reported from grasslands also. The extensive sampling in South Africa following the generic revision indicates that only two of the species, *G. meikleae* and *G. sibyna*, are entirely restricted to forest; the remaining species in the genus have now also been recorded from fynbos, grassland, savanna or thicket habitats. Griswold (1994) also proposed that the 10 species of *Phanotea* were largely restricted to closed canopy forests, except for three species also found in fynbos and one (*P. peringueyi*) known only from caves. Following SANSA, only one species, *P. knysna*, is known only from forests. The others have now also been recorded from fynbos, grassland, savanna and/or thicket habitats too. Despite the fact that zoropsids are not restricted to forests, most species can still be regarded as essential components of forest spider assemblages.

2.4 Fynbos – Tantalizing correlates of diversity

Fynbos covers only about 6.7% of South Africa (about 85 000 km^2) but has the largest number of plant species of any biome in the country (about 7500). Most fynbos is found along the coast and in the Cape Fold Mountains between Nieuwoudtville in the north-west and Port Elizabeth in the east (Fig. 2). The Fynbos Biome includes both fynbos and Renosterveld vegetation.

The Fynbos Biome of South Africa has been the focus of considerable attention due to the high levels of plant diversity and endemism. While this may be true for the field of Botany, the invertebrate fauna, and spiders in particular, has remained comparatively poorly studied. To date, only four studies have been published on the spider diversity of the biome (Coetzee et al., 1990; Visser et al., 1999; Haddad & Dippenaar-Schoeman, 2009; Mukherjee et al., 2010). However, several studies are currently underway, including a long term survey that started in 2004 in the Cederberg Mountains, where invertebrates are sampled in 17 sites along an altitudinal gradient (Botes et al. 2006). Initial results points to the discovery of several new species along the transect (Seshothela & Dippenaar-Schoeman, 2011). Presently 61 families represented by 251 genera and 636 species are known from the Fynbos Biome,

with 189 species being endemics and 102 near endemics. The Salticidae with 240 spp. are the most diverse, followed by the Gnaphosidae (176) and Thomisidae (133).

Data from intensively sampled fynbos localities, such as the De Hoop Nature Reserve where 252 spider species were recorded (Haddad & Dippenaar-Schoeman, 2009), indicate that the fauna is less diverse that the Savanna Biome, where species counts regularly exceed 275 species. However, family diversity is the highest so far recorded in South Africa, with 54 families recorded from the reserve. When one considers that fynbos is a structurally less complex vegetation type than savanna, this diversity is quite remarkable.

Perhaps most interesting is the high levels of endemism in the Fynbos Biome reported for some families. For the family Corinnidae, for example, 10 of the 15 species recorded from De Hoop Nature Reserve are fynbos endemics, of which several have only recently been described (Haddad, 2006; Haddad & Lyle, 2008; Lyle & Haddad, 2010; Lyle, 2011). The same was found in the Cederberg, where three of the four species of Ammoxenidae sampled are new fynbos endemics (Seshothela & Dippenaar-Schoeman, 2011).

2.5 Nama and Succulent Karoo

The Nama Karoo Biome is the second-largest biome in South Africa and the distribution of this biome is determined primarily by rainfall that varies between 100 and 520mm per year, with the dominant vegetation being grassy, dwarf shrubland. It is bordered to the west side by the Succulent Karoo Biome that covers a flat to gently undulating plain, with some hilly and "broken" veld, mostly situated to the west and south of the escarpment, and north of the Cape Fold Belt (Fig. 2). Rainfall in the Succulent Karoo Biome varies between 20 and 290 mm per year and the vegetation is dominated by dwarf, succulent shrubs that are particularly prominent. There is little difference between the soils of the Succulent Karoo and Nama Karoo Biomes - both are lime-rich, weakly developed soils on rock.

Presently 464 spp. from 50 families are known from the Nama Karoo, of which 74 are endemics and 77 near endemics. The Gnaphosidae are the most diverse with 76 spp., followed by the Salticidae (49) and Thomisidae (32). Only 44 families have been recorded from the Succulent Karoo Biome so far, represented by 117 genera and 219 spp., of which 49 are endemics and 40 near endemics. The families Gnaphosidae (34 spp.), Lycosidae (25 spp.) and Salticidae (13 spp.) are the most diverse. Both of these biomes remain the most neglected in terms of qualitative long-term sampling in South Africa, despite considerable effort to sample and understand the scorpion fauna of these biomes. Most sampling was done on an *ad hoc* basis between 1890-1930 by arachnologists stationed in the South African Museum, resulting in a fair number of species being described (e.g. Tucker, 1923). Published results are mainly from collecting undertaken in the Mountain Zebra National Park (Dippenaar-Schoeman, 1988), Karoo National Park (Dippenaar-Schoeman et al., 1999a), Swartberg Nature Reserve (Dippenaar-Schoeman et al., 2005b) and Nama Karoo grassland (Haddad & Dippenaar-Schoeman, 2005).

Logistical constraints due to the distance that needs to be travelled by suitably qualified collectors from their bases, which are predominantly in the eastern half of the country, hampered new SANSA collections. The common perception is that these dry arid biomes have a low diversity of arachnids and are difficult to sample in, thus giving a low species yield per unit effort. This is clearly reflected in the number of sampling records from these biomes, and most notably, the presence of two degree-square grids from which no spiders have yet been recorded (Fig. 2). What needs to be appreciated is that while both reasons have some validity, the fauna that exists in these biomes is uniquely adapted to these arid

habitats, and likely shows high levels of endemism. These biomes are thus of considerable interest biogeographically, phylogenetically and from a conservation perspective. Jenkins et al. (2011) also point to the importance of these arid areas in developing our understanding of the effects of global climate change.

Due to the low vegetation, fewer plant- and web-dwelling spiders are recorded, but a rich fauna of ground dwellers is present. There are a number of endemics found in ground dwelling families such as the Ammoxenidae (12 spp.) and several trapdoor spider families: Cyrtaucheniidae (8); Ctenizidae (13); Idiopidae (7) en Nemesiidae (11) (Dippenaar-Schoeman, 2002). More than 100 species of Gnaphosidae alone are known from these biomes, most of which were described by Tucker (1923) in his revision of the family in South Africa, with several endemics. Other ground dwelling spiders such as the genus *Tyrotama* (4 species) of the Hersiliidae (Foord & Dippenaar-Schoeman, 2005) and ground-dwelling Eresidae (Dippenaar-Schoeman, 1989) have several species that are endemic to these arid biomes.

2.6 Thicket, between a rock and a hard place

The Thicket Biome is found from the west coast to KwaZulu-Natal, with most of the biome being found in the Eastern Cape. It makes up 2.5% of the area of South Africa (nearly 31 500 km^2) and the vegetation ranges from shrubland to low forest. There are many evergreen and succulent trees and shrubs and many of the plants have spines or thorns. Analysis of the spider assemblages did, however, suggest that this biome overlaps considerably with other biome types, and could potentially be grouped together with the fauna of the Savanna Biome. No published surveys are known from this biome but several surveys are presently underway. To date 55 families represented by 206 genera and 464 spp. have been recorded from thicket, of which 90 are endemics and 96 near endemics. The Thomisidae with 62 spp. are the most diverse, followed by the Salticidae (38) and Araneidae (37).

2.7 Agro-ecosystems

South Africa has about 12% arable land and some of these areas were sampled to determine the spider diversity. Spiders are one of the most ubiquitous predator groups in agroecosystems (Van den Berg & Dippenaar-Schoeman, 1991a) and inventories in South Africa have provided valuable baseline information on species in agroecosystems (Table 2). As predators, spiders have a two-fold function. Not only do they feed directly on their prey, but their presence also causes indirect mortality of arthropods. The presence of spiders could disturb larvae who then drop from the plant and die. The webs spun over the surface of leaves also seems to make them less suitable for oviposition and feeding. Therefore, while considerable effort has been put into baseline surveys in agroecosystems in South Africa, there is a large scope for further experimental work on the biological control potential of the dominant agobiont spiders in each agroecosystem.

The family Salticidae has been shown to be an important component of agrobiont assemblages. In a knock-down study of three macadamia orchards in the Mpumalanga Lowveld in South Africa, 73% of the sampled spiders were salticids (jumping spiders). Salticids were also the most diverse (17 species), of which four species represented more than 61% of all spiders collected. *Thyene coccineovittata* was the most abundant species and represented 30% of all the spiders collected, followed by *T. natali* with 14%, *Viciria alba* with 9% and *Tusitala guineensis* with 8%. These four species were present throughout the year in all three orchards sampled (Dippenaar-Schoeman et al., 2001a, b).

Salticidae were also the dominant spider family in pistachio orchard canopies in the arid Northern Cape, although their abundance varied between the three orchards sampled: Green Valley Nuts 1 (81.2%), Remhoogte (58.8%) and Green Valley Nuts 19 (82.4%). It seems that factors such as tree canopy size, presence or absence of bark and dry leaves, climatic conditions, and orchard size may be the main influences on spider abundance (Haddad et al., 2005). The fauna of ground covers was dominated by Salticidae in Green Valley Nuts 1 (37.2%) and Remhoogte (35.0%), while Oxyopidae dominated the ground cover fauna of the youngest orchard, Green Valley Nuts 19 (31.9%) (Haddad et al., 2004a). Four families, Linyphiidae, Lycosidae, Salticidae and Gnaphosidae, dominated the ground-dwelling fauna of the orchards, although the abundance of each differed considerably between sampling methods (pitfall trapping and active searching) and also between orchards (Haddad & Dippenaar-Schoeman, 2006b).

Crop	**Province**	**Methods**	**Family**	**Genus**	**spp.**	**References**
Citrus	Eastern Cape, Mpumalanga, Limpopo, North West	Hand, beating	21	35	197	(Dippenaar-Schoeman, 1998)
Cotton	North West, Mpumalanga, Limpopo	Whole-bag; hand; pittraps	31	92	127	(Van den Berg et al., 1990; Van den Berg & Dippenaar-Schoeman, 1991b; Dippenaar-Schoeman et al., 1999b)
Bt cotton	Limpopo	Pittraps; scouting	21	49	54	(Mellet et al., 2006)
Avocado	Mpumalanga	Fogging	26	68	90	(Dippenaar-Schoeman et al., 2005a)
Macadamia	Mpumalanga	Fogging	21	57	80	(Dippenaar-Schoeman et al., 2001a, b)
Pistachio	Northern Cape	Fogging; sweeping; hand; pittraps	31	83	143	(Haddad et al., 2004a; Haddad et al., 2005; Haddad & Dippenaar-Schoeman 2006b)
Strawberries	Gauteng	Hand	14	20	32	(Dippenaar-Schoeman, 1979)

Table 2. Crops in South Africa that have been surveyed for spiders and for which agrobiont spider species have been identified.

Preliminary data of spider prey items included Thysanoptera (Phlaeothripidae), Coleoptera (Bruchidae, Coccinellidae, Chrysomelidae), Diptera (Tephritidae, Muscidae, Cecidomyiidae), Acari (Tetranychidae), and various Lepidoptera larvae. Laboratory and field experiments on *Heliophanus pistaciae* (Salticidae), the dominant arboreal agrobiont species, indicated that it has potential as a biological control agent of the false cinch bug, *Nisius natalensis* (Hemiptera: Lygaeidae), one of the minor pests of pistachio nuts in South Africa (Haddad et al., 2004b).

2.8 Public education, participation and awareness (SANSA)

This constitutes another important component of the CBD and SANSA, aimed to increase awareness about arachnids to wider society through a variety of activities that includes an extensive website[3] containing information on the activities of SANSA. Additional information is also available on: 1) all the arachnid orders; 2) survey results; 3) medically important spiders; 4) arachnid research; 5) virtual museum entries; and 6) copies of newsletters and reports.

Target audiences are also identified and information is disseminated in the appropriate communication medium and language through books, CD's, posters, magazine and newspaper articles, pamphlets, TV and radio (Dippenaar-Schoeman & Jocqué, 1997; Dippenaar-Schoeman, 2002; Dippenaar-Schoeman & Van den Berg, 2010; Holm & Dippenaar-Schoeman, 2010). In addition to several radio and television appearances by Dippenaar-Schoeman, the Spider Educare Programme based at the National Collection of Arachnida has conducted road shows to schools, lectures to societies and clubs, and courses presented at Universities for the past ten years.

The online Virtual Museum of South African Arachnids presently houses about 3400 images received from 80 photographers throughout the country. Not only is it a wonderful display of photographs, but it also provides valuable information on species' behaviour, colour patterns and prey. Colour patterns in particular are lost when the spiders are killed and preserved in alcohol. The Virtual Museum has provided the spider images for two full colour books that have recently been published (Dippenaar-Schoeman & Van den Berg, 2010; Holm & Dippenaar-Schoeman, 2010).

3. Conclusion

Resources for surveys will always be limited and funding will have to be directed at answering targeted questions. Three biomes that require particular attention are the Thicket, Nama and Succulent Karoo Biomes. The Fynbos is also of particular significance as it seems to match the better known savanna biome in terms of spider diversity based on preliminary survey results. The hypothesized restricted distributions of species to Afromontane forests have also been rejected because of contemporary discoveries of these taxa in other biomes employing techniques that target specific microhabitats.

Two taxa, the families Theridiidae and Lycosidae, comprise the bulk of what constitutes a taxonomic impediment to a better understanding of diversity patterns in spiders. The family Salticidae is another important family, dominant in all ecosystems, that fortunately has and will continue to receive considerable taxonomic attention. The potential of this family as a surrogate of spider diversity, in conjunction with families such as Thomisidae and Gnaphosidae, could be explored, should the taxonomic impediment of taxa mentioned earlier persist.

We view the future research as a combination of optimized and standardized inventories in the degree squares of the four biomes identified. This will provide both cost efficient and comparable results. These inventories will, however, have to include protocols that target cryptic biotopes. The by-catch of other invertebrate taxa also provides the opportunity to collaborate with specialists on these taxonomic groups generating data on a "shopping basket" of taxa, as has been shown by a study that was done using the ant by-catch of one of the SANSA surveys (Schoeman & Foord, In press). These inventories have to be

[3] www.arc.agric.za quick link SANSA

complemented by targeted studies at regional and local scales over larger temporal scales if we are to fully understand the dynamics of spider assemblages in South Africa.

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9

Relationships Between Bird Species Richness and Natural and Modified Habitat in Southern Mexico

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1. Introduction

Principles implicitly addressed in most landscape level investigations of bird communities focus mainly on the arrangement of habitat patches, corridors, and matrix elements within landscapes; and patch area and isolation effects on dispersal, colonization, and local extinction (Forman, 1995). Ecologists are increasingly examining ecological patterns and processes at a scale that makes easier to understand the distribution and abundance of organisms contained within the habitat patches that compose the landscape (Forman & Gordon, 1986, Flather & Sauer, 1996, Bolger et al., 1997). Species interactions may vary for species within patches that adjoin different patch types (i.e., edge effects; Paton, 1994); for species in habitat patches of similar composition, but of differing patch sizes or distributions (i.e., habitat fragmentation effects; Robinson et al., 1995); for species requiring source-sink dynamics among patches in a landscape (i.e., metapopulations; Pulliam, 1988); and for species in habitat patches of similar composition but located within different landscape matrices (Renjifo, 1999).
Many studies of the effects of forest fragmentation on bird communities have been conducted in fragments surrounded by agricultural lands, and principles of island biogeography theory (MacArthur & Wilson, 1967) are usually invoked to explain patterns of species richness (Opdam, 1991). Birds are important model organisms for such studies because their taxonomy and distribution are well known, and because inventory and census methods are well developed (Ralph et al., 1995). However, in other situations, the surrounding habitat is not totally unsuitable for birds, and its characteristic determine how island-like the fragment will be (Hinsley et al., 1995, Stouffer & Bierregaard, 1995). In such cases, habitat fragmentation creates a mosaic of habitat patches of different quality, with forest fragments providing high quality habitat, and the matrix providing lower quality habitat (Wiens, 1994). For example, in North America, forest fragmentation has had an array of effects on neotropical migratory birds through habitat loss, small forest-patch size, reduced proximity of patches, more edge effect, and negative interactions with species surrounding nonforest patches (Faaborg et al., 1995, Freemark et al., 1995, McGarigal & McComb, 1995, Robinson et al., 1995).

The distribution and diversity of bird communities in the tropical forests of Mexico and Central America have certainly been affected by a high degree of deforestation and therefore habitat fragmentation, but little quantitative or comparative data exist (Stiles, 1983, Flores-Villela & Geréz, 1994, Ceballos, 1995, Challenger, 1998). Some studies have identified landscape and habitat structural characteristics associated with the distribution of bird species richness in forest fragments that may be used to predict patterns of species richness in tropical deciduous forest patches (Gillespie & Walter, 2001), because different bird communities occur in response to changes in vegetation structure and species composition following logging (Morrison, 1992, Aleixo & Vielliard, 1995).
The Central Depression of Chiapas, located in southeastern Mexico, is an important area for conservation because highlights key characteristics of Middle American tropical deciduous forests: high level of endemism and the convergence of two biogeographically important migratory routes (the Gulf and the Pacific ones), thus, contains species that have migrated to the dry forest through each of these corridors. Also, there is a high turnover rate (beta diversity) between areas of tropical deciduous forest, which is also important for species conservation (Janzen, 1988, Escalante et al., 1993, Stattersfield et al., 1998). The area has also global importance for avian endemism (Stattersfield et al., 1998), and as a well-defined ecoregion (NT0211; Olson & Dinerstein, 1998, Myers et al., 2000), a Terrestrial Priority Site (Arriaga et al., 2000), an important bird area (IBAS; Arizmendi & Márquez, 2000), and the presence of some Natural Protected Areas including National Parks and Biosphere Reserve (i.e., Sumidero Canyon, El Zapotal; CONANP, 2011).
The understanding of the relationships and factors that influence bird community structure provides valuable information on the impact of habitat disturbance on populations, which is important for the conservation of these species. The goal of this contribution was to investigate differences in the species richness and composition of the bird communities in a mosaic natural and modified habitat and to evaluate how forest habitats perform to preserve species in the Central Depression of Chiapas. The results will be used to inform about appropriate strategies for the conservation of both the remnants of the original forest and the habitats created by humans with the species that inhabit them.

2. Material and methods

2.1 Study area

The study area is located among the Municipalities of San Fernando, Tuxtla Gutiérrez, Chiapa de Corzo, Osumacinta and Chicoasén in the Central Depression region in Chiapas, southern Mexico (Table 1, Fig. 1). The climate is warm sub-humid with rainy summer (June to October), being May the hottest month, to moderate sub-humid in altitudes above the 1000 m (FORTAM, 1984, García, 1996). Mean annual temperature is 18-24 °C and mean annual precipitation varies between 500-2500 mm (FORTAM, 1984, INEGI, 2004, 2006). Annual precipitation shows a marked seasonality. The rainy season begins in mid-May, causing a surge of foliage and regrowth in natural vegetation areas as well as in crops and pasture grasses. This period normally lasts until the end of September. The dry season begins in December, lasting until May. The area includes a complex mixture of tropical habitats that have been classified on the basis of the physiognomic characteristics of the vegetation (Rzedowski, 1988, Reyes-García & Souza, 1997) and on the basis of land management. The natural and semi-natural habitats include tropical deciduous forest, tropical semideciduous forest, tropical oak forest, riparian forest, secondary forest, abandoned tropical forest with

distinct successional stages (secondary forest), and agriculture fields, living fences, cattle pasture, shaded coffee plantations, urban and suburban areas (Miranda, 1975, FORTAM, 1984, Reyes-García & Souza, 1997). Common tree species in study area included *Pistacia mexicana*, *Cochlospermum vitifolium*, *Ceiba* sp, *Bursera bippinata, B. simaruba*, *Zuelania guidonia*, *Gyrocarpus* sp, *Acacia cornigera, A. pennatula*, *Haematoxylum* sp, *Lysiloma* sp, *Alvaradoa amorphoides*, *Swietenia humilis*, *Ficus* sp, *Fraxinus purpusii*, *Sideroxylon celastrinum* and *Heliocarpus reticulatus* (Miranda, 1975, Reyes-García & Souza, 1997).

Study sites	Municipality	Geographical coordinates	Elevation (m)	Habitats types
1	Chicoasén	16°57´N, 93°08´W	819	Tdf, Sf, C, Af, Lf
2		16°58´N, 93°10´W	1056	Tdf, Tsf, Sf, Rf, C, Af, Lf
3	Osumacinta	16°56´N, 93°05´W	583	Tdf, Sf, C, Af, Lf
4		16°55´N, 93°04´W	855	Tdf, Sf, C, Af, Lf
5		16°53´N, 93°07´W	630	Tdf, Tsf, Sf, Rf
6	San Fernando	16°54´N, 93°09´W	1068	Tdf, Tsf, Sf, Af
7		16°52´N, 93°11´W	968	Tdf, Sf, C, Af, Lf
8		16°50´N, 93°13´W	1085	Tdf, Sf, C, Af, Lf, Ur
9		16°50´N, 93°12´W	970	Tdf, Sf, C, Af, Lf
10		16°51´N, 93°12´W	862	Tdf, Sf, C, Af, Lf, Ur
11		16°48´N, 93°10´W	835	Tdf, Tsf, Sf, To, Rf, C, Af, Lf
12		16°49´N, 93°11´W	868	Tdf, Sf, To, C, Af, Lf
13		16°49´N, 93°09´W	890	Tdf, Sf, C, Af, Lf
14		16°49´N, 93°12´W	1050	Tdf, Sf, C, Af, Lf
15		16°50´N, 93°11´W	875	C, Af, Lf, Ur
16		16°48´N, 93°11´W	883	Tdf, Sf, C
17		16°47´N, 93°10´W	710	Tdf, Sf, C
18		16°54´N, 93°10´W	995	Tdf, Tsf, Sf, Rf
19	Tuxtla Gutiérrez	16°45´N, 93°06´W	535	Sf, Ur
20		16°45´N, 93°08´W	550	Ur
21		16°45´N, 93°05´W	508	Sf, Ur
22		16°47´N, 93°05´W	845	Tdf, Sf, C, Af, Lf
23	Chiapa de Corzo	16°42´N, 93°01´W	395	Sf, Rf, C, Af, Lf
24		16°41´N, 92°59´W	412	Sf, Rf, C, Af, Lf, Ur

Table 1. Characteristics of study sites in Chiapas Central Depression. Habitat types: Tdf (tropical deciduous forest), C (Cattle pastures), Lf (living fences), Rf (gallery forest), Tsf (Tropical semideciduous forest), Sf (secondary forest), Af (Agricultural fields), To (Tropical oak forest), Ur (Urban and suburban areas).

2.2 Bird data

Base data were collected in the field from February 2003 to November 2004 by sampling by point counts (Hutto et al., 1986, Ralph et al., 1995) that were used to asses species richness and abundance in each habitat. The number of point counts per habitat (4-8 points) was proportional to depended on the extent of different habitat types (between major coverage of the habitat sampled highest number of points). At each count station, the number of individuals of each species detected by sight and sound were recorded during a 5 min count

period. Each count lasted for 5 minutes with a 5-minute interval between points. Birds detected at ≥ 100 m were recorded but not used in analyses to reduce the possibility of counting the same individual twice in consecutive points. Birds detected when not conducting counts were also recorded and used to calculate total species richness. No counts were conducted on days when visibility was poor, or under windy or rainy conditions. Counts were conducted between 0700 and 1100 in the morning, and 1600 and 1900 in the afternoon (i.e., during the highest bird activity). No survey was conducted during unfavorable weather conditions (rainy, windy and mist days) because birds were less detectable under those conditions (O'Connor & Hicks, 1980, Robbins, 1981).

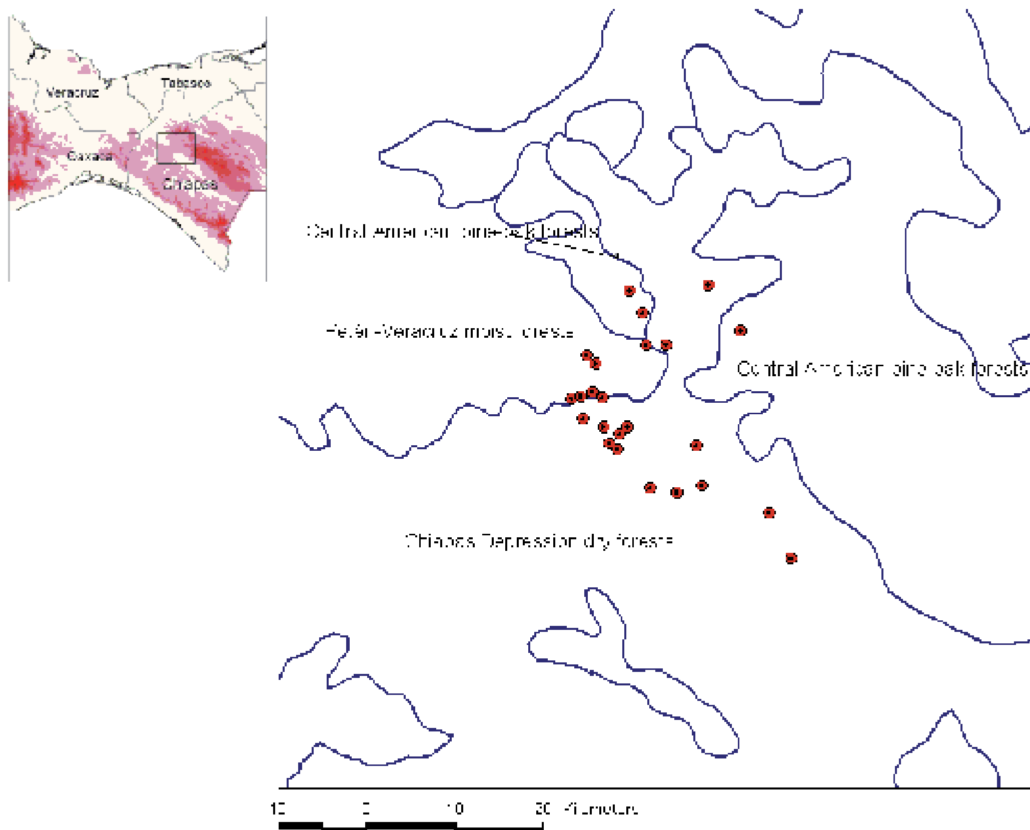

Fig. 1. Map of Tehuantepec Isthmus region in southern Mexico, the quadrangle depicts study region (a). Underlying map is average elevation (http://www.conabio.gob.mx). Study sites (dots) in the Central Depression of Chiapas, Mexico (b). Labels correspond to Ecoregions of World Wildlife Fund (http://www.wwf.org).

Bird species richness was calculated as the total number of species recorded in each habitat. Two estimates of relative abundance (including both visual and aural detections of both sexes) for each species were obtained for each habitat: the average number of individuals per point count, and frequency of occurrence during monthly samples. These two measurements of relative abundance assume that birds are recorded more often in areas where they are more abundant (Renjifo, 2001). The average number of individuals per point count was based on all

point counts conducted within a study habitat, and frequency of occurrence was based on presence or absence over all monthly samples. Relative abundances of neotropical or neartic migrants were based upon samples during months when they were present in the study area: January-April and October-January (i.e., winter visitors, summer residents). Each species was classified by a habitat guild (forest interior, generalist, and forest edge). We calculated species richness (We referred to total species richness as the total number of species per habitat) and abundance (bird abundance was obtained as the mean number of individuals detected in the total points counts per habitat) at each study site for forest interior species, generalist species, and forest edge species. Habitat guild classification was based Ehrlich et al. (1988) and also supported by other studies in fragmented forest (Brooks & Croonquist, 1990, Murcia, 1995, McIntyre, 1995, Rodewald & Yahner, 2001). Bird species were categorized into seven broad diet categories (carnivore, insectivore, nectarivore, frugivore, granivore, omnivore and aquatic) based upon primary components of the diet or subdiet obtained directly from field information and with supplemental information from literature (*i.e.*, Ortiz-Pulido et al., 1995, Arizmendi et al., 1990, Ramírez-Albores, 2010).

2.3 Analysis

We used aerial photographs (scale 1:75,000; INEGI, 2001) to map land-use types of study sites and we performed direct surveys throughout the area for confirmation of site suitability. Sampling intensity was stratified among different sites based on the extent and cover proportions of different habitat on the INEGI image. Each sites was surveyed an equal number of times (sites were visited one time each month). At each site, at least 90-100% of the nonforest cover within 1 km of the study site consisted of only one disturbance type (primarily agricultural fields, cattle pastures and urbanization). We determined forest cover from classified thematic mapped imagery using ARC/INFO geographic information system software (ESRI, 1999). We calculated species richness by habitat guild: forest specialist, generalist, and early successional species, at each study site. Species richness was analyzed separately by a multiple regression analysis to assess if there was any differential response to forest disturbance characteristics, based on the species level of dependence on arboreal cover proportion. Stepwise regression analysis was performed on log-transformed total number of species, and on resident and migrant species. Bird abundance data were log (e) x + 1 transformed previous to the analyses to reduce the skewness of the data, resulting in a more interpretable analysis. An *F*-test probability value of 0.05 and 0.001 was used in all cases. Differences in species richness and guild structure of bird communities, represented as the species richness in different foraging guilds, were compared among habitat types using one-way analysis of variance (ANOVA). Tukey´s multiple range test was used for post-hoc comparisons among habitat types (Zar, 1999). The Similarity of species composition between habitats types was measured using Sorenson´s similarity index ($IS=2S/N_1+N_2$, where S is the number of common species, N_1 is the number of species of habitat 1, and N_2 is the number of species of habitat 2; Ravinovich, 1981). To improve the knowledge of the geographic distribution of each individual species we used a set of maps of all species of landbirds of Mexico (Navarro-Sigüenza & Peterson, 2007) constructed by ecological niche modeling (Nix, 1986, Peterson, 2001). Maps depict the potential distributions of the species using the Genetic Algorithm for Rule-set Production (GARP; Stockwell & Noble, 1992), in its PC implementation DesktopGARP (Scachetti-Pereira, 2003), using as primary source the data points contained in the Atlas of the Birds of Mexico data base (Navarro-Sigüenza et al., 2003). For generating the

models a set of 19 climatic variables, derived from temperature and precipitation (Hijmans et al., 2005; http://www.worldclim.org), and three topographic (Hydro1k project; http://eros.usgs.gov) was used. Individual summaries of distributions of species were summed to produce species richness maps for total species, summer resident species, winter resident species (Navarro-Sigüenza & Peterson, 2007). From the GARP maps, we derived predicted numbers of resident and migrant species using ArcView (version 3.2; ESRI, 1999). We also compared species richness values for each grid cell (resolution 0.05°) with GIS data layers summarizing Terrestrial Prioritary Regions (Arriaga et al., 2000, CONABIO, 2004) to assess whether areas recognized as priority under diverse criteria coincide with areas of greatest species richness. All statistical analyses were permormed using STATISTICA® 10 and SPSS® 19.5.

3. Results

3.1 Bird species composition

A total of 279 species of 45 families was recorded from the 24 sites (Appendix 1). Of these, 193 were permanent residents and 86 were migrant species (including one occasional, two summer residents, 18 transients and 65 winter visitors). In general, the average bird richness during the study period was of 131 species/month; however, the monthly bird species richness ranged from 100 to 161 (Fig. 2). The fewest species were found in May and the most in March, April, December and January (Fig. 2). The composition of the bird community associated with percentage of disturbance in the study sites, according to habitat preferences, corresponding to 30.2% (N = 84) for forest specialists, 10.4% (N = 29) of early successional species and 39.5% (N= 110) forest generalists (Fig. 3). The distribution of each category in the sites showed greater richness of specialists and forest generalists. Forest specialist species richness ($F_{1,22}$= 5.98, r= 0.46, P= 0.02) and generalist ($F_{1,22}$= 17.53, r= 0.66, P= 0.0003) were negatively associated with percentage of disturbance (Fig. 3). Early succesional species richness ($F_{1,22}$= 4.21, r= 0.40, P= 0.05) was slightly related to disturbance within study sites. Diet or subdiet composition of bird communities in the study sites was: 82 were insectivores, 72 insectivores/frugivores, 39 carnivores, 20 granivores/fruigivores, 14 nectarivores and 13 granivores (Appendix 1).

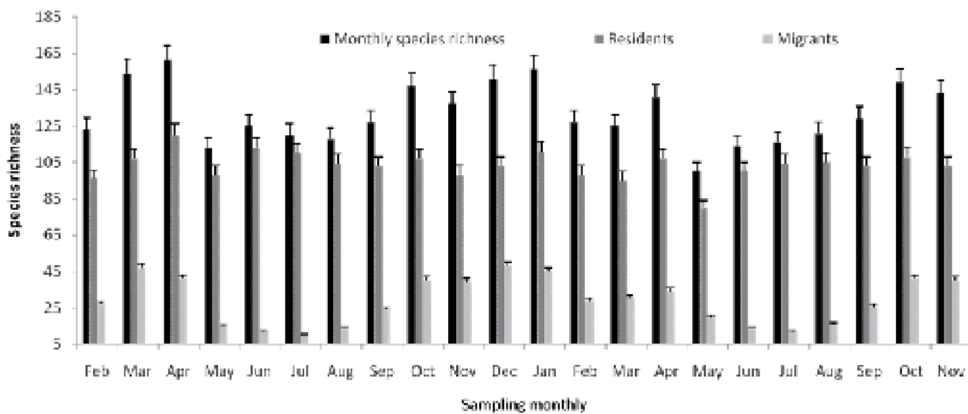

Fig. 2. Monthly species richness during study period. Mean and standard error for data pooled over visit and point count shown (errors bars represent 95% confidence intervals).

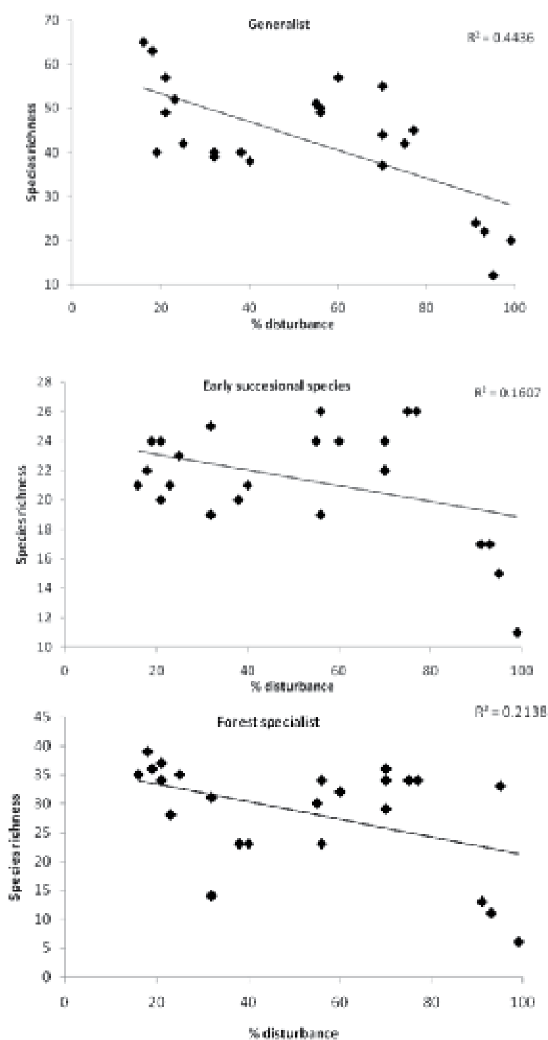

Fig. 3. The relationships between the extent disturbance (%) and bird species richness at the study sites.

3.2 Comparison among habitat types

Tropical deciduous forest (203) had the highest number of species, whereas tropical oak forest (51) and aquatic and semiaquatic habitats (24) had the fewest species (Fig. 4). Of the total bird species recorded (278), 20 were exclusively found in tropical deciduous forest, four of tropical semideciduous forest, two of urban/suburban areas and one of cattle pastures (Appendix 1). To analyzed comparative the habitat types with bird species richness and mean abundance, and we found significant differences ($F_{8,125} = 70.6$, $P < 0.0001$, $F_{8,125} = 106.2$, $P < 0.0001$, respectively). As migration status in the different habitat types, also significant differences between residents species ($F_{8,125} = 79.1$, $P < 0.0001$) and migratory species ($F_{8,125} = 45.3$, $P < 0.0001$).

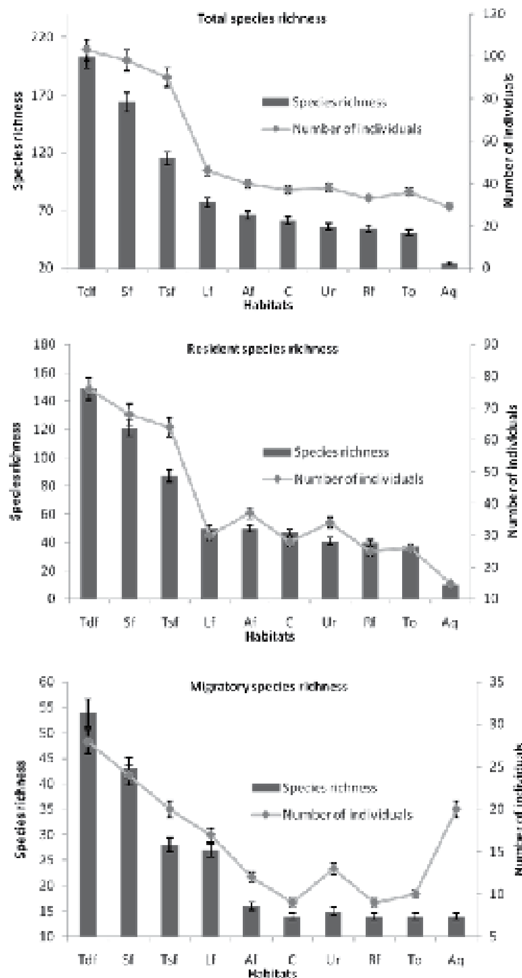

Fig. 4. Species richness of birds in different habitat types at the study sites. Mean and standard error for data pooled over visit and point count shown (errors bars represent 95% confidence intervals). Habitat types: Tdf (tropical deciduous forest), C (cattle pastures), Lf (live fences), Rf (gallery forest), Tsf (tropical semideciduous forest), Sf (secondary forest), Af (agricultural fields), To (tropical oak forest), Ur (urban and suburban areas) and Aq (aquatics and subaquatics).

Carnivores bird species were better represented in the tropical deciduous forest (17), agricultural fields (18) and pastures (17; Fig. 5), while the lowest numbers occurred in the tropical oak forest (5) and urban/suburban zones (4) and there were no species in living fences ($F_{8,125}$ =53.9, P<0.0001). Insectivores-frugivores species were more abundant in the tropical deciduous forest (64) than in agricultural fields (3) and pastures (6; $F_{8,125}$ =35.4, P<0.0001). The lowest number was recorded in living fences and pastures with one species each, presenting significant differences between habitats ($F_{8,125}$ =47.2, P<0.0001). Tropical deciduous forest (70) had a greater number of insectivores species than gallery forest (10; $F_{8,125}$ =80.1, P<0.0001). Tropical deciduous forest had the highest number of

nectarivores (14), compared to the tropical oak forest, cattle pastures and agricultural fields where there were no records of these species, the differences between habitats ($F_{8,125}$ =38.0, P<0.0001).

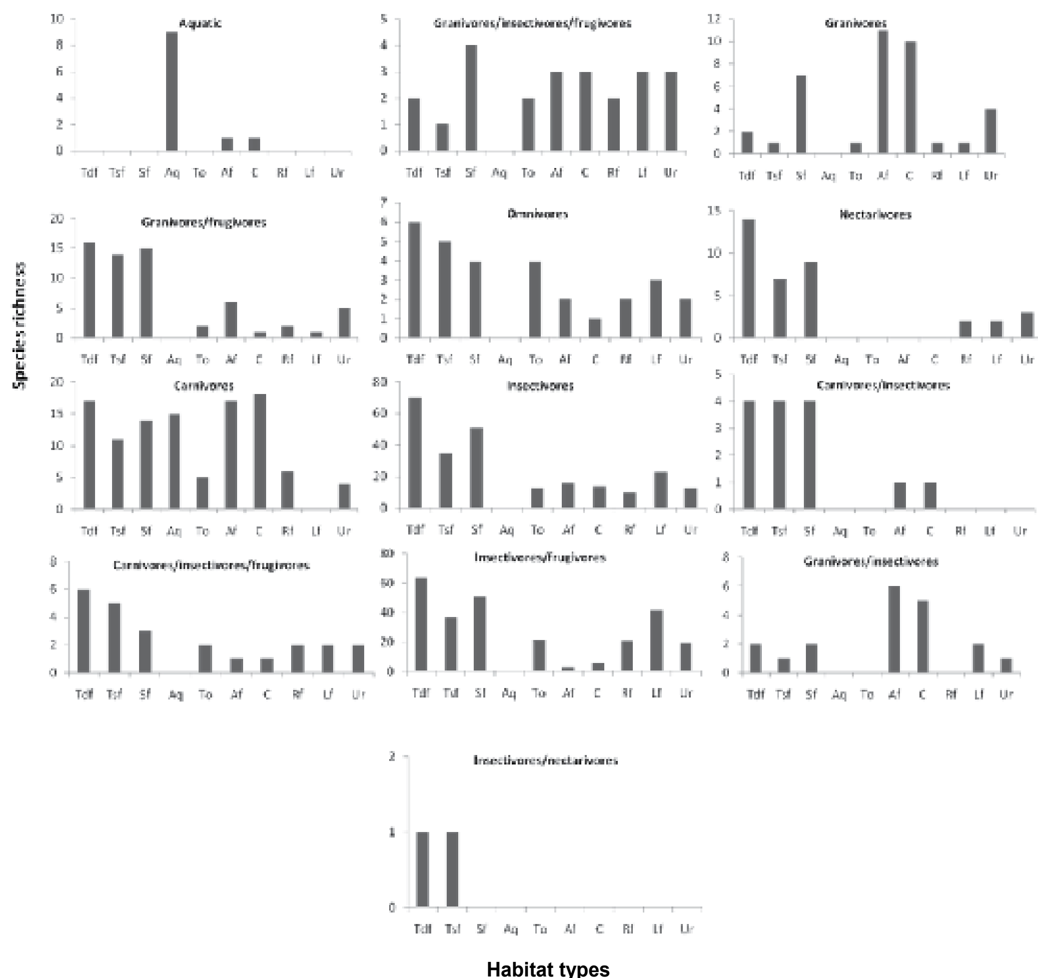

Fig. 5. Diet or subdiet composition of bird communities in different habitat types of study area. Habitat types: tropical deciduous forest), C (cattle pastures), Lf (living fences), Rf (gallery forest), Tsf (tropical semideciduous forest), Sf (secondary forest), Af (agricultural fields), To (tropical oak forest), Ur (urban and suburban areas) and Aq (aquatics and subaquatics). Diet categories: I (insectivore), C (carnivore), O (omnivore), N (nectarivore), A (aquatic), G (granivore), F (frugivore).

Similarity of species composition between habitat types indicates that the highest values were among cattle pastures and agricultural fields (0.81), followed by tropical deciduous forest and secondary forest (0.75) (Table 2). The fewest values were among gallery forest and cattle pastures (0.15), gallery forest and agricultural fields (0.19), and tropical semideciduous forest and cattle pastures (0.19; Table 2).

	Tropical semideciduous forest	Tropical oak forest	Secondary forest	Gallery forest	Cattle pastures	Agricultural fields	Living fences	Urban/suburban areas
Tropical deciduous forest	0.69	0.33	0.75	0.32	0.22	0.23	0.46	0.34
Tropical semideciduous forest		0.44	0.61	0.36	0.19	0.24	0.35	0.39
Tropical oak forest			0.42	0.57	0.34	0.26	0.51	0.46
Secondary forest				0.34	0.31	0.36	0.54	0.40
Gallery forest					0.19	0.15	0.53	0.49
Cattle pastures						0.81	0.24	0.37
Agricultural fields							0.22	0.32
Living fences								0.45

Table 2. Matrix similarity of bird species, based on Sorenson´s Index, among habitat types surveyed in Chiapas Central Depression.

3.3 General patterns of bird diversity

Species per 1 km^2 cell in the map of the region (Fig. 6) can do a high geographic consistency of the patterns. The richest areas of the study area form a strip that runs in an east-west from the eastern part of the Petén-Veracruz moist forest, following to northern part of the Chiapas Depression dry forest, and continues the Central America pine-oak forest. The richest cells within this region are precisely in the northern part of Chiapas Depression. Two cells differ with high values of richness, which are north of the Central Depression of Chiapas. We can say that there is a continuous strip of high species richness throughout the study area in east-west. In this sense, are evident two regions: the northern part of the Central Depression of Chiapas, and Gulf Coastal Plain. In fact, this latter may represent a decrease in species richness west-east (Fig. 6). The figure 6 helped to identify the species richness of areas of greatest concentration of diversity; the southern region presented the lowest concentration with a maximum of 62 species. The prediction map of migratory species richness shows the greatest number of species concentration mainly in the southern and northeastern. Most species are concentrated in the dry and moist forest, which is apparently a different distribution pattern observed in the Central America pine-oak forest.

4. Discussion

Of a total of 656 bird species occurring in Chiapas according to Álvarez del Toro (1980) and Palomera-García et al. (1994), the species recorded in the study area (Central Depression of Chiapas) corresponds to 42% (279 species; Appendix 1). This high richness is a result of a complex array of habitats, convergence of two important migratory routes (of the Gulf and Pacific), as well as biogeographic (biotic provinces) and physiographic heterogeneity (Arriaga et al., 2000). Bird species richness found in study sites is similar to that in other tropical forest regions in Mexico, such as La Mancha on the coast of Veracruz (250 species; Ortiz-Pulido et al., 1995) and Chamela in Jalisco (270 species; Arizmendi et al., 1990). The study sites showing greater species richness (especially in tropical deciduous forest) are

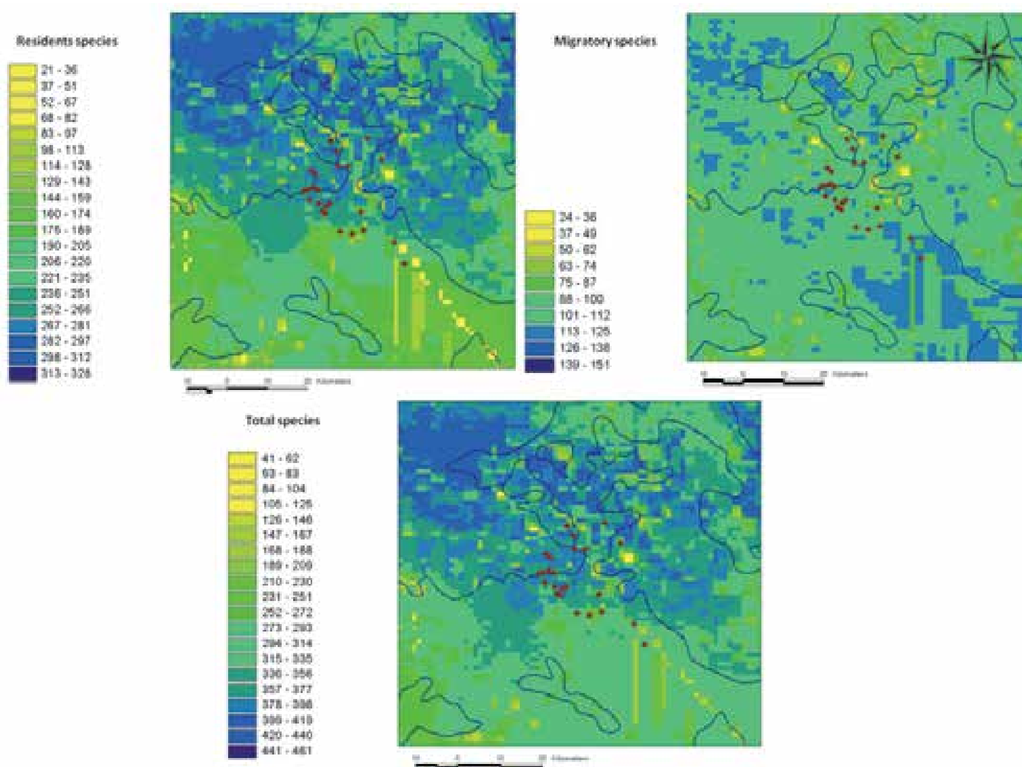

Fig. 6. Maps representing of modeled species richness in the study sites at the Central Depression of Chiapas.

different in forest cover but diverse in habitat types associated with tropical forest are areas that contained continuous secondary forest, the same pattern found in forest fragments in southern Brazil (Anjos, 2001).

The 195 species (70%) were considered residents; as the number of resident species may be higher due to birds with less conspicuous behavior in certain periods of the year and/or difficulty in detecting those (Krügel & Anjos, 2000). Karr et al. (1982) mentioned that in some tropical environments the migratory species are capable of producing changes in the composition of bird communities. In this study migratory species (30%) played a minor role in the observed changes in the bird community. According to Arizmendi et al. (1990) and Moya-Moreno (1990), it is possible that in the study area altitudinal and latitudinal movements are correlated with fluctuations in the abundance of species. For example, some rare species are clearly features temperate environments whose populations are dispersed to other locations during times of scarcity of resources, or are migratory in passing that occur in small amounts within Chiapas. However, these seasonal changes in abundance, possibly also associated with seasonal phenology of the deciduous forest were not assessed, so that needed to be discussed in detail later.

Species richness was greater in April, October, and December, surely due to the presence of migratory species and to the beginning of reproductive activity, which make birds more detectable. On the other hand, a lower richness was found in May and August, a period in which migratory species were absent and birds were quiet, making them difficult to detect.

Seasonal variation of the avifauna in the present study was similar to that found in other tropical forest regions (Chamela region in Jalisco, Mexico and Maringá in Paraná, Brazil; Ornelas et al., 1993, Krügel & Anjos, 2000, respectively) where the species richness was greater from October to November. Our results show that species composition did not differ significantly across the 24 study sites, and similarities of different levels among the sites were common. This could suggest that most tropical forest patches still have suitable habitats that ensure availability of food, nesting sites, and protective cover for the species but are still vulnerable to persistent encroachment evident around them. In the long term this could jeopardize the ability to sustain particular bird species, especially forest-dependent bird, threatened and endemic.

A considerable amount of species associated with secondary forest, open areas, clearings and forest edges remain abundant and are likely to increase in regions with small isolated forest fragments (Bierregaard & Lovejoy, 1989, Thiollay, 1992). Generalist birds, which change their diet from fruit to insects or vice-versa, are also favored in small patches (Willis, 1979). Mota (1990) found increasing, devastation of pristine areas. Although some general tendencies were observed for certain bird groups, the effects of forest fragmentation are certainly different for each species. A study in forest fragments (in Maryland) suggested that the impacts of forest fragmentation on bird communities are complex, species specific and not related only to fragment area or fragment isolation (Lynch & Whigham, 1984). The increase in species richness with fragmentation was primarily due to the addition of several migrants that were associated with edge habitats and secondary forest.

These species showed a lower frequency possibly because they were represented by few individuals, and are more sensitive to forest change and fragmentation than more widespread species, as patterns that has been shown before (Fjeldsa, 1999, Renjifo, 2001). Priority species (i.e., endemic and threatened) are important contributors to biodiversity because their restricted distributions make them globally rare and particularly vulnerable to population declines or extinction (Terborgh & Winter, 1983, Diamond 1986). Species with small ranges are also less abundant at a local scale than large-range species (Brown, 1995). The birds may demonstrate a differential response to forest fragmentation (Hobson & Bayne, 2000, Fahrig, 2003) or that probably bird species richness in the study area can be affected by other factors, such as floristic diversity, and vegetation composition and structure (Gillespie & Walter, 2001). Other effects, such as the extent and nature of the fragments edges, fragment connectivity, or fragment shape (Bierregaard et al., 1992, Laurance & Bierregaard, 1997, Cornelius et al., 2000), might be more important than forest cover in predicting the number of species found in the area (Ramírez-Albores, 2010). As for other studies, it is expected that the effect of forest cover would affect bird species richness (Kattan et al., 1994, Laurance & Bierregaard, 1997).

Diet composition was similar among habitat types, with greatest representation by insectivores and insectivores/frugivores, and decreasing representation by nectarivores and granivores. According to Petit et al. (1999) and Karr (1990), this distribution of foraging guild memberships is typical of that found in tropical forest. Tropical forest fragments resulting from human disturbance of a continuous forest are isolated more rapidly. The remaining areas suffer of progressive degradation due to isolation, which, in the long term, jeopardizes the survival of several species. In tropical environments, modified habitats are very important to a lot of carnivores, granivores and insectivores species as a temporary or permanent supply of these resources depending on their phenology and seasonality (Loiselle & Blake, 1994). On the other hand, the habitats with more complex vegetation

structure and formed by several layers of coverage are mainly species of insectivores, frugivores and nectar habits (Rappole et al., 1993). The results in this study are consistent with the above, as modified habitats had a higher proportion of species and individuals of carnivores and granivores habits compared to the original habitats (i.e., tropical deciduous and semideciduous forest).

In general, the variety of habitats present in the study region seems to contribute a high proportion of species, especially considering the number of species occurring in tropical deciduous forest (203). This may be due to the structural complexity that makes an ecosystem with greater species richness in Mexico (Ceballos & García, 1995, Ceballos et al., 2010), and the fact of having a greater horizontal and vertical stratification with respect to others, thus generating increased availability of habitats and ecological niches (Blake & Loiselle, 1991, McIntyre, 1995, Villard et al., 1999), as the plant structure determines the amount and distribution of resources used by birds. The differences in diversity and richness found indicate that the tropical deciduous forest, tropical semideciduous forest, and secondary forest show a greater richness compared to other modified habitats (i.e., agricultural fields, cattle pasture). This coincides with other studies conducted in tropical environments and indicates that the original habitat loss directly affects the presence, abundance and persistence of species (Kattan et al., 1994, Laurance & Bierregaard, 1997). The results of this study suggest that species richness and diversity of habitats ranging from the study. Natural habitats (tropical deciduous forest, tropical semideciduous forest and secondary forest) appear to be attractive to a larger number of bird species, as both the richness and diversity were higher in these, which is consistent with other studies (Estrada et al., 1997, Petit et al., 1999, Blake & Loiselle, 2001, Bojorges & Lopez-Mata, 2005).

Modified habitats (agricultural fields, living fences, cattle pasture) had a significant contribution to the bird species richness in the study area. These habitats provide roosting sites and food resources (Lynch, 1989). This is consistent with that reported by Estrada et al. (1997), which brings a richness of 226 in the region of Los Tuxtlas (Veracruz, Mexico), finding 79% of the species found in forest areas, 80% farmland, 43% in living fences and only 5% in grassland/pasture. In addition, Petit et al. (1999) in the central area of Panama found that species richness in modified habitats (i.e., shade coffee plantations, residential areas, grasslands and pine plantations) is equal or similar to the natural habitat. However, live fences exhibited the highest species richness (77 species) from modified habitats (i.e., agricultural fields, cattle pasture), probably because their plant structure is more complex and diverse. This is consistent with other studies (i.e., Villaseñor, 1993, Villaseñor & Hutto, 1995, Morales, 2002), which state that living fences can be very attractive to a large number of individuals and species of birds, and also can support high densities as they provide food resources, roosting sites and shelter (Villaseñor & Hutto, 1995). For example, in the study area, some birds prefer to use corridors or live fences instead of open or cleared areas (Wegner & Merriam, 1979) and turnover rates are significantly more frequent along corridors connected to original habitats or with other corridors (Hass, 1995, Machtans et al., 1996). In the case where the original habitat remains, the complexity of vegetation provides alternative sites for some species, partially offsetting the fragmentation and allowing the persistence of resident and migratory species (Morales, 2002).

The similarity between the habitats types of study area indicates the existence of a high turnover of species and an apparent high connectivity between them. Suggesting that both the configuration of the environment (i.e., landscape, habitat and microhabitat) and the available amount thereof would not be equally important in the distribution of species (Karr

1990) and could confer changes in the composition of the community birds (Blake & Loiselle, 2001). Although the conservation of bird species depends on a clear understanding of their habitat requirements and the physical and biotic processes that keep (Askins, 2000), has been established that the combination of natural and modified habitats leading to new opportunities differential exploitation of space (Willson, 1974) and diversity of bird species is related to landscape diversity, so that conservation of the latter ensures the preservation of species diversity (Bôhning-Gaese, 1997).

The distribution of birds in different physiographic regions of Chiapas is highly heterogeneous (Rangel-Salazar et al., 2005), and may also occur heterogeneity within each region, or even between adjacent physiographic regions (González-Domínguez, 1998). As the behavior of the birds in the Central Depression of Chiapas can be shared or influenced by other regions such as Montañas del Este, Altiplano Central, and even by the Sierra Madre de Chiapas, giving it the ability to host species of these regions (Altamirano, 2004). Biogeographic research biotic transition zones are an essential part of the study of the processes that govern the distribution and diversity of organisms (Williams et al., 1996). In this regard, species richness captures a fundamental aspect of spatial patterns of biodiversity (Koleff et al., 2003). Studying diversity patterns among the cells used in the present analysis helps to generate hypotheses about the processes that contribute to defining the current distribution patterns in the Isthmus, as the spatial turnover of species may reflect deterministic processes such as adaptation of species to different conditions, speciation, and responses to weather events or other historical effects (Condit et al., 2002).

5. Conclusions

Given the continued fragmentation of natural habitats and according to the results of this study, addition and maintenance of natural and modified habitats are necessary for survival and reproduction of many species of birds in the study area. The study area, like many other regions of the country is being affected by anthropogenic factors, particularly the expansion of the agricultural frontier, forest fires, population growth and livestock, which directly affects wildlife populations wild. Studies of diversity and species richness are approximations that represent the basis to further evaluate information by monitoring the changes associated with environmental factors and especially anthropogenic. And the visualization of the biogeographic patterns over changes in species richness according to changes land permits to locate the sites that have been modified over time. This method facilitates the identification of priority areas for conservation because key to the survival of species groups threatened and endemic. Understanding the patterns of richness is closely linked the establishment of actions at the federal, regional and local levels, as they reflect as conditions of land use change are affecting populations. The need to make a stock assessment and particular requirements of each species; can support the planning, implementation and evaluation. Additional conservation actions can help to assure that these viable long-term populations are sufficient to retain species.

6. Appendix

Bird species record from 24 sites of the Central Depression Chiapas. Taxonomy and order species follow AOU (2010). Migratory status: resident (R), migratory (M; including winter visitor, summer resident and transit). Habitat types: Tdf (tropical deciduous forest), C (cattle

pastures), Lf (living fences), Rf (gallery forest), Tsf (tropical semideciduous forest), Sf (secondary forest), Af (agricultural fields), To (tropical oak forest), Ur (urban and suburban areas) and Aq (aquatics and subaquatics). Diet categories: I (insectivore), C (carnivore), O (omnivore), N (nectarivore), A (aquatic), G (granivore), F (frugivore).

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Crypturellus soui	R	O	x									
Crypturellus cinnamomeus	R	O	x	x								
Dendrocygna autumnalis	R	A										x
Anas discors	M	A										x
Ortalis vetula	R	GF	x	x	x	x						
Penelope purpurascens	R	GF	x	x								
Colinus virginianus	R	G			x			x	x			
Tachybaptus dominicus	R	A										x
Podilymbus podiceps	M	A										x
Pelecanus occidentalis	M	C										x
Phalacrocorax brasilianus	R	C										x
Anhinga anhinga	R	C										x
Ardea herodias	M	C										x
Ardea alba	M	C										x
Egretta thula	M	C										x
Egretta caerulea	M	C										x
Egretta tricolor	M	C										x
Bubulcus ibis	R	I						x				
Butorides virescens	M	C										x
Nycticorax nycticorax	M	C										x
Nyctinassa violacea	R	C										x
Coragyps atratus	R	C	x	x	x	x	x	x	x		x	
Cathartes aura	R	C	x	x	x	x	x	x	x		x	
Pandion haliaetus	M	C	x	x	x							
Elanus leucurus	R	C						x	x			
Rostrhamus sociabilis	R	C	x		x							
Ictinia mississippiensis	M	C	x	x		x		x	x			
Accipiter striatus	M	C	x	x	x			x	x		x	
Accipiter cooperii	M	C	x	x	x	x		x	x			
Buteogallus anthracinus	R	C	x		x			x	x			
Buteo magnirostris	R	C	x		x	x		x	x			
Buteo nitidus	R	C	x		x			x	x			
Buteo brachyurus	R	C	x		x							
Buteo swainsoni	M	C	x		x				x			
Buteo albicaudatus	R	C	x	x								
Buteo jamaicensis	R	C	x		x			x	x		x	
Caracara cheriway	R	C						x	x			
Herpetotheres cachinnans	R	C						x	x			
Falco sparverius	R	C						x	x			

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Falco columbarius	M	C			x			x	x			
Falco femoralis	R	C						x	x			
Falco peregrinus	M	A						x	x			
Charadrius vociferus	M	A										x
Himantopus mexicanus	R	A										x
Jacana spinosa	R	A										x
Actitis macularius	M	A										x
Tringa solitaria	M	A										x
Columba livia	R	G									x	
Patagioenas flavirostris	R	GF	x	x	x							
Patagioenas nigrirostris	R	GF	x	x				x				
Zenaida asiatica	R	GF	x	x	x			x			x	
Zenaida macroura	M	GF	x	x	x			x				
Columbina inca	R	G			x	x		x	x		x	
Columbina passerina	R	G			x			x	x			
Columbina minuta	R	G	x		x		x					
Columbina talpacoti	R	G			x			x	x			
Claravis pretiosa	R	GF	x		x							
Leptotila verreauxi	R	GF	x	x	x		x	x				
Geotrygon montana	R	GF	x		x							
Aratinga holochlora	R	GF	x		x							
Aratinga nana	R	GF	x		x						x	
Aratinga canicularis	R	GF	x	x	x			x			x	
Amazona albifrons	R	GF	x	x								
Amazona autumnalis	R	GF	x	x							x	
Coccyzus minor	R	CI	x	x	x							
Piaya cayana	R	CIF	x	x	x							
Tapera naevia	R	CIF	x					x				
Dromococcyx phasianellus	R	CI	x	x								
Morococcyx erythropygus	R	CI	x	x	x							
Geococcyx velox	R	CI			x			x	x			
Crotophaga sulcirostris	R	GIF			x			x	x	x	x	
Tyto alba	R	C	x					x	x			
Megascops guatemalae	R	C	x	x	x							
Pulsatrix perspicillata	R	C	x	x				x	x			
Glaucidium brasilianum	R	CI	x	x	x							
Ciccaba virgata	R	C	x	x								
Chordeiles acutipennis	R	I	x	x	x						x	
Nyctidromus albicollis	R	I	x	x	x						x	
Caprimulgus ridgwayi	R	I	x	x	x							
Streptoprocne zonaris	R	I	x	x	x	x		x	x			
Chaetura vauxi	R	I	x	x	x	x		x	x		x	
Aeronautes saxatalis	R	I	x	x	x	x		x	x			

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Panyptila sanctihieronymi	R	I	x	x	x	x		x	x			
Phaethornis longirostris	R	N	x		x							
Phaethornis striigularis	R	N	x									
Florigusa mellivora	R	N	x	x								
Colibri thalassinus	R	N	x		x						x	
Chlorostilbon canivetii	R	N	x	x	x						x	
Amazilia beryllina	R	N	x	x			x			x		
Amazilia tzacatl	R	N	x		x		x			x	x	
Amazilia yucatanensis	R	N	x		x							
Amazilia viridifrons	R	N	x	x	x							
Eupherusa eximia	R	N	x	x	x							
Lamprolaima rhami	R	N	x	x								
Heliomaster longirostris	R	N	x		x							
Tilmatura dupontii	R	N	x	x	x							
Archilochus colubris	M	N	x									
Trogon melanocephalus	R	IF	x	x								
Trogon violaceus	R	IF	x	x								
Momotus mexicanus	R	CIF	x	x		x	x					
Momotus momota	R	CIF	x	x			x					
Megaceryle torquata	R	C					x					x
Megaceryle alcyon	M	C					x					x
Chloroceryle amazona	R	C					x					x
Chloroceryle americana	R	C					x					x
Aulacorhynchus prasinus	R	GF	x		x							
Pteroglossus torquatus	R	GF	x		x							
Ramphastos sulfuratus	R	GF	x		x							
Melanerpes aurifrons	R	IF	x	x	x	x					x	
Sphyrapicus varius	M	I	x	x	x							
Picoides scalaris	R	I	x		x						x	
Colaptes rubiginosus	R	IF	x	x	x							
Dryocopus lineatus	R	I	x	x	x							
Campephilus guatemalensis	R	I	x	x	x							
Sclerurus guatemalensis	R	I	x									
Synallaxis erythrothorax	R	I	x									
Automolus ochrolaemus	R	I	x		x							
Dendrocincla homochroa	R	I	x									
Dendrocolaptes sanctithomae	R	I	x									
Xiphorhynchus flavigaster	R	I	x	x								
Lepidocolaptes souleyetii	R	I	x	x	x							
Taraba major	R	I	x		x					x		
Thamnophilus doliatus	R	I	x		x			x		x		

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Cercomacra tyrannina	R	I	x		x							
Grallaria guatemalensis	R	I	x	x								
Ornithion semiflavum	R	I	x									
Camptostoma imberbe	R	I	x		x					x	x	
Myiopagis viridicata	R	I	x		x							
Elaenia flavogaster	R	IF	x		x							
Leptopogon amaurocephalus	R	I	x		x							
Oncostoma cinereigulare	R	I	x	x	x							
Poecilotriccus sylvia	R	I	x	x								
Rhynchocyclus brevirostris	R	I	x		x					x		
Xenotriccus callizonus	R	I	x		x							
Contopus cooperi	M	I	x		x							
Contopus pertinax	R	I	x		x	x	x		x	x		
Contopus virens	M	I	x		x							
Contopus cinereus	R	I	x		x	x	x		x	x		
Empidonax virescens	M	I	x		x							
Empidonax traillii	M	IF	x	x	x							
Empidonax albigularis	M	IF	x		x	x	x			x		
Empidonax minimus	M	I	x		x							
Sayornis nigricans	R	I	x		x		x					
Pyrocephalus rubinus	R	I							x	x		
Rhytipterna holerythra	R	I	x		x							
Myiarchus tuberculifer	R	IF	x						x	x		
Myiarchus cinerascens	M	IF	x	x	x							
Myiarchus nuttingi	R	IF	x		x	x	x			x		
Myiarchus tyrannulus	R	IF	x	x	x	x	x		x	x	x	
Pitangus sulphuratus	R	CIF	x	x	x	x		x	x	x	x	
Megarhynchus pitangua	R	CIF	x	x	x		x			x	x	
Myiozetetes similis	R	IF	x	x	x	x	x		x	x	x	
Myiodynastes luteiventris	M	IF	x	x	x	x	x		x	x	x	
Legatus leucophaius	M	I	x				x					
Tyrannus melancholicus	R	IF	x		x	x	x		x	x	x	
Tyrannus vociferans	M	IF	x						x	x		
Tyrannus verticalis	M	IF			x				x	x		
Tyrannus tyrannus	M	I			x			x		x		
Tyrannus forficatus	M	IF			x			x		x		
Pachyramphus aglaiae	R	IF	x		x							
Tityra semifasciata	R	IF	x	x	x						x	
Vireo griseus	M	IF	x		x					x		
Vireo bellii	M	I	x							x		
Vireo solitarius	M	IF	x	x	x	x	x			x	x	
Vireo huttoni	R	I	x							x		
Vireo gilvus	M	IF	x	x	x					x		

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Vireo leucophrys	R	IF	x									
Vireo philadelphicus	M	I	x	x	x					x	x	
Vireo olivaceus	M	IF	x	x	x		x			x	x	
Vireo flavoviridis	M	IF	x	x								
Hylophilus decurtatus	R	I	x	x	x					x		
Cyclarhis gujanensis	R	IF	x	x	x					x		
Calocitta formosa	R	O	x	x	x	x	x	x		x		
Cyanocorax yncas	R	O	x	x	x	x				x		
Cyanocorax morio	R	O	x	x	x	x						
Progne chalybea	R	I	x	x	x							
Tachycineta albilinea	M	I	x	x	x	x		x	x			
Stelgidopteryx serripennis	R	I	x	x	x	x		x	x		x	
Riparia riparia	M	I	x				x					
Petrochelidon pyrrhonota	M	I						x	x		x	
Petrochelidon fulva	M	I						x	x		x	
Hirundo rustica	M	I						x	x		x	
Catherpes mexicanus	R	I	x	x			x					
Thryothorus maculipectus	R	I	x	x	x	x				x		
Thryothorus pleurostictus	R	I	x	x	x	x	x	x		x		
Thryothorus modestus	R	I	x	x	x			x		x	x	
Troglodytes aedon	R	I	x									
Cistothorus platensis	R	I	x									
Uropsila leucogastra	R	I	x	x								
Henicorchia leucophrys	R	I	x		x							
Microcerculus marginatus	R	I		x								
Ramphocaenus melanurus	R	I	x		x							
Polioptila caerulea	R	I	x		x	x	x			x	x	
Polioptila plumbea	R	I	x	x	x	x	x			x		
Catharus aurantiirostris	R	IF	x									
Catharus minimus	M	IF	x									
Catharus ustulatus	M	IF	x	x								
Hylocichla mustelina	M	IF		x								
Turdus grayi	R	IF	x	x	x	x	x			x	x	
Dumetella carolinensis	M	IF	x		x							
Mimus gilvus	R	IF			x			x		x		
Bombycilla cedrorum	M	IF	x		x	x				x		
Oreothlypis pinus	M	I	x		x							
Oreothlypis celata	M	IF	x									
Oreothlypis ruficapilla	M	IF	x		x	x	x			x	x	
Parula americana	M	I	x							x		
Parula pitiayumi	R	I	x							x		
Dendroica petechia	M	IF	x			x	x			x	x	
Dendroica pensylvanica	M	IF	x									

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Dendroica magnolia	M	IF	x	x	x	x	x			x	x	
Dendroica coronata	M	IF		x								
Dendroica virens	M	IF	x	x	x	x	x			x	x	
Dendroica townsendi	M	IF	x							x		
Dendroica occidentalis	M	IF	x	x	x					x		
Dendroica fusca	M	I	x									
Dendroica graciae	R	IF	x	x		x				x		
Mniotilta varia	M	I	x	x	x	x				x	x	
Protonotaria citrea	M	I	x									
Helmitheros vermivorum	M	I		x								
Seiurus aurocapilla	M	I	x	x	x							
Seiurus noveboracensis	M	I	x	x	x							
Oporornis formosus	M	I	x	x								
Oporornis tolmiei	M	I	x									
Geothlypis trichas	M	I						x	x			
Geothlypis poliocephala	R	I						x	x			
Wilsonia citrina	M	I	x		x					x		
Wilsonia pusilla	M	IF	x	x	x	x	x			x	x	
Wilsonia canadensis	M	IF	x		x					x		
Euthlypis lachrymosa	R	I	x	x	x		x					
Basileupterus rufifrons	R	I	x		x					x		
Icteria virens	M	I			x					x		
Eucometis penicillata	R	IF	x		x							
Thraupis episcopus	R	IF			x					x		
Thraupis abbas	R	IF	x	x		x	x			x		
Cyanerpes cyaneus	R	IN	x	x								
Saltator coerulescens	R	IF	x		x					x		
Saltator atriceps	R	IF	x		x					x		
Volatinia jacarina	R	G						x	x			
Sporophila torqueola	R	G						x	x		x	
Oryzoborus funereus	R	G						x	x			
Tiaris olivaceus	R	G			x			x	x	x		
Arremonops rufivirgatus	R	GI	x	x	x			x	x	x	x	
Aimophila botterii	R	G						x	x			
Aimophila rufescens	R	GI						x	x			
Piranga rubra	M	IF	x	x	x							
Piranga ludoviciana	M	IF	x	x	x	x				x		
Habia rubica	R	IF	x	x	x							
Habia fuscicauda	R	IF	x	x	x							
Pheucticus chrysopeplus	R	IF	x	x	x	x	x				x	
Pheucticus ludovicianus	M	IF			x	x						
Granatellus venustus	R	IF	x	x	x							
Granatellus sallaei	R	IF	x	x	x							

Species	Migratory status	Diet or subdiet	Habitat types									
			Tdf	Tsf	Sf	To	Rf	Af	C	Lf	Ur	Aq
Cyanocompsa parellina	R	G	x	x	x			x				
Passerina caerulea	R	GI						x	x	x		
Passerina cyanea	M	GI	x					x	x			
Passerina versicolor	R	GIF	x		x			x	x			
Passerina ciris	M	GI			x			x				
Sturnella magna	R	GI						x	x			
Dives dives	R	GIF	x	x	x	x	x			x	x	
Quiscalus mexicanus	R	O	x	x	x	x	x	x	x	x	x	
Molothrus aeneus	R	GIF			x	x	x	x	x	x	x	
Icterus prosthemelas	R	IF	x		x					x		
Icterus wagleri	R	IF	x		x					x		
Icterus maculialatus	R	IF	x									
Icterus spurius	M	IF			x			x		x		
Icterus mesomelas	R	IF	x		x					x		
Icterus pustulatus	R	IF	x	x	x	x	x			x	x	
Icterus gularis	R	IF	x	x	x		x			x	x	
Icterus galbula	M	IF	x				x			x	x	
Amblycercus holosericeus	R	IF	x	x								
Cacicus melanicterus	M	IF	x									
Psarocolius wagleri	R	GF	x	x								
Psarocolius montezuma	R	GF	x		x							
Euphonia affinis	R	IF	x		x		x			x	x	
Euphonia hirundinacea	R	IF	x	x	x	x	x			x		
Euphonia elegantissima	R	IF	x	x	x	x				x		
Carpodacus mexicanus	R	G						x	x		x	
Spinus psaltria	R	GF			x	x	x	x	x	x	x	
Passer domesticus	R	O									x	

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10

Anuran Amphibians: A Huge and Threatened Factory of a Variety of Active Peptides with Potential Nanobiotechnological Applications in the Face of Amphibian Decline

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1. Introduction

All anurans produce venomous skin secretions composed by a complex mixture of bioactive peptides used against potential predators and pathogens that have evolved in a predator-prey interaction and defence against a microbial invasion scenario. Each new species studied reveal new molecules, homologous to hormones, neurotransmitters, antimicrobials, as well as several others with unknown biological activity. The vast majority of species have yet to be studied. Recently, these secretions have also been reported as a rich source of multiple antimicrobial peptides against multidrug-resistant strains of bacteria, fungi, protozoa, and virus, including cancer, providing several instructive lessons for the development of new and more efficient nanotechnology based therapies for infectious disease treatment. However, new drugs arising from the identification and analysis of bioactive peptides from anuran biodiversity are threatened by amphibian decline. Nearly one-third of amphibian species are globally threatened with extinction or extinct due the effects of climate change, reduction and modification of natural habitats, pollution, as well as emerging diseases. Unfortunately, conservation efforts have not been sufficient enough to counter balance the decline in amphibian species. As a result, several species have already become extinct before their peptidome can be evaluated, and others could disappear, which would seriously inhibit understanding required for the development of important new therapies against the superbugs and degenerative diseases. This situation requires drastic strategies in order to build robust anuran peptide libraries and biological anuran tissue banks in order to conserve part of this biological richness. In this chapter, the knowledge of anuran peptide and its potential for the development of new and more effective therapies based on a nanotechnological approach against superbugs that is threatened by amphibian decline are presented.

2. Anuran amphibians: Origen, evolution and distribution

Modern amphibians belong to the subclass Lissamphibia, super order Salientia, and can be scientifically subdivided into three orders: *Anura*, which includes frogs and toads, is the largest group with more than 6,000 species; *Caudata*, which includes salamanders and newts, with 608 species; and *Gymnophiona*, the least-known group, which are commonly referred to as caecilians, with 189 species. According to the AmphibiaWeb database, numbers of new species have grown rapidly over the last 20 years or so. Since 1985 the total number of recognized species has increased by over 60%, one reflex of the growing interest in biodiversity knowledge. Currently, each new area researched shows new species, one example is the Amazon Forest, where between 1999 to 2009, 216 new species were discovered (Thompson, 2010).

The origin of amphibians can be traced back to the Devonian period (about 416 to 359 million years ago). They were developed from a common ancestor similar to the modern day coelacanth, considered as the "missing link" between fish and tetrapods (Long & Gordon, 2004). When amphibians first appeared, Earth's terrestrial area was essentially one giant landmass inhabited by plants and insects. Amphibians were the first vertebrates to make the transition from water to land (Mattoon, 2001). Somehow, a type of bony fish evolved into a creature that had four legs, could breathe atmospheric oxygen instead of dissolved oxygen, and had a body structure that allowed it to manoeuvre without the support of water (Mattoon, 2001). During the Carboniferous Period (around 359 to 299 million years ago) amphibians moved up in the food chain and occupied the ecological position that presently belongs to crocodiles. These amphibians were notable for their ability to use the mega insects on land and many types of fish as an energy source. However, during the Triassic Period (250 to 200 million years ago), the better land-adapted proto-crocodiles began to compete with amphibians for food and space (Mattoon, 2001), which, in turn, reduced their energy sources significantly, leading the amphibians to a dramatic reduction in their average size, and consequently a dropping position in the food chain. Modern anurans originated from these amphibians that had to adapt to new environment challenges in order to survive extinction.

The anuran order is the most diverse group of vertebrates, with more than 6,000 known species, a total, which is being added to annually by the discovery of new species. This order is subdivided into three suborders: *Archaeobatrachia,* which includes four families of primitive frogs; *Mesobatrachia*, which includes five families of more evolutionary intermediate frogs; and *Neobatrachia*, so far the largest group, which contains the remaining families of modern frogs, including most common species throughout the world (Table 1). The families Leptodactylidae, Hylidae and Ranidae belonging the Neobatrachia suborder are the richest in number of species.

Anurans are to be found in both tropical and subarctic regions with the exception of some ocean islands, a few deserts and Arctic and Antarctic regions (Figure 1) (Frost et al., 2008). The majority of anuran species are found in the tropical rainforests. According to the Brazilian Herpetological Society, Brazil has at least 847 anuran species (Brazilian Herpetological Society [SBH], 2011), approximately 15% of the world anuran fauna, this represents the greatest number of amphibians for any country on Earth, and is closely followed by Colombia. Both South American countries have received extensive survey efforts in recent decades, and although both countries can be expected to add significantly to their totals, the level of increase is likely to be less than in some of the other highly diverse

countries (International Union for Conservation of Nature and Natural Resources [IUCN], 2011). Within South America, Peru in particular is relatively poorly researched and is almost certain to rise very substantially in its species total (IUCN, 2011).

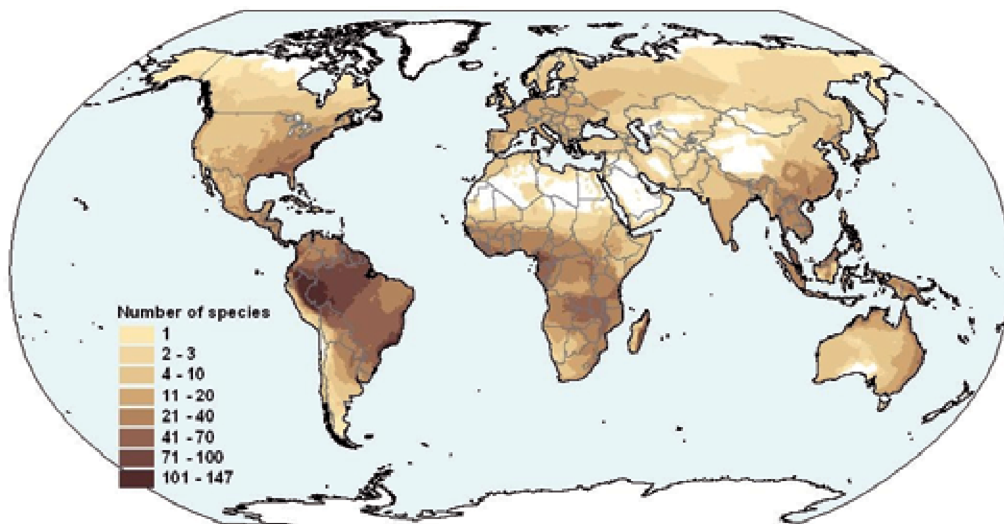

Fig. 1. Global patterns of amphibian diversity are shown. This diversity map clearly shows certain areas of high global diversity, including tropical South America and tropical West Africa. However, the problem of uneven survey efforts around the world complicates interpretation of this map. Regions such as Indonesia, New Guinea and the Congo Basin are especially likely to be under represented on this map due to lack of adequate surveys (Extracted from IUCN, 2011; Copyright 2011 International Union for Conservation of Nature and Natural Resources - Red List Unit).

3. Amphibian decline: The biodiversity crisis

According to the International Union for the Conservation of Nature (IUCN), amphibians may be the only major group currently at risk globally. IUCN assesses the status of species on a global scale and maintains a database of species that face a high risk of global extinction: the IUCN Red List of Threatened Species. The IUCN Red List, recent detailed worldwide assessment and subsequent updates show that nearly one-third of species (32.4%) are either globally extinct or threatened with extinction (Critically Endangered, Endangered and Vulnerable), representing 2,030 species (IUCN, 2011). McCallum (2007) estimates that current rates of extinction are 211 times the background extinction rate for amphibians, and rates would be as high as 25,000–45,000 times greater if all of the currently threatened species become extinct. If this is allowed to continue, the projected losses would constitute the largest mass extinction since the disappearance of the dinosaurs, which many scientists argue would be the sixth great mass extinction (Wake & Vredenburg, 2008).
Several long-term studies performed on intact natural ecosystems such as Yellowstone National Park and Sierra Nevada of California in United States (Noss et al., 2002; Vredenburg et al., 2007), Eungella National Park in Australia (McDonald, 1990), and

Monteverde Cloud Forest Preserve in Costa Rica (Pounds et al., 1997) show a worldwide decline in amphibian species in the last two decades. Populations of many species of frogs have declined dramatically in relatively undisturbed habitats at high altitudes and anthropized areas throughout the world (Blaustein & Wake, 1990, 1995; Blaustein et al., 1994; Bradford, 1991; Campbell, 1999; Carey, 1993; Collins & Storfer 2003; Crump et al., 1992; Czechura & Ingram, 1990; Hero et al., 2005; Kiesecker et al., 2001; McDonald, 1990; McMenamin et al., 2008; Pounds et al., 2006; Pounds, 2001; Reading, 2007; Richards et al., 1993; Skerratt et al., 2007; Stuart et al., 2004; Young et al., 2001). A map produced by IUCN shows the global distribution of threatened amphibians (Figure 2) revealing that the greatest concentration of threatened amphibian are in relatively limited areas dominated by species living within specific ranges, often living in mountainous areas. Many of these species have been subjected to severe habitat loss, and exposure to the fungal disease chytridiomycosis (Frost et al., 2008; IUCN, 2011).

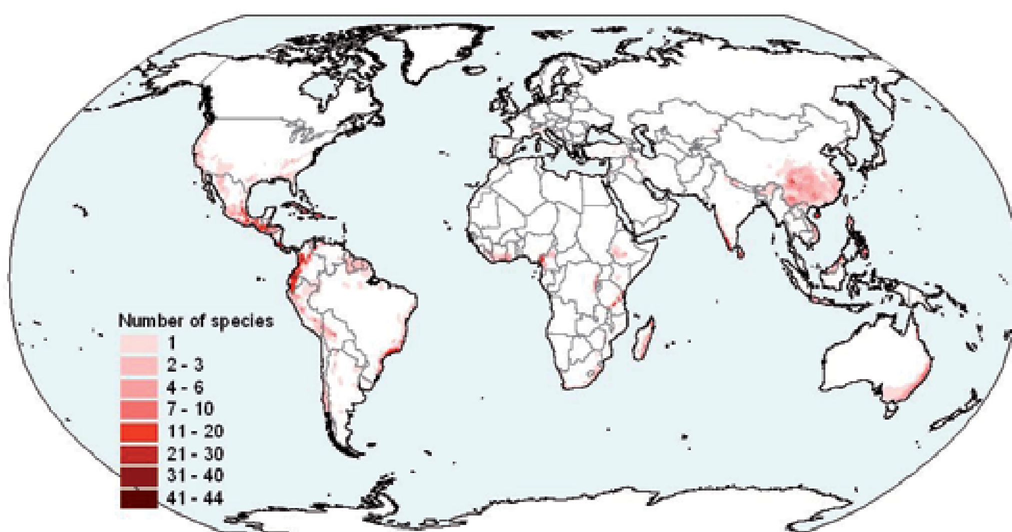

Fig. 2. Global distribution of threatened amphibians. Number of threatened species is show in red. Important concentrations of threatened species are to be found from Southern Mexico to Ecuador and Venezuela, as well as the Greater Antilles, Atlantic Forests of southern Brazil, upper Guinea forests of western Africa, forests of western Cameroon and eastern Nigeria, Albertine Rift of eastern central Africa, Eastern Arc Mountains of Tanzania, Madagascar, western Ghats of India and Sri Lanka, Borneo and Philippines, eastern Australia, central and southern China (Extracted from IUCN, 2011; Copyright 2011 International Union for Conservation of Nature and Natural Resources Red List Unit).

Atmospheric and water pollution, pathogens, exotic species, UV irradiation, and habitat destruction and/or modification have all contributed to the current amphibian decline (Alford & Richards, 1999; Blaustein et al., 2003; Collins & Storfer, 2003). Climatic change poses an additional serious threat to populations as is seen by precipitous decline of amphibian populations in remote and preserved areas. This data indicates that this phenomenon is linked to landscape and environmental changes brought about by global climatic change (Alford et al., 2007; Beebee, 1995; Carey & Alexander, 2003; McMenamin et

al., 2008; Pounds et al., 2006; Reading, 2007; Wake, 2007). According to McMenamin and co-workers (2008), changes in climate can affect amphibian populations in many ways, three of which we detail here (Figure 3).

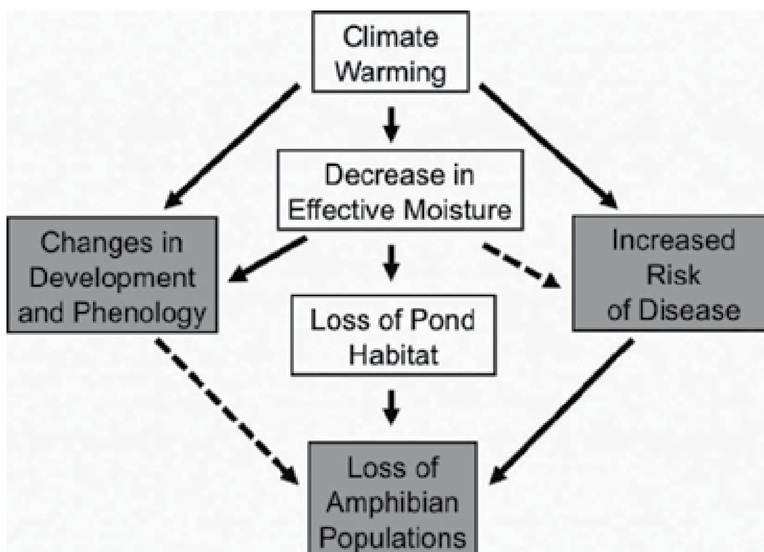

Fig. 3. Flow chart of global climatic change impacts on amphibian populations. These mechanisms are affecting amphibian populations worldwide. Solid lines denote clearly established relationships. (Extracted from McMenamin et al., 2008, Copyright 2011 National Academy of Sciences, U.S.A.).

Some examples of amphibian populations loss are catastrophic, as has been observed in Monteverde Cloud Forest Preserve in Costa Rica by Pounds and co-workers (1997) that performed a 5-year study involving daily monitoring of a large amphibian fauna demonstrating that 20 species of frogs, representing 40% of the total population, have been lost at the Preserve. What is especially notable about this case is that these observations discount the hypothesis of habitat destruction or modification, the most common reason for species disappearance, because the Preserve has a highly protected status. According to Wake & Vredenburg (2008), the start of this decline was observed in the late 1980s, where at the same time disappearances of species of the unique gastric brooding frogs from Australia (Rheobatrachus) occurred in protected areas in the Australian wet tropics (McDonald, 1990). According the same authors, at first all of these declines recorded were enigmatic, but eventually two primary causal factors emerged: the infectious disease chytridiomycosis and global warming (Lips et al., 2006; Pounds et al., 2006).

The chytridiomycosis is an emerging panzootic fungal disease caused by the chytrid fungus *Batrachochytrium dendrobatidis*. This disease was first described in 1998 from moribund and dead adult amphibians collected at sites of mass deaths in Australia and Panama between 1993 to 1998 (Berger et al., 1998). Symptoms of this amphibian lethal disease include abnormal posture, extension of hind limbs, convulsions, lethargy, and loss of attempt to escape danger; roughening of the skin; gross lesions consisting of abnormal epidermal shedding and ulceration; hemorrhages in the skin, muscle, or eye; hyperemia of digital and ventrum skin, and congestion of viscera (Berger et al., 1999; Daszak et al., 1999). According

to Berger and co-workers (1998), three mechanisms by which chytridiomycosis causes death have been proposed: epidermal hyperplasia impairs essential cutaneous respiration or osmoregulation; a fungal toxin is absorbed systemically; and a combination of both factors (Berger et al., 1998, Pessier et al., 1999).

In 2006, Pounds and co-workers hypothesized that climate change, precipitation, and increased temperature have acted synergistically in favour of the growth of the infectious chytrid fungus. This hypothesis is based on a situation where global warming has shifted temperatures closer to the presumed optimal conditions for *B. dendrobatidis*.

According the scientists of the *Intergovernmental Panel on Climate Change* (IPCC), human activities are the main cause of climate change, and will be responsible for the estimated temperature rise during the next century, that is projected to be between 2°C to 4°C, but rising as high to 7°C for much of the United States and Europe, with even higher temperatures expected in northern Eurasia, Canada, and Alaska (Parry et al., 2007). This change will produce a devastating effect on amphibian species. Impacts of the different warming scenarios are all dramatic and severe, where the first event predicted by the IPCC panel, ''Amphibian Extinctions Increasing on Mountains'', is now an empirical fact (see: http://www.ipcc.ch/graphics/ar4-wg2/jpg/ts6.jpg).

Multiple factors acting synergistically are contributing to the loss of amphibians. The association of extrinsic forces, such as global warming and increased climatic variability that increases the susceptibility of high-risk species (those with small geographic ranges, low fecundity, and specialized habitats), with habitat modification and destruction, use of fertilizers and pesticides, introduction of pollutants and exotic organisms, have severely impacted upon amphibians (Hayes et al., 2002; Sodhi et al., 2008; Wake & Vredenburg, 2008). According to Cunningham and co-workers (2006), the emergence of new infectious diseases produced by the expansion of human populations into new habitats have consequences for many other species, such as the case of chytridiomycosis in amphibians.

The IUCN has been producing lists of threatened species since the 1960s (Burton, 2003; Scott et al., 1987) reporting the very serious situation facing amphibians globally, which may be indicative of the state of freshwater species as a whole. Amphibians are declining more quickly than either birds or mammals (Stuart et al., 2004). The IUCN Red List of Threatened Species shows that at least 1,622 of the known anuran species on Earth are known to be threatened with extinction (IUCN, 2011). In 2008, a total of 120 amphibian species are listed as Critically Endangered (Possibly Extinct), and the majority of these could have disappeared since 1980 (Baillie et al., 2004; Vié et al., 2009). Because the amphibian extinctions are happening so fast and only a few areas on earth have been monitored by an insufficient number of scientists, it is difficult to obtain a complete current picture of the amphibian population status (Maas, 2011). The indications show that the extinction of amphibians is the most serious wave of all extinctions currently taking place, but the situation may be even graver than the numbers suggest (Baillie et al., 2004; Crawford et al., 2010; IUCN, 2011).

Several families of amphibians appear to be disproportionately threatened, in particular the Hynobiidae (Asian salamanders), Plethodontidae (lungless salamanders), Astylosternidae (Cameroonian stream frogs), Bufonidae (true toads), Rhacophoridae (Asian tree frogs), Leptodactylidae (typical Neotropical frogs), Leiopelmatidae (New Zealand frogs), Nasikabatrachidae (Indian burrowing frog), Rhinodermatidae (Darwin's frogs), and Sooglossidae (Seychelles frogs). Both members of the Rheobatrachidae (gastric-brooding frogs) are now Extinct, representing the loss of an entire vertebrate family (Baillie et al., 2004). It is important to note that some biologists class them within Myobatrachidae under

the subfamily Rheobatrachinae, but others place them within their own family, Rheobatrachidae (Heyer & Liem, 1976).
In spite of the massive deaths, some amphibian species appear to have an innate capacity to withstand chytridiomycosis infection. Even within species that generally succumb, some populations survive, possibly demonstrating that these anuran populations are being subjected to a selection process. According to Wake & Vredenburg (2008), despite these alarming estimates, some anuran species, particularly these that are invasive, are apparently doing very well in many parts of the world, and many thrive in landscapes heavily modified by human activities, such as the Cane Toad (*Rhinella marina*), the American Bullfrog (*Rana catesbeiana*), and the Clawed Frog (*Xenopus laevis*). They have shown they are not afflicted by chytridiomycosis.
This massive loss of anuran species diversity will produce a severe impact upon the ecosystem and human life brought about because amphibians consume huge quantities of invertebrates, including humanity's most vilified pests; play a crucial role in global ecosystems, both as predator and prey, help maintain healthy functioning environments; some species are an important protein source in many subsistence cultures and are traded in their millions as food and pets; the skin secretions that protect amphibians against predators and infection have been found to contain important pharmaceutical compounds that show potential in treating a variety of illnesses from HIV to cancer. One of these dramatic examples is the Golden Toad *Bufo periglenes* from Costa Rica, extinct since 1989 (Baillie et al., 2004), before its interesting chemical composition and potential applications could be evaluated by researches from different scientific areas.

4. Anuran skin protective adaptations

The anuran skin presents morphofunctional and behavioral protective adaptations against a number of adverse factors in the terrestrial environment (Barra & Simmaco, 2005). The cutaneous glands present in the skin play an essential role in respiration, reproduction, protection against desiccation and defence against predators and infection by microorganisms on the body surface (Toledo & Jared, 1995). Secretions produced by these glands have a key role in the protection by the presence of complex chemical composition with noxious or toxic substances with diverse pharmacological effects, which constitute an important source of biologic active compounds against bacteria, fungi, protozoa, virus and cancer (Calderon et al., 2009, 2010, 2011). However, the majority of the anuran species have not had their gland content examined by science and so remain unknown.
The cutaneous gland ultrastructural characterization of all living amphibians demonstrates that they usually belong to four main types located in the spongious dermis differing from others in size and secretory activity, and can be classified as: mucous, serous (granular or poison), lipid (or wax), and mixed (seromucous) glands (Almeida et al., 2007; Brizzi et al., 2002; Duellmann & Trueb, 1994; Lacombe et al., 2000).
Each gland presents specific action in homeostasis behavior: lipid glands promote the impermeabilization of the skin in order to decrease water loss (Castanho & De Luca, 2001); mucous glands produce mucus to support cutaneous functions, such as respiration, reproduction, thermoregulation, and defence (Toledo & Jared, 1995); serous glands, that are the largest and most widely distributed over the animal’s body surface, act as a main element in amphibian passive chemical defence (Lacombe et al., 2000; Toledo & Jared, 1995). Thus, the mixed gland contains both mucous and serous cells (de Brito-Gitirana, 2004).

The serous glands produce a wide variety of noxious or toxic substances with diverse pharmacological effects on microorganisms, vertebrate, and invertebrate species (Toledo & Jared 1995; Lacombe et al., 2000). The serous glands exhibit remarkable polymorphism, having been classified into two classes, type I and II (Delfino et al., 1998; Lacombe et al., 2000). Type I glands exhibit a poorly developed smooth endoplasmic reticulum (Lacombe et al., 2000) and present two subtypes, Ia and Ib. Type Ia has dense granules that characterize the biosynthesis of proteinaceous products for exocytosis, which engage both rough endoplasmic reticulum and Golgi apparatus (Delfino, 1991). Type Ib has vesicles holding a lucent material in the fluid serous secretion on the anuran skin (Toledo & Jared, 1995). Type II glands present a well-developed smooth endoplasmic reticulum that is potentially engaged in the biosynthesis of peptides (Blaylock et al., 1976; Lacombe et al., 2000). These peptides are produced as prepropeptides, which have to be processed into mature peptides by the removal of the signal and acidic components, and then stored in the granules (Nicolas & El Amri, 2009).

Some anurans, such as the bufonidaes (toads) have a pair of peculiar glandular structures symmetrically disposed in a post-orbital position named as parotoid glands (Young, 1985). These glands are composed of large aggregations of granular glands responsible for the production and storage of a thick and creamy secretion, which contribute to protection against predators and parasites (Clarke, 1997; Croce et al., 1973; Duellman & Trueb, 1994; Sakate et al., 2000). The parotoid gland is an integument region, in which three exocrine glandular types occur: mixed glands, smaller granular glands and larger granular glands. The mixed gland is formed by mucous and serous cells while the small granular glands contain a homogene acidophilic intake. The larger granular glands produce a basophilic and alcianophilic material, and are responsible for the macroscopic protuberances designed as parotoid glands. Thus, the end product released by the parotoid glands is a mix of secretions produced by the three glands (Almeida et al., 2007).

It is accepted that the release of the gland content onto the skin surface is mediated by a holocrine mechanism that involves rupture of the plasmatic membrane and extrusion of the granules through a duct opening onto the surface (Nicolas & El Amri, 2009). Immunofluorescence analysis of *Phyllomedusa bicolor* (Hylideae) dermal glands using an antibody to the acidic propiece region of the preprodermaseptin/preprodeltorphins-derived peptide family [ENENEENHEEGSE] demonstrated that the fluorescence-positive reaction is restricted to the serous glandular content, indicating their specific role in the biosynthesis and secretion of dermaseptins and deltorphin peptides (Lacombe et al., 2000). Additionally, mass spectrometry image (MALDI-image) performed with the skin of *P. hypochondrialis* (Hylideae) indicated that the serous glands present specialization in the peptide production and storage (Brand et al., 2006b).

In spite of the large number of anuran species from different genera, a great deal of attention is being paid to the study of neotropical hylid frogs that belong to the subfamily Phyllomedusinae, as an excellent source of peptides. In 1985, Vittorio Erspamer also stated that ''No other amphibian skin can compete with that of the Phyllomedusae'' (Erspamer et al., 1985). The initial efforts on Phyllomedusa skin secretions by V. Erspamer followed by other scientists around the world during the last four decades has revealed a complex profile of biologically active peptides with antimicrobial, hormonal, and neuro activities (Calderon et al., 2011). These peptides differ significantly among species within this genus leading to an interesting molecular diversity, possibly associated with specific differences presented in the specie niche, such as interactions with the environment, predators, and

pathogens that characterize hylid species evolution (Amiche et al., 1993; Bevins & Zasloff, 1990).

5. Frog skin active peptides family: Defence against pathogens and predators

The complex chemical composition of anuran skin secretions constitutes a rich chemical warehouse of a wide number of natural biologically active compounds, such as amines, steroid derivatives, alkaloids and peptides. Peptides from anuran skin secretion are grouped into the Frog Skin Active Peptide (FSAP) family. The FSAP family can classify into three main groups according to their primary activity: antimicrobial peptides (AMPs); smooth muscle active peptides; and nervous system active peptides (Calderon et al., 2011; Erspamer et al., 1981). The secondary activities of FSAPs were not considered in this systematization. The first group acts as a skin anti-infective passive defence barrier, the second and the third groups cause the disruption of the predator homeostasis balance (Calderon et al., 2011). However, the biological activity of several peptides from anuran skin remains unknown.

The antimicrobial peptides (AMPs) compose the innate immunity system of anurans against microbial invasion (Giuliani et al., 2008; Radek & Gallo, 2007; Zasloff, 2002) effective against multidrug resistant strains of bacteria, fungi, protozoa, and virus including cancer, and provide instructive lessons for the development of new and more efficient nanotechnological-based therapies for infectious and degenerative diseases treatment (Calderon et al., 2011; Rinaldi, 2002). Many AMPs possess a wide range of activity showing effectiveness against diverse microorganism strains. One example is the dermaseptin family of AMPs and their analogs from the skin of Phyllomedusinae species. Dermaseptins have in vitro lytic activity against a broad spectrum of free-living microorganism strains, including wall-less, Gram-negative and Gram-positive bacteria, fungi, protozoa, and virus, as shown above (Table 1). Despite the sequence similarities, the dermaseptins differ in their action efficiency (Nicolas & El Amri, 2009; Rivas et al., 2009). However they present rapid and irreversible antimicrobial effect and no toxic effects in mammalian cells in vitro (Kustanovich et al., 2002; Navon-Venezia et al., 2002).

In additiony to antimicrobial activity, some dermaseptins present other additional biological functions that have unclear relations with pathogen clearance, e.g., dermaseptin B2 (adenoregulin) stimulates the binding of agonists to A1 adenosine receptors and also enhances the binding of agonists to several G-protein coupled receptors in rat brain plasmatic membrane through a mechanism involving enhancement of guanyl nucleotide exchange at G-proteins (Shin et al., 1994); Dermaseptin-B4 stimulates insulin release by acute incubation with glucose-responsive cells (Marenah et al., 2004); Dermaseptin-S1 stimulates the production of reactive oxygen species and release of myeloperoxidase by polymorphonuclear leukocytes (Ammar et al., 1998).

Gram-negative *Salmonella typhimurium,* wall-less *Mycoplasma gallisepticum* and *M. mycoides* show resistance to dermaseptin B9 from *P. bicolor* (Fleury et al., 1998).

Antimicrobial peptides are part of the innate immunity system of anurans against microbial invasion (Giuliani et al., 2008; Zasloff, 2002; Radek & Gallo, 2007). Crafted by evolution into an extremely diversified array of sequences and folds, AMPs share a common amphiphilic 3-D arrangement (Giuliani et al., 2008). This feature is directly linked to a common mechanism of action that predominantly develops upon interaction of peptides with cell membranes of target cells (Giuliani et al., 2008). The mechanisms of action of AMPs in microbial membranes are complex and still relatively unknown, but they constitute a

Microorganisms susceptible to dermaseptins	Dermaseptins active against microorganism	Species where the dermaseptin was identified	References
Wall less bacteria			
Acholeplasma laidlawii	B9	*P. bicolor*	Fleury et al., 1998
Spiroplasma apis	B9	*P. bicolor*	Fleury et al., 1998
Spiroplasma citri	B9	*P. bicolor*	Fleury et al., 1998
Spiroplasma floricola	B9	*P. bicolor*	Fleury et al., 1998
Spiroplasma melliferum	B9	*P. bicolor*	Fleury et al., 1998
Gram-negative bacteria			
Aerornonas caviae	B1	*P. bicolor*	Strahilevitz et al., 1994
	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Acholeplasma laidlawii	B9	*P. bicolor*	Fleury et al., 1998
Acetobacter calcoaceticus	O1	*P. oreades*	Brand et al., 2002
Escherichia coli	B1, B9	*P. bicolor*	Fleury et al., 1998; Strahilevitz et al., 1994
	D1, D2, D3, D4, D5	*P. distincta*	Batista et al., 1999
	H1	*P. hypochondrialis*	Brand et al., 2006b; Conceição et al., 2006
	O1	*P. oreades*	Brand et al., 2002; Leite et al., 2008
	T7	*P. tarsius*	Silva et al., 2000
	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Neisseria gonorrhoeae	S4	*P. sauvagii*	Rydlo et al., 2006; Zairi et al., 2009
Pseudomonas aeruginosa	B9	*P. bicolor*	Fleury et al., 1998
	D1, D2, D3, D4, D5	*P. distincta*	Batista et al., 1999
	H1	*P. hypochondrialis*	Brand et al., 2006b; Conceição et al., 2006
	O1	*P. oreades*	Brand et al., 2002; Leite et al., 2008
	T7	*P. tarsius*	Silva et al., 2000
Gram-positive bacteria			
Corynebacterium glutamicum	B9	*P. bicolor*	Fleury et al., 1998
Enterococcus faecalis	D1, D2, D3, D4, D5	*P. distincta*	Batista et al., 1999
	T7	*P. tarsius*	Silva et al., 2000
Micrococcus luteus	H1	*P. hypochondrialis*	Conceição et al., 2006
Nocardia spp	O1	*P. oreades*	Leite et al., 2008
Nocardia brasiliensis	B1	*P. bicolor*	Strahilevitz et al., 1994
	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Staphylococcus aureus	B1, B9	*P. bicolor*	Fleury et al., 1998; Strahilevitz et al., 1994
	D1, D2, D3, D4, D5	*P. distincta*	Batista et al., 1999
	H1	*P. hypochondrialis*	Brand et al., 2006b; Conceição et al., 2006
	O1	*P. oreades*	Brand et al., 2002; Leite et al., 2008
	T7	*P. tarsius*	Silva et al., 2000
Streptococcus dysgalactiae	O1	*P. oreades*	Leite et al., 2008
Streptococcus uberis	O1	*P. oreades*	Leite et al., 2008

Microorganisms susceptible to dermaseptins	Dermaseptins active against microorganism	Species where the dermaseptin was identified	References
Fungi			
Aspergillus fumigatus	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Arthroderma simii	B1	*P. bicolor*	Strahilevitz et al., 1994
	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Cryptococcus neofonnans	B 1	*P. bicolor*	Strahilevitz et al., 1994
	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Candida albicans	B 1	*P. bicolor*	Strahilevitz et al., 1994
	O1	*P. oreades*	Leite et al., 2008
	S1, S2, S4	*P. sauvagii*	Mor & Nicolas 1994; Zairi et al., 2008
Candida tropicalis	D1, D2	*P. distincta*	Leite et al., 2008
	O1	*P. oreades*	Leite et al., 2008
Candida guilliermondii	D1, D2	*P. distincta*	Leite et al., 2008
	O1	*P. oreades*	Leite et al., 2008
Microsporum canis	B1	*P. bicolor*	Strahilevitz et al., 1994
	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
Tricophyton rubrum	S1, S2	*P. sauvagii*	Mor & Nicolas, 1994
	B 1	*P. bicolor*	Strahilevitz et al., 1994
Protozoa			
Leishmania major (Pro)	S1, S4	*P. sauvagii*	Feder et al., 2000; Gaidukov et al., 2003; Kustanovich et al., 2002
Leishmania mexicana (Pro)	S1	*P. sauvagii*	Hernandez et al., 1992; Mor & Nicolas 1994b
Leishmania amazonensis (Pro)	O1	*P. oreades*	Brand et al., 2006b
	H1	*P. hypochondrialis*	Brand et al., 2006b
Leishmania amazonensis (Epi)	H5	*P. hypochondrialis*	Brand et al., 2006b
Leishmania chagasi (Pro)	H5	*P. hypochondrialis*	Zampa et al., 2009
Plasmodium falciparum (Trf)	S3, S4	*P. sauvagii*	Ghosh et al., 1997; Krugliak et al., 2000
Trypanosoma cruzi (Try)	O1	*P. oreades*	Brand et al., 2002
	D1, D2	*P. distincta*	Brand et al., 2002
Virus			
HSV-1	S4	*P. sauvagii*	Belaid et al., 2002
HIV-1	S4	*P. sauvagii*	Lorin et al., 2005; Zairi et al., 2009

Table 1. Microorganisms susceptible to dermaseptins from anuran species belonging the genus Phyllomedusa.

promising and attractive proposition as new antimicrobial therapeutics (Calderon et al., 2009, 2010, 2011). Interestingly, the mechanism of interaction between AMP and microbial membrane inhibits a fast adaptation of parasites to the peptide action, as it requires a wide change in its membrane structure or composition, demanding a significant great metabolic change in a short period of time, in contrast to drugs of intracellular action (Phillips, 2001).

The emergence, increased prevalence and rapid spread of extremely multidrug resistant pathogenic microorganisms together with the increased use of immunosuppressive

therapies, and the association with HIV co-infection present a serious challenge to public health systems around the world. The lack of therapeutic options against these pathogens has stimulated research into new bioactive molecules from the biodiversity as a source of more efficient (low toxicity and major potency) mechanisms for infection control (Calderon et al., 2009; Vaara, 2009).

The interest in the development of new forms of anti-infective agents such as those based on AMPs from anuran skin as therapeutic agents has been increased (Rinaldi, 2002; Xiao et al., 2011). Thus, they are likely to be active against pathogens and even those that are resistant to conventional drugs. Many peptides have been isolated and shown to be effective against multi-drug resistant pathogens. According to Xiao and co-workers (2011), more than 500 AMPs have been identified from amphibians. This number of peptides described is insignificant when compared to all the potential represented by the amphibian global fauna, that are composed of much more than 6,000 species, with increases new species every year. According to Jared & Antoniazzi (2009), from the toxinology viewpoint, is possible imagine that with more than 6,000 species, should be at least 6,000 kinds of poison and hundreds of thousands of new bioactive molecules to be discovered.

Of a total of 49 anuran families (Frost, 2011) only 10 have had part of their peptides identified, as can be observed in Table 2. Only members of the Ascaphidae, Bombinatoridae, Hylidae, Hyperoliidae, Leiuperidae, Leptodactylidae, Myobatrachidae, Pipidade, Ranidae families have been examined in order to discover new bioactive peptides. Members of the families Hylidae and Ranidae have received more attention, with a high number of peptide families characterized. The abundance of AMPs in frog skin is remarkable and constitutes a rich source for the design of new pharmaceutical molecules. Unfortunately, several anuran species have become extinct due to the events related to the amphibian decline before their bioactive molecules have had a chance to be discovered, such as the golden toad *Bufo periglenes* (Bufonidae) (Figure 4).

Suborder	**Family (conservation status)**	**Peptide identified**
Archaeobatrachia	Ascaphidae	Ascaphin, Bradykinin, Skin secreted peptide, Tryptophyllin
	Bombinatoridae	Bombesin, Bombinin, Bradykinin, Maximin, Tryptophyllin, Thyroliberin
	Discoglossidae (**EX**)	Alytesin
	Leiopelmatidae (**CR**)	none
Mesobatrachia	Megophryidae (**CR**)	none
	Pelobatidae	none
	Pelodytidae	none
	Pipidae (**CR**)	Antimicrobial peptide, Caerulein, Dorphin, Leap2 protein, Levitide, Magainin, Midkine, Midkine, Peptide pGQ, Peptide PYLa/PGLa, Pleiotrophin, Xenopsin, Xenoxin
	Rhinophrynidae	none
	Scaphiopodidae	none

Suborder	Family (conservation status)	Peptide identified
Neobatrachia	Allophrynidae	none
	Aromobatidae (**CR**)	none
	Arthroleptidae (**CR**)	none
	Brachycephalidae	none
	Brevicipitidae	none
	Bufonidae (**EX**, **EW**, **CR**)	Neurotensin, Seritocin
	Calyptocephalellidae (**CR**)	none
	Centrolenidae (**CR**)	none
	Ceratobatrachidae (**CR**)	none
	Ceratophryidae (**CR**)	none
	Ceuthomantidae	none
	Craugastoridae (**EX**, **CR**)	none
	Cycloramphidae (**CR**)	none
	Dendrobatidae (**CR**)	none
	Dicroglossidae (**EX**, **CR**)	none
	Eleutherodactylidae (**CR**)	none
	Heleophrynidae (**CR**)	Bradykinin
	Hemiphractidae (**CR**)	none
	Hemisotidae	none
	Hylidae (**EX**, **CR**)	Antimicrobial peptide, Aurein, Bioactive peptide, Bradykinin-potentiating peptide, Bradykinin, Caeridin, Caerin, Caerulein, Citropin, Dahlein, Deltorphin, Dermadistinctin, Dermaseptin, Dermatoxin, Dermorphin, Electrin, Fallaxidin, Frenatin, Hylaseptin, Hylin, Hyposin, Litorin, Maculatin, Novel peptide, Peptide TRP, Peroniin, Phyllocaerulein, Phyllokinin, Phyllomedusin, Phylloseptin, Pseudin, Rothein, Rubellidin, Skin secreted peptide, Splendipherin, Tryptophyllin, Uperin
	Hylodidae (**CR**)	none
	Hyperoliidae (**CR**)	Caerulein-like, FMRFamide-related, Galensin, Hylambatin, Kasseptin, Kassinakinin, Kassinatuerin, Kassinin, Kassinin, Kassorin, Tachykinin
	Leiuperidae (**CR**)	Bradykinin, Phyllokinin, Physalaemin
	Leptodactylidae (**CR**)	Aggression-stimulating peptide, Leptoglycin, Ocellatin, Ranaspumin
	Limnodynastidae (**CR**)	none
	Mantellidae (**CR**)	none

Suborder	Family (conservation status)	Peptide identified
Neobatrachia	Micrixalidae (**CR**)	none
	Microhylidae (**CR**)	none
	Myobatrachidae (**EX**, **CR**)	Bombesin, Crinia-angiotensin, Deserticolin, Dynastin, Fletcherin, Kassinin, Riparin, Rugosauperolein, Signiferin, Substance P-like, Uperin, Uperolein
	Nasikabatrachidae	none
	Nyctibatrachidae	none
	Petropedetidae (**CR**)	none
	Phrynobatrachidae	none
	Ptychadenidae	none
	Pyxicephalidae (**CR**)	none
	Ranidae (**EX**, **CR**)	Atrial natriuretic factor, Bombesin, Bradykinin, Brevinin, Calcitonin, Chensinin, Gaegurin, Galanin, Granuliberin, Guentherin, Hydrin, Japonicin, Lectin-like, Melittin-like, Neurokinin, Neuromedin, Neurotensin, Nigrocin, Odorranain, Orexigenic neuropeptide, Palustrin, Peptide tyrosine arginine, Ranacyclin, Ranakinin, Ranalexin, Ranamargarin, Ranatachykinin, Ranatensin, Ranatuerin, Rugosin, Temporin, Tigerinin, Vasoactive intestinal peptide
	Ranixalidae (**CR**)	none
	Rhacophoridae (**EX**, **CR**)	none
	Sooglossidae	none
	Strabomantidae (**CR**)	none

*According to the IUCN (2011), of the 6,260 amphibian species assessed, nearly one-third of species (32.4 %) are globally threatened or extinct, representing 2,030 species. Thirty-eight of the 2,030 species are considered to be Extinct (EX), and one Extinct in the Wild (EW). Another 2,697 species are not considered to be threatened at present, being classified in the IUCN Categories of Near Threatened or Least Concern, while sufficient information was not available to assess the status of an additional 1,533 species. It is predicted that a significant proportion of these Data Deficient species are likely to be globally threatened (IUCN, 2011; Frost et al., 2008).

Table 2. Anuran families ordered by suborder according to Frost (2011) with current status informed by IUCN* and peptide family described for each one deposited in the UniProtKB/Swiss-Prot. Current status are designated by the presence of extinct species (**EX**), extinct species in the wild (**EW**), and/or critically endangered species (**CR**) according to Frost and co-workers (2008).

Since the first peptide was isolated from the Phyllomedusa skin, the Phyllokinin, a bradykinyl-isoleucyl-tyrosine O-sulfate from *P. rohdei* in 1966 by Erspamer's research group (Anastasi et al., 1966), the number of anuran peptides discovered has increased exponentially (Calderon et al., 2011), but is still far from its real potential, which is evidenced by the observation that for every new anuran species studied new peptides are found, with homologies to hormones, neurotransmitters, antimicrobials, and several other peptides with unknown biological activity.

Fig. 4. Golden toad *Bufo periglenes* (Bufonidae) (**EX**) from Costa Rica (male), also called the Monteverde golden toad, or the Monte Verde toad. First described in 1966 (Savage, 2002), is considered extinct by the IUCN since 1989 (IUCN, 2011), before its content of bioactive molecules could be researched (Image from the U.S. Fish and Wildlife Service's online digital media library, public domain).

Nowadays, it is possible to carry out transcriptome analysis in order to build a robust cDNA library only with the secretions from a single living specimen (Chen et al., 2003b). The emergence of modern high-throughput molecular technologies involving *de novo* peptide sequencing via tandem mass spectrometry, cDNA cloning, and pharmacological screening applied to peptide discovery allowed fast structural data analysis and the generation of peptide sequence libraries, which in turn increased the capacity of peptide characterization, thus reducing the amount of samples needed (Shaw, 2009), which reduces significantly the impact on the amphibian populations researched by the reduction of the number of individuals necessary to perform bio prospection research.

6. The resistance crisis: Increasing need for new antimicrobials

Recently, antibiotic-resistant infections have reached unprecedented levels, some public health specialists and scientists have been warning that the antibiotic-resistant microorganisms strains, or superbugs, outstrip our ability to fight them with existing drugs. It is estimated that in 2007 approximately 25,000 patients died in the European Union, Iceland and Norway from an infection due to antibiotic-resistant bacteria that is able to outsmart even the newest antibiotics, such as *Staphylococcus aureus*, methicillin resistance (MRSA); *S. aureus*, vancomycin intermediate resistance and vancomycin resistance

(VISA/VRSA); *Enterococcus* spp. (e.g. *Enterococcus faecium*), vancomycin resistance (VRE); *Streptococcus pneumoniae*, penicillin resistance (PRSP); Enterobacteriaceae (e.g. *Escherichia coli*, *Klebsiella pneumoniae*), third-generation cephalosporin resistance; Enterobacteriaceae (e.g. *K. pneumoniae*), carbapenem resistance; and Non-fermentative Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*), carbapenem resistance. In addition, infections due to any of these antibiotic-resistant bacteria resulted in approximately 2.5 million extra hospital days and extra in-hospital costs of more than EUR 900 million (European Centre for Disease Prevention and Control/European Medicines Agency [ECDC/EMEA] Joint Working Group, 2009). The situation has reached to a critical point.

One example of this situation is the emergence and rapid spread of extremely multiresistant pathogenic microorganisms endowed with new antibiotic resistance mechanisms such as *New Delhi metallo-beta-lactamase-1* (NDM-1), an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics, including the carbapenem family of antibiotics (except aztreonam), one of last resort for many bacterial infections, such as *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (Kumarasamy et al., 2010; Nordmann et al., 2011; Richter et al., 2011). This gene has been identified in strains that possess other resistance mechanisms contributing to their multidrug resistance patterns, because these bacteria have often been referred to in the news media as "superbugs" because infections caused by them are difficult to treat successfully (Raghvendra et al., 2011). Most isolates with NDM-1 enzyme are resistant to all standard intravenous antibiotics for treatment of severe infections (Health Protection Agency [HPA], 2009a,b). It has been recently extensively reported from the United Kingdom, India and Pakistan and, albeit to a lesser extent, from a number of other countries worldwide (Nordmann et al., 2011).

The emergence of multiresistant pathogenic microorganisms, increased use of immunosuppressive therapies, and the association with HIV co-infection, represent a serious public health problem with high mortality and morbidity rates, such as *Cryptococcus*, *Cryptosporidium* and *Leishmania* (Abu-Raddad et al., 2006; Pukkila-Worley & Mylonakis, 2008; Rivas et al., 2009; Vaara, 2009). The critical problem represented by the limited therapeutic options for increasing multidrug resistance in Gram-negative bacteria, in particular *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, have forced infectious disease clinicians and microbiologists to reappraise the clinical application of polymyxin antibiotic, a cyclic peptide with a long hydrophobic tail, discovered more than 50 years ago (Li et al., 2006). Polymyxin is usually active *in vitro* (though not *vs. Morganella morganii*, an intrinsically resistant species) but of uncertain clinical efficacy, especially in pneumonia, owing to poor lung penetration. The Antibiotic Resistance Monitoring & Reference Laboratory (ARMRL) from the HPA Centre for Infections with the pharmaceutical industry is urgently reviewing the activity of both experimental and outdated antibiotics in order to develop alternative chemotherapies for NDM-1 (HPA, 2009a,b).

One of the greatest accomplishments of modern medicine has been the development of antibiotic therapies for potentially fatal infections by multidrug-resistant pathogenic microorganisms. Unfortunately, over the past two decades, the discovery and development of novel antibiotics has decreased while pathogen resistance to those currently available has increased (Li et al., 2006).

According to the European Centre for Disease Prevention and Control (ECDC) and the European Medicines Agency (EMEA) with contributions from the international network Action on Antibiotic Resistance (ReAct), there is a need for more development of antibiotics that are effective against multidrug-resistant bacteria. The ECDC/EMEA Joint Working

Group, using data from the European Antimicrobial Resistance Surveillance System (EARSS) and two commercial databases of antibacterial agents in clinical development worldwide (Adis Insight R&D and Pharmaprojects) concluded that there is a gap between the burden of infections due to multidrug-resistant bacteria and the development of new antibiotics to tackle the problem; and that a European and global strategy to address this gap is urgently needed (ECDC/EMEA Joint Working Group, 2009).

Limited therapeutic options against these pathogens demand urgent prospection of new bioactive molecules from the biodiversity as a source for more efficient (low toxicity and major potency) mechanisms of microorganism killing (Calderon et al., 2009; Vaara, 2009). The discovery of new lead compounds is important to subsidize the development of new chemicals with structural characteristics for large-scale production by the pharmaceutical industry at a feasible cost. The sources from the biodiversity, such as the skin of several amphibian and other vertebrate and invertebrate animals, plants, and microorganisms, have proved to be an inexorable source of antimicrobial molecules, with a broad spectra of activity (Calderon et al., 2009), specially against the drug-resistant pathogens described before, in which the AMPs have highlights in their potential therapeutical application as exposed in Table 1 (Gomes et al., 2007; Hancock, 1997; Hancock & Lehrer 1998; Koczulla & Bals, 2003).

One interesting example is the cationic alpha-helical peptide Ascaphin-8 (GFKDLLKGAAKALVKTVLF-NH2), from the skin secretion of the primitive anuran *Ascaphus truei* (Archaeobatrachia: Ascaphidae) (Conlon et al., 2004). This AMP shows broad-spectrum antibacterial activity against clinical isolates of beta-lactamase producing bacteria such as *Escherichia coli* (MIC=1.5-6 microM) and *Klebsiella pneumoniae* (MIC=12.5-25 microM), as well as a group of miscellaneous beta-lactamase producing strains of *Citrobacter*, *Salmonella*, *Serratia*, and *Shigella* spp (Eley et al., 2007). According to Eley and co-workers (2007), Ascaphin-8 is also toxic to human erythrocytes ($LC_{(50)}$= 55 microM), however, the L-lysine-substituted analogs Lys10, Lys14, and Lys18 also displayed potent antibacterial activity while showing very low hemolytic activity ($LC_{(50)}$> 500 microM). This result shows that peptide engineering could reduce toxicity of haemolytic AMPs, which makes possible the development of a drug delivery system association to improve the efficiency of Ascaphin-8 analogs to be used as a therapeutic peptide antibiotic against multidrug-resistant pathogenic microorganisms.

7. Peptide antibiotics: Nanotechnological approaches against superbugs

According to Marr and co-workers (2006), therapeutic peptide antibiotics will have advantages over conventional antibiotics due to their diverse potential applications, such as single antimicrobials, in combination with other antibiotics for a synergistic effect, or as immunomodulatory and/or endotoxin-neutralizing compounds (Zasloff, 2002). In particular, the most potent agents have an unusually broad spectrum of activity against most Gram-negative and Gram-positive bacteria, and also to fungi and even a variety of viruses, such as dermaseptins (Table 1). One of their advantages is their ability to kill multidrug-resistant bacteria (Marr et al., 2006). Compared with conventional antibiotics, these bacteria-killing peptides are extremely rapid and attack multiple bacterial cellular targets (Brogden, 2005).

Despite their obligatory interaction with the plasmatic membrane, some peptides are able to perforate plasmatic membrane at their minimal inhibition concentration (MIC), a number of AMPs translocate across the membrane and affect cytoplasmic processes, including

inhibition of macromolecular synthesis, particular enzymes or cell division, or the stimulation of autolysis (Marr et al., 2006). Minimal inhibitory concentrations and minimal bactericidal concentrations often coincide (less than a two-fold difference), indicating that killing is generally bactericidal, a highly desirable mode of action (Marr et al., 2006). Furthermore, AMPs are not hindered by the resistance mechanisms that occur with currently used antibiotics (Zhang et al., 2005). Indeed, killing can occur synergistically with other AMPs and conventional antibiotics, helping overcome some barriers that resistant bacteria have against currently used antibiotics (Marr et al., 2006).

Until then, many efforts have been carried out in order to use the AMPs in the development of new infection-fighting drugs applicable to new treatments of nosocomial infections and multidrug-resistant infections (Amiche et al., 2000), due to the skill of the AMPs to kill multidrug resistant strains of microorganism by a mechanism unlikely to induce antibiotic-resistance. The development of new antimicrobials based on AMPs hold promises to medicine at the end of the classical antibiotic age by the emergence of the multidrug-resistant microorganisms (Alanis, 2005; Arias & Murray, 2009; Nordmann et al., 2011).

Even with the expected advantages in the use of AMPs as new antimicrobials for the post-antibiotic age, several impediments to therapeutic peptides arise. According to Marr and co-workers (2006), the main problem at the present moment is the cost of manufacturing peptides, which is economically unfeasible for the amounts of AMPs needed compared to other antibiotics, preventing the widespread clinical use of AMPs as a common antibiotic, and the shortage of studies thoroughly examining systemic peptide pharmacodynamic and pharmacokinetic issues, including peptide aggregation problems, the in vivo half-life of peptides (and particularly their susceptibility to mammalian proteases), and the required dosing frequency (Marr et al., 2006). Due to the specific characteristics of the AMPs, that differentiate them from other antibiotics, the development of new strategies for the therapeutic use of AMPs in medicine are necessary in order to reduce the amount of AMPs necessary to promote the therapeutic infection suppression effect, including the addition of striking affinity to specific targets, efficiency at very low concentrations and negligible toxicity (Marr et al., 2006).

From this viewpoint, the nanotechnological approach has become an efficient and viable alternative to promote the therapeutic application of AMPs by the use of nanocarriers in order to: protect the AMP from degradation; enhance AMP absorption by facilitating diffusion through epithelium; modification of pharmacokinetic and tissue distribution profile; and/or improving intracellular penetration and distribution.

According to Couvreur & Vauthier (2006), over the past 30 years, the explosive growth of nanotechnology has burst into challenging innovations in pharmacology, which is in the process of revolutionizing the delivery of biologically active compounds.

The main application of nanotechnology in cancer and infectious diseases pharmacology collaborate with the development of several approved forms of drug-targeting systems for the treatment of certain cancer and serious infectious diseases (Couvreur & Vauthier, 2006). One of the main examples is the Ambisome®, a formulation of amphotericin B in liposome, which was marketed in 1996 (NeXstar now Gilead, Foster City, CA, USA). Before the nanostructured formulation, the toxicity of the leading compound against leishmaniasis and fungus, was 50- to 70-fold higher (Adler-Moore & Proffitt, 1993). This allowed the administration of more than 5-fold of the drug compared with conventional treatments. Thus, today it is considered the most efficient treatment for leishmaniasis and other fungal infections (Dupont, 2002; Ringden, 2002; Croft & Coombs, 2003).

Nanotechnology also seems to be a promising alternative to overcome the problems of the administration of peptides and of the new drug molecules coming out of the discovery pipeline. Nanotechnological based drug-targeting system carrying AMPs can be targeted to a precise location which would make the AMP much more effective, reducing the amount necessary to promote the antimicrobial action, as well as the chances of possible side-effects and production costs, making the therapeutic peptide antibiotics feasible economically compared to other antibiotics.

In recent years, significant efforts have been devoted to the development of nanotechnological tools capable of enhancing the assembly and immobilization of AMPs in a synergistic way in biomedical devices (Huguenin et al., 2005; Siqueira et al., 2006; Zampa et al., 2007; Zucolotto et al., 2006, 2007).

The structural and physico-chemical properties of the AMPs, such as the presence of a a-helix fold and distribution of positive charges along the chain have allowed their use as active material in the development of bio-nanostructures with a potential application in therapeutics by the pharmaceutical industry and diagnosis (Zampa et al., 2009). These nanostructures include cationic nanoparticles, formed by the conjugation of cholesterol and AMPs, able to cross the blood–brain barrier for treatment of fatal Cryptococcal meningitis in patients with late-stage HIV infection (Wang et al., 2010); Polymyxin B conjugates with Au nanoparticles and CdTe quantum dots with improved antimicrobial activity and reduced toxicity to mammalian cells (Park et al., 2011); nanostructured thin films with immobilized AMPs as an agent intended to combat and prevent infection and formation of Staphylococcus biofilm (slimelike communities) related implant failure (Shukla et al., 2009); or as sensor elements for detection of Leishmania cells using cyclic voltammetry (Zampa et al., 2009).

The use of the AMPs through nanotechnological innovation approach could provide an entirely novel way to treat and prevent infection and new systems for the detection and identification of infectious parasites. Nanotechnology could provide new ways to use lower amounts of AMPs with extreme efficiency in the infection suppression, by improving the cell, tissue, or organ's specific biodistribution and increasing AMP potency by the association with nanotechnological structures. It is expected that in the forthcoming years nanotechnology will promote the emergence of new products for control and prevention of multidrug-resistance microbe infection arising from the identification and analysis of AMPs from anuran amphibian biodiversity.

8. Final considerations

Anuran amphibians are an enormous source of bioactive molecules with potential application for the development of new nanotechnological based therapies against multidrug-resistant microorganisms in the modern day public health system crisis. However, the emergence of chytridiomycosis, climate change, pollution, and destruction and/or alteration of natural habitats are producing a devastating effect on biodiversity causing the amphibian decline. One-third of the anuran species are extinct or threatened with extinction before its content of bioactive molecules, specially the antimicrobials, can be discovered. Without a concerted effort, biodiversity and humans could be dealing with the "nightmare scenario" of a worldwide spread of untreatable infections and disappearance of species with potential solutions to combat superbugs. A united push to inspect and preserve the biodiversity in order to produce subsides for the development of new drugs is urgently needed.

9. Abbreviations

AMP	Antimicrobial peptide
ARMRL	Antibiotic Resistance Monitoring & Reference Laboratory
CR	Critically Endangered
EARSS	European Antimicrobial Resistance Surveillance System
ECDC	European Centre for Disease Prevention and Control
EMEA	European Medicines Agency
Epi	Epimastigote form
EW	Extinct in the Wild
EX	Extinct
FSAP	Frog Skin Active Peptide
HIV-1	Human Immunodeficiency Virus 1
HPA	Health Protection Agency
HSV-1	Herpes Simplex Virus 1
IPCC	Intergovernmental Panel on Climate Change
IUCN	International Union for Conservation of Nature and Natural Resources
$LC_{(50)}$	Lethal Dose, 50%
Lys	Lysine
MALDI-image	Matrix Assisted Laser Desorption Ionization-Image
MIC	Minimal Inhibition Concentration
MRSA	*Staphylococcus aureus*, methicillin resistance
NDM-1	New Delhi metallo-beta-lactamase-1
Pro	Promastigote form
PRSP	*Streptococcus pneumoniae*, penicillin resistance
Trf	Trophozoite form
Try	Trypomastigote form
UniProt	The Universal Protein Resource
VISA	*Staphylococcus aureus*, vancomycin intermediate resistance
VRE	*Enterococcus faecium*, vancomycin resistance
VRSA	*Staphylococcus aureus*, vancomycin resistance

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11

Brine Shrimp Diversity in China Based on DNA Barcoding

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1. Introduction

Taxonomy, the science that deals with the study of identifying, grouping, and naming organisms according to their established natural relationship, is the basis of all biological studies. Biological and observation-based classification is still generally the best known form of taxonomy since 1735 when Carl Linnaeus published the great book - Species Plantarum, and it is an empirical science mostly based on morphological difference. With the development of science and technology, scientists have discovered many methods to identification new species and other tools or definitions for species classification, such as biochemical identification (Farmer et al. 1985), cytotaxonomic identification (Le Berre et al. 1985), chromosomal DNA fingerprinting (Owen 1989), restriction fragment length polymorphism (RFLP) (Sakaoka et al. 1992), and PCR-based DNA fingerprints (Matsuki et al. 2003). Among others, molecular or genetic approaches to identify species have been proposed and extensively used (Yamamoto 1992; Zhou et al. 2003).

1.1 DNA barcoding

The study of biodiversity lays the foundation for all biological studies, especially the classification of species, and the ways to do it have never stopped since Linnaeus. Traditional morphology-based taxonomy has its limitations, such as when facing mimetic polymorphism, and it mainly depends on the expertise of taxonomists, and there is little doubt that evidence at molecular levels should be complementary and of necessary. As the development of molecular biology, the idea of molecular taxonomy has been propounded and gradually accepted by related scientific communities. The standard molecular identification system was initiated during 1990s by using PCR-based and sequencing-based approaches (Frézal et al. 2008). Taken the advantage of the two powerful technologies in accuracy and convenience, DNA sequence signatures provide adequate “barcodes” for species identification, and “DNA barcoding” has been widely used in studies for speciation (Ghebremedhin et al. 2008; Sullivan et al. 1996), phylogenetics andevolution (Göker et al. 2009;Wood et al. 2000), and molecular ecology (Govan et al. 1996; Valentini et al. 2009) as well as for the classification of both pathogenic microbes (Beckmann 1999) and normal microbiomes (Holzapfel et al. 2001).

DNA barcoding is an ultimate and direct approach for molecular taxonomy, depending on the complexity of sequence signatures used, especially in distinguishing species with nearly

identical morphological features, thereby helping to establish legitimate phylogenetic relationships and to reveal evolutionary histories. The concept of DNA barcoding was first advocated by Arnot in 1993 (Arnot et al. 1993) and its new era began in 2003, marked by the establishment of the Consortium for the Barcode of Life (CBOL, http://barcoding.si.edu). This initiation was put forward and promoted by researchers at the University of Guelph in Ontario, Canada in 2004. The aim of this project is to create a universal protocol for an eukaryotic species inventory based on a standard molecular approach. Up to this date, the Consortium has more than 150 member organizations from 45 countries, including natural history museums, zoos, botanical gardens, university departments as well as private companies and governmental organizations (Frézal & Leblois 2008; Schindel et al. 2005). The DNA Barcode of Life Database (BOLD, http://www.boldsystems.org), an informatic workbench aiding the acquisition, storage, analysis, and publication of DNA barcode records, has been developed since 2004 and was officially established in 2007 (Ratnasingham et al. 2007). This database provides an integrated bioinformatic platform for the acquaintance, collection, and analysis of basic barcoding data and facilitates the development of DNA barcoding.

1.1.1 What is DNA barcoding?

In theory, nucleotide sequences of nuclear and organellar origins are natural 'barcodes' that are unique to each organism on earth. Therefore, a 15-bp nucleotide sequence creates 4^{15} (1 billion) combinations that would be sufficient for the differentiation of the estimated 10-15 million species (Butchart et al. 2010; Perrings 1996). However, practically, species are related and their genome sequences are often homologous, depending on their evolutionary distances. In addition, the rates of molecular evolution vary dramatically across taxa and even at different positions in a given genome. In the latter case, the main task of DNA barcoding is to find a sequence fragment that is evolutionarily less selected and serves as a unique barcode for species identification.

There had not been a universal barcode sequence for all species yet, especially across distant lineage boundaries, but several candidate genes are commonly used for phylogenetic analysis, such as mitochondrial 16S rRNA gene, mitochondrial cytochrome *b* gene, and the mitochondrial cytochrome c oxidase subunit 1(*COI*) which can serve as the core of global bio-identification system for animals (Hebert et al. 2003a, 2003b). A 648-bp segment at 58-705 from the 5'end of this gene is chosen as the barcode segment. *COI* gene is an ideal model to evaluate the evolution rate, as its third-position nucleotides show a high incidence of base substitutions but its amino acid sequence changes rather slowly as compared to other mitochondrial genes. As a result, on the one hand, the evolution of this gene is rapid enough for identification of not only closely related species, but also phylogeographic groups within a single species, and on the other hand, it is possible to place an unidentified species into higher taxonomic categories (from phyla to orders) (Hebert et al. 2003a). Although a unified opinion has not been reached on a single barcoding DNA segment chosen for taxological studies (Lin et al. 2009), the *COI* gene-based identification system has been proven superior within taxonomic groups of Protista (Chantangsi et al. 2007; Evans et al. 2007) and animals, including gastropods (Hebert et al. 2003b; Remigio et al. 2003), ants (Smith et al. 2005), butterflies (Hebert et al. 2004a), birds (Hebert et al. 2004b), spiders (Greenstone et al. 2005), fish (Ward et al. 2005), worms (Ferri et al. 2009; Jennings et al. 2010), Crustacea (Lefébure et al. 2006), and very recently primates (Nijman et al. 2010).

1.1.2 Examples of DNA barcoding applications

DNA barcoding has been successfully used for the taxonomy of invertebrate and vertebrate animals as well as microbes, including bacteria (Siddall et al. 2009), fungi (Kelly et al. 2011; Stockinger et al. 2010), Protista (Chantangsi et al. 2007; Evans et al. 2007), and algae taxonomies (Saunders 2005). In the past three years, an increasing number of studies has been focused on DNA-barcoding of plants (He et al. 2010; Kress et al. 2007; Lahaye et al. 2008). Since there is not yet a universally accepted DNA barcode for plants, many strategies have been proposed, based either on a single chloroplast segment (Hollingsworth et al. 2009; Lahaye et al. 2008) or a combination of multiple segments (He et al. 2010; Kress & Erickson 2007). Examples of DNA barcoding studies are summarized in Table 1 including DNA barcodes for animals, plants, fungi, and protists. As mentioned previously, there are advantages and limitations among the barcodes with respect to specific applications.

Organism group	DNA barcode	References
Animals	COI, 28SrRNA, cob	(Hebert et al. 2004a; Hogg et al. 2004; Ward et al. 2005; Zhang et al. 2011)
Plants	COI, trnL, matK, rbcL, trnH-psbA, ITS	(Kress et al. 2005; Savolainen et al. 2008; Shaw et al. 2011; Specht et al. 2007)
Fungi	COI, ITS, LSU, mtSSU, beta-tubulin	(Porter et al. 2008; Schussler et al. 2010; Seifert et al. 2007; Summerbell et al. 2007; Tedersoo et al. 2008)
Protists	COI, ITS	(Brodie et al. 2006; Keeling et al. 2010; Pawlowski et al. 2010; Saunders 2005; Stern et al. 2010)

Table 1. Applications of DNA barcoding technology

1.1.3 Advantages and drawbacks of DNA barcoding

There are several obvious advantages in the currently used DNA barcoding system. First, it uses a standard procedure that can be applied universally to relevant research fields. It is of great utility in conservation biology and can also be applied to samples where traditional morphological methods are unable to define, including species identification based on eggs and larval (Wang et al. 2008) and analysis of stomach contents or excreta to determine food webs. Another advantage of DNA barcoding comes from the rapid and cost-efficient acquisition of molecular data, enabling large-scale species identification (Frézal & Leblois 2008), whereas conventional taxonomy is time consuming, and in some cases it is almost impossible to apply (Rusch et al. 2007). Therefore, it is important to be able to improve large surveys aiming at unknown species detection and identification of pathogenic species with medical, ecological, and agronomical significance (Ball et al. 2008; Barth et al. 2006). Particularly, DNA barcoding becomes necessary when morphological traits do not adequately discriminate species (Caron et al. 2009; Guo et al. 2010; Kauffman et al. 2003; Kumar et al. 2006) or if species have polymorphic life cycles and/or exhibit pronounced phenotypic plasticity (Pegg et al. 2006; Randrianiaina et al. 2007).

However, controversies about DNA barcoding still remain. Although DNA barcoding was proposed initially as a method for species identification, to better achieve this goal, it needs

be validated intensively, especially in choosing the best candidate sequences that are both universal and highly variable among species. The first question is: what are these sequences: nuclear, mitochondrial, or chloroplast? An idea to use a simple sequence from mtDNA has been dismissed. It is not adequate to be used as a sole source for species-definition due to following genetic factors: reduced effective population size and introgression, maternal inheritance, recombination, inconsistent mutation rate, heteroplasmy, and compounding evolutionary processes (Meier et al. 2006; Rubinoff et al. 2006). Until now, there has not been an universal DNA barcode for all organisms and we have not found a single gene that is conserved enough and also exhibits appropriate divergence for all species regardless where they come from (Hickerson et al. 2006; Rubinoff et al. 2006; Song et al. 2008). The validity of DNA barcoding therefore lies on establishing reference sequences from taxonomically confirmed specimens, which will acquire an integration of morphological and molecular based taxonomy data, as well as decent cooperation among sample collection, such as museums, zoos, and research institutes (De Hoog et al. 2008). This approach is closest to what has been termed "integrative taxonomy" (Dayrat 2005; Will et al. 2005). DNA sequences in combination with traditional character sets are used in a complementary fashion to define and describe species (Heethoff et al. 2011; Padial et al. 2010; Pereira et al. 2010).

1.1.4 Recent progresses in DNA barcoding

Recently, the approach of DNA barcoding has been greatly revived to increase accuracy and sensitivity, and the major improvements are focused on using more than one barcoding strategies for a better identification of specific species (Aliabadian et al. 2009; Ferri et al. 2009; Lin et al. 2009; Nassonova et al. 2010). Shatters et al improved DNA barcoding by using different regions of *COI* gene to do biotype-specific barcoding (Shatters et al. 2009). As the sequencing technology developed rapidly in the past few years, sequence-based DNA barcoding also advanced rapidly, such as cap analysis of gene expression (CAGE) using an ultra high-throughput sequencer (Maeda et al. 2008), to show biodiversity (Creer 2010; Fonseca et al. 2010; Mitsui et al. 2010), and the ArkChip strategy for highly-resolved patterns of intraspecific evolution and a multi-species (Carr et al. 2008). Several new techniques have been implemented, and all based on the sequencing of individual DNA molecules (with or without an amplification step) in massive and parallel ways (Table 2, Figure 1). The high accuracy, throughput, and efficiency make the identification of genome sequences unique to different species and life forms easy.

The processes that apply next-generation sequencers to DNA barcoding are expected to be more complex than what has been anticipated. For instance, the classical DNA barcode is defined to be a fragment around 650bp but the effective read lengths of the next-G sequencers are actually shorter than it at present time. Progress has been made in recent studies, where smaller DNA fragments, called mini-barcode, of *COI* gene or rDNA were used for accurate species identification (Hajibabaei et al. 2006; Pawlowski & Lecroq 2010). Researches show that more than 90% and 95% success rates were achieved by using 100-bp and 250-bp barcodes, respectively (Meusnier et al. 2008). Although biodiversity studies based on next-G sequencing technologies were emerged in 2006, (Ley et al. 2006; Sogin et al. 2006), most of the studies have been done with the Roche/454 system (Hajibabaei et al. 2011; Meyer et al. 2007; Porazinska et al. 2009) and mainly for environmental samples (Deagle et al. 2010;Fire et al. 2007; Hajibabaei et al. 2011). More recently, the upgrading

speed of different sequencing platforms, such as those of Illumina and Life Technologies, has been very impressive and the read lengths of these new versions of sequencers are getting longer (Table 2). They may also one day be used for biodiversity study when their read length is increased to ~100bp and more.

Sequencer	Company	Read length	Reads per run	Total output	Time per run
Solexa	Illumina	75 bases	60 million	4 Gb	6.5 days
SOLiD	Life Technologies	50 bases	85 million	4 Gb	6 days
454 GS FLX	Roche Diagnostics	500 bases	1 million	0.5 Gb	8 h

Note: The recent machine and software upgrades from Illumina (such as HiSeq2000) and Life Technologies (such as 5500XL) promise ~100-fold increases in the total outputs of raw data.

Table 2. Comparison of next-generation sequencing platforms.

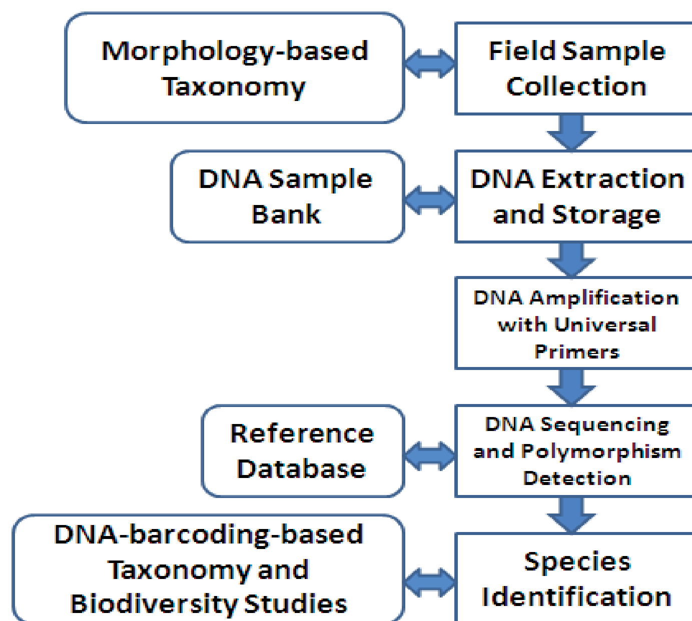

Fig. 1. Methodology for analyzing biodiversity based on high-throughtput DNA sequencing.

1.2 A case study on *Artemia* (Crustacea, Anostraca) in China

Brine shrimp or *Artemia* (Crustacea, Anostraca) is a worldwide living species well-adapted to survive in very harsh hypersaline environments, such as salty lakes and lagoons (Clegg et

al. 2009), it typically shows enormous diversity at the genus level in terms of their ability to survive under different ionic compositions, climatic conditions, and altitudes. In this case study, *Artemia* species are served as ideal model organisms for biodiversity study in inland hypersaline lakes (Camargo et al. 2005; Castro et al. 2006; Hand SC 1982; Maniatsi et al. 2009). In addition, the morphological variations displayed among *Artemia* populations also provide excellent materials for studying adaptive genetic polymorphisms at molecular levels. During the past two decades phylogenetic relationships among *Artemia* species have been established by combined studies based on cross-breeding, morphological differentiation, cytogenetics, nuclear (including allozymes and other nuclear DNA sequences) (Badaracco G 1995; Baxevanis et al. 2006; Sun Y 2000) and mitochondrial (mtDNA) DNA markers (Badaracco G 1995).

Seven sexual species have been described thus far, as well as numerous parthenogenetic populations. Five species are found in Eurasia: *A. salina* (Mediterranean area), *A. urmiana* (Iran), *A. tibetiana* (Tibet), *A. sinica* (van Wely et al.), and *A. spp* (Old World). The New World species are *A. franciscana* and *A. Persimilis*; the former are widely distributed in most part of America, while A. persimilis is restricted to certain locations in Chile and Argentina (Clegg et al. 2009). *A.franciscana, A.tibetiana,* and *A.sinica* are the main *Artemia* species that inhabit in China (Figure. 2). *A.tibetiana* dwells in the Tibetan Plateau, with the altitude of ~ 4,500m above the sea level. Living under the harsh condition of hypoxia, low temperature, high solar radiation, and lack of biological production, it requires a modified and adapted energy metabolism for survival. In 1980s, a large quantity of *A.franciscana* was released in the most part of salt field in the Bohai Bay. As a dominant species, *A.franciscana* replaced the local species, *A.sinica*, rapidly and has become the primary species in the Bohai Bay since. As a result, *A.sinica* is almost disappeared completely in sea shores of Eastern China.

Fig. 2. The distribution of *Artemia* in the world.

2. Biodiversity of *Artemia* populations in China

The phylogeny of various *Artemia* samples from different habitats around the world was reported previously, and our focus now is on the biodiversity of *Artemia* species in China, especially that of the Tibetan Plateau. All strains used in this study are also kept as cysts at the Laboratory of Aquaculture & Artemia Reference Center (ARC) with ARC code numbers (Wang et al. 2008), including six populations represented five salt lakes from Nima (ARC 1609), Yangnapeng Co (ARC 1610), Lagkor Co (ARC 1348), Jingyu lake (ARC 1524), and Co Qen Lakes (ARC 1526 and ARC 1612) of the Tibetan Plateau (Table 3).

ARC #	Location	Year of harvesting
1348	Lagkor Co, Tibet, China	1996
1524	Jingyu Lake, Xinjiang, china	2001
1526	Co Qen,Tibet, China	2001
1612	Co Qen, Tibet, China	2001
1609	Nima, Tibet, China	1999
1610	Yangnapengco, Tibet, China	2002

Table 3. List of Artemia species in China and their locality and ARC codes.

2.1 Phylogenetic analysis of *Artemia* species in China based on *COI* gene barcoding

A 648-bp segment of the mitochondrial *COI* gene was selected as the standard barcode to establish phylogenetic relationships among *Artemia* species from major habitats, including species from the Tibetan Plateau (Figure 3, Wang et al. 2008). We built a phylogenetic tree based on *COI* gene, which separates the populations into five stable clades. Three of them are composed of species from China, and the first clade contains genotypes from populations collected in the Bohai Bay areas of Eastern China and also one sample from Vinh Chau, Vietnam, which shows a high sequence similarity to *A. franciscana*. It is evident for a large-scale invasion of *A. franciscana* in the Bohai Bay (Van Stappen 2007). The second clade is made of *A.tibetiana* genotypes from populations in Tibet and Southwestern China, with high sequence similarity to *A. urmiana*. The third clade belongs to *A. sinica*, which mainly contains populations from Inner Mongolia in the Central North of China. The fourth and fifth clades correspond to *A. persimilis* and *A. Salina,* respectively, and they are not found in this study as major populations in China.

Investigating the amino acid variations, we found two consistent amino acid changes in COI between high and low altitude species we collected in China: 153A/V and 183L/F. These sequence alterations may provide clues for further functional studies such as to determine if the adaptation to high altitude had resulted in the fixation of such mutations. We also used Ka/Ks calculator to estimate Ka/Ks (Zhang et al. 2006) with the aim to reveal sequence signatures of natural selection in *COI* gene. When using *A. franciscana* as a reference, *A. tibetiana* has significantly higher Ka/Ks ratios, which imply relatively stronger selective pressure on this species. Two variations that alter amino-acid sequences between the high and low altitude populations shared by the high altitude group were also detected. The sequence from sample 1612 has the highest Ka/Ks ratio, and its mutation spectra suggests a relatively stronger selection posed on this population and its synonymous mutations provide clues that the population is rapidly diverging, which is most likely due to environmental changes during last three million years rather than genetic drift.

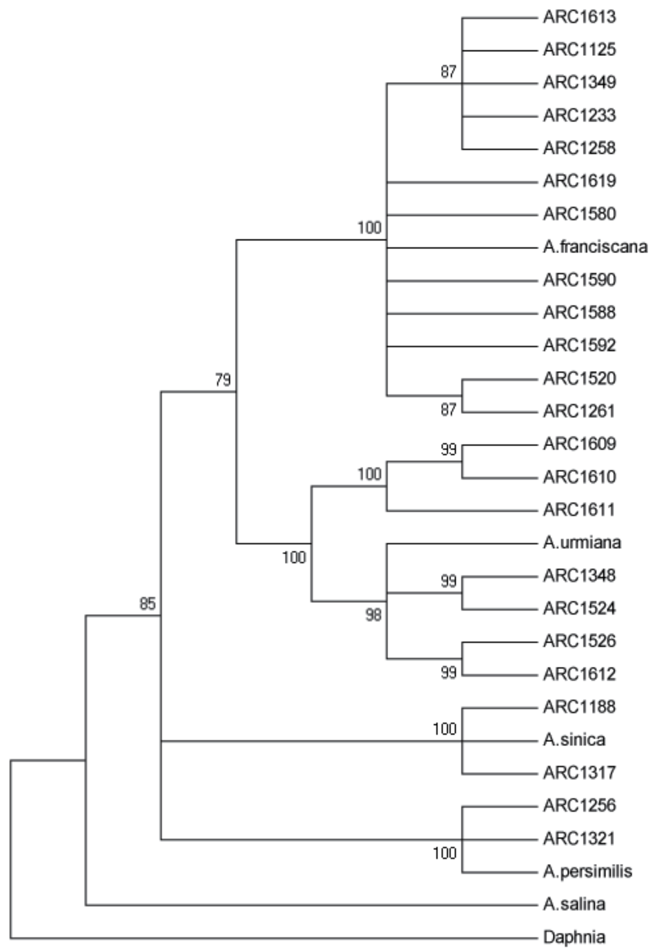

Fig. 3. A phylogenetic tree based on neighbor-joining method. The tree is constructed based on a sequence fragment of *COI* gene (Adapted from Wang et al.).

We further obtained high-quality sequences from individual adults of the six Tibetan populations and calculated the Kimura-2-Parameter distances (Table 4). For phylogentic tree construction, we used the consensus sequences when sequence heterogeneities are encountered among a minor set of samples.

	1348	1524	1526	1609	1610	1612
N	20	18	9	20	20	8
Min	0	0	0.38	0	0	2.55
Max	7.76	1.31	11.07	4.92	9.56	12.01
Mean	2.3	0.51	4.17	2.49	3.42	7.07
S.D.	0.34	0.17	0.52	0.45	0.5	0.79

Table 4. Kimura-2-Parameter distances of samples from Tibet (Adapted from Wang et al 2008).

2.2 Sequencing and comparative analysis of *Artemia* mitochondrial genomes

Based on the obvious divergence of *COI* gene, we speculated that environmental selection may bring more variations to other mitochondrial encoding genes involved in energy metabolism during the long-term selection that may affect structures and activities of the ATPase subunits or even other components of the mitochondrial respiratory chain complexes. Therefore, we decided to take *Artemia* species in Asia as our model and acquired whole mitochondrial genome sequences of *Artemia tibetiana* collected from the Tibetan Plateau and carried out comparative analysis involving other lower altitude *Artemia* species, *A. franciscana, A. urmiana*, and *A.sinica*, and aim to observe specific characteristics of the mt genome sequences of *A. tibetiana*.

We indeed acquired and annotated five mitochondrial genomes, including two ecotypes of *A. tibetiana*, one each from *A. urmiana*, *A.franciscana*, and *A.sinica*. The *A. tibetiana* samples were collected from Nima (ARC 1609) and Yangnapeng Counties (ARC 1610) of the Tibetan Plateau with the altitude higher than 4,000m. *A. urmiana*, which had a very close phylogenetic relationship with *A. tibetiana* based on previous DNA barcoding study, were collected from Urmia Lake of Iran (ARC 1227) at an altitude of 1275m above the sea level. *A.sinica*, another native species in China which is collected from Yimeng of Inner Mongolia (ARC 1188), where it has an altitude of ~1000m above the sea level and a climate of dry, windy, and sandy. We also have one ecotype of *A.franciscana* is collected from Huangnigou, Shangdong in China (ARC 1590). The length variations are mainly found in the non-coding region (known as the D-loop region).All *Artemia* mitochondrial genomes encode 37 genes including 2 rRNAs, 22 tRNAs, and 13 polypeptides that are subunits of the respiratory chain complexes residing on the inner mitochondrial membrane.

Comparative analysis of mitochondrial DNA (mtDNA) of these *Artemia* species shows that the nucleotide variation ratio is higher between *A. tibetiana* and *A. franciscana* and much lower between *A. tibetiana* and *A. urmiana* or *A.sinica*. Among the 13 protein-coding genes, *ND* gene family has more nucleotide variations than other genes. ND6 varies the most both between *A. tibetiana* and *A. franciscana* (T-F) and between *A. tibetiana* and *A. urmiana* (T-U), and the same situation is observed between *A. tibetiana* and *A.sinica* (T-S). When analyzing the amino-acid changes, *ATP8* gene has higher variation rates, second only to the *ND* gene family. In addition, COI is the most conservative protein in amino-acid sequence among the 13 polypeptides. The complexes IV and V contain more variations than other complexes. With Ka/Ks Calculator, ATP8 has a high Ka/Ks ratio, just lower than that of *ND4* when *A. tibetiana* and *A. urmiana* are separated from *A. franciscana*, while *ATP6* possesses higher evolutionary rate between *A. tibetiana* and *A. Urmiana* (data not shown)

3. Conclusion

Consequently, our results on DNA barcoding and comparative analysis reveal the current distribution of *Artemia* species in China and phylogenetic relationship among them, providing insights into the adaptive evolution of DNA sequences of *Artemia*. Based on phylogenetic and divergence analyses of the selected samples from different regions of the world, it is possible that the high altitude group of *Artemia* are descendents of a local ancestral species in the Himalayas which diverged genetically as the Tibetan Plateau arose stepwise over approximately the last three million years (Tapponnier et al. 2001).

The comparative studies among different *Artemia* species reveal complex sequence diversities that are expected to have functional relevance, such as energy metabolism and environmental

adaptation. The highest number of adaptive variations in ATP8 implies that it is under selective pressure during long-term geographical isolation when *A. tibetiana* separated from their common ancestor together with the rise of Himalaya Mountains. It was reported that the *ATP8* gene encodes a core subunit of F0 in ATPase that synthesizes ATP based on a proton-gradient that results from H+ pumping into the intermembrane space (da Fonseca, Johnson et al. 2008). It was also suggested that ATP8 may play regulatory roles in ATP synthesis among different species since it has highly variable sites in the protein-coding sequence (da Fonseca, Johnson et al. 2008). Moreover, the Ka/Ks ratio in ATP6 is also relatively high when we compared the 13 protein-coding mitochondrial genes of *A. tibetiana* to those of *A. urmiana* and *A.sinica*. It is known that ATP6 plays an important role in the assembly of F0 (Hadikusumo, Meltzer et al. 1988) and the highly variable sites are found in the predicted loop regions where the sequences are less selected in terms of its overall function. The high variation rates found among the ATPase subunits imply a strong selective pressure on the *Artemia* energy metabolism system from the high plateau environment.

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12

Fishes of the Atlantic Rain Forest Streams: Ecological Patterns and Conservation

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1. Introduction

Fishes constitute more than one half of the species of vertebrates, with around 32,000 living species (Froese & Pauly, 2011). Approximately 40% of this global fish diversity lives in freshwater environments, which represents less than 1% of the surface of the Earth (Dudgeon et al., 2006). In the Neotropical Region, freshwater fishes constitute a taxonomically distinct fauna that extends throughout the continental waters from Central Mexico to the southernmost tip of South America. This zoogeographical region is known to harbor the richest and most diverse freshwater fish fauna of the whole planet (Géry, 1969; Vari & Malabarba, 1998; Lundberg et al., 2000; Albert et al., 2011).

The Atlantic Rain Forest is one of the richest biomes in the Neotropics mainly due to the variety of habitats throughout the range of the forest types and subtypes, which originally covered a wide stripe of the Brazilian coastline (Morellato & Haddad, 2000). Considering the fact that the definition of the limits and forest types of the Brazilian Atlantic Rain Forest is controversial and beyond the scope of this study, this vegetation domain was considered here in a narrower sense, comprising the coastal forest formations between 6–30^{0} S, with elevations from sea level to approximately 1,000 meters. In this sense, this forest is dispersed along degraded landscapes, embracing some of the largest and oldest Brazilian urban areas, where more than 150 million people live.

The Atlantic Rain Forest constitutes one of Brazil´s most important vegetation domains, because of its historical relationship with the colonization of the country, and also in view of the role that it plays in the conservationist scenario (Silva, 2003). In the broadest and most generic sense of the forest formations, this biome is one of the most biodiverse and endangered ecosystems in the world (Myers et al., 2000).

The region bounded by this forest has a high percentage of fish species with restricted distribution, as a result of the great number of independent coastal drainages (or groups of basins), and the isolating effect of mountain ranges and seawater among coastal rivers (Bizerril, 1994; Menezes et al., 2007). In fact, according to our survey a great amount (70%) of the freshwater fishes can be considered exclusive to the coastal drainages of this vegetation domain The high rate of speciation and high degree of geographic endemism is an important factor that needs to be considered in the conservation policies of the Atlantic Rain Forest remains, as this biome is located in the most populated regions of the country,

making these aquatic environments especially vulnerable to anthropogenic impacts (Menezes et al., 2007).

In this chapter, despite the fact that a standard definition for the term stream is difficult and sometimes controversial, streams were defined as naturally flowing surface waters that are contained in a channel with definite boundaries and hydrodynamics, with channel widths up to approximately 40 m. This definition allowed the inclusion of Atlantic Rain Forest streams of many different characteristics (Fig. 1), all characterized by mosaics of different habitats and microhabitats determined by the system's response to spatial and temporal variation in the input of water, flow regime, depth, sediment, debris, and biotic structure.

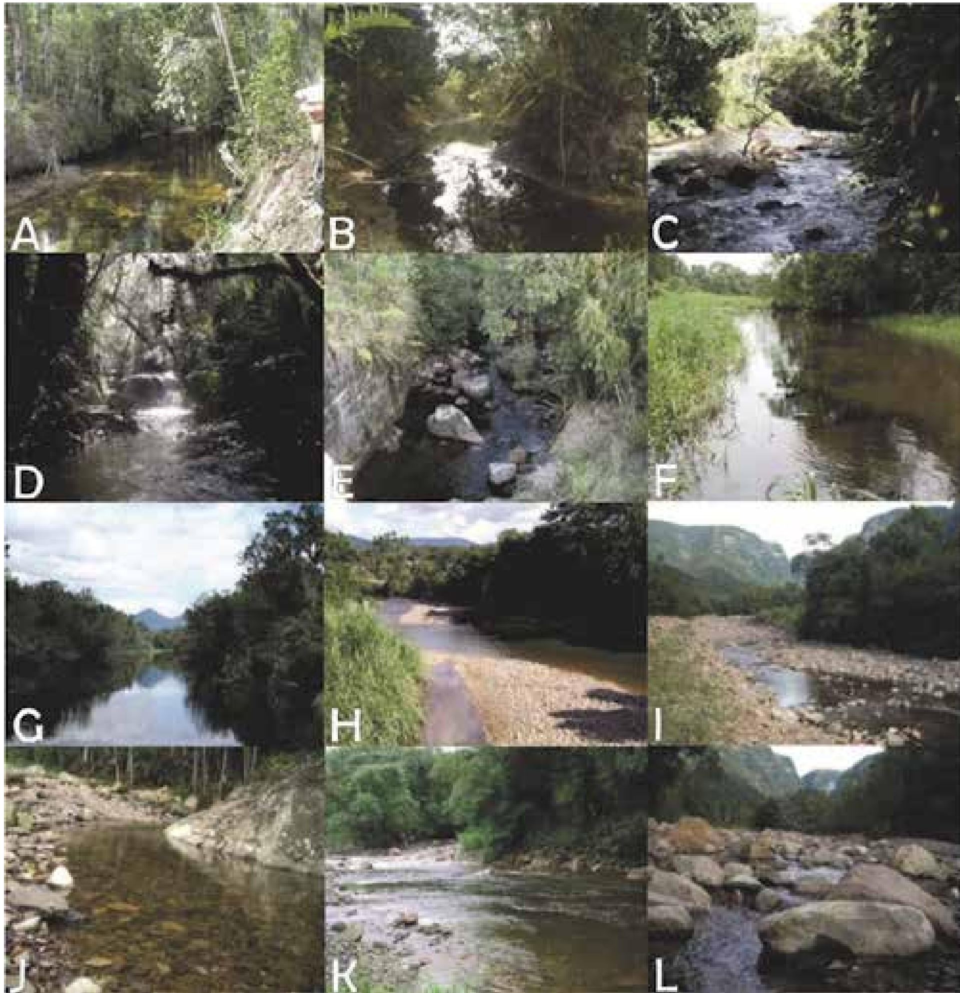

Fig. 1. Atlantic Rain Forest streams habitats along the Brazilian coastline. A. Mocuba River (Bahia); B. Guaibimzinho River (Bahia). C. São João River (Espírito Santo); D. Ubatiba River (Rio de Janeiro). E. São João River (São Paulo). F. Das Pombas River (Paraná); G. Guaraguaçu River (Paraná); H. Nhumdiaquara River (Paraná); I. Ararangua River (Santa Catarina); J. Itapocu River (Santa Catarina); K. Mampituba River (Rio Grande do Sul); L. Do Boi River (Rio Grande do Sul).

This chapter investigates ecological patterns of stream fish community in the Atlantic Rain Forest of Brazil. Several streams that flow through this ecosystem are inhabited basically by small-sized fish species, which dwell in streams or shallow water of rivers, showing sometimes a high rate of speciation and a high degree of geographic endemism. The aim of this chapter is to demonstrate the great biodiversity and high endemism of fish species in the Atlantic Rain Forest streams, and also assess general patterns in assemblage structure and composition, feeding habits and reproductive strategies. Conservation issues and impacts concerning the anthropic pressure were also explored.

2. Stream fish composition and structure

Species richness registered in the Atlantic Rain Forest streams is high. The taxonomic biodiversity is represented by 269 species belonging to 89 genera and 21 families (based on, Menezes & Weitzman, 1990; Lucena & Lucena, 1992; Bizerril, 1994; Schaefer et al., 1997; Pereira & Reis, 2002; Reis & Schaefer, 1998; Weitzman & Malabarba, 1999; Costa, 2002; Bertaco, 2003; Bertaco & Lucena, 2006; Oyakawa et al., 2006; Menezes et al., 2007; Buckup et al., 2007; Lucinda, 2008). Regardless of been relatively poor at higher taxonomic levels (one class), the ichthyofauna is dominated by ostariophysian fishes (205 species), and the most represented orders are Siluriformes (114 species) and Characiformes (83 species) (Fig. 2). These groups also constitute the main fish component of other Neotropical freshwater environments.

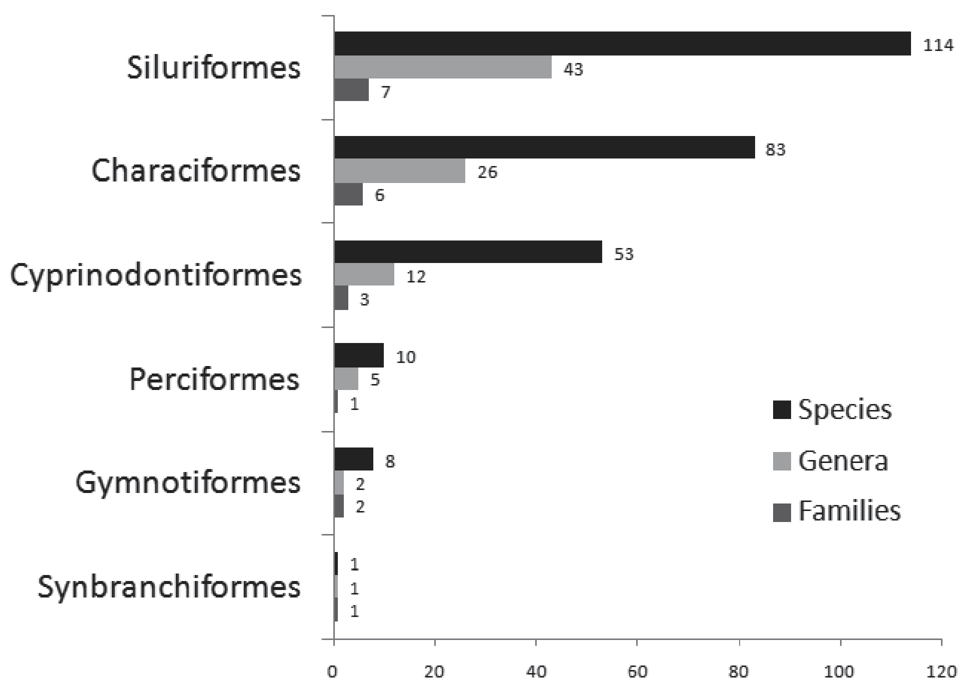

Fig. 2. Number of species, genera and families of freshwater fishes of the Atlantic Rain Forest streams.

Characins (tetras and relatives), loricariids (armored catfishes and relatives), trichomycterids (candirus), rivulids (killifishes), and poeciliids (guppies) are strongly dominant. Some species can be found in streams with rapids and rocky bottom, while others live in temporary aquatic environments during rainy seasons. The most speciose genera are *Trichomycterus*, with 19 species, *Phalloceros*, with 18 species, and *Astyanax*, comprising 15 species (Fig. 3).

The Neotropical catfish family Trichomycteridae comprises more than 200 species of small-sized fishes (de Pinna & Wosiacki, 2003) which in general inhabit fast-flowing rocky streams (Arratia, 1983; de Pinna, 1998). Despite the remarkable range of habitats and feeding habits, there is little information on trichomycterids natural history in the Atlantic Rain Forest. Available evidences for the genus *Trichomycterus* suggest that the group is represented by generalist predators of aquatic invertebrates (Trajano, 1997; Rolla et al., 2009), and individuals can be found in streams with rapids and rocky bottom, living usually beneath rocks (Menezes et al., 2007) and buried under sand or leaves.

The livebearing fishes of the family Poeciliidae are characterized by internal fertilization, viviparity, and marked sexual dimorphism. The neotropical genus *Phalloceros* comprises twenty-two species distributed throughout southern and southeastern river basins of South America (Lucinda, 2008). *Phalloceros* species live primilarly near shore in open sunny areas of quiet backwaters of streams, where aquatic vegetation provides cover (Menezes et al., 2007).

The genus *Astyanax* is diverse and widespread in freshwaters of South America, including at least 136 described species (Froese & Pauly, 2011). *Astyanax* is likely a non-monophyletic genus, and the taxonomic status of some species from the Atlantic Rain Forest and adjacent areas is not completely clear (Menezes et al., 2007). *Astyanax* species can be observed in clear and turbid waters, in segments with pools and riffles, within a great variety of substrates such as stones, rocks, sand or mud.

Besides characins, catfishes, killifishes, and guppies, marine species such as sleepers (Eleotridae), mullets (Mugilidae), snooks (Centropomidae), pipefishes (Syngnathidae), and gobies (Gobiidae) make up the list of species that so far have been registered in the Atlantic Rain Forest biome drainages. Some species can be found in streams and in aquatic systems considered as a transitional environment between the estuarine-riverine and the estuarine mixing zones, such as *Dormitator maculatus*, *Poelicia vivipara*, *Gobionellus oceanicus*, *Ctenogobius shufeldti*, *Awaous tajasica*, and *Microphis brachyurus*. The ability to tolerate salinity fluctuations is common to some of this marine/estuarine species, but their adaptability and distribution vary according to the physiologic tolerance of each species.

Coastal Atlantic Rain Forest streams can be recognized as a very distinct area in terms of its ichthyofauna. The predominance of small-sized fish species of tetras, armored catfishes, candirus, killifishes, and guppies, which dwell in streams or shallow water of rivers, and the high rate of speciation and degree of geographic endemism, may indicate a certain degree of uniformity in the characterization of local and regional stream fish assemblages. Main differences in regional diversity of fish community among streams are related to large-scale factors such as biogeographical history, elevation and stream hierarchical organization in the watershed, while local diversity variations seem to be mostly due to site-specific factors (environmental conditions). Differences in fish community are caused by the natural variability of ecosystems, but also by human disturbances like habitat alteration, non native species introduction and pollution.

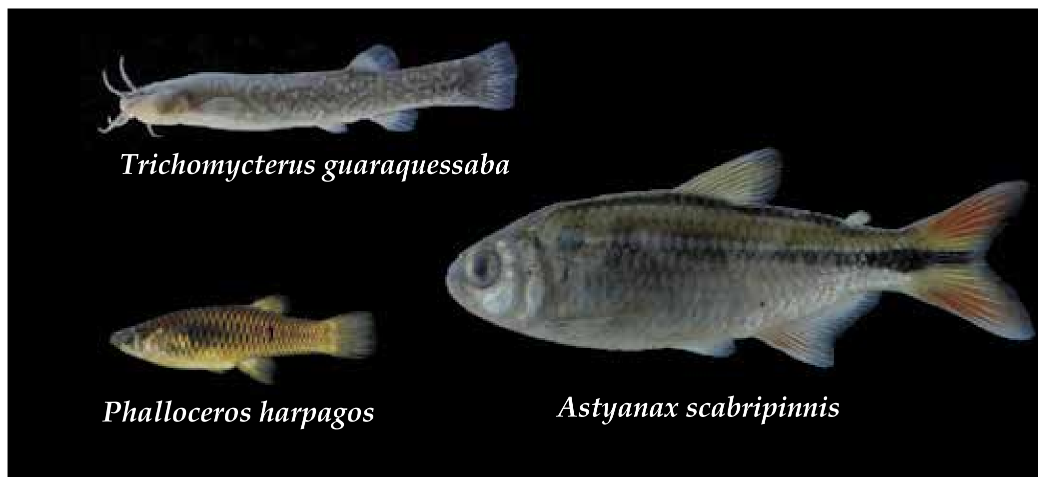

Fig. 3. Atlantic Rain Forest stream species of *Trichomycterus* (72 mm SL), *Phalloceros* (43 mm SL), and *Astyanax* (100 mm SL).

3. Reproduction

Reproduction biology of Atlantic Rain Forest stream fishes have been studied by several authors (Mazzoni & Caramaschi, 1997; Menezes et al., 1998; Amaral et al., 1998; Aranha & Caramaschi, 1999; Mazzoni & Petito, 1999; Sabaj et al., 1999; Mazzoni et al., 2002, 2005; Mazzoni & Iglesias-Rios, 2002a, 2007; Braga et al., 2006; 2008; Mazzoni & Silva, 2006; Becker et al., 2008; Vitule et al., 2007, 2008a). However there still is a large gap on this subject.

Reproductive aspects of fishes are characterized by a wide plasticity that is unique among vertebrates, with several evolutionary transitions between external fertilization and copulation. In general, fishes have a fixed sex (gonochoristic) but there are hermaphroditic ones such as *Synbranchus marmoratus*, a diandric (both primary and secondary male development) protogynous species (females turn into males) (Lo Nostro & Guerrero, 1996). Most teleost species are ovuliparous (external fertilization and development) (e.g. *Rhamdia quelen*, *Hyphessobrycon griemi*, *Gymnotus carapo*, *Crenicichla* spp.), and in some species the common reproductive mode is oviparity, where fertilization is internal but the development is external. This seems to be the case of the characin subfamily Glandulocaudinae, but there is no evidence of actual internal fertilization, only internal insemination (Burns et al., 1995).

Viviparity (internal fertilization and development with birth of live young) is also rare but common to the family Poeciliidae (e.g. *Phalloceros* spp., *Poecilia vivipara*, *Phalloptychus januarius*). This type of reproduction allows the reproduction to be relatively independent from environmental variables, turning this onto an interesting adaptation on such an instable environment as neotropical streams.

Among external fertilizing stream fishes some characins such as *Astyanax* species spawn their eggs into the water column where males release sperm. Other common breeding behaviours are deposition of egg under rocks, into cavities and scattering on the substratum. A few external fertilizing species present parental care. It is common among Loricariinae (armoured catfishes) where males search for shelter to protect their eggs, and in some cases eggs are attached to the body of females. Cichlids are also well kwon for building nests where they protect their eggs and young.

Another important life history pattern of reproduction regards to semelparity (single reproductive event in their life) or iteroparity (species reproduce several times during a lifetime). To date there are no records of a semelparous species at Atlantic Rain Forest streams. Some rivulids such as *Leptolebias aureoguttatus* that live on stream´s adjacent temporary ponds are annual, which means that individuals spawn several times before dying in these temporary aquatic environments during rainy seasons, and eggs survive dry periods buried in the substrate, not characterizing semelparity.

To avoid predation and to enhance fertilization and dispersal fishes have a wide variety of temporal and spatial patterns of spawning. They either release their eggs all at once in the same location (total spawners, e.g. *Characidium* spp., *Geophagus brasiliensis*, *Deuterodon langei*) or scatter them along the year and/or throughout the stream (partial spawner, e.g. *Rhamdia quelen*, *Hypostomus luetkeni*, *Pseudotothyris obtusa*). In the tropics, day length and temperature are slightly uniform and most species opt to breed throughout the whole year, but in the Brazilian coastal rivers rain regime is recognized to be the most important factor influencing species that spawn their eggs at once (Menezes & Caramaschi, 1994; Amaral et al., 1998; Vitule et al., 2008a).

3.1 Reproductive period

Neotropical stream fishes usually present a relatively long reproductive period (e.g. *Harttia* sp., *Phalloceros* spp., *Poecilia vivipara*, *Phalloptychus januarius*, *Jenynsia lineata*, *Characidium pterostictum*, *Hypostomus luetkeni*, *Geophagus brasiliensis*, *Astyanax hastatus*) (Mazzoni & Caramaschi, 1997; Menezes et al., 1998; Aranha & Caramaschi, 1999; Mazzoni & Iglesias-Rios, 2002a; Becker et al., 2008), with some species breeding throughout the whole year (e.g. *Rhamdia quelen*, *Astyanax janeiroensis*) (Gomiero et al., 2007). This pattern is attributed to an adaptive response to unstable environments such as these neotropical stream that are subject to flash floods, a frequent natural hydrological disturbance. This way the continuous input of newborn would assure a higher rate of survival and therefore the maintenance of a viable population in a stochastic environment.

Conversely, there are some species that exhibit a very seasonal and restricted breeding period, usually reproducing during spring and summer (e.g. *Pimelodella pappenheimi*, *Bryconamericus microcephalus*, *Mimagoniates microlepis*, *Deuterodon langei*) (Amaral et al., 1998; Mazzoni & Silva, 2006; Braga et al., 2006, 2008; Vitule et al., 2008a). Flash floods occur mainly on early months of the year, so it seems that these species are adapted to these events and they spawn just before summer rains. Those studied populations present an important difference regarding a latitudinal gradient. With the exception of *Bryconamericus microcephalus* population studied by Mazzoni & Silva (2006) the other populations that presented a seasonal reproduction period are situated at a subtropical region. These habitats with more defined seasons and therefore more variation on photoperiod and temperature may be shaping this reproductive characteristic in different ways. Therefore a macroecological study is needed to unveil a potential pattern in these populations.

3.2 Sexual dimorphism

The most common form of sexual dimorphism among fish are body size and length-weight relationship. An increase in fecundity is an advantage provided by large females whereas larger males are more successful in territorial confrontations and thus increase reproductive fitness (e.g. *Mimagoniates* spp.). Among nest guarding species larger males

(e.g. *Harttia* sp.) is also an advantage as they would be more successful at protecting its offspring. As previously mentioned, high fecundity is an important feature among Atlantic Rain Forest stream fishes and so larger females seem to be the general pattern found on these habitats. Examples of some species that present this characteristic are *Rhamdia quelen*, *Characidium* spp. *Astyanax janeiroensis*, *Deuterodon langei* and *Bryconamericus microcephalus*. Secondary sexual characters are also found in Glandulocaudinae and Poeciliidae. Males of Glandulacoudinae (e.g. *Mimagoniates* spp., *Glandulocauda* spp.) display in their caudal fin, glandular tissues associated to modified scales which presumably pumps pheromone to attract females. Poeciliidae males are easily distinguished for their modified anal fin that is called gonopodium which is used to inseminate females of the species.

4. Ontogenetic migration

Another pattern found in Atlantic Rain Forest stream fishes is a longitudinal segregation. In some populations a greater amount of small individuals (in proportional terms) are found at lower portions of the stream while larger individuals (in proportional terms) concentrate at the higher portions (Fig. 4). It is hypothesized that during flash floods, eggs, larvae and small individuals along with rocks, branches and other aquatic fauna, get carried out to the lower portions of the stream. As they grow, they increase their swimming capability and become able to explore upstream habitats, where they reproduce and complete the cycle. This dynamic ensure the long term permanence of the population along the whole hydrographic basin. Opposed to a spawning migration as observed in larger characin species inhabiting large neotropical rivers, this ontogenetic migration is not restricted to the reproduction period at wet seasons and can be observed along the whole year. This pattern was observed for *Hypostomus punctatus*, *Astyanax janeiroensis*, *Deuterodon langei, Geophagus brasiliensis,* and *Mimagoniates microlepis* (Menezes & Caramaschi, 2000; Mazzoni & Lobón-Cerviá, 2000; Mazzoni et al., 2004; Braga et al., 2007; Vitule et al., 2008a). This is probably a pattern derived mainly by ecological factors with low phylogenetic interference.

Several hypotheses of patterns and main drivers of this segregation must be tested in different streams and with more species and direct field experiments (Fig. 5). For example, the prediction that due to the stream bed inclination, the average size of individuals within a population increases towards higher altitudes and is more accentuated as altitude variation increases. The average length is also expected to decrease as distance from stream spring increases due to water carrying of small individuals. Also flash flood intensity and/or frequency may be responsible for a decrease of the average length at upstream sites. As pointed out by Vitule et al. (2008a) for *Deuterodon langei*, reproductive intensity should decrease along the longitudinal gradient as an indirect effect of size segregation, but the young proportion increases at lower habitats as small individuals are carried out and larger ones (adults) persist or swim back to higher habitats. As this is a flash flood dependent dynamic as the water velocity increases, the size segregation becomes more evident. In a climate change scenario it is clear that as temperature rises, flash floods become more often and/or intense, possibly increasing the average speed of the water and compromising several species ability to settle at upstream sites. Apparently this is not far from happening as shown by recent events in the Atlantic Rain Forest (Fig. 6). These hypotheses reinforce the importance of preserving the entire portion of the stream as each part represents an important habitat for each stage of the life of stream fish.

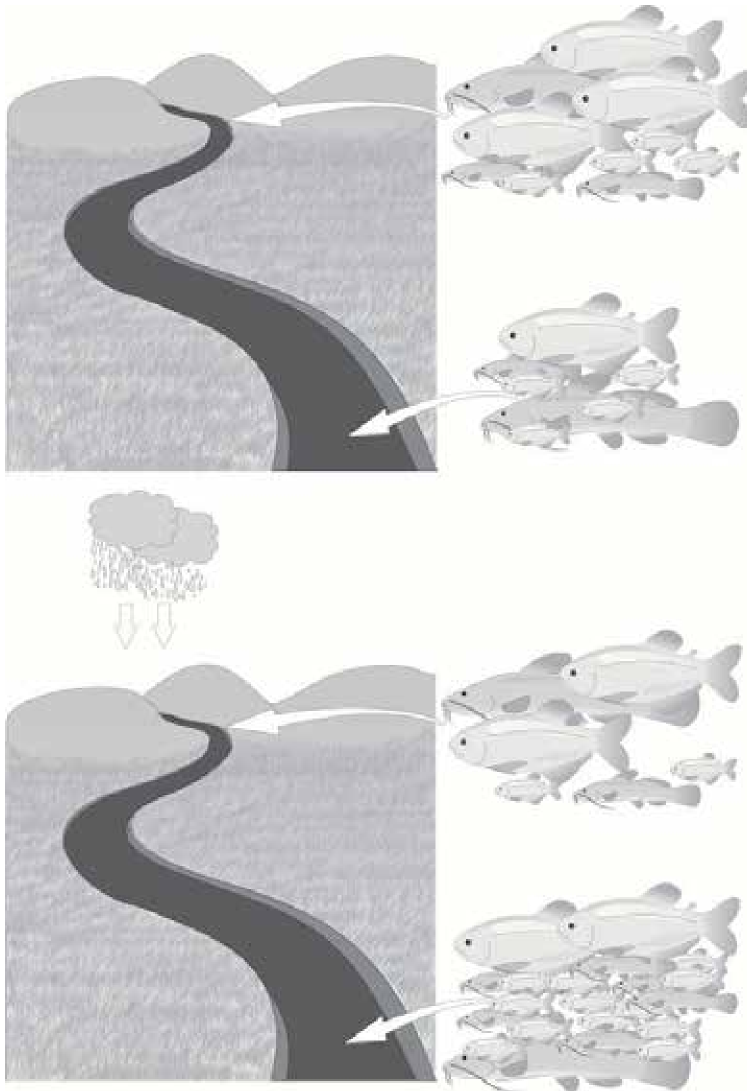

Fig. 4. A hypothetical scenario of ontogenetic migration. After a disturbance (flash flood), a greater amount of small individuals (in proportional terms) are found at lower portions of the stream while larger individuals (in proportional terms) concentrate at the higher portions (Illustration by Igor Kintopp).

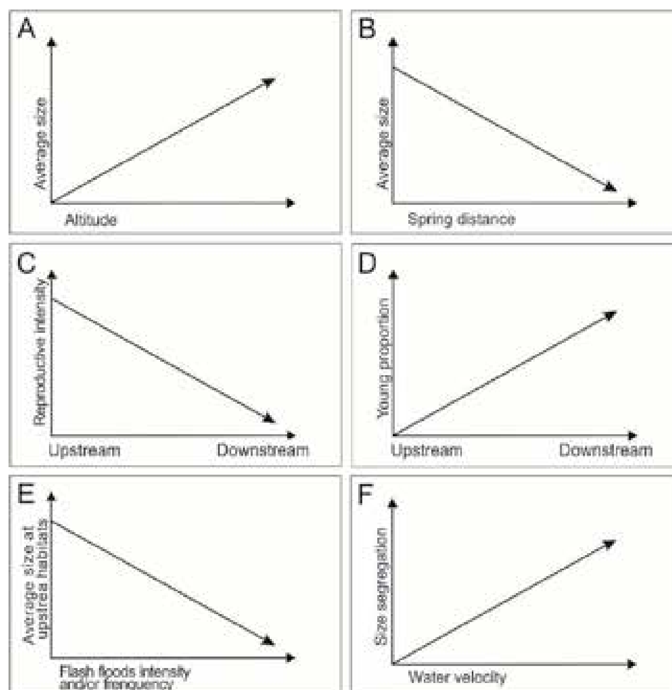

Fig. 5. Hypotheses of patterns and main drivers of the ontogenetic migration. A. Average size of individuals within a population increases towards higher altitude; B. Average size of individuals within a population decreases with spring distance; C. Population's reproductive intensity decreases along the longitudinal gradient from upstream to downstream; D. Proportion of young individuals increases along the longitudinal gradient from upstream to downstream; E. Average size of the individuals within a population decreases with higher flash flood intensity and/or frequency; F. Size segregation becomes more evident as water velocity increases (Illustration by Igor Kintopp).

Fig. 6. On March 11, 2011 the Paraná coast (Brazil) was affected by heavy rains, causing floods in the cities of Morretes, Antonina and rural region, blocking highways to Paranaguá city. This rain caused one of the most catastrophic destructions at the Jacarezinho and Sambaqui River on the last decades.

5. Feeding

Feeding ecology of Atlantic Rain Forest stream fishes has been studied by several authors (e.g. Buck & Sazima, 1995; Aranha et al., 1998, 2000; Esteves & Lobon-Cerviá, 2001; Vilella et al., 2002; Vitule & Aranha, 2002; Deus & Petrere, 2003; Fogaça et al., 2003; Mazzoni & Rezende, 2003; Rezende & Mazzoni, 2003, 2006; Barreto & Aranha, 2006; Abilhoa et al., 2007, 2010a,b; Botelho et al., 2007; Mazzoni & Costa, 2007; Gomiero & Braga, 2008; Vitule et al., 2008; Rolla et al., 2009; Mazzoni et al., 2010). Based on the analysis of 55 species' gut contents in the above studies, stream-dwelling fishes in Atlantic Rain Forest exhibit diverse feeding habits, and "omnivory" (i.e. predation in >1 trophic level, with an opportunistic use of resources) seems to be the predominant feeding strategy. Diet analysis also revealed a wide range of food items and allowed the recognition of five major trophic groups: omnivorous (47.2%), insectivorous (21.8%), herbivorous (20%), carnivorous non-insectivorous (5.4%), and detritivorous (5.4%). These general groups have been divided into more restricted trophic guilds (sometimes artificial groups) which makes data compelling and metanalysis more difficult and less accurate.

Trophic plasticity of fishes seems to favour the utilization of a large array of food resources. The carnivorous guild, for example, shows different capture tactics according to the microhabitat and prey behaviour. While some benthic invertebrate predators are non-selective (generalists), others are specialized and use sit-and-wait predation (*Characidium* spp.) (Casatti & Castro, 1998) and substrate speculation (heptapterids catfishes) as feeding strategies. Piscivorous fishes have their diets based on the pursuit (e.g. *Oligosarcus* spp.) or ambush (e.g. *Hoplias malabaricus*) of preys, although they may complement their diets with allochthonous items (mainly terrestrial insects). In spite of the fact that insectivorous fishes are a type of carnivores, they are commonly placed in their own category. In this case, most small-sized Atlantic Rain Forest characins are mainly insectivorous (Costa, 1987; Sabino & Castro, 1990; Aranha et al., 1998; Vitule et al., 2008b), feeding on autochthonous (aquatic immature and aquatic adult insects) and allochthonous food material. Several fish species feed primarily on terrestrial insects (Costa, 1987; Allan, 1995; Casatti & Castro, 1998; Aranha et al., 1998; Sabino & Zuanon, 1998; Abilhoa et al., 2009), collecting insects that fall into the stream from the riparian vegetation. The fusiform body shape, eyes dorsolaterally placed on the head, and sometimes the upward turned mouth (e.g. *Mimagoniates* spp., *Rachoviscus* spp., *Rivulus* spp.), indicate surface picking behaviour as a feeding strategy for small-jawed characins, by which the fish swims upstream and catches terrestrial items on the water surface (Sazima, 1986).

Besides insects, the input of terrestrially derived organic material (e.g. plants, seeds, fruits, and other invertebrates) is also considered important for fish feeding in Atlantic Rain Forest streams, where riparian forest blocks part of sunlight and the primary productivity is relatively low. As the importance of autochthonous production of organic matter in rivers is generally expected to increase downstream from the headwaters because of the widening of the stream channel, which allows more light to reach the water, small forested streams depend on the allochthonous resources coming from the riparian forest to maintain the predominantly heterotrophic biota (Vannote et al., 1980). Riparian input may be the primary energy source to consumers in low-order streams worldwide (Minshall et al., 1985).

The guild of herbivorous comprises usually armored catfishs. Loricariids have a dorsoventrally flattened body, a sucker-like mouth, and comb-like tooth plates, morphological specializations that enable them to scrape attached algae and diatoms from the substrate.

These bottom feeders can also suck up organic matter (detritus), a poor nutritionally source of food compound by large amounts of associated microorganisms (Gerking, 1994).
On the other hand, detritus can turn into an important resource during dry seasons once the availability of other food items decrease. For *Deuterodon langei* it was detected an association between abundance and occurrence of detritus and microscopic algae, thus suggesting that the greater the proportion of detritus the greater will be the proportion of microalgae (Vitule et al., 2008b). In this sense, the abundance of microscopic algae can be related to the presence of detritus in benthic food resource such as periphyton, which increase with gradual drying (Vitule et al., 2008b). The only detritivorous specialized fish in the Atlantic Rain Forest seems to be the curimatid *Cyphocharax santacatarinae*. All these species above mentioned possess a long, narrow and twisted (coiled in Loricariids) digestive tract, morphological adaptations of the digestive system reported for several herbivorous/detritivorous neotropical fishes (Delariva & Agostinho, 2001, Vitule et al., 2008b).
Additionally, most studies on the feeding ecology of fish have shown that the increase of feeding activity can be related with the rainy period, when prey availability is higher, and several species shift their diets according to the ontogenetic development (Vitule & Aranha, 2002; Abilhoa et al., 2007; Vitule et al., 2008b; Abilhoa et al., 2009), what seems to be related with morphological (e.g. buccal opening) an behavioral (e.g., locomotion capability) changes during development (Wooton, 1999).
In recent years, it has become apparent that some omnivorous stream fishes (e.g. *Bryconamericus* sp., *Phalloceros* sp.) that ingested great amount of allochthonous material, predominantly assimilated carbon derived from algae (Brito et al., 2006). The utilization of stable isotope analysis indicates that stomach content studies provide only instantaneous information on these species' diet, but does not account for long-term patterns of mass transfer (Woodward & Hildrew, 2002). Thus traditional dietary inference probably failed in giving a clear indication of the origin of sources of carbon and their flow through food webs (Peterson & Fry, 1987). Despite the strong tendency towards omnivory among fishes indicated by gut contents analysis, we think that many fundamental questions associated with the trophic ecology and the investigations of energy transfer in neotropical streams are necessary to be accessed in order to clarify this paradigm.
Stomach content analysis is often considered to provide a short-term information about ingested prey, and may be influenced by differences in digestion rates of prey resulting in under-representation or bias (Hyslop, 1980). Stable isotope analysis, on the other hand, is considered to provide long-term information about resource intakes (Phillips & Gregg, 2003). We therefore suggest that mixing models using stomach contents and stable isotopes can provide a robust and synthetic approach to understand the trophic ecology in Atlantic Rain Forest ecosystems in the near future. Several other methodologies such as non-lethal methods (regurgitation and direct observations) and fatty acid analysis exist but are rarely used. Alternative methodologies can be of great interest and use for these Neotropical stream fishes once non-lethal techniques can constitute a unique opportunity to study the feeding ecology of endangered species.

6. Threats, impacts and conservation

6.1 Threats and impacts

The Atlantic Forest (*lato sensu*) embraces a large number of species (~1–8% of the world's species) and a high number of endemic species (Myers et al., 2000). An up to date

assessment revealed a massive number of endemic species, such as 8000 tree species (40% of the total), 200 birds (16%), 71 mammals (27%), 94 reptiles (31%), and 286 amphibians (60%), not to mention least-known taxonomic groups (Metzger, 2009). The Atlantic Rain Forest has lost nearly 93% of its primary vegetation and is considered a hotspot for conservation. Habitat loss due to illegal deforestation and urbanization along with the introduction of non-native species and pollution are major threats to these areas.

Freshwater habitats represent round 0.8% of the Earth's surface and supports more than 6% of all described species. Its biodiversity comprise a precious natural resource in terms of economic, cultural, aesthetic, scientific and educational value (Dudgeon et al., 2006) both in global and regional scales. Unfortunately, freshwaters ecosystems are the most threatened ones (Abell, 2002) mostly due to the water demands and human negative impacts on ecological integrity.

Therefore, to understand the ecosystem dynamics and improve conservation in this important hotspot, basic ecological studies are fundamental, especially with the increasing advance of human activities and its negative impacts to the water, its fauna and humans themselves. In addition, many local anthropogenic problems have synergetic effects with global changes, but these associations are difficult to predict (Fig. 7). Proper management (e.g. forestry code enforcement, reducing non-native species introduction, landscape planning) will be decisive for conservation of the Atlantic Rain Forest basins and its fish fauna. We believe that we still have a huge gap in understanding stream fish biodiversity. Some important specific objectives for the near future are: (i) to exchange and disseminate scientific, technical, and practical information about endangered species and their conservation priorities; (ii) to recognize current and future practices in the conservation of the Atlantic Rain Forest fish fauna.

6.1.1 Information gaps and conservation efforts

Information gaps on biodiversity jeopardize stream fish conservation efforts, as there is little understanding about species distribution, abundance, dispersal processes, metapopulation structures, population viability, and many other basic biological data. We need also to comprehend specific adaptations and how populations, communities, and ecosystems are affected by anthropogenic alterations. We predict that survey intensification into least studied areas can reveal a wealth of diversity. This intensification is highly important as Abell (2002) argues that: "there is insufficient empirical evidence to convince some policymakers".

In general, there is an imperative apprehension on the possibility that the functioning of ecosystems might be threatened by biodiversity loss. Many eminent ecologists argue that, diversity tends to stabilize community dynamics and/or increase productivity (Odum, 1953; MacArthur, 1955; Elton, 1958). Obviously ecosystem functioning links are complex (Cameron, 2002). Therefore fish biodiversity and net productivity in streams of the Atlantic Rain Forest can have intricate links due to its richness and complexity and a change in only one of these variables can have an impact on one another. In spite of that there are no comprehensive studies about relationships between stream fish biodiversity and ecosystem services, we believe that it's highly important to improve studies using stream fish richness or other kinds or measures of diversity (e.g. species, genetic, community, functional, evenness, Shannon–Weaver, etc.) as a proxy of biodiversity and its importance or relationship for ecosystem services. In sum, if fish biodiversity has an influence on Atlantic Rain Forest ecosystem functioning, it could affect ecosystem services, goods and human welfare in a near future. As a result, improving basic and applied research about connection

between biodiversity and ecosystem functioning is of direct relevance to public policy and to the "real world".

Knowledge on endemic areas and species distribution or extinction rates and conservation is still scarce and inaccurate. The application of biogeographical tools, principles, theories and analyses is fundamental for an effective conservation of fishes inhabiting these ecosystems. Recently, some core challenges to conservation of freshwater fish were recognized (Olden et al., 2010), such as: (1) Testing new ecological theories for this specific fauna; (2) Quantifying with accuracy the extinction risk and loss of genetic, taxonomic (e.g. species) and functional biodiversity; (3) Evaluating the magnitude of extinction debt for freshwater fishes; (4) Elucidating the patterns and drivers of freshwater fish invasions; (5) Forecasting the future biogeography of the Atlantic Rain Forest fishes; (6) Understanding the interactive or synergetic effects of multiple environmental and ecological stressors; (7) Identifying and quantifying new features of the small scale biodiversity (8) Identifying and quantifying fish faunal homogenization and the emergence of novel assemblages; (9) Promoting and improving scientific rigour in conservation strategies and conservation planning strategies for freshwater fish species and (10) explain all this and spread to society in general.

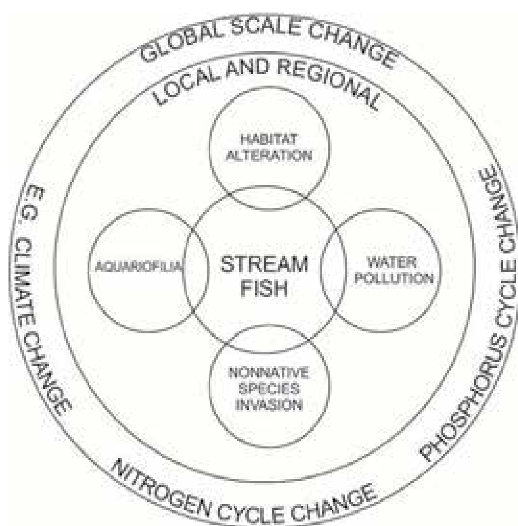

Fig. 7. Local and regional anthropogenic negative impacts and their potential interactive impacts to the Atlantic Rain Forest fish biodiversity. Environmental global changes such as climate change, nitrogen and phosphorous cycle changes can affect and amplify all these threats (Illustration by Igor Kintopp).

Despite the fact that biodiversity assessments provide data on global priority regions for freshwater conservation (e.g. Abell et al., 2008), accurate local scale priorities remain unknown around the World (Nogueira et al., 2010). Scale refining of biodiversity assessments and the translation of these into conservation priorities remains a major challenge to biodiversity science. In Atlantic Rain Forest streams biodiversity science depends directly on improvement of accurate basic data like species occurrence with high taxonomic and geographic resolution. Implementation of local scale conservation actions is also in a weak position by the reality that most conservation assessments tend to treat freshwater ecosystems as formerly separate hydrographic basins and/or smallest biogeographical units even within the same basin or sub-

basin. In sum, it's highly important to divide areas into units with similar environmental/geophysical conditions as well as similar fish assemblages, natural communities and ecosystem dynamics.

6.1.2 Climate change

There are many potential direct (more obvious) and indirect impacts of climate change or "global warming" on freshwater fish, especially into hotspots like the Atlantic Rain Forest coastal freshwater habitats. First of all the melting of polar ice caps and thermal expansion of seawater will result in a sea level rise that would inundate foremost important habitats in the Atlantic Forest, and/or cause modifications on disturbance regimes. Second, temperature is a major variable in all freshwater systems because of its widespread effects on the life history, physiology, behavior and ecology of most freshwater organisms. Moreover, fish have evolved with their current local micro climate and hydrological conditions like disturbance regime (e.g. frequency and intensity of flash floods). The homeostasis or optimal physical and biological range of each individual, species or population is determined by temperature as they are all hexothermic or "cold-blooded (Schmidt-Nielsen, 1990). In other words, all fish have their ideal temperatures that are necessary for optimal or efficient metabolism, reproductive success, and disease or parasites resistance (Schmidt-Nielsen, 1990).

Temperature increase is a major concern since environmental thermal conditions may encroach on suboptimal conditions for many local adapted stream fishes, or bring them closer to their incipient lethal temperatures. Moreover, climate change can affect fish populations through its influence on rain regimes, disturbance intensity and physical factors such as limnology and water chemistry (e.g. pH, oxygen solubility, hydrological regimes, and primary net productivity). Even small increases in temperature (~ 1 - 2°C) can be sufficient to have major effects on tropical fish physiology; reproduction in particular, especially when combined with an altered hydrologic regime and other anthropogenic stressors (e.g. invasive species). Facing climate changes, tropical fish populations can achieve new equilibrium dictated largely by the new energetic cost, so some species may increase or decrease in abundance, others may experience new interactions, range expansions or contractions, and many species may face extinction. In this sense, climate changes are new challenges to survival and reproductive success by influence of many ecological parameters and human stressors. We argue that conservation biologists have not yet developed a sufficient understanding of the precise impacts of the climate changes for all tropical fish species, especially for the Atlantic Rain Forest streams, but certainly they exist and are imperative.

Unfortunately, accurate risk assessment is impeded by contingency: the impacts of climate changes on tropical fish species vary over time and space under the influence of local environmental variables, interspecific interactions and evolutionary changes. But certainly, some potential impacts of climate change, such as species extinctions, are large and irrevocable. Finally, removing other stressors from natural systems is a necessary and important proactive management strategy for tropical fish conservation.

6.1.3 Habitat destruction and alteration

The increase of human activities, if poorly managed, can generate significant negative impacts on water bodies and wildlife. The destruction of habitat as a result of expansion of urban areas near water bodies may cause changes in species composition, favoring species better adapted to degraded environments, or even completely extinguish some species, most

notably the rare and endangered ones. This occurs due to direct and/or indirect effects, such as channel rectification, substrate dredging, illegal occupation of riparian forest and adjacent areas, and construction of landfills and illegal dumps. All these processes can cause dramatic ecological changes, for instance, a significant decrease on energy exchange between riparian and aquatic environments. Changes in water flow dynamics can lead to profound alteration on aquatic fauna, reducing productivity, variability and environmental heterogeneity. For example, the high input of organic matter may lead to eutrophication or to its demise and reduced physical complexity (Allan, 1995).

In many rivers, certainly in small ones, much of the energy present in the food chain comes from terrestrial habitats. Therefore changes in terrestrial habitats may act as an important source of impacts to the aquatic environment (Allan, 1995), and to the land itself, due to their interdependence. On the other hand, the process of dredging, construction and formation of artificial channels produce different changes in the environment, not only in water but also in the adjacent terrestrial environment. There are countless negative effects to the natural ecosystem, for example, flooding of forested areas, changes on physical and chemical properties adjacent to the aquatic environment and changes in structure and composition of communities in small streams. Furthermore, the modification of natural hydrologic regimes and biological invasions are two synergistic and major threats to freshwater biota (Johnson et al., 2008).

In streams, as in large rivers, impoundments negatively affect flooding patterns, flow regime, sediment transport, trophic structure and species composition. Dams and associated impoundments also have major influence on the β and γ diversity along small basin network. Dam effects can reduce hydrologic connectivity between neighboring aquatic habitats by preventing fish migration up or downstream and affecting recruitment. On the other hand, impoundments can allow dispersal of undesirable fish into systems outside of their natural range, facilitating invasion and contributing to biotic homogenization process through habitat homogenization, where local riverine biota is replaced by cosmopolitan lentic species (Rahel, 2002). In the Neotropical Region, the increase on dam numbers has altered hydrology and is affecting the freshwater fish fauna, which was previously subject to geographic constraints by a multiplicity of physiographic barriers. Besides the fact that predicting long-term cumulative environmental impacts on aquatic ecosystems is a challenging mission, the potential problem of eliminating natural obstacles in streams and its consequence has not received attention during environmental impact studies of hydropower facilities.

6.1.4 Water pollution

The most common source of pollution includes agriculture, industries and discharge of untreated sewage. The direct or indirect consequences of domestic dump and industrial effluents, often highly toxic can cause profound changes in the diversity of fish, favoring species with greater adaptability, or completely extinguishing all species of fish. The enrichment of water with nutrients (especially phosphorus and potassium) can cause eutrophication. In this process, the richness and diversity are profoundly diminished, leaving only species highly resistant to low oxygen concentration (Allan, 1995).

6.1.5 Introduction of non-native species and invasions

Introduced fishes have a long history of globally catastrophic cases, including examples in the Atlantic Rain Forest (Vitule, 2009), being a precursor of a prominent global biotic homogenization. A species of fish can be considered non-native, even at the level of basins or

sub-basins. This makes the perception and/or detection of introduced fish even more complex in nations like Brazil, due to its continental dimensions and rich continental aquatic environments. In this sense, fish introductions become serious threats as these organisms are very widespread, mobile and of difficult perception and/or detection. It is a common fact that introductions of this group of organisms are perceived only when they are already in advanced stages of the invasion process and the damage is irreversible. Some non-native fish to the Atlantic Rain Forest streams may be considered "invisible" (e.g. less exposed than the majority of the introduced terrestrial organisms). Often, cultural aspects and time of release contribute to the "invisibility" of the problem. Some fish species that were translocated from other continents (e.g. carp, tilapia, and trout) a long time ago are considered "native" and/or even important for some small human groups that don't understand its potential or real impacts (Vitule, 2009; Vitule et al., 2009).

Despite the recent increase in publicity about the subject and its problems worldwide, the numbers of non-native species introductions may have a tendency to exponential increases in aquatic environments of the Neotropical Region. In the case of introduction of non-native species with huge invasive potential, lack of action can result in serious and irreversible problems. Introduced species may be restricted and avoided, and if they invade a new environment where they are unwanted and/or may cause harm, it should be eradicated. If not possible, they should be studied in long term, monitored and maintained at acceptable low population's levels. In other developed countries, committed a long time ago to this issue, there are examples of success in all types of actions mentioned above, although, even in developed and rich countries, there are few investments, studies and efforts. If there is a lack of serious interest and investment worldwide, the scenario is much worst in developing countries such as Brazil. For example, even in the scientific field the theme of aquatic invasions into the Atlantic Rain Forest streams is very under-explored in Brazil. However, information about troubles that may arise from biological invasions is building up (Vitule, 2009). Given the magnitude of the problem, and disproportion in the search for answers and impacts, a review of the theme with suggestions for actions is presented in Vitule (2009).

A clear and highly problematic example of introduction is the release of the trout *Oncorhynchus mykiss* in southern Brazil. Inexplicably, this is a project of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), with support from United Nations Program for Development in Brazil (Vitule, 2009). It therefore seems rather serious, rather serious, since *O. mykiss is officialy* listed in the Global Invasive Species Database as one of the 100 worst "alien species" and it is considered a potential pest (Froese & Pauly, 2011). In previous example, we see again a complete distortion in the costs/benefits relationship and a total disregard by the authorities with respect to natural biodiversity. There are analogue cases that have been occurring in several streams and states of Brazil. Deliberated and illegal releases are very easy to identify. With a simple internet search, the release of great amounts of fish can be documented with photos of ecovandalism events. Easily any one can find sites that sell non native fish species (fingerlings or adults). Such virtual mechanisms can be easily tracked, hunted and restricted the same way as done with child pornography or other illegal sites.

6.1.6 Aquarium trade and illegal commerce

Although not officially recorded, it is a fact that all small beautiful species treated here appear in books and/or international aquarium sites. It is strange that these species

sometimes listed in the local Red List are used as aquarium animals at other countries and/or continents. In this sense, its use is inappropriate, because these species may have been caught through illegal collecting, characterizing animal trafficking and/or biopiracy. These are impacts that must be considered and investigated thoroughly.

6.2 Conservation

Recently, foremost multi-taxa projects have amplified the ecological understanding of major causes and consequences of Atlantic forest alterations (Metzger, 2009). Unfortunately, this is not a homogenous reality for all sub-areas or regions and/or kind of habitats and organism. In our view there are discrepant disproportions in the ecological and conservation research between terrestrial and aquatic ecosystems. Much of the knowledge on Atlantic Rain Forest stream fish conservation is dispersed and fragmented and the need to assemble the information is crucial. We believe that enhancement about specific studies on stream fish ecology and conservation biogeography can provide clear recommendations for biological conservation in a near future. Another top high priority should be perhaps to take on its stream physical and biological restoration, but a successful stream restoration, will depend on our knowledge about many of the basic ecological processes embracing fish fauna remains unknown. We hope that in the future the general knowledge obtained in Atlantic Rain Forest can help conservation efforts in other tropical areas.

6.2.1 Case study

A case study of some threatened species of the coastal plain in the state of Paraná, southern Brazil: an example of what happens to Atlantic Rain Forest stream fish.

Despite the scarcity of information, the national (Machado et al., 2008) and Paraná (Abilhoa & Duboc, 2004) lists of threatened fishes present endemic species of Atlantic Rain Forest coastal plain streams of Paraná: *Mimagoniates lateralis, Spintherobolus ankoseion, Rachoviscus crassiceps,* and *Scleromystax macropterus.* Due to the observation of populations decline, reduced distribution areas and/or strong anthropogenic pressures, these species were grouped into categories of threat according to IUCN, but unfortunately none of which actually appears on the official list of the IUCN. These species are all exclusive of low altitude water bodies extending from Paraná to north of Santa Catarina. Available information, although sparse, show that these populations occur in areas near large urban centers, and are therefore under strong pressure. Despite its importance and high degree of endemism, there are no official records of individuals of these species in captivity for conservational finalities. However, as stated earlier, all species mentioned have appeared in books and worldwide aquarium web sites. For those reasons if we wish to improve the success of conservation projects, it is extremely important to gather basic information concerning species ecology, including habitat preferences and environmental tolerances. In the near future, we hope that modern molecular techniques will discriminate evolutionary diversity within species, aiding conservation efforts.

7. Conclusions and perspectives

In the Atlantic Rain Forest, natural physical isolation and dispersal limitation that contributed to the high freshwater diversity can also enhance extinction rates along with anthropogenic changes. At last, this chapter does not want to exhaust this subject; we expect that our

compilation and ideas will offer a useful guideline for identifying challenges, key research questions and new paths of investigation into the Atlantic Rain Forest fish domain. The balance between the practice of backward reflection and forward-looking is critical to advance the knowledge about freshwater fishes in this important region to global biodiversity.

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Diversification of Circum-Mediterranean Barbels

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1. Introduction

The Mediterranean Basin is one of the 25 most biodiverse regions on Earth. It is considered a biodiversity hotspot for its high numbers of endemic vascular plants, birds, mammals, reptiles, and amphibians, sometimes restricted to small distribution areas (Médail & Quézel, 1999; Mittermeier et al., 1998; Myers et al., 2000). The Mediterranean has had a long and complex geomorphologic history, being a relic of the Mesozoic Tethys Ocean. The Tethys had disappeared by the end of the Eocene (34 Ma) due to the collision of the Indian and Asian plates (Rögl, 1999). The orogenic movements raised new mountain ranges in the Taurides, the Hellenides, the Dinarides and finally the Alps by the Middle/early Late Miocene (Hsü et al., 1977). This orogeny separated the borning Mediterranean and a central/eastern European inland sea – the Paratethys Sea (Hsü et al., 1977; Rögl, 1999). Landbridge connections and seaway passages between the Mediterranean and Paratethys, and between them and the Indian and Atlantic oceans, were then intermittent throughout the Miocene until the final opening of the Strait of Gibraltar ending the Messinian Salinity Crisis (Agustí et al., 2006; Hsü et al., 1977; Krijgsman et al., 1999; Rögl, 1999). This complex geomorphological scenario has allowed multiple faunal and floral exchanges between neighboring regions (e.g. Agustí et al., 2006; Benammi et al., 1996; Pickford et al., 1993, 1995); this melting pot might have contributed to the extraordinary diversity observed nowadays. For instance, the Middle East has been an important region for freshwater fish interchange between Africa, Asia and Europe (Durand et al., 2002). Another relevant aspect is whether persistence (i.e. low extinction), diversification (i.e. high speciation), or both, are responsible for high species diversity in the Mediterranean (e.g. Reyjol et al., 2007).

Among vertebrate groups, primary freshwater fishes probably constitute the majority of living endemisms in the Mediterranean region and include several species with restricted distribution ranges. Certain regions in the northern Mediterranean have been identified as important biodiversity hotspots for riverine fish (Reyjol et al., 2007) and the same is likely true for southern Mediterranean ones. This is explained by the limited dispersal routes of freshwater-restricted species, living within the confines imposed by salt water on one hand, and land on the other. Such qualities make primary freshwater fishes ideal models for the study of biogeographical history, landscape evolution and processes driving diversification in general (Briggs, 1995). Cyprinid fishes are a prime example. They are the most diverse family within the order Cypriniformes and naturally inhabit freshwaters of all continents except for Antarctica, Australia, and South America (Banarescu & Coad,

1991). Therefore, they can offer invaluable insights on the historical biogeography of the Mediterranean Basin.
Two major biogeographical scenarios have been proposed to explain current distribution of Mediterranean cyprinids. The classical northern river dispersal hypothesis states that primary freshwater fishes reached Europe from Asia when the Turgai Sea dried out, and then continuously dispersed southwards from Europe to Africa by river rearrangements, throughout the Miocene and Pliocene (Almaça, 1976, 1988; Banarescu, 1960, 1992). On the other hand, the southern sea hypothesis proposes that cyprinids colonized different regions across the Mediterranean. Some proponents of the latter favor a dispersalist scenario during the Messinian Salinity Crisis (Bianco, 1990), others a vicariant one from the Middle East, to Africa, to Iberia through intercontinental land bridges during the formation of the current North African coast in the early Pliocene (Doadrio, 1990), or a combination of both dispersal and vicariance (Zardoya & Doadrio, 1999).
In the present work, the timing and pattern of diversification of circum-Mediterranean barbels is re-examined using molecular phylogenies and the latest fossil data available to shed light on historical biogeography of the region and how it shaped the evolution of freshwater fishes.

2. Barbels as a model system in Mediterranean biogeography studies

After decades during which the genus *Barbus* was used for many different barbines, from Asia, Africa or Europe alike, *Barbus s. str.* was restricted to 'true' barbels. 'True' barbels are a group of tetraploid fishes distributed throughout freshwaters of Europe, southwestern Asia and northern Africa (Berrebi et al., 1996; Collares-Pereira & Madeira, 1990; Howes, 1987; Ráb & Collares-Pereira, 1995). They are composed of two lineages based on morphology (Doadrio, 1990) and molecular evidence (e.g. Gante, 2009; Machordom & Doadrio, 2001a). These lineages, *Barbus* and *Luciobarbus*, are now considered separate genera by several authors, nomenclature that is followed here. Based on mitochondrial phylogenies, Tsigenopoulos & Berrebi (2000) showed that the monotypic tetraploid genus *Aulopyge* is sister to *Barbus* and *Luciobarbus*. Soon after, Durand et al. (2002) and Tsigenopoulos et al. (2003) found that the hexaploid genus *Capoeta* nests within *Luciobarbus*. Thus, the delimitation of 'barbel' is loosened to include the Balkanic *Aulopyge huegelii* and the Middle Eastern *Capoeta* in the present analysis.
Barbels are medium- to large-sized bottom dweller fishes, adapted to a variety of habitats from standing water lakes to fast-flowing montane rivers. Taken together, these four genera have a very wide geographical range, from the Black Sea, Caucasus and Middle East to the Iberian Peninsula in the West, from northwestern Africa in the South to Russia in the North. In the Mediterranean region they are only not found in Libya and Egypt. Interestingly, the four genera are mostly allopatric except for the Caucasus, Middle East, and small areas in Anatolia, Greece, and Iberia. In particular, *Luciobarbus* is found in the Caucasus, Middle East, Anatolia, Greece, northwestern Africa and Iberia. This truly circum-Mediterranean distribution is ideal for the study of historical biogeography of the region, since it allows tracing time and direction of colonization events between different regions. Also, barbels as a whole are a diverse group with dozens of species with distributions usually restricted to specific basins. This offers the opportunity for repeated sampling from each region. Finally, a sizeable collection of mitochondrial sequences of barbels and allied genera has been deposited in public repositories by many authors over the years. They are an invaluable resource.

Fig. 1. Sampling sites of *Barbus*, *Luciobarbus*, *Capoeta* and *Aulopyge huegelii* analyzed in the present study. Numbers refer to Table 1. Color codes refer to geographical range of the species irrespective of generic placement: Northern Africa, Anatolia, Middle East and Caucasus, Greece, central and eastern Europe, and Balkans, Italy, and Iberia.

3. Improved fossil data and calibration of barbel phylogenies

Some previous studies of historical Mediterranean biogeography – of parts or the whole area – have made use of calibrated molecular phylogenies of barbels. Most of the studies relied on molecular clocks calibrated with known geological events, such as the openings of the Strait of Gibraltar and/or the Strait of Korinthos (Machordom & Doadrio, 2001b; Mesquita et al., 2007; Tsigenopoulos et al., 2003, 2010; Zardoya & Doadrio, 1999). The results from the different studies varied slightly, depending on which particular node was calibrated, but most importantly they might have inadvertently biased results for accepted vicariant events or those perceived as more likely.

Other studies have calibrated molecular phylogenies using published rates for other organisms (Durand et al., 2002; Tsigenopoulos & Berrebi, 2000; Tsigenopoulos et al. 2010). The use of (a range of) possible mutation rates is commonly accepted, in particular if fossils are not available for specific groups. Nevertheless, mass-specific metabolic rate and temperature influence the rate of molecular evolution in poikilotherm fishes (Estabrook et al., 2007). Using a ‘universal’ rate might have as a consequence over- or underestimation of the real rate of molecular evolution of the particular study organism. Interestingly, of the abovementioned studies that calibrated phylogenies either using known geological events or published rates, perhaps all but one underestimated the age of *Barbus* and *Luciobarbus* according to current fossil data.

One last study calibrated a molecular phylogeny with fossil data (Gante et al., 2009), but it was restricted to a very small area of the Mediterranean Basin. Therefore, given the current scenario just described, there is the need for a new analysis using updated fossil information, across the entire Mediterranean Basin, to re-evaluate the timing and pattern of diversification of circum-Mediterranean barbels.

Latest fossil data available in Böhme & Ilg (2003) were used to calibrate a molecular phylogeny. Divergence times and their credibility intervals (highest posterior density: HPD)

were estimated using a bayesian MCMC approach implemented in BEAST v1.6.1 (Drummond & Rambaut, 2007). *Barbus* fossils of Burdigalian age are now known from several localities in what is presently Central Europe and Anatolia (oldest: 19.0 Ma). This wide distribution area suggests that the genus had already diversified by the Early Miocene. Likewise, *Luciobarbus* fossils of Burdigalian age are known from Anatolia (oldest: 17.7 Ma). These dates set hard lower bounds for the diversification of each group. Additionally, *Luciobarbus* fossils of Messinian age are known from the Iberian Peninsula (oldest: 5.8 Ma), which represents a lower bound for the diversification of Iberian *Luciobarbus* as in Gante et al. (2009). The upper age is a soft bound free to vary following a lognormal distribution (Ho, 2007) set in real space with average of 1.0 and standard deviation of 0.5. Each gene used (see below) was a distinct data partition, with unlinked substitution models, and following relaxed uncorrelated lognormal clock models and a General Time Reversible model of evolution. Third codon positions were treated separately from 1st and 2nd codon positions. A speciation birth-death tree prior was used, since a Yule speciation prior assumes complete taxon sampling. Analysis was run for 25,000,000 generations, sampled every 2,500 generations, first 1,001 trees discarded as burn-in.
A total of 80 taxa were analyzed for the mitochondrial regions cytochrome *b* (1,141 bp) and ATPsynthases 6/8 (842 bp). As target ingroup, for the reasons explained above, representatives of *Barbus* (n=16), *Luciobarbus* (n=29), *Capoeta* (n=3), and *Aulopyge huegelii* from throughout the distribution area of the group were included. Additional cyprinins (n=31) originating in Asia and Africa were included in the analysis to provide a geographic, as well as phylogenetic context. Since the birth-death tree prior used assumes balanced sampling, outgroup species with varying divergence levels were selected (Fig. 1; Table 1).

Species	**Fig. 1**	**River**	**Locality/Region**	**ATP synthase6/8**	**Cytochrome *b***	**Source**
Aulopyge huegelii	34	Busko Lake	Bosnia-Herzegovina	AF287359	AF287415	a
Barbus balcanicus	37	Judrio	Gorizia (Italy)	AF287368	AF287424	b
Barbus cf. *balcanicus*	31	Aliakmon	Kaloneri (Greece)	AF287392	AF287439	a
Barbus barbus	36	Danube	Lutzmannsburg (Austria)	AB238965	AB238965	c
Barbus carpathicus	35	Dîmbovitza	Dragomiresti (Romania)	AF287397	AF287441	a
Barbus cyclolepis	27	Erithropotamus	Mikro Derio (Greece)	AF287372	AF090782	a,d
Barbus euboicus	28	Maniklotiko	Oxilothos (Greece)	AF287378	AF090785	a,d
Barbus haasi	40	Esca	Isaba (Spain)	AY004687	AF045976	b,e
Barbus macedonicus	26	Axios	Axiopolis (Greece)	AY004720	AY004753	b
Barbus meridionalis	39	Tordera	Barcelona (Spain)	AF287386	AF045977	a,e
Barbus peloponnesius	30	Thiamis	Parapotamus (Greece)	AF287390	AF287438	a
Barbus plebejus	38	Roggia	Udine (Italy)	AY004717	AY004750	a
Barbus prespensis	32	Prespa Lake	Psarades (Greece)	AF287400	AF090790	a,d
Barbus rebeli	33	Aoos	Komitsa (Greece)	AF287401	AF090791	a,d
Barbus sperchiensis	28	Sperchios	Lamia (Greece)	AF287374	AF090783	a,d
Barbus strumicae	26	Agiaki	Kastanies (Greece)	AF287375	AF090784	a,d
Barbus thessalus	25	Pinios	Omolio (Greece)	AF287365	AF090781	a,d
Capoeta angorae	22	Seyhan	Turkey	–	AF145950	f
Capoeta capoeta	19	Sevan Lake	Armenia	–	AF145951	g

Table 1. (Continued)

Species	**Fig. 1**	**River**	**Locality/Region**	**ATP synthase6/8**	**Cytochrome *b***	**Source**
Capoeta trutta	17	Tigris	Turkey	–	AF145949	f
Luciobarbus albanicus	24	Trichonis	Panetolio (Greece)	AY004690	AY004723	b
Luciobarbus antinorii	13	Bichri	Fatnassa (Tunisia)	AY004692	AY004725	b
Luciobarbus biscarensis	11	El Abiod	Arris (Algeria)	AY004693	AY004726	b
Luciobarbus biscarensis amguidensis	14	Imirhou	El Tassili, Iherir (Algeria)	AY004691	AY004724	b
Luciobarbus bocagei	42	Duratón	Carrascal del Río (Spain)	AY004695	AY004728	b
Luciobarbus brachycephalus	18	Terek	Kislar (Russia)	AY004696	AY004729	b
Luciobarbus callensis	12	Kebir	Ain Assel (Algeria)	AY004680	AF045974	b,d
Luciobarbus capito	18	Terek	Kislar (Russia)	AY004681	AF045975	b,d
Luciobarbus comizo	43	Tajo	Colmenar de Oreja (Spain)	AY004702	AY004735	b
Luciobarbus esocinus	16	Tigris	Diyarbakir (Turkey)	–	AF145934	f
Luciobarbus graecus	23	Kifisos	Orhomenos (Greece)	AY004684	AF090786	b,d
Luciobarbus graellsii	41	Gállego	Ipiés (Spain)	AY004683	AF045973	b,d
Luciobarbus guiraonis	44	Buyent	Pego (Spain)	AY004685	AF045972	b,e
Luciobarbus ksibi	3	Kasab	Essaouira (Morocco)	AY004705	AY004738	b
Luciobarbus labiosa	6	Ifrane	Azrou (Morocco)	AY004700	AY044733	b
Luciobarbus lepineyi	1	Noun	Iguissel (Morocco)	AY004706	AY004739	b
Luciobarbus longiceps	15	Tiberias Lake	Israel	–	AF145942	f
Luciobarbus magniatlantis	4	Bounual	Bounual (Morocco)	AY004714	AY004747	b
Luciobarbus massaensis	2	Assaka	Assaka (Morocco)	AY004707	AY004740	b
Luciobarbus microcephalus	45	Estena	Navas de Estena (Spain)	AY004686	AF045971	b,e
Luciobarbus moulouyensis	7	Moulouya	Boumia (Morocco)	AY004709	AY004742	b
Luciobarbus mursa	20	Arax	Armenia	–	AF145943	g
Luciobarbus nasus	5	Oum Er Rbia	El Borj (Morocco)	AY004711	AY004744	b
Luciobarbus pallaryi	8	Guir	Boudenib (Morocco)	AY004712	AY004745	b
Luciobarbus mystaceus	21	Keban Dam Lake (Euphrates river)	Elazig (Turkey)	–	AF145938	f
Luciobarbus sclateri	46	Alhama	Granada (Spain)	AY004688	AF045970	b,e
Luciobarbus setivimensis	10	Aissi	Azouz (Algeria)	AF317412	AY015991	b
Luciobarbus sp.4	9	Tifrit	Balloul (Algeria)	AY004710	AY004743	b
Luciobarbus subquincunciatus	21	Euphrates	Elazig (Turkey)	–	AF145937	f
Barbonymus gonionotus	–	Moon	Ubon (Thailand)	AB238966	AB238966	c
‚Barbus' anoplus	–	Incomati	Ngodwana (South Africa)	AF287361	AF287417	a
‚Barbus' bigornei	–	Kaba	Kouloundela (Guinea Conakry)	AY004719	AY004752	b
‚Barbus' bynni bynni	–	Nile	Egypt	AF287366	AF287420	a
‚Barbus' fritschii	–	Zamrine	Khouribga (Morocco)	AF287380	AF287429	a

Table 1. (Continued)

Species	Fig. 1	River	Locality/Region	ATP synthase6/8	Cytochrome *b*	Source
‚Barbus' gurneyi	–	Mgeni	Kwazulu-Natal (South Africa)	AF287383	AF287432	a
‚Barbus' motebensis	–	Marico	North Western Province (South Africa)	AF287387	AF287435	a
‚Barbus' paludinosus	–	Mooi	North Western Province (South Africa)	AF287388	AF287436	a
‚Barbus' serra	–	Upper Olifants	Western Cape (South Africa)	AF287460	AF287446	a
Carasobarbus canis	–	David	Bet Shean (Israel)	AF288484	AF288486	a
Carassius auratus	–	–	Asia	EF483931	EF483931	h
Cyprinion kais	–	Tigris	Diyarbakir (Turkey)	–	AF180860	g
Cyprinion macrostomus	–	Tigris	Diyarbakir (Turkey)	–	AF180826	g
Cyprinus carpio	–	Lake Biwa	Japan	AP009047	AP009047	i
Gymnocypris przewalskii	–	Qinghai Lake	China	AB239595	AB239595	c
Labeo bata	–	–	India	AP011198	AP011198	j
Labeo batesii	–	Loa Loa	Gabon	AB238967	AB238967	c
Labeo senegalensis	–	–	Africa	AB238968	AB238968	c
Neolissochilus hexagonolepis	–	Trishuli	Central Region, Nepal	EF588118	EF588174	k
Procypris rabaudi	–	Yangtze	Mudong (China)	EU082030	EU082030	l
Pseudobarbus afer	–	Blindekloof	Eastern Cape (South Africa)	AF287405	AF287449	a
Pseudobarbus asper	–	Vlei	Western Cape (South Africa)	AF287407	AF287451	a
Pseudobarbus phlegeton	–	Noordhoeks	Western Cape (South Africa)	AF287408	AF287452	a
Puntius conchonius	–	Aquarium	Asia	AY004718	AY004751	b
Puntius ticto	–	Mae Kok	Chang Rai (Thailand)	AB238969	AB238969	c
Puntius titteya	–	Aquarium	Asia	AF287411	AF287455	a
Schizothorax zarudnyi	–	Sistan	Southeastern Iran	EF588136	EF588192	k
Sinocyclocheilus altishoulderus	–	–	China	FJ984568	FJ984568	m
Sinocyclocheilus grahami	–	–	China	GQ148557	GQ148557	m
Tor tambroides	–	Phetchaburi Province	Thailand	EF588111	EF588167	k
Varicorhinus maroccanus	–	Oum Er Rbia	El Borj (Morocco)	AF287413	AF287457	a

Sources are: a: Machordom & Doadrio (2001b), b: Machordom & Doadrio (2001a), c: Saitoh et al. (2006), d: Zardoya & Doadrio (1999), e: Zardoya & Doadrio (1998), f: Tsigenopoulos et al. (2003), g: Durand et al. (2002), h: Lee (unpublished), i: Mabuchi et al. (2006), j: Saitoh et al. (2011), k: Nguyen et al. (2008), l: Zhang et al. (2009), m: Wu et al. (2010).

Table 1. List of species included in the present study. Geographical origin is indicated when known and shown in Fig. 1 for species of *Barbus*, *Luciobarbus*, *Capoeta* and *Aulopyge*. Color codes refer to geographical range of the species irrespective of generic placement: Northern Africa, Anatolia, Middle East and Caucasus, Greece, central and eastern Europe, and Balkans, Italy, Iberia, Asia, and central and southern Africa. Accession numbers of the two mitochondrial genes analyzed are shown.

4. Phylogeny of barbels

4.1 Relationships among genera and major groups

The phylogenetic relationships of circum-Mediterranean barbels have been thoroughly explored over the last decades. The wide phylogeny of barbels obtained here based on mitochondrial cytochrome *b* and ATPsynthase 6/8 genes, which includes several additional allied Asian and African cyprinines, is shown on Fig. 2. It is consistent with phylogenies obtained in previous studies based on partially overlapping sets of taxa (Durand et al., 2002; Gante et al., 2009; Machordom & Doadrio, 2001a, 2001b; Mesquita et al., 2007; Tsigenopoulos & Berrebi, 2000; Tsigenopoulos et al., 2002, 2003, 2010; Zardoya & Doadrio, 1998, 1999).

Circum-Mediterranean *Barbus s. str.* forms a strongly supported monophyletic group composed of two barbel lineages (Machordom & Doadrio, 2001a). These mitochondrial lineages, *Barbus* and *Luciobarbus*, now considered distinct genera, are in agreement with previous morphological evidence (Doadrio, 1990), and recent nuclear data (Gante, 2009). *Barbus* and *Luciobarbus* are sister to *Aulopyge* as initially suggested by Howes (1987) and Tsigenopoulos & Berrebi (2000). Altogether, they are likely sister to a group constituted by Middle Eastern *Cyprinion*, and Asian genera such as *Schizothorax* (Durand et al., 2002) and *Gymnocypris* (Fig. 2). This relationship is not well supported by available molecular evidence and would benefit form added sampling effort, both in terms of taxa and markers.

Mitochondrial phylogenies strongly indicate that 'true' barbels are not closely related to other African and Asian barbels. Rather, African diploids are a distinct paraphyletic group, with African tetraploids nested within them, suggesting a tetraploidization event from African diploids, and independent from the one that originated circum-Mediterranean barbels (Fig. 2; Machordom & Doadrio, 2001a; Tsigenopoulos et al., 2002). Likewise, hexaploid cyprinins found in Africa and the Middle East constitute an independent evolutionary lineage whose origin is still not well understood (Fig. 2). These have recently been lumped into *Labeobarbus* (Tsigenopoulos et al., 2010). Together with *Labeo*, they indicate multiple independent colonization events of Africa from Asian ancestors, possibly at different times.

4.2 Phylogenetic diversity of circum-Mediterranean barbels

Even without exhaustive sampling, the genera *Aulopyge*, *Barbus* and *Luciobarbus* show strikingly different degrees of diversity. *Aulopyge* is a species-poor genus composed of only one very specialized extant species, *A. huegelii*, compared to the species-rich *Barbus* and *Luciobarbus*. *Aulopyge heugelii* is an (almost) scale-less species with an elongated urogenital opening which functions as an ovipositor (Kottelat & Freyhof, 2007). It inhabits the Dinaric karst in the Balkanic region. Perhaps the lack of diversity within the genus can be explained by its biology and the degree of specialization attained and/or by its habitat, being imprisoned within the karstic labyrinth and not able to colonize other regions.

The genus *Barbus* is composed of at least four mitochondrial lineages (Fig. 2). It is restricted to the north Mediterranean region and is particularly diverse in Greece where species belonging to three out of the four identified lineages are found. Low diversity of *Barbus* in central and eastern Europe is likely related to the last Ice Ages, when glacier formation drove local populations/species to extinction, followed by rapid re-colonization by a restricted pool of founders (Kotlík & Berrebi, 2001).

The genus *Luciobarbus* is the most diverse and widespread of all. It is composed of at least seven mitochondrial lineages showing very good geographic concordance (Fig. 2). This pattern indicates that *Luciobarbus* speciated *in loco* after seeding by ancestral species.

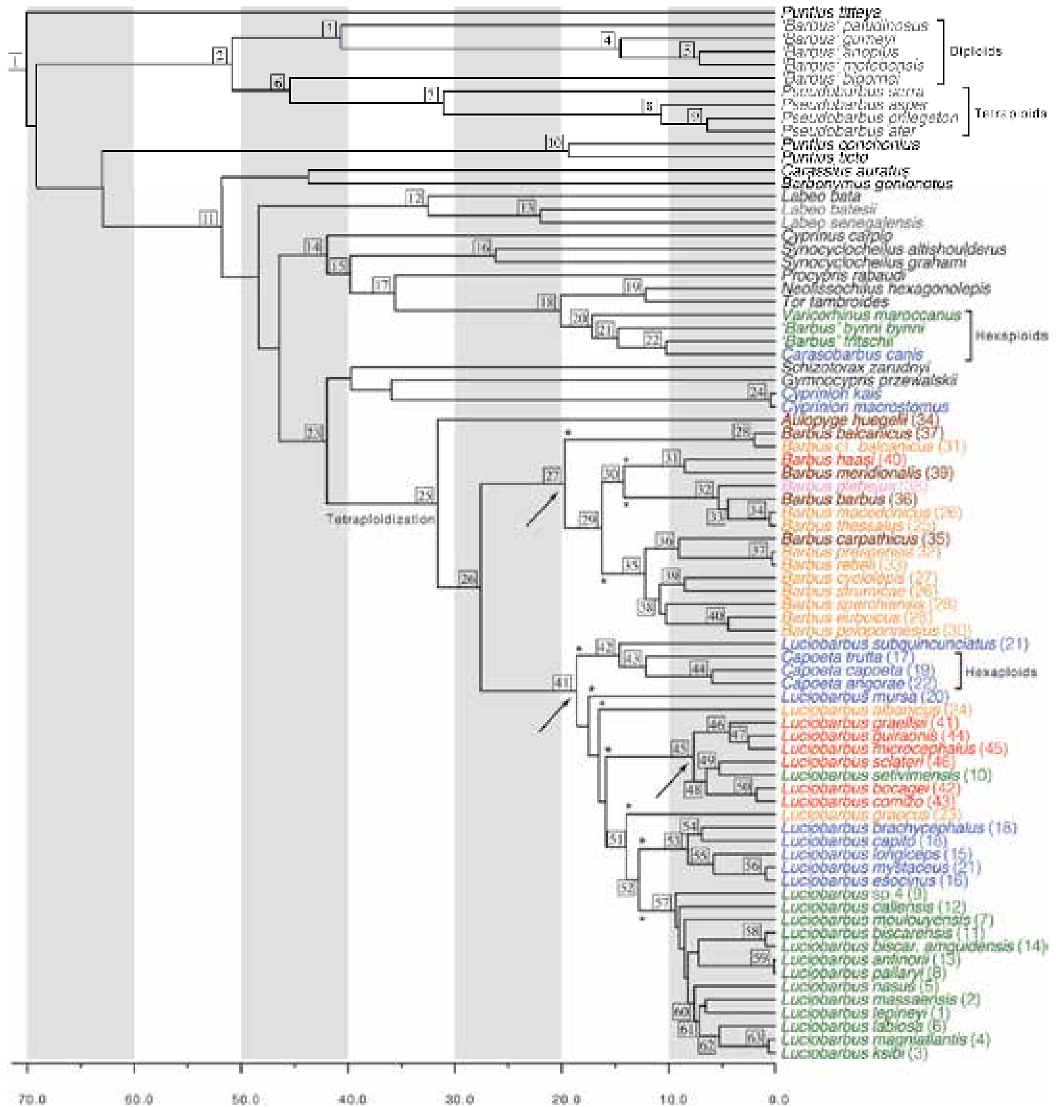

Fig. 2. Bayesian phylogeny calibrated using fossils of *Barbus*, *Luciobarbus* and Iberian *Luciobarbus* (arrows). Nodes with posterior probabilities above 0.8 are numbered in boxes and shown in Table 2. Asterisks (*) label lineages identified within *Barbus* and *Luciobarbus*. Color codes refer to geographical range of the species irrespective of generic placement: Northern Africa, Anatolia, Middle East and Caucasus, Greece, central and eastern Europe, and Balkans, Italy, and Iberia, Asia, and central and southern Africa. Numbers after species names refer to Fig.1 and Table 1.

Interestingly, one of these lineages comprises the hexaploid genus *Capoeta*. *Capoeta* appears to be monophyletic and has likely evolved from ancestors of (the tetraploid?) *Luciobarbus subquincunciatus*. Together, they form a strongly supported monophyletic group deep in the *Luciobarbus* lineage (Durand et al., 2002; Tsigenopoulos et al., 2003). *Luciobarbus* has a very wide distribution, only not being native to central and eastern Europe, and Italy (Doadrio,

Node No.	Age (Ma)	95% HPD	Posterior Probability
1	70.0	57.0–89.9	1
2	50.9	41.0-62.2	1
3	40.6	31.5-51.2	1
4	14.5	10.4-18.8	1
5	7.1	4.7-10.1	1
6	45.4	35.5-56.6	0.88
7	31.1	23.0-40.4	1
8	10.6	7.4-14.3	1
9	6.4	4.0-9.1	1
10	19.4	12.6-27.3	1
11	51.8	44.2-60.3	1
12	32.5	25.5-40.4	1
13	22.0	16.3-29.3	1
14	42.0	35.2-48.5	1
15	39.8	33.3-46.3	0.86
16	26.2	20.0-33.0	1
17	35.6	29.4-41.8	1
18	20.1	15.9-24.5	1
19	12.2	8.0-17.1	1
20	17.2	13.4-21.2	1
21	14.8	11.3-18.6	0.91
22	10.3	7.1-13.7	1
23	42.0	36.2-49.1	0.86
24	0.5	0.1-1.2	1
25	31.6	27.4-36.2	1
26	27.6	24.6-31.2	1
27	19.7	19.2-20.5	1
28	1.9	1.1-3.2	1
29	16.3	14.0-18.4	0.98
30	14.2	11.4-16.8	0.80
31	8.5	5.6-11.6	1
32	5.4	3.6-7.3	1
33	4.4	2.9-6.2	0.95
34	0.6	0.2-1.1	1
35	12.2	10.1-14.5	1
36	9.0	6.6-11.5	1
37	0.3	0.1-0.7	1
38	10.8	8.7-12.9	1
39	8.5	6.2-10.7	1
40	4.4	2.7-6.3	1
41	18.6	17.4-19.7	1
42	14.6	10.9-18.0	0.99
43	12.1	8.6-16.0	0.99
44	6.0	3.3-9.0	1
45	7.7	6.4-9.4	1
46	4.3	2.9-5.8	1

Table 2. (Continued)

Node No.	Age (Ma)	95% HPD	Posterior Probability
46	4.3	2.9-5.8	1
47	2.5	1.5-3.7	1
48	6.5	5.0-8.1	0.84
49	5.3	3.7-7.1	1
50	1.8	1.0-2.9	1
51	14.0	11.5-16.3	1
52	12.8	10.7-15.1	0.97
53	8.2	5.7-10.6	1
54	6.9	4.6-9.3	0.94
55	5.8	3.6-8.4	1
56	0.9	0.3-1.8	1
57	9.3	7.6-11.2	1
58	0.9	0.4-1.6	1
59	0.1	0.0-0.3	1
60	8.2	6.7-9.7	0.99
61	7.6	5.6-8.6	0.85
62	7.1	3.8-6.8	1
63	0.7	0.3-1.2	1

Table 2. Ages and their 95% HPD of tree nodes (Fig. 2) with posterior probability above 0.8.

1990). It is very diverse in northern Africa, where one lineage radiated and where a colonizer from Iberia, *L. setivimensis*, can be found (Machordom & Doadrio, 2001b). *Luciobarbus* is also relatively diverse in the Middle East and Caucasus, where at least three lineages occur, concordant with multiple colonization routes scenario (Almaça, 1990). *Capoeta* is also a very species-rich genus, with about 20 species distributed from western Asia to Anatolia (Banarescu, 1999; Turan et al., 2008).

Besides the inferred radiation within Africa, *Luciobarbus* has undergone rapid speciation early in its existence – most of the lineages identified date back to early *Luciobarbus* diversification (Tsigenopoulos et al., 2003). These polytomies (as the ones identified in the African lineage) do not likely represent a lack of information content in the data (soft polytomies), since the phylogenetic signal before and after these splits is very strong (Fig. 2). Therefore, these polytomies should represent legitimate radiation events (i.e. hard polytomies).

Regarding regional relationships within *Luciobarbus*, the northern African lineage, the Middle Eastern/Caucasus lineage and the Greek *L. graecus* form a strongly supported group. This is in conflict with the view that Iberia could have been seeded by northern African *Luciobarbus* (Doadrio, 1990; Gante et al., 2009) or the other way around (Almaça, 1990). Likewise, a hypothetical relationship between *Capoeta* and Iberian *Luciobarbus* (Tsigenopoulos et al., 2003) is not supported by the data. This lends the exact origin and relationships of Iberian *Luciobarbus* a mystery.

In contrast to this abundance of fast speciation in *Luciobarbus*, only a few short internodes are present in *Barbus* lineage. Whether this pattern reflects a difference in biology between *Barbus* and *Luciobarbus* is unclear. Interestingly, though, poorly supported nodes show some overlap in time, suggesting a possible external (environmental) driver. This hypothesis would need proper testing with a much more exhaustive taxon sampling.

Regarding regional relationships within *Barbus*, there is a much weaker correlation between lineages and geography than that seen in *Luciobarbus*. Such pattern indicates less isolation between Greece, central and eastern Europe, Italy and Iberia.

5. Dating the diversification of barbels

The dating strategy followed here differs from that of most other studies that included circum-Mediterranean barbels. Here, up to date fossil data (Böhme & Ilg, 2003) was used to calibrate a molecular clock, instead of biogeographical events or 'standard' mutation rates. As a consequence, the dates estimated in the present work are substantially older than previous estimates. For instance, the time of splitting between *Barbus* and *Luciobarbus* has been estimated to have occurred 5.5 Ma (Machordom & Doadrio, 2001b), 7.3 Ma (Tsigenopoulos et al., 2010) or ≈8 Ma (Tsigenopoulos et al., 2003; Zardoya & Doadrio, 1999) using the Messinian Salinity Crisis as the driver of speciation of the Iberian *Luciobarbus* lineage. It was estimated to have occurred 10.3 Ma (Tsigenopoulos et al., 2010) or 10.6–12.8 Ma (Tsigenopoulos & Berrebi, 2000) using previous estimates of mutation rates. Since the oldest known fossils of *Barbus* and *Luciobarbus* are 19.0 Ma and 17.7 Ma, respectively, those ages are certainly an underestimation of the real time of divergence between these two genera. In contrast to the abovementioned estimates, according to the calibration used here, *Barbus* and *Luciobarbus* diverged 27.6 Ma (95% HPD: 24.6–31.2 Ma). Since the calibration was applied to the nodes (without the stem), it is possible this age could be somewhat overestimated if earlier lineages diversifying within both *Barbus* and *Luciobarbus* (and represented in the fossils found) got extinct and are missing from the molecular phylogeny. Nevertheless, other sources of evidence support the new estimates shown here. For instance, divergence of *Varicorhinus* is estimated to have occurred 17.2 Ma (95% HPD: 13.4–21.2 Ma), which is supported by fossils of 17.8 Ma found in central Europe (Böhme & Ilg, 2003). Furthermore, the estimated time of divergence of *L. setivimensis* is 5.3 Ma (95% HPD: 3.7–7.1 Ma), which is exactly coincident with the re-opening of the Strait of Gibraltar by the end of the Messinian (Krijgsman et al., 1999).

According to the molecular clock calibration presented here, divergence of the lineage leading to *Aulopyge* happened 31.6 Ma (early Oligocene, Rupelian) and divergence between *Barbus* and *Luciobarbus* occurred 27.6 Ma (late Oligocene, Chattian). After a long period of stasis (or possibly high extinction) diversification within *Barbus* and *Luciobarbus* took place 19.7 Ma and 18.6 Ma, respectively (early Miocene, Burdigalian). The lineage leading to *B. haasi* and *B. meridionalis* split 14.2 Ma (middle Miocene, Langhian–Serravalian boundary). Nevertheless, this date should be carefully interpreted since the node support is rather low (BI = 0.80). Indeed, fossil teeth and vertebrae of *Barbus* have been found in Iberian sediments with 16–17 Ma (Doadrio 1990). *Barbus haasi* and *B. meridionalis* subsequently diverged at 8.5 Ma (late Miocene, Tortonian). The lineage leading to the Italian *B. plebejus* (and *B. tyberinus*; Tsigenopoulos & Berrebi, 2000) split 5.4 Ma (late Miocene, Messinian). *Capoeta* split from *L. subquincunciatus* 14.6 Ma (middle Miocene, Langhian) and started diversifying 12.1 Ma (middle Miocene, Serravalian). Between 18.6 Ma and 14.0 Ma (Miocene, Burdigalian–Langhian boundary) a series of fast cladogenetic events took place within *Luciobarbus*. One of them involved the ancestor of Iberian taxa, which only started diversifying 7.7 Ma (late Miocene, Tortonian–Messinian boundary). Again, like in the case of the apparent evolutionary stasis experienced by *Barbus* and *Luciobarbus* immediately after their split, it is possible that such long branch represents high extinction rather than low speciation rate. This time of divergence of Iberian *Luciobarbus* is coincident with the split of the Iberian *B. haasi*. As mentioned above, *L. setivimensis* split from its Iberian sister 5.3 Ma (Miocene-Pliocene, Messinian–Zanclean boundary). At 12.8 Ma (middle Miocene, Serravalian) the northern African and Middle Eastern *Luciobarbus* split. Rapid radiation within the northern African lineage took place between 9.3 Ma and 8.2 Ma (late Miocene, Tortonian). Therefore, *Aulopyge*, *Barbus* and *Luciobarbus* originated during the Oligocene, all lineages (including

Capoeta) arose in the Miocene and no radiation events date to the Messinian Salinity Crisis of the Mediterranean.

6. Paleogeography of the Mediterranean Basin and diversification of barbels

Seeding of the Mediterranean with a tetraploid barbel lineage most likely occurred during the late Eocene or early Oligocene (Fig. 2). This dating is consistent with an Asian origin of cyprinids and colonization of Europe at the closing of the Turgai Strait in the Eocene–Oligocene boundary (Almaça, 1990; Banarescu, 1992; Briggs, 1995; Rögl, 1999). Progression towards the west was possible due to the emergence of a large landmass that extended across the Balkans, Anatolia and Iran (Rögl, 1999). The carbonate rocks with more than 8,000 m that form the Karst Dinarides were deposited for more than 270 Ma and raised during the Alpine orogeny (Velic, 2007), suggesting that present-day karst habitat inhabited by *Aulopyge* was already present, in the place this oldest barbel lineage is presently found. Such a colonization scenario through southwestern Asia was also hypothesized for Leuciscins (Perea et al., 2010).

The split between *Barbus* and *Luciobarbus* in the late Oligocene could have been driven by the fragmentation of this landmass (Rögl, 1999). In a time of intense tectonic activity in the Mediterranean, the opening of the Slovenian corridor is a likely candidate, fragmenting *Barbus* to the north and *Luciobarbus* to the south. The timing of diversification within these genera at 20 Ma is coincident with the closure of the Slovenian seaway (Rögl, 1999). This reunited landmass might have allowed access to regions where the oldest fossils of *Barbus* and *Luciobarbus* have been found.

The time of origin of the lineage leading to *L. subquincunciatus* and *Capoeta*, which are found in the Middle East and Caucasus, is coincident again with a transitory fragmentation of this landmass. In the middle Miocene, seaway corridors opened between Arabia, south Anatolia and eastern Anatolia, and possibly along a suture between the Balkanides and the Rhodopes (Rögl, 1999). The branching out of *Capoeta* occurred at the time when oceanic circulation between the Indian and Atlantic oceans stopped, in the Serravalian (Rögl, 1999).

By then, the main lineages within *Barbus* and *Luciobarbus* had already originated. Explaining their current distribution is no easy task with current paleogeographical and paleontological evidence. Fossils of *Barbus* of Burdigalian age are found from Turkey to Iberia (Böhme & Ilg, 2003; Doadrio, 1990), which corresponds to the present distribution of the genus, except for Italy. Colonization of Italy during the Messinian Salinity Crisis is a likely scenario. Equally old fossils of *Luciobarbus* have been only found in Turkey and it is not before the Tortonian they are found in central Europe (Böhme & Ilg, 2003). This could be due to taxonomic bias, since *Luciobarbus* has not been recognized for as long as *Barbus*, or it could reflect a real trend of *Luciobarbus* biogeography. Nevertheless, the presence of fossils in central Europe in Tortonian times, where it is now absent, opens new routes for *Luciobarbus* dispersal. In particular, it is known that during Alpine orogeny, marine influence in the North Alpine Molasse ended in the middle Miocene (Langhian; Hsü et al., 1977; Krenmayr, 1999; Rögl, 1999). Barbels could have used this basin as a means of southwestward dispersion to Iberia, independent from the colonization of northern Africa. Alternatively, they could have used slightly different pathways via the *Gomphotherium* landbridge connecting Africa and Eurasia (Rögl, 1999), as suggested by Perea et al. (2010) to explain vicariance of Peloponessus and Magreb *Tropodophoxinellus*. Since the distribution areas of Leuciscinae and Cyprininae are similar, as well as inferred dates of groups occupying those regions, it is likely they shared common migratory routes. The subsequent radiation of northern African *Luciobarbus* is likely related to complex paleogeomorphology of the Rif massif (e.g. Alvinerie et al., 1992; Machordom &

Doadrio, 2001a). Starting around 7.8 Ma the marine corridors between Iberia and northern Africa became restricted until the establishment of a land bridge around 5.6 Ma (Messinian) (Garcés et al., 1998, 2001; Krijgsman et al., 1999; Martín et al., 2001; van Assen et al., 2006). Dispersal and subsequent vicariance of *L. setivimensis* between Betic and Riffian massifs has occurred during the Messinian Salinity Crisis. Nevertheless, speciation events are not concentrated in this period, nor are the inferred radiations. Other authors have recently ruled-out a "Lago Mare dispersal" for leuciscin cyprinids (Levy et al., 2009; Perea et al., 2010). This period seems to have been used for transfer between adjacent areas (e.g., Iberia – northern Africa, central Europe – Italy) rather than a Mediterranean-wide colonization by barbels.

7. Conclusion

According to the fossil calibrated molecular phylogeny presented here, divergence of the circum-Mediterranean barbel lineages occurred during the Oligocene. Divergence within *Barbus* and *Luciobarbus* took place throughout the Miocene, including spreading to new areas. Altogether, colonization of the Mediterranean region by barbels must have been a very dynamic process we are just starting to understand, as indicated by the presence of many fossils in regions where the genera are presently not found. A good example is the presence of several *Luciobarbus* fossils in Libya, Italy, Austria, and Slovakia.

Greater insight will likely continue coming from paleontological and paleogeographical data, and that should be accompanied by new biological data of extant species. In particular, all of these scenarios are only based in non-recombinant mitochondrial DNA markers. The coming decade should see the rise of nuclear phylogenies and an improved understanding of barbel biogeography in the Mediterranean region.

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14

Biogeography and Population Connectivity of Coral Reef Fishes

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1. Introduction

Most fundamentally, the distribution of populations and species is a function of both habitat availability and dispersal ability. Understanding the patterns that result from the interaction of these factors is one of the central aims of ecology. It has long been recognized that populations are not evenly distributed throughout the entire geographic range of the species. Populations occupy spatially distinct patches of suitable habitat that are separated from each other by areas of unsuitable habitat. Depending on factors such as the distance between populations, the nature of the intervening environment, and the relative mobility of the organisms in question, areas of unfavorable habitat may or may not prevent movement of individuals among patches. Given a long enough period of isolation between populations of the same species, speciation or local extinction may result. The degree of connectivity among populations therefore has major ecological and evolutionary implications.

Though the implications of connectivity have long been acknowledged and explored in terrestrial organisms, variable connectivity patterns are only beginning to be recognized as an important driver of present-day population dynamics in the marine realm. It had long been assumed that, due to the lack of visible barriers and the presumed ability of larvae to passively disperse great distances by riding ocean currents, marine organisms dispersed freely and had high levels of population connectivity throughout their ranges. Recent studies in coastal habitats, however, have revealed that the larvae of many coral reef fish species actually settle much closer to their natal reefs than previously thought (Almany et al., 2007; Jones et al., 1999; Kingsford et al., 2002; Planes et al., 2001, Swearer et al., 1999; Taylor & Hellberg, 2003). Similarly, population structure has been found in pelagic species such as cod (Bentzen et al., 1996; Ruzzante et al., 2000) and tuna (Block et al., 2005; Carlsson et al., 2004).

The existence of patchy marine populations, especially if the patches are not connected by migration or dispersal, has enormous implications for biodiversity conservation and management. For instance, isolated populations are more prone to extinction than are more connected ones (Munday, 2004). In this chapter we present an overview of the distribution and population connectivity patterns of coral reef fishes and the methods that have been used to quantify them. We argue that widespread variability in predicted and observed patterns can be explained via the interaction between reef fish life history traits and oceanographic conditions. We emphasize throughout the importance of understanding the

interactions among evolutionary, ecological, and physical processes in structuring contemporary distribution and dispersal patterns. Finally, we address how a better knowledge of connectivity among reef fish populations holds the potential to dramatically improve management and conservation of threatened coral reef ecosystems.

2. Coral reef fish biogeography: The link between evolutionary and ecological processes

Biogeography, or the study of the distribution of organisms in space and time, asks which species occur where, and why or why not (MacArthur and Wilson, 1967). The answers to these questions are complex, since contemporary distribution patterns of organisms reflect biological and physical processes operating at multiple spatial scales, on both evolutionary and ecological time scales. We focus here on the study of coral reef fishes, specifically the ten families that are considered to be characteristic of modern coral reef ecosystems: Acanthuridae (surgeonfish), Apogonidae (cardinalfish), Bleniidae (blennies), Carangidae (jacks), Chaetodontidae (butterflyfish), Holocentridae (squirrelfish), Labridae (wrasses), Mullidae (goatfish), Pomacentridae (damselfish and clownfish), and Scaridae (parrotfish) (Bellwood, 1996). Coral reefs have existed in some form since the Ordovician period (Wood, 1999), and the biogeographic and taxonomic patterns observed in coral reef fish families today reflect a long and complex history of geological, oceanographic, and biological interactions (Bellwood & Wainwright, 2002). The contemporary distribution and population structure of coral reef fishes appear to be similarly mediated by both geography and life history. In our subsequent discussion of emergent biogeographic and taxonomic patterns, we use the term "reef fish" to refer specifically to tropical coral reef fish.

2.1 Temperature controls the distribution of suitable habitat

The global dispersal of coral reef fishes is broadly controlled by the availability of suitable habitat, which is dictated by the shape of the latitudinal temperature zone around the Equator. The Tropical Zone (Figure 1) was formed by dramatic cooling at high latitudes long ago in the Earth's geological history, and it remains constrained today by the flow of major ocean currents (Briggs, 2007). Oceanography also influences reef fish distributions within the confines of this tropical zone. On the western sides of the Atlantic and Pacific Ocean basins, the North and South Equatorial currents turn toward higher latitudes, bringing warm water and tropical organisms with them. On the eastern sides of the ocean basins, however, the major currents turn toward the tropics, transporting colder water from higher latitudes toward the Equator. This oceanographic pattern ensures that the western regions of each basin have a larger area of tropical marine habitat (Briggs, 2007), and therefore greater species diversity, than do the eastern regions.

The Tropical Zone (TR) defines the latitudinal availability of suitable tropical habitat. Within this temperature zone, several major faunal boundaries have contributed to marine speciation events in the past and continue to act as barriers to reef fish dispersal in the present: the Red Sea land bridge, the Indo-Pacific Barrier, the Eastern Pacific Barrier, and the Isthmus of Panama. Together with temperature restrictions, these dispersal barriers have produced five longitudinal biogeographic regions: the Indian Ocean, the Indo-West Pacific, the Eastern Pacific, the Western Atlantic, and the Eastern Atlantic (Bellwood & Wainwright, 2002; Briggs, 2007).

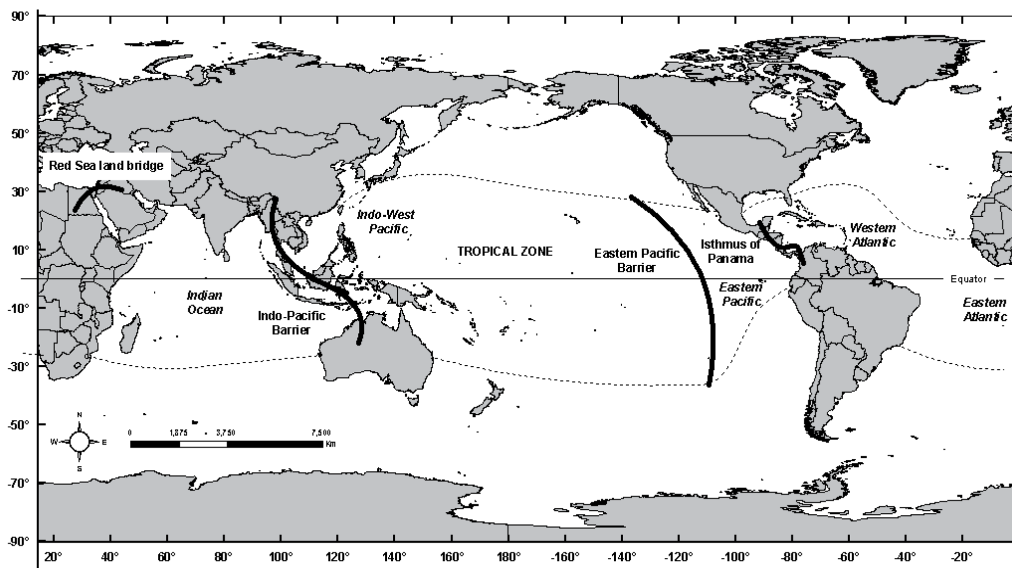

Fig. 1. Major physical factors affecting the global distribution of reef fishes, and resulting biogeographic regions.

Despite these divisions, there is broad taxonomic overlap of reef-associated fishes worldwide. Of the ten families that are considered to be characteristic of coral reef systems (Bellwood, 1996), few are restricted to only one region, and in most cases overlap is extensive at the genus level (Briggs, 1974). The Western Atlantic region, with 700 species of fish (Rocha, 2003), is essentially a more species-poor version of the tropical Pacific, which hosts more than 4000 fish species (Springer, 1982). The reef fish fauna of the Western Atlantic and the Eastern Pacific biogeographic regions are very similar, probably reflecting their connection prior to the closure of the Isthmus of Panama (Bellwood & Wainwright, 2002).

2.2 Historical barriers to dispersal

Over evolutionary history, geological processes have created a series of environmental barriers to dispersal that progressively separated reef fish taxa among the ocean basins. Cooling at high latitudes "locked in" the tropics 37 million years ago, restricting the movement of tropical species to a latitudinal band around the Equator now known as the Tropical Zone (Briggs, 2007). Later, the Atlantic and Indian oceans were cut off from each other when Africa collided with Eurasia 12-18 million years ago, and the uplift of the Isthmus of Panama around 5 million years ago separated the Atlantic from the Pacific. The last tropical connection between the ocean basins was effectively closed 2 million years ago by the formation of the Benguela upwelling, a cold-water barrier, off the Atlantic coast of southern Africa (Bellwood & Wainwright, 2002). These and other historical barriers to dispersal (Figure 1) prevented the exchange of individuals among populations, causing both extinctions and speciation events (Bellwood & Wainwright, 2002; Palumbi, 1994).

Biogeographic barriers that resulted in historical vicariance events can continue to hinder dispersal among populations. For instance, the Pacific has been divided into three major biogeographic regions: the Indian Ocean, the Indo-West Pacific, and the Eastern Pacific. The

barriers between these provinces include the world's largest uninterrupted expanse of deep ocean, the 4000 - 7000 km wide Eastern Pacific Barrier, which isolates the Eastern Pacific from the rest of the Pacific, and the Indo-Pacific Barrier, the dense mass of islands and continents (including Australia) that separates the Indian and Pacific Oceans (Figure 1). The latter barrier is enhanced during low sea level stands, when there are fewer passages between islands, and by strong upwelling that is likely to reduce the habitat available for tropical species (Barber et al., 2002).

2.3 How biogeography helps us to understand population connectivity

The present-day distribution of coral reef fishes is heavily influenced by physical and geological events in the history of the earth that defined the boundaries of the tropical region and the various ocean basins. In addition to the evolutionary-scale processes that have structured the distribution of tropical marine species, ecological processes act to maintain current distribution patterns (Bellwood & Wainwright, 2002). For instance, the scale of dispersal of reef fishes influence how populations within a species are spatially distributed (James et al., 2002). Due to the lack of visible obstructions in the marine realm and the presumed ability of larvae to passively disperse great distances, the prevailing paradigm for decades was that marine fish populations mixed freely and had high levels of connectivity throughout their ranges. In other words, the popular view was that marine populations were largely open, connected by demographically significant movement of individuals (immigration and emigration) (Hixon et al., 2002).

Biogeography tells us that species are not uniformly distributed throughout their ranges, and it is obvious that the long-held belief in freely dispersing marine populations discounts temporal and spatial variability in environmental conditions and dispersal patterns among sites. It became apparent only recently that barriers to dispersal, aside from delimiting distribution patterns in evolutionary time, could also structure populations of marine species on ecological time scales. The assumption of range-wide population openness has been challenged by a litany of recent studies showing that coral reef fish larvae, even of widely distributed species, often settle closer to their natal reefs than previously thought (Almany et al., 2007; Jones et al., 1999; Jones et al., 2005; Kingsford et al., 2002; Leis, 2002; Planes et al., 1998; Planes et al., 2001, Swearer et al., 1999; Taylor & Hellberg, 2003).

The nature of connectivity has important ecological and evolutionary implications. At one extreme, if reef fish populations are highly connected among patches, they may be more able to withstand local disturbances that degrade or remove habitat, and in the long term they may be less prone to local extinction. Under such open population models, we may expect only slow rates of evolution due to the very large effective population sizes. At the other extreme, if populations are isolated from one another, they may be more prone to local extinction as a result of habitat degradation or loss, since they are not supplemented by immigration of individuals from elsewhere. Further, over long periods of time genetic differences may build up such that reef fish populations become reproductively isolated, with the capacity to form new species.

The influence of connectivity on speciation is particularly relevant when discussing how historical context informs present-day population connectivity in reef fishes. Within the five major biogeographic regions of the tropics (the Indo-West Pacific, the Eastern Pacific, the Western Atlantic, the Eastern Atlantic, and the Indian Ocean), reef fish species are not evenly distributed. Rather, they have become concentrated into relatively small areas of

very high biodiversity. The Coral Triangle (also known as the East Indies Triangle) and the southern Caribbean are centers of diversity for the Indo-West Pacific and Western Atlantic regions, respectively. In the biogeographic provinces of the Coral Triangle and the southern Caribbean, at least 10% of the fauna are endemic species (Briggs, 1974). In the Coral Triangle in particular, speciation rates are so remarkable that Mora et al. (2003) observed that there seemed to be a continuous flow of species from the East Indies outward across the Indian and Pacific oceans.

Why are these particular areas of the tropics, specifically the Coral Triangle, effectively functioning as species factories? It is known that scales of dispersal influence speciation rates (Palumbi, 1994). Multiple, smaller scale barriers to gene flow clearly operate within the larger biogeographic regions, isolating populations by preventing dispersal among them. Barriers to dispersal that create population divisions within species on an ecological scale will become evolutionary-scale if they persist and lead to speciation or extinction. Biogeographical patterns of species distributions therefore simply reflect population structure that has been reinforced over evolutionary time. Indeed, studies are increasingly revealing that, even without obvious barriers to dispersal, populations of species that are morphologically alike are often divergent enough to be considered separate, or sibling, species (Knowlton, 1993).

The recognition that marine populations are not uniformly well mixed throughout their ranges has fundamentally changed the way biologists and ecologists think about the distribution of marine species, especially coral reef fishes. It is no longer enough to recognize habitat patchiness as a driver of population dynamics. We are now compelled to evaluate the scale of patchiness, or the relative openness, of each marine population in question, which may or may not correlate with the availability of suitable habitat. In other words, the question has become about the temporal and spatial scales of mixing among populations. Quantifying this connectivity is crucial for understanding local adaptation and speciation, population replenishment, and the likelihood of local extinction. While there are still examples of populations that appear to be highly open (Eble et al., 2011) as well as highly closed (Jones et al., 2005), the majority of reef fish populations likely lie somewhere along a continuum, and the important task going forward will be identifying where different organisms occur along this spectrum.

3. Quantifying connectivity by estimating larval dispersal

Despite its critical ecological importance, population connectivity remains notoriously difficult to quantify directly. Most coral reef fishes have a biphasic life cycle with a dispersive larval phase and a relatively less mobile adult phase. Movement from one site to another is thus accomplished mainly via larval dispersal, as opposed to adult migration. The pelagic stage of reef fishes is critical for connecting populations among patchy habitats but is arguably the least understood phase of their life cycle (Planes, 2002). In particular, the smallness of larvae relative to their vast and complex fluid environment (Mora & Sale, 2002) has hindered accurate quantification of dispersal and connectivity.

Both direct and indirect attempts have been made to measure larval dispersal. For decades, a popular method of tracking taxa such as whales (Ray et al., 1978) and tuna (Fink and Bayliff, 1970) has been tagging, in which a satellite or radio tag is physically attached to or implanted into the animal. These tags potentially yield a detailed record of movement over time. For obvious reasons, this approach is not possible with reef fish larvae, but another form of tagging has been attempted. A mark-recapture method has been developed that

involves tagging the otoliths of larval fishes by saturating the surrounding water with a chemical, such as tetracycline, that becomes incorporated into the calcium carbonate structure of the otolith (Jones et al., 2005; Thorrold et al., 2002). Later analyses of juvenile fish otoliths reveal the chemical signature. Such methods, however, are often prohibitively time-consuming and expensive, with low returns due to extensive larval mortality and dilution in the ocean. For instance, Jones et al. (1999) tagged over 10 million developing embryos of a damselfish (*Pomacentris amboinensis*) on the Great Barrier Reef in Australia. Of 5,000 juveniles subsequently settling at the same location, the authors retrieved 15 marked individuals (Jones et al., 1999), a return rate of just 0.00005%.

The many difficulties associated with direct tracking of larvae have encouraged the development of indirect, more efficient methods of estimating larval dispersal. Monitoring ocean currents is a relatively straightforward way to predict larval dispersal and adult migration (Cowen, 2002; Cowen et al., 2000; Lobel, 1997), but a major handicap of this approach is that it relies on the assumption that larvae drift passively with currents, which is not always true (Gerlach et al., 2007; Kingsford et al., 2002). In addition, long-term mean current patterns may differ from local or infrequent oceanographic conditions that could affect dispersal.

3.1 Genetics: From phylogeography to molecular ecology

It was first suggested in 1975 that genetic variation could be the best approach for assessing dispersal and migration among geographically separated populations of reef fishes (Ehrlich, 1975). In tandem with advances in the field of molecular ecology, in the past several decades the analysis of genetic information has emerged as a means to understand the distribution and diversity of marine species and populations (Bohonak, 1999; Burton, 1996, 2009; Grosberg and Cunningham, 2001; Hellberg et al., 2002; Shulman, 1998; Slatkin, 1987). This indirect method is possible because the dispersal of propagules maintains gene flow between geographically separated populations (Hellberg et al., 2002; Shanks et al., 2003). Genetics-based methods can infer gene flow, and therefore dispersal, through spatial variation in allele and genotype frequencies (Hedgecock et al., 2007; Planes, 2002). Therefore, the same factors that influence dispersal, or larval "flow", can be assumed to also influence gene flow (but see Section 3.2 for a discussion of caveats to this assumption).

On evolutionary time scales, the study of phylogeography assesses genetic diversity among species to examine historical processes that may be responsible for contemporary geographic distributions (Rocha et al., 2007). Phylogeographic approaches rely on genetic markers that reflect deep historical linkages among taxa in order to build gene genealogies, or trees, that help to explain contemporary species-level relationships. For instance, the Barcode of Life is a recent initiative to catalog diversity based on a region of the genome that varies in an easily identifiable, species-specific manner (Hebert et al., 2003).

Because biogeographic and ecological processes are linked, genetic information can also be used to evaluate whether connections exist among spatially segregated populations of the same species (Shulman & Bermingham, 1995); that is, are populations demographically open or closed (Mora & Sale, 2002) on ecological time scales? In theory, predictable recruitment and connectivity patterns are expected to drive stable genetic structure among populations (Purcell et al., 2006). If larvae are dispersed randomly and widely away from the parental populations, then no consistent genetic structure will occur. On the other hand, if larvae are returned to the parental population, then gene flow between geographically

separated populations will be restricted, and genetic structure will arise (Shulman & Bermingham, 1995).

Several well-known theoretical models of population genetic structure have existed since well before the application of genetic information to estimating larval dispersal. These models describe expected patterns of structure, given a certain set of conditions. For instance, the so-called isolation by distance pattern can arise in species with sufficiently large geographic ranges relative to their dispersal ability. Wright (1943) introduced this model to describe how genetic differences could accumulate over time, given restricted dispersal. In conjunction with the stepping-stone model of population structure (Kimura and Weiss, 1964), the isolation by distance model has developed to describe the population structure among organisms whose dispersal ability is constrained by distance, such that gene flow is most likely to occur between neighboring populations (Slatkin, 1993). Together, these approaches link gene flow with geographic distance (Planes and Fauvelot, 2002).

Predicting the population genetic structure that may result from a given pattern of larval dispersal, however, is not so straightforward, as there are many influences on dispersal besides geographic distance. It is important to explicitly consider all of the factors that influence dispersal, and therefore gene flow. Certain life history and behavioral traits, oceanographic conditions, and historical barriers to dispersal all influence whether geographically disjunct populations of coral reef fishes are connected via planktonic larval dispersal.

The variation in the magnitude of observed genetic structure may depend critically on the life history of the study species (Gerlach et al., 2007), such as the timing and location of spawning relative to currents, gyres, and tides (Pelc et al., 2009). Egg type and reproductive strategy also play a role (Leis & McCormick, 2002). The pattern of high gene flow for reef fish with a pelagic larval phase is supported by numerous examples of reef fishes that show no significant population structure over distances spanning thousands of kilometers (Craig et al., 2007; Horne et al., 2008; Klanten et al., 2007; Palumbi, 1994; Planes & Fauvelot, 2002; Riginos and Victor, 2001). Species with non-pelagic larvae such as clownfish and damselfish, meanwhile, tend to show strong genetic structure indicative of high levels of local recruitment (Jones et al., 1999). In a study of gene flow in eight reef fish species, Shulman & Bermingham (1995) found no evidence of genetic structure across the Caribbean except in species that lacked pelagic larvae.

The length of time that larvae spend in the water column has also been implicated as a factor affecting dispersal and genetic structure (Bowen et al., 2006; Weersing & Toonen, 2009). Reef fishes typically have larval lives lasting for weeks or months, seemingly long enough to achieve dispersal great distances away from the source population. It seems intuitive that the longer a larval propagule stays in the water column, the farther it will travel. However, there is no consistent correlation between pelagic larval duration (PLD) and geographic range size (Lester et al., 2007; Lester & Ruttenberg, 2005; Victor & Wellington, 2000), or between PLD and population genetic structure (Weersing & Toonen, 2009). Previously reported correlations appear to have been driven by species lacking a pelagic larval phase (Eble et al., 2011). Reproductive strategy may thus be a stronger influence than PLD on dispersal. Larvae can also actively participate in their own dispersal through evolved behaviors such as "smelling home" (Gerlach et al., 2007) and navigating toward sensed targets, which in sum can promote local retention.

Superimposed on the patterns of variable life history traits is the larger pattern of abiotic influences on dispersal, including oceanographic features such as currents, eddies, tides,

and fronts (Cowen, 2002). It is well known that larvae of marine species can accumulate at fronts (Roughgarden et al., 1991). Regional currents, as well as secondary or indirect currents, are also important (Visram et al., 2010). As our understanding of ocean physics improves, the role of advection and diffusion in larval dispersal is also beginning to be appreciated (Largier, 2003). Points, jets, and retention zones can cause variable larval transport along coastlines (Gaylord and Gaines, 2000; Largier, 2004; Richards et al., 1995). Seasonal shifts in current patterns and episodic events such as relaxation of upwelling (Largier, 2004) may have significant consequences for larval transport and recruitment. At large scales (greater than 300 km), studies have linked areas of strong genetic structure in fishes and corals to major oceanographic and environmental features (Baums et al., 2006; Galarza et al., 2009; Galindo et al., 2006; Pelc et al., 2009).

Finally, studies have shown that even marine species with high dispersal potential break into genetically similar groups within biogeographic provinces (soldierfish, Craig et al., 2007; surgeonfish, Planes and Fauvelot, 2002). For instance, Barber et al. (2000) observed a sharp, localized genetic break among stomatopod populations in Indonesia, suggesting a marine counterpart to the Wallace's line that separates terrestrial fauna there. These results demonstrate that biogeographic provinces that were formed millions of years ago can still function as barriers to dispersal today, even in widely ranging species (Barber et al., 2000).

3.2 The double-edged sword of molecular ecology

Predicting patterns of genetic structure is inherently difficult, and empirical measurements often fail to correspond to predictions. In the Pacific, biogeographic divisions do not appear to affect the dispersal of some reef fish species, possibly due to the extensive mixing and unstable circulation generated by El Niño events and other phenomena (Muss et al., 2001). Limited genetic subdivision across reefs of the Pacific has been demonstrated in a number of reef fishes, such as surgeonfish (DiBattista et al., 2011; Eble et al., 2011; Horne et al. 2008; Klanten et al., 2007), grouper (Rivera et al., 2011), parrotfish (Bay et al., 2003), snapper (Evans et al., 2010), and angelfish (Schultz et al., 2007). Some species have been shown to traverse even biogeographic barriers that were thought to be insurmountable, such as the Eastern Pacific Barrier (Lessios & Robertson, 2006; Figure 1). Conversely, sometimes population genetic structure is observed despite an apparent lack of barriers to dispersal (Planes et al., 1998; Taylor and Hellberg, 2003; Toonen et al., 2011).

These discrepancies have several possible explanations related to the power of genetic analyses to resolve ecologically meaningful patterns. If genetic structure is present, but weak, then deciding whether populations are open or closed at the scale in question can be difficult (Hepburn et al., 2009). Moreover, though some of the factors producing population genetic structure are intimately related to demographic processes, others are not. Though it is commonly assumed that genetic structure reflects a balance between gene flow and genetic drift, migration and dispersal are not the only influences on genetic differentiation. Other forces such as natural selection and historical contact between populations can influence the allele frequencies used to evaluate levels of dispersal (Bohonak, 1999; Planes, 2002). For instance, even if gene flow is occurring, natural selection can be a major force maintaining genetic differentiation among populations (Mora & Sale, 2002). Patterns over evolutionary time (natural selection and historical contact) can therefore influence the genetic structure observed in ecological time, and this is cause for caution when interpreting

genetic data as a reflection of demographic patterns. Furthermore, because an exchange of just one individual per generation can result in genetic homogeneity (Planes, 2002), populations may be linked genetically without an obvious demographic connection (Kool et al., 2010). When interpreting genetic patterns in the context of ecology, then, it is critical to discriminate based on temporal scale, delineating evolutionary versus ecological connectivity.

Adding complexity to studies of connectivity is selecting genetic markers, a process that is rife with controversy even within the molecular ecology community (Fauvelot et al., 2007; Hellberg, 2007, 2009). Because different markers have different spatial and temporal powers of resolution, great care must be taken in both selecting markers and interpreting patterns of genetic structure obtained from analyses of variation in those markers. Two types of markers that are commonly used in genetic studies, allozymes and mitochondrial DNA (mtDNA), are not very sensitive to genetic drift at the appropriately short temporal scales (Hellberg, 2009). These types of markers are useful for evaluating the evolutionary relationships of closely related populations and for inferring connectivity over longer temporal scales. Demographic studies, on the other hand, require that markers are able to drift distinctively among populations at temporal scales that are relevant to population replenishment. For these types of studies, other DNA markers called microsatellites are promising alternatives to the more traditionally used allozymes and mtDNA. Microsatellites are gaining popularity among molecular ecologists because they have a high mutation rate and are probably neutral to selection (Hellberg, 2009). Their mutation rate is high enough to result in a large amount of variation among unrelated individuals within a population, but low enough so that changes usually do not occur more often than every few generations (Hartwell et al., 2008). Microsatellites can therefore detect subtle differentiation among closely related individuals, which is useful for determining ecologically relevant connectivity as opposed to more historical population subdivisions (Hellberg, 2009). Ideally, to begin to address these differences, population connectivity studies should at least compare results obtained from multiple markers.

Finally, different methods of characterizing genetic structure and estimating gene flow make different assumptions about the equilibrium status of populations, migration patterns, population structure, and the attributes of genetic markers (Grosberg & Cunningham, 2001). Sampling protocols are widely variable, as are geographic and temporal scales surveyed. In addition to the inherent limitations of using population genetic structure to infer dispersal, this lack of a cohesive approach further complicates the interpretation of genetic structure analyses.

There are several ways to counteract the pitfalls inherent in a molecular ecology approach. More direct genetic methods for tracking larvae, such as parentage analysis (Christie et al., 2010; Jones et al., 2005; Planes et al., 2009; Saenz-Agudelo et al., 2009), are gaining traction, but this approach is really only feasible with strongly site-attached taxa such as clownfish, where brooded larvae can be easily associated with a parental source. Other recent genetic advances include the ability to resolve the high temporal variation in reproduction and recruitment patterns that commonly occurs among Caribbean and Pacific reef fishes (Sale et al., 2005). At small scales, this temporal variation produces a complex pattern called chaotic genetic patchiness (Johnson & Black, 1982), which remained unexplained until recently (Selkoe et al., 2010). It is important to detect the causes of this variability, as spatial and temporal variation in connectivity may itself help to promote long-term stability in populations (Hogan et al., 2010).

3.3 Integrating multiple types of data: An example from the Caribbean

There is a growing recognition that, given the complexity of larval dispersal, genetic data are most useful when analyzed in tandem with models, oceanographic data, or other approaches (Galindo et al., 2006; Rivera et al., 2011; Selkoe et al., 2008). In the absence of inexpensive, effective methods of directly measuring larval dispersal, such integrated approaches represent the next best strategy to obtain ecologically meaningful data, as emphasized in the following example from the Caribbean.

The Caribbean is an essentially closed ocean basin with relatively stable current systems since the shoaling of the Isthmus of Panama around three million years ago (Briggs, 2007). The basin is biogeographically isolated from the southwestern tropical Atlantic (e.g., Brazil) by the inter-regional barrier of the Amazon outflow, and from the eastern tropical Atlantic (e.g., western Africa) by sheer distance (Floeter et al., 2008). Within the Caribbean, species distribution patterns suggest that there are few biogeographic divides for reef fish, with most species ranges spanning the entire basin. As such, species richness across Caribbean islands is predicted best by island size and distance from neighbors, rather than by taxon-specific dispersal histories (Sandin et al., 2008). The biogeography and oceanography of the Caribbean would seem to indicate a strong potential for population connectivity, and therefore genetic similarity, across the basin. Given its stepping-stone geography and a total distance along a current track of 4500 km, Shulman & Bermingham (1995) suggested that it could take as few as 13 generations for a novel haplotype to spread throughout the basin. Correspondingly, studies have shown high rates of gene flow in reef fish leading to genetic similarity within taxa throughout the Caribbean (Lacson, 1992; Shulman & Bermingham, 1995).

In contrast, a model by Kool et al. (2010) showed that, while reef fish populations in the Caribbean became increasingly genetically connected with one another over time, relative differences between populations persisted, providing the basis for the development of genetic structure. Similarly, a biophysical model by Cowen et al. (2006) identified four broadly defined, distinct regions of population isolation: the eastern Caribbean, the western Caribbean, the Bahamas-Turks and Caicos Islands, and the region at the periphery of the Panama-Colombia gyre. These regions correspond to genetic and morphological clines observed across a range of marine organisms (Cowen et al., 2006). Given the significant degree of structure expected to be present in the Caribbean from these model simulations (Cowen et al., 2006; Kool et al., 2010), why are strong breaks between populations not more evident in observations of natural populations (Purcell et al., 2006; Shulman & Bermingham, 1995)? This discrepancy may occur because sampling a limited number of individuals decreases the amount of visible structure (Kool et al., 2010). That empirical studies may be inherently limited by sampling power relative to model simulations is therefore an important consideration in evaluating results of population genetic studies.

4. How do we account for this variability? A model of passive and active dispersal

When considering the pronounced variation in the biotic and abiotic factors determining dispersal, it is unsurprising that published estimates of connectivity and dispersal in coral reef fishes vary widely (Cowen et al., 2006; Purcell et al., 2006; Gerlach et al., 2007; Hepburn et al., 2009) and that no general relationship between spatial scale and the likelihood of population-level genetic divergence has emerged (Hepburn et al., 2009). Given the complexities and simplifying assumptions involved in equating gene flow with dispersal, this variability is

partly a relic of the genetics methods used to estimate dispersal. The majority of marine species have high rates of gene flow over evolutionary time scales (Hedgecock et al., 2007). Determining the extent to which populations are connected on an ecological scale, despite high gene flow at an evolutionary scale, remains the single greatest challenge for revealing ecologically meaningful patterns of larval dispersal (Botsford et al., 2009).

The case study from the Caribbean illustrates this dichotomy between evolutionary gene flow and ecologically relevant dispersal. Even though studies have shown genetic similarity across the Caribbean, suggesting basin-scale mixing among reef fish populations (Geertjes et al., 2004; Lacson, 1992; Shulman & Bermingham, 1995), the ecologically meaningful pattern of larval dispersal is likely at the regional scale (Cowen et al., 2006). Isolation in reef fish populations at this scale has also been shown in other oceanographically and geographically complex regions such as the Indo-Pacific (Drew et al., 2008).

The paradigm shift in marine population connectivity means that, on ecological time scales, reef fish populations are now considered to be substantially closed, rather than broadly open (Mora & Sale, 2002). Effective dispersal is much smaller than potential dispersal extrapolated from ocean current speed and pelagic larval duration (Planes, 2002). We may therefore expect a relatively narrow dispersal kernel for most species, with most offspring settling near to the location where they were produced (Figure 2). This pattern of enhanced local retention (Roberts, 1997) can be explained by active, as opposed to passive, mechanisms limiting the distances that most offspring disperse. Reef fish can employ a suite of spatial, temporal, and behavioral adaptations that interact with local oceanographic conditions to enhance local retention of larvae (Kingsford et al., 2002; Sponaugle et al., 2002; Figure 2).

In the passive model, larval density is best approximated as a normal distribution generated by a random-walk model of movement away from natal reef. Local retention of larvae is fairly evenly balanced with widespread dispersal. This pattern is produced if adult fishes do not spawn preferentially near ocean currents that would keep larvae close to shore, if spawning occurs regardless of lunar phase, and if larvae drift passively with ocean currents once spawned. In the active model, however, local retention of larvae is enhanced, and widespread dispersal is substantially reduced (to about 10% of offspring). This pattern can arise from spawning aggregations near ocean currents that retain larvae near the coast (Lobel, 1989), from spawning timed to a particular lunar phase, and from active swimming behaviors of larvae that allow them to respond to stimuli from their natal reef (Gerlach et al., 2007). It can be difficult to resolve the spatial and temporal scales at which each factor is most important, but this is critical to understanding marine population connectivity, since the interaction of life history and oceanography produces an emergent pattern that is not obvious with empirical genetics work alone (Cowen et al., 2006; Kool et al., 2010).

Dispersal in general is important in selecting habitat, finding unoccupied sites to settle, escaping locally deteriorating environments, founding new populations, and bet hedging on the part of the parental population (Strathmann et al., 2002). However, none of these objectives requires long-distance dispersal, and many actually can be accomplished more successfully by dispersing shorter distances. For instance, long-distance dispersers do not make the best colonizers of new habitat. They may be able to reach new areas more often than shorter distance dispersers, but once they arrive, their highly dispersive larvae will simply be exported from the site (Strathmann et al., 2002). Marine populations therefore achieve closure by developing life histories that retain offspring as members of the reproductive unit (Strathmann et al., 2002).

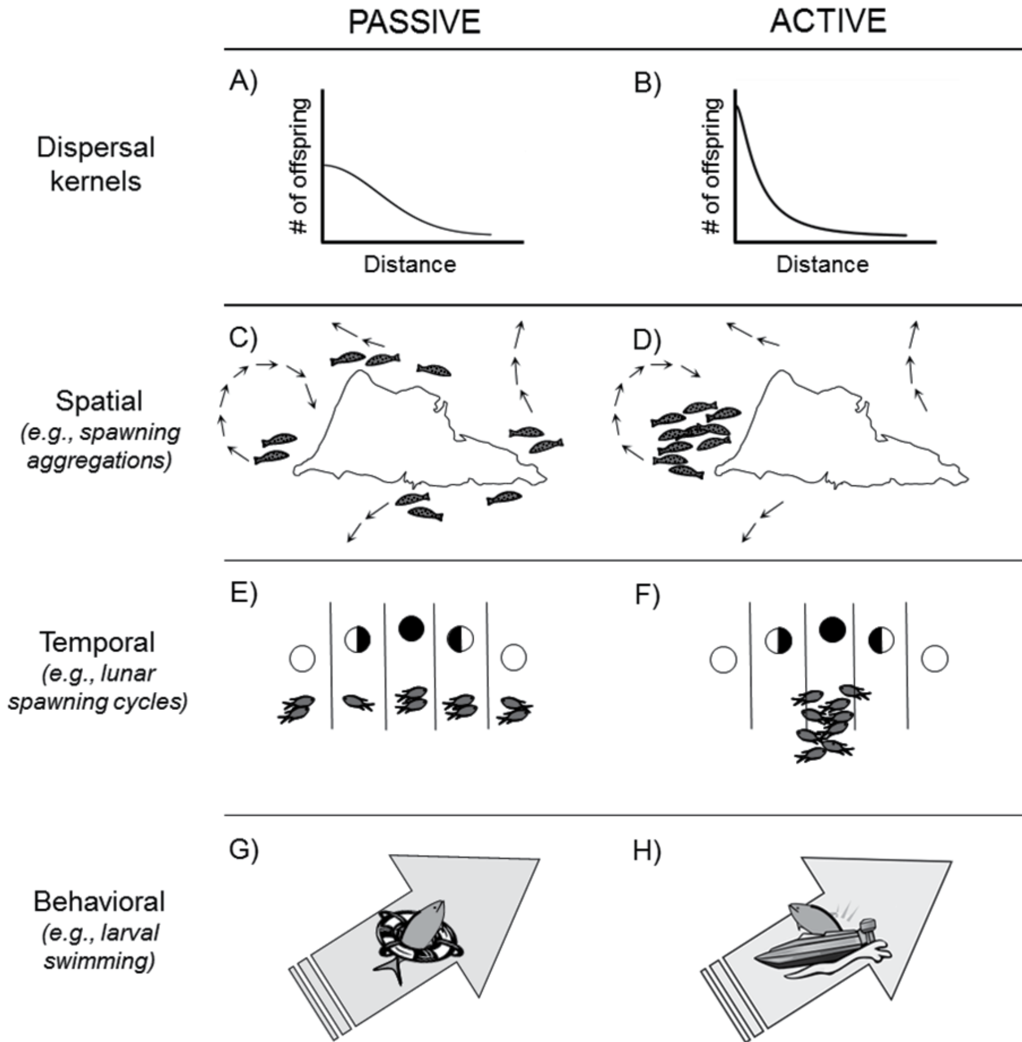

Fig. 2. Passive versus active models of dispersal in coral reef fish. (A & B) Dispersal kernels describing the density of offspring with increasing distance from a natal reef. Active processes tend to increase the density of offspring near to the source reef (as noted by leptokurtic distribution). A number of species-specific mechanisms can generate limited dispersal. (C & D) Spawning fish can aggregate in areas near favorable ocean currents, for example retention eddies. (E & F) Adult fish can time spawning during times of slack tides, reducing advection of larvae from natal reefs. (G & H) Larval fish can employ active behaviors (e.g., ballooning across depth strata or swimming) to maintain position near to natal reefs.

If local retention of larvae is so important, why then do about 10% of reef fish larvae disperse far from home? A traditional explanation of widespread dispersal suggests that it

could result from the relatively long larval life of many reef fishes, stepping-stone dispersal, travel by larvae in major currents and coastal countercurrents, and/or changes in the direction of major currents over time scales of hundreds to thousands of years (Shulman & Bermingham, 1995). In contrast, widespread dispersal in reef fishes could also simply be a byproduct of selective pressures that actually favor dispersal over shorter distances, closer to the parental population (Strathmann et al., 2002). For instance, the pelagic phase could have itself developed as an ontogenetic migration into the water column so that larvae can avoid demersal predators, access richer food resources, and/or escape parasites (Johannes, 1978; Swearer et al., 2002). The relatively small percentage of widely dispersing larvae therefore represents "the ones that got away", perhaps carried off by ocean currents. Widely dispersing larvae therefore comprise the tail of a dispersal distribution centered around the parent population. Wide dispersal could also represent an intentional temporary migration away that is followed by a return back to the source area. In other words, larvae that are transported away from their parents will not necessarily settle away from their parents (Strathmann et al., 2002). Even if initially transported several hundred kilometers away, the majority of larvae will recruit close to home (Planes, 2002; Strathmann et al., 2002).

As discussed in earlier sections, genetic similarity between populations can be reinforced by as few as one migrant (or larval propagule) per generation (Planes, 2002). The very existence of long-distance dispersal therefore has genetic consequences, perhaps leading to homogeneity over a large area. However, this model of dispersal posits that demographic (and therefore, ecologically relevant) consequences are driven primarily by patterns of local dispersal and subsequent recruitment (Strathmann et al., 2002).

4.1 What defines "home"?

Though the 90% close to home/10% far away model of reef fish larval dispersal may be generally true, the geographic distance defining "close to home" depends on the interaction between life history and oceanography, which in turn depends on location and taxa. For instance, even in the absence of any apparent barriers, some Pacific reef fishes with a high dispersal capacity demonstrate high genetic divergence among populations (Fauvelot & Planes, 2002; Planes et al. 1996; Planes & Fauvelot, 2002; Riginos and Victor, 2001). This may be related to the degree of isolation of Pacific islands, which makes them good candidates for studies of elevated self-recruitment and other dispersal patterns (Planes et al., 1996; Treml et al., 2008). Because Pacific islands are so far away from each other relative to Caribbean islands, it is logical to expect that local (within-island) retention on ecological scales is more important in the Pacific than in the Caribbean, since longer distance dispersal is unlikely to be sufficiently great to allow larvae to settle at a very distant island. In other words, "close to home" for a reef fish in the Pacific may be at the within-island or within-reef scale, where "close to home" in the Caribbean may be among several relatively closely spaced islands or reef habitats. In addition, the definition of "close to home" is also variable among taxa. Because damselfish and clownfish are brooders, their "close to home" is their coral territory or anemone, respectively. What qualifies as "home" is likely to cover a wider area for species that spawn into the water column. Furthermore, life history traits such as egg size, larvae size, pelagic larval duration, and larval growth rates can vary within reef fish families among different ocean basins (Thresher and Brothers, 1989), supporting the idea that life history traits related to reproduction have evolved to optimize local oceanographic conditions.

5. Connectivity is relevant to the management and conservation of coral reef ecosystems

Coral reefs, which have been called the rainforests of the sea (Connell, 1978), are some of the most biodiverse and productive ecosystems on the planet. They contain an estimated 25% of all marine species, including thousands of species of fish. Millions of marine species live primarily or exclusively in association with coral reefs. In addition, the economic, social, and cultural importance of coral reefs to humans cannot be overestimated. Over 1 billion people worldwide depend directly on reef resources such as fish, creating an intimate link between reef ecosystem functionality and human wellbeing.

Despite their immense biological and ecological significance, as well as their demonstrated importance to humans, coral reefs are one of the most highly impacted ecosystems on the planet (Halpern et al., 2008). Stressors such as climate change, overfishing, and pollution are causing distributional shifts and biodiversity loss in coral reef ecosystems in many regions of the world (Birkeland, 2004; Graham et al., 2006, 2007; Hughes et al., 2003; Jones et al., 2004; Munday, 2004; Munday et al., 2008; Pandolfi et al., 2003; Wilson et al., 2006, 2008, 2009). In the face of these numerous threats, an improved knowledge of evolutionary and ecological patterns of reef fish connectivity is needed in order to design effective marine protected areas that both conserve biodiversity and enhance fisheries (Almany et al., 2007, 2009; Jones et al., 2009; Palumbi, 2003; Russ, 2002; Sale et al., 2005). In addition, identifying ecological connectivity patterns between fragmented populations can indicate the resilience of species and ecosystems to changing environmental conditions (Jones et al., 2009, 2010; McCook et al., 2009; Planes et al., 1996; Steneck et al., 2009). For instance, because dispersal sustains populations with new recruits (Shanks et al., 2003), high connectivity can both buffer populations from local extirpation and, in the event of a population decline, facilitate post-disturbance recovery.

6. Conclusions and directions for future research

Reef fish populations are heterogeneously distributed in space and time, reflecting both the evolutionary origins and ecological maintenance of distribution patterns. Biogeography can be used to inform contemporary patterns of distribution and dispersal. The recognition that reef fish populations could experience high levels of local recruitment and limited larval dispersal was an important paradigmatic shift that refocused studies onto understanding the degree of connectivity among populations, which has important ecological and evolutionary implications. The fact that reef fish dispersal occurs primarily via larval propagules, however, means that connectivity has resisted easy quantification. In order to achieve a more complete picture of population connectivity patterns among coral reef fishes, it must be a research priority to better understand the spatial and temporal variability in larval dispersal and recruitment dynamics across taxa, as well as across ocean basins. This will necessarily involve the development of more sophisticated biophysical models that should be used in conjunction with empirical approaches. Methods that relate genetic structure to estimates of dispersal have been useful, but due to their limitations, they represent only a fraction of the suite of tools that will be necessary for resolving demographically relevant connectivity patterns in reef fishes. A generalized model of dispersal distance based on our current understanding of these patterns proposes that the majority of larvae stay close to home rather than dispersing widely.

Future research should also bear in mind the influence of anthropogenic factors on connectivity patterns in marine populations. Climate change-related increases in ocean temperatures and changes in weather patterns are projected to alter the speed and direction of major currents, which will affect the physical transport of larvae (Cowen & Sponaugle, 2009). In addition, increasing habitat fragmentation may play a role in the widely reported local-scale genetic structure in coral reef fishes (Salas et al., 2010), and the benefits of local retention and larval transport are likely to erode in degraded environments (Jones et al., 2009). The disruption of natural patterns of larval retention and connectivity has thus been proposed as a key factor threatening reef fish populations in the coming century (Jones et al., 2009). Given the vulnerability of coral reef ecosystems to global change and other stressors, a more explicit understanding of population connectivity patterns in reef fishes is crucial to their effective management and conservation.

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15

Diversity of Wild Mammals in a Megalopolis: Mexico City, Mexico

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1. Introduction

Mexico City, the capital of Mexico, is located on the Southern-Central part of the country and it is one of the most important megalopolis in the world. This city lies within the Distrito Federal, bordering with the state of Mexico on the north, east and west and with the state of Morelos on the south. It is one of the 32 political divisions of Mexico and the smallest one, comprising an area of 1,485 km^2 (Fig. 1). Mexico City belongs to the Basin of Mexico together with parts of the states of Hidalgo, Puebla, Tlaxcala and México. México City and the rest of the metropolitan area is one of the largest cities in the world with more than 30 million inhabitants (INEGI, 2010), just behind Tokyo, Japan and forward New York and the Philadelphia area, USA (34.3 and 22 respectely; Brinkhoff, 2011). The rapid growth of its population has led to the loss of original habitats and to the transformation of natural sites and therefore and in spite of being considered as a region with high levels of biodiversity to the local extinction of wildlife species. Mammals are one of the most important components of biodiversity, particularly in México. Several inventories and studies have reported the occurrence of mammals in the Valley of Mexico in the past years. Some of the most remarkable contributions to this subject are by far those of Villa-R (1952) and Hall (1981). However, important mammalian information for the Valley of México was also gathered by Ceballos & Galindo (1984) and Villa & Cervantes (2003). Moreover, the paper published by Ramírez-Pulido *et al.* (1986) more than a quarter of a century ago about mammals of the Distrito Federal contributed important information to the knowledge of the species richness of local mammals species. In addition, several papers reports on mammals collected in diverse areas of the Distrito Federal (López-Forment, 1989; Sánchez *et al.*, 1989; Negrete, 1991; Negrete & Soberón, 1994; Castro-Campillo, 1992; Chávez & Ceballos, 1992, 1994; Chávez, 1993 a, b; Álvarez *et al.*, 1994; Monroy *et al.*, 1999; CONANP-SEMARNAT, 2006; Navarro, *et al.*, 2007; Bárcenas & Medellín, 2007; Gómez-Jiménez, 2009). Research papers referriing especially to particular taxonomic groups (Villa-R, 1966; Álvarez & Ramírez-Pulido, 1972; Polaco *et al.*, 2002; Carraway, 2007) also mentioned the presence of mammals in México City and confirmed the outstanding contribution of mammals to the biodiversity of the Distrito Federal.

Despite this large number of reports, the available information on mammals of Mexico City was scattered and incomplete. Therefore, in order to properly documents the species

richness of mammals in Mexico City, to make decisions about its protection, and to prevent a further loss of species due to the urban sprawl that has endangered all existing ecosystems in the Valley of Mexico, a synthesis on the subject as well as an updated inventory of the mammal fauna of this region was necessary. The result of this research will undoubtedly serve also as a tool for the development of programs aimed to the conservation and sustainable use of the biodiversity of México City and the remaining The Distrito Federal region.

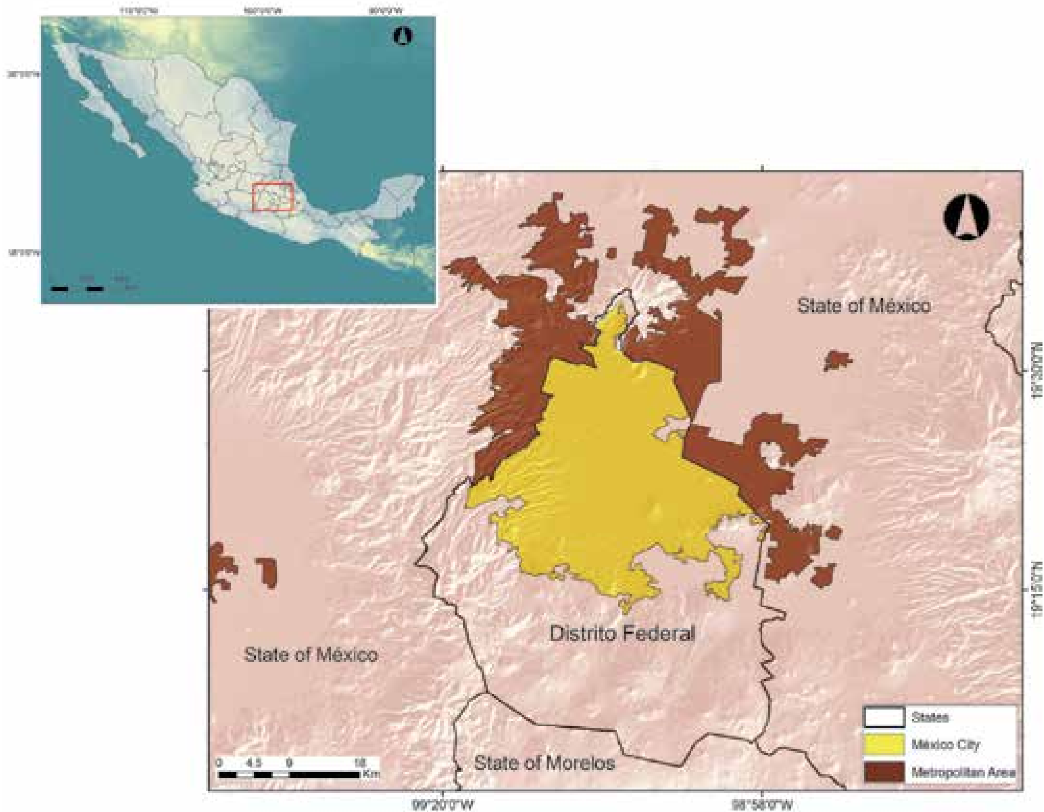

Fig. 1. Mexico City and the rest of the metropolitan area lie in the northeastern and central Distrito Federal.

1.1 Topography

The Distrito Federal topography is defined by a valley whose average altitude is 2,400 m, surrounded by high mountains whose highest elevation 3,930 m- is located on the Ajusco volcano. This city is surrounded by the mountains of Sierra de Guadalupe (2,780 m) and Cerro del Tepeyac in the northern part of the entity. In the central-eastern part is bordered by the mountains of Santa Catarina and Cerro de la Estrella, whereas the mountain range of the Sierra de las Cruces and the volcanoes Ajusco, Xitle, Chichinautzin, Teuhtli and Tláloc are located in the southern part of México City. This mountainous system belongs to the Neovolcanic Axis, also known as Sierra Ajusco-Chichinautzin. The topography of the Valley of México also includes hills such as Peñón de los Baños, Peñón Viejo and Cerro de Chapultepec.

A few years ago several permanent rivers and streams flowed through The Distrito Federal, including the rivers Magdalena, La Piedad, Becerra, Michoacán, Tacubaya, Churubusco, Consulado, San Joaquín, and Los Remedios. They used to drain into the ancient Texcoco Lake; however, today they are intubated and run under streets and avenues; the water of some streams is stored in dams such as Anzaldo and Canutillo. Today the only remaining lakes in the Valley of Mexico are Zumpango, Texcoco, Xochimilco and Chalco.

1.2 Climate

The Distrito Federal climate ranges from mild to cold and wet alpine tundra in the higher parts of the southern mountains. The urban area has a rainy temperate climate, with temperatures ranging from 0 ° C at the beginning of the year to 28 ° C in late spring, being the Ajusco ridge having the coldest one. The wet season runs from May to November, with most rainfall between June and August, with a different pattern because of the altitude. The northern part of the Distrito Federal is dry and warm in contrast to the southern part that is rather mesic due to the presence of forested mountains.

1.3 Vegetation

The Distrito Federal has approximately 149, 830 ha, and depending on the land use and population activities, the land is divided into two areas: urban land and conservation land. The former is found in the northeastern part of the entity which corresponds to Mexico City, whereas the latter comprises about 59% of the Distrito Federal and is located in the southwest section of this entity (SEMARNAT, 2010; Rivera & Espinosa, 2007). Natural vegetation is still found in half of the conservation land, while the rest correspond to agricultural and urban land According to Rivera & Espinosa (2007) the Distrito Federal has the same six types of vegetation described for the Basin of Mexico (Reiche, 1926; Miranda & Hernández, 1963; Rzedowski *et al.*, 2001). The vegetation map and soil conservation followed CONABIO (1999) and the vegetation description is after to Rivera & Espinosa (2007). Another two types of vegetation ocurring in the Distrito Federal are the cloud forest and the aquatic and subaquatic vegetation, but they do not appear in maps due to their small extension and scale restrictions.

1.3.1 Coniferous forest: Oyamel (*Abies religiosa*)

The tree stratum composed by oyamel *Abies religiosa* is scarce but *Roldana angulifolia* prevails, and sometimes *Ribesciliatum*, *Symphoricarpos microphyllus*, *Cestrum anagyris*, *Solanum cervantesii* and *Physalis coztomatl*, among others. The herbaceous stratum is composed by *Arracacia atropurpurea*, *Sigesbeckia jorullensis*, *Alchemilla procumbens*, *Stellaria cuspidata* and *Euphorbia furcillata*. Almost all the forests in this region show different stages of damage and a large part of the original landscape has become urbanized. The high demographic density imposes a negative pressure against these resources, principally on forest management issues (wood extraction), creating spaces for urbanization, agricultural management or pastures for cattle management (cows and sheep). Oyamel forests are mainly located in the western part of The Distrito Federal, in the following political delegations: Cuajimalpa, Álvaro Obregón and Magdalena Contreras (Cañada de Contreras); there are also small spots in the political delegations of Tlalpan and Milpa Alta. This type of vegetation is mainly located at altitudes between 2500 and 3500 m (Rivera & Espinosa, 2007).

1.3.2 Coniferous forests: Pines (*Pinus*)

This kind of vegetation is composed of pine trees: Pinus hartwegii, P. montezumae, P. pseudostrobus, P. teocote, P. rudis, besides Salix paradoxa and Juniperus monticola, along with other tree species such as Alnus jorullensis, Quercus laurina, Arbutus xalapensis and shrubs such as Buddleja cordata. There is practically no vegetation strata at high altitudes whereas at lower altitudes we have Solanum cervantesii and Barkleyanthus salicifolius wich are notorious, Alchemilla procumbens, Salvia prunelloides, Stipa ichu, Muhlenbergia quadridentata and Festuca tolucensis are abundant in places where bunch grasslands conform the herbaceous stratum, these grasslands are quite frequent, also containing dominant grasses like Muhlenbergia macroura, M. quadridentata, M. robusta, Festuca tolucensis and F. amplissima, together with the herbs Penstemon tenuiflora, Piqueria trinervia and Zephyranthes fosteri, among others. This is the most extended kind of vegetation in the Distrito Federal at altitudes between 2700 and 3800 m, and it mainly occurs in the following political delegations: Cuajimalpa, Álvaro Obregón, Magdalena Contreras, Tlalpan and Milpa Alta (Rivera & Espinosa, 2007).

1.3.3 Oak (*Quercus*) forest

The dominant oak species are: Quercus rugosa, Q. laeta, Q. crassipes and Q. castanea. In smaller numbers we find: Q. obtusata, Q. candicans, Q. crassifolia and Q. dysophylla. They are frequently found with Pinus leiophylla, P. rudis, Clethra mexicana, Arbutus xalapensis, Garrya laurifolia, Prunus serotina ssp. capuli and Ceanothus coeruleus. The shrub stratum is abundant, with the most common species being: Solanum cervantesii, Cestrum anagyris, Monnina ciliolata, Bouvardia ternifolia, Acaciella angustissima and Croton adspersus. In the herbaceous stratum it is common to find Penstemon roseus, Peperomia campylotropa, Polygala alba, Castilleja tenuiflora and Ageratina pazcuarensis, among others. The presence of climbing plants such as Smilax mora-nensis, Dioscorea galeottiana, Passiflora exsudans, Clematis dioica and Bomarea hirtella is common in this forest. Oak forests are located in the political delegations of: Cuajimalpa, Álvaro Obregón, Magdalena Contreras, Xochimilco, Gustavo A. Madero, at altitudes between 2300 and 3000 m. Some spots of these plant species in Milpa Alta and Tlalpan, are located at altitudes of 3300 m (Rivera & Espinosa, 2007).

1.3.4 Sarcocrassicaule (Xerophitic) ticket

Xerophytic thicket and say it is located in the driest areas of the Distrito Federal between 2300 and 3060 m (Rivera & Espinosa, 2007). The dominant species is *Pittocaulon praecox*. Others species are: *Buddleja cordata*, *Dodonaea viscosa*, *Montanoa tomentosa*, *Schinus molle* and *Wigandia urens*, all of which form a rich tree stratum. Some of the abundant shrubs are represented by *Verbesina virgata*, *Bouvardia ternifolia* and *Sedum oxypetalum*. In the herbaceous stratus we can find: *Commelina coelestis*, *Arracacia tolucensis* var. *multifida*, *Anagallis arvensis*, *Begonia gracilis*, *Muhlenbergia robusta*, *Pseudognaphalium oxyphyllum*, *Asclepias linaria*, *Dahlia coccinea*, *Sarcoglottis schaffneri*, *Lepechinia caulescens* and *Manfreda pringlei*. These thickets are located in the political delegations of Gustavo A. Madero (Sierra de Guadalupe), Iztapalapa (Cerro de la Estrella and Sierra de Santa Catarina), Tláhuac (Sierra de Santa Catarina), Tlalpan (Pedregal de San Ángel), Xochimilco and Milpa Alta.

1.3.5 Halophytic and gypsophytic vegetation

These are located in the southern part of the Valley of Mexico, in soil with high amounts of salt and alkaline within areas affected by constant flooding. Some of the typical species of

this type of vegetation are *Distichlis spicata*, *Muhlenbergia* spp. and *Atriplex* spp. (CONABIO, 1999).

1.3.6 Cloud forest

This type of vegetation occurs only in a very small area in the Cañada de Contreras or Cañada de los Dínamos, the National Park Desierto de los Leones, the Parque Ecológico of the Mexico City and other spots with sharp edges and in the bottom of some ravine, between 2500 and 2700 m. Among the dominant species of trees we can find *Clethra mexicana*, *Cornus disciflora*, *C. excelsa*, *Meliosma dentata*, *Symplocos citrea*, *Viburnum stenocalyx*, *Rhamnus mucronata*, *Sambucus nigra* var. *canadensis* and *Quercus laurina*. We can also find *Abies religiosa*, *Cupressus lusitanica*, *Prunus serotina* ssp. *capuli* and *Quercus rugosa*. In the shrub stratus, we can find *Archibaccharis asperifolia*, *Cestrum anagyris*, *Ageratina aschenborniana* and *Iresine ajuscana*. At the edge of creeks, we can find the following species: *Acer negundo* var. *mexicanum*, *Ilex tolucana* and *Alnus acuminata*. Climbing plants are *Valeriana clematitis*, *Philadelphus mexicanus*, *Lonicera pilosa*, *Archibaccharis hirtella*, *Solanum appendiculatum*, *Clematis dioica*, *Didymaea alsinoides* and *Smilax moranensis*. *Tillandsia andrieuxii*, *T. violacea*, *Peperomia galioides* are also found in this forest. Different kinds of ferns are common in the herbaceous stratum, along with *Bidens ostruthioides* and *Peperomia hispidula* (Rivera & Espinosa, 2007).

1.3.7 Aquatic and subaquatic vegetation

The Broad-leaf cat-tail, Typha latifolia is the most abundant species, though Polygonum amphibium, Cyperus semiochraceus, Hydrocotyle ranunculoides, Pistia stratiotes, Berula erecta, Hydromystria laevigata and Jaegeria bellidiflora are also common in the edges of Xochimilco canals and ditches as well as in other spots of The Distrito Federal. Floating vegetation is very common in these places, and it mainly consists of thick layers of Lemna spp. and Azolla filiculoides, which occasionally cover all the canals. These canals are mainly located in the political delegations of Xochimilco and Tláhuac, at altitudes of 2250 m, on the slopes of the southern mountains of The Distrito Federal (Rivera & Espinosa, 2007).

1.3.8 Agricultural, cattle and forestal management

The agricultural areas are located in the southern and southeastern part of The Distrito Federal, and they were created as a result of deforestation. There are induced pastures most of them derived from bunch grasslands also called alpine or subalpine bunch grassland and are associatted to the *Pinus hartwegii* forest.

These pastures are maintained by the continuous shepherding of cattle and by controlled burning, which stimulate their growth during the rainy season. Some representative species of these grasses important in pastures for livestock are: *Bouteloua* spp. *Aristida* sp. *Muhlenbergia macroura, Festuca tolucencis*, *F. amplissima* and *Stipa ichu.* Most agricultural zones are seasonal, where we may also find corn, bean, chile, oats, broad bean and nopal. In the lowest areas of The Distrito Federal there are other pastures of secondary origin, commonly mixed with the xerophitic bushes. There may occur *Aristida adscensionis*, *Bouteloua simplex* and *Hilaria cenchroides*, isolated specimens of *Schinus molle* and *Mimosa biuncifera*. This vegetative association is located in the political delegations Cuajimalpa, Álvaro Obregón, Magdalena Contreras, Tlalpan and Milpa Alta, between 2800 and 3860 m. In some areas like Xochimilco the cultivation of vegetables and floriculture is important (SEMARNAT, 2010; Rivera & Espinosa, 2007).

2. Materials and methods

To create an updated inventory of mammals of Mexico City we gathered data from collecting work in the field, from visiting mammalian collections in the Valley of Mexico, and from consulting the literature and databases available in web sites. Collected specimens were conventionally prepared as museum specimens and taxonomically determined following conventional identification keys (Hall, 1981; Medellín *et al.*, 1997; Villa & Cervantes, 2003). Voucher specimens were stored and catalogued in the mammalian collection "Colección Nacional de Mamíferos (CNMA)" of Instituto de Biología, Universidad Nacional Autónoma de Mexico, at Mexico City.
All resulting data were stored and managed in a database created in Microsoft Access 2003, where were incorporated 50 fields of geographical and biological information according to the guidelines of Darwin Core (Ver. 3.0) and MaNIS/HerpNet/ORNIS Georeferencing Guidelines (Wieczorek, 2001).

2.1 Updated list, distribution and conservation status

The list of the inventory was elaborated only with taxa adequately documented, at least with one voucher specimen cataloged in a biological collection. An exception to this was the coyote, *Canis latrans* (Aranda, 2010a; Farías, 2010) and *Nasua narica* (Aranda, 2010b). The nomenclature and classsification at species level we followed was that by Wilson & Reeder (2005), while for subspecies level we consulted the list of Ramírez-Pulido *et al.* (2005). For taxa of the family Heteromyidae and Soricomorpha we followed Hafner *et al.* (2007) and Carraway (2007) respectively. We reported the category of extinction risk of the taxa according to both the Norma Oficial Mexicana 059 (SEMARNAT, 2010), and the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). The endemic condition (E) of a species and its condition as a monotypic or polytypic taxon is indicated (SEMARNAT, 2010; Carraway, 2007).

2.2 Collecting locaties

Specimens collecting locaties were verified because many were wrong, incomplete, or their names were ambiguous. Therefore, it was necessary to check maps, gazeteers, literature, field diaries and catalogues to accurately identify the localities referred. The names of the locaties were standardized and the geographic coordinates were calculated using a conventional guide for georeferencing (Wieczorek, 2001); topographic maps at scales 1:50,000 and 1:100,000 were used (INEGI, 2001; Secretaría de Comunicaciones y Transportes, 1987, respectively); in those cases when the data were taken directly from a Global Positioning System (GPS) unit (Garmin Co. Inc.). The information obtained was visualized using Arcview software and the digital map of The Distrito Federal was provided by the Unidad de Informática para la Biodiversidad (UNIBIO).

3. Results

The results produced 5,724 records of mammals from Mexico City in The Distrito Federal and some near localities bordering on the other states confoming the Valley of Mexico. The specimens correspond to different preservation types such as skin, skull, skeleton and alcohol cataloged in 17 mammalian collections, seven of them are domestic and ten from the United States of America. The collection name, acronym (Hafner *et al.*, 1997; Lorenzo *et al.*,

2006) and the number of specimens held are as follows: Colección Nacional de Mamíferos, Instituto de Biología, UNAM, (CNMA, 1,622); Universidad Autónoma Metropolitana, Iztapalapa, (UAMI, 1,489), Colección de Cordados, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, (ENCB, 951), Colección de Fotocolectas Biológicas del Instituto de Biología, UNAM (CFB: FB, 43); Museo de Zoología "Alfonso L. Herrera", Facultad de Ciencias, UNAM, (MZFC, 33), Instituto Nacional de Antropología e Historia (INAH, 12); Museo Dugès, Universidad de Guanajuato, Gto, Mexico (MADUG-MA,1); University of Michigan, Museum of Zoology (UMMZ 567); Smithsonian Institution National Museum of Natural History (USNM, 421); University of Kansas, Museum of Natural History (KU 270); The Field Museum of Natural History (FMNH, 139); Brigham Young University, Monte L. Bean Life Science Museum (BYU, 62); University of California, Berkeley, Museum of Vertebrate Zoology (MVZ, 48); Michigan State University Museum (MSU, 29); Yale University Peabody Museum (MAM, 23); Texas Tech University, Museum of Texas Tech University (TTU, 9); and Harvard University Provider (MCZ, 5). The database containing the whole data set will be available through the CNMA at the web site of Instituto de Biología, Universidad Nacional Autónoma de Mexico (http//unibio.ibunam.mx).

3.1 Collected specimens by species and collecting periods

The number of specimens cataloged by mammalian collection varied from 1 to 1,622; the collection period recorded was 1830 – 2011, excluding several years with no collecting. Some years have few collectings (1 – 25 specimens) whereas other years show intense collecting activity: 1892 (240), 1944 (359), 1947 (468), 1949 (196), 1980 (226), 1985 (210) and 1996 (485), 1997 (482), 1998 (264), 1999 (280). The decades of the 1940s and 1990s correspond to periods of the greatest number of specimens collected (Fig. 2). On the other hand, the oldest mammal records for the Distrito Federal correspond to the following carnivores: *Bassariscus astutus astutus (B[assaris] astuta ,* Lichtenstein, 1830), **Mustela frenata* (Lichtenstein, 1831), **Procyon lotor hernandezii* (*Pr*[*ocyon*] *hernandezii ,* Wagler, 1831), and the skunk **Mephitis macroura* (Lichtenstein, 1832). The first and second specimens were collected near Mexico City, the third specimen in Tlalpan, and the last one in the hills NW of Mexico City.

There are also four species of bats with old records, a specimen of *Nyctinomops macrotis (Nyctinomus drepressus*, Ward, 1891*;* 9246 CNMA*)* that was collected in Tacubaya in 1887). A second specimen of this species was collected in Mexico City´s Cathedral (270 MADUG-MA),there is no date on the label but the collector´s notes (Dugès)indicate that it was collected between 1870-1910; it also indicates that the museum only keeps this mammal from the Distrito Federal. From the Iztapalapa region we have*Tadarida brasiliensis*, *Molossus ater* (Herrera, 1895) and *Myotis velifer* and two from Valley of Mexico (in USNM and MCZ respectely; Miller & Allen, 1928), all bat specimens are kept in alcohol. The analysis of the data-base generated shows that 1892 was the year with the highest number of new records from The Distrito Federal, with a total number of 25, some of which are: one marsupial *Didelphis virginiana,* two rabbits, *Sylvilagus cunicularius* and *S. floridanus*, two shrews,*Cryptotis parva* (50762 USMN **Blarina soricina,* Merriam, 1895) and *C. alticola*; one bat *Tadarida brasiliensis* and two carnivores, *Spilogale putorius* (50825 USMN, **Spilogale a. angustifrons,* Howell, 1902). After a period of 51 years in which only two more species appear, in 1943 there are again new records of species with a slow but constant increase, with a maximum number of 6 species in one year. (Fig. 3). In addition, our data set shows that approximately 170 collectors have contributed the specimens collected in Mexico City and surroundings. Similarly, the representation of specimens by species found was highly

variable (1 – 1,175; Table 1). As expected, few species (6 taxa, 7.4%) were represented by a high number of specimens, particularly the mouse species, such as *Peromyscus melanotis* (1,175), *Peromyscus gratus gratus* (649), *Neotomodon alstoni* (512), *Microtus mexicanus mexicanus* (456), *Peromyscus difficilis felipensis* (354), *Peromyscus maniculatus labecula* (236). In contrast there were 14 species represented only by one specimen: one shrew (*Sorex orizabae*), 10 bats (*Artibeus lituratus palmarum*, *Pteronotus parnelli mexicanus*, *Natalus stramineus saturatus*, *Eumops perotis californicus*, *Molossus rufus*, *Nyctinomops laticaudatus ferruginea*, *Lasiurus intermedius intermedius*, *Corynorrinus townsendii australis*, *Idionycterys phyllotis* and two carnivores, *Nasua narica* and *Taxidea taxus berlandieri*.

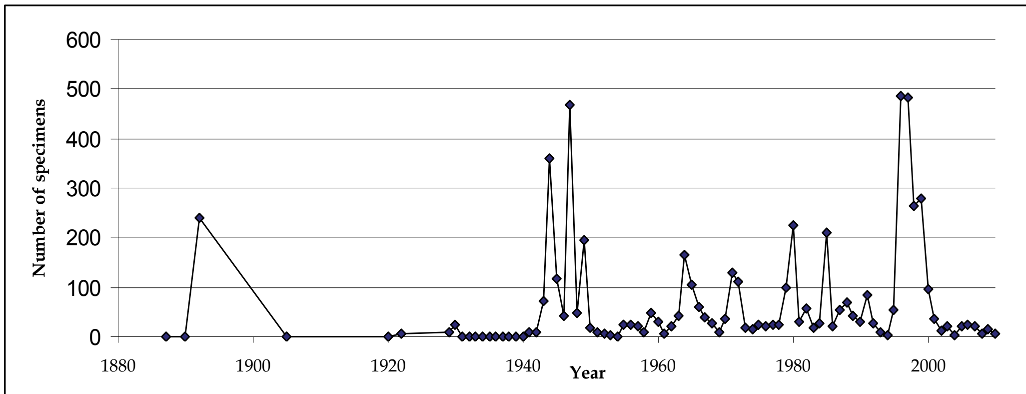

Fig. 2. Representation of the number of specimens collected by year in Mexico City and other localities of Distrito Federal.

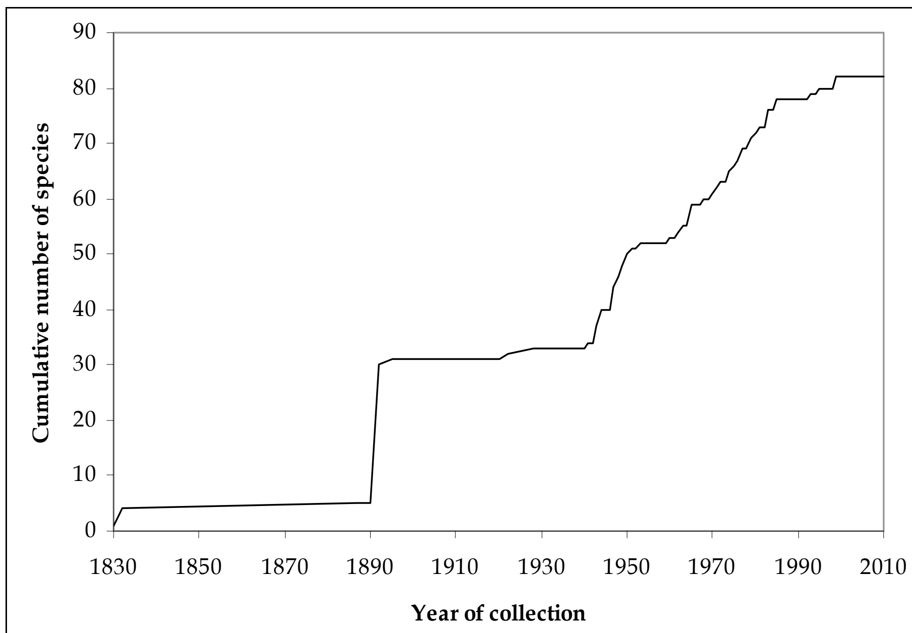

Fig. 3. Accumulation curve of mammal species registered in Mexico City and other localities of the Distrito Federal from 1830 to 2011.

ORDER DIDELPHIMORPHIA
Didelphis virginiana californica (62): **CNMA:** 5, 437-438, 796, 3785-3787, 4165, 4254, 8736, 11305, 16491, 16863, 23070, 30722-30723, 31342, 33417, 34642-34645, 34780, 34858, 37131, 40199, 42908-42910, 43372-43377, 44049-44054, 44085, 45113-45116; **ENCB:** 339, 5426, 10942, 40020, 40730, 41531, 42380, 43363; **MZFC:** 3465, 3468; **KU:** 27979, 66268; **UAMI:** 5171; **USNM:** 50062-50064.
ORDER CINGULATA
Dasypus novemcinctus mexicanus (5): **CNMA:** 16738**; CFB-FB**: 1699, 1732; **KU:** 28280, 28281.
ORDER LAGOMORPHA
Romerolagus diazi (172): **CNMA:** 341-350, 353-358, 401, 3800-3803, 4166, 6797, 12479-12486, 13504, 14577, 15221-15222, 16888-16951, 16955-16958, 16960, 16962, 18287-18288, 26427, 26430-26431, 28525-28526, 30731, 34335-34339, 34824, 34840, 34856, 35586, 39776-39777, 45929-45930; **CFB-FB**: 1997; **ENCB:** 1468-1472, 10244-10249, 19283; **INAH:** 1558, 4381; **KU:** 30815, 102028-102033; **MCZ:** 59284; **MZFC:** 465-469; **UMMZ:** 96526, 103246, 111225-111226, 112316-112318; **USNM:** 143611, 146901, 146918-146919, 146935-146941.
Sylvilagus cunicularius cunicularius (21): **CNMA:** 1063, 16525, 16739, 16744-16745, 18294, 18296-18299; **ENCB:** 5354; **KU:** 28277; **USNM:** 50069-50071, 50073-50074; **UAMI:** 2757, 8226-8227, 8341.
Sylvilagus floridanus orizabae (77): **CNMA:** 1056-1062, 1064-1069, 1133, 7166, 16740-16743, 16876-16884, 18289-18293, 19595-19596, 19602, 30732, 34841, 44513-44522, 44091-44092; **CFB-FB**: 1709; **KU:** 28273-28274; **UAMI:** 8228-8230, 8342-8350, 13196-13197; **UMMZ:** 112315; **USNM:** 50072, 50075-50080, 51111, 143612.
ORDER SORICOMORPHA
Cryptotis (1): **CNMA** 45805.
Cryptotis alticola (12): **CNMA:** 951, 6118; **UAMI:** 13208*, 13209, 13210*-*13212,* 14610, 14611*, 14612, 14619*; **UMMZ:** 93367*, *94597**; **USMN***: 50763.*
Cryptotis parva soricina (5): **CNMA:** 439; **ENCB:** 29010; **UAMI:** 5; **USNM:** 50760, 50762.
Sorex (2): **CNMA** 45806; **CFB-FB**: 2404.
Sorex oreopolus (6): **CNMA:** 440, 19591, 31955; **UAMI:** 13245, 13247, 14613.
Sorex orizabae (1): **ENCB:** 5316**.
Sorex saussurei saussurei (103): **CNMA:** 847, 12791, 12792, 42911, 42912, 43368-43371, 44055-44062; **ENCB:** 581, 41701; **UAMI:** 13237*, 13238, 13239-13244*, 13246*, 13248*, 13249*, 14614*, 14615*, 14617*, 14618*, 14620*-14644*, 14646*, 14648*-14655*, 14657*, 14658**, 14659-14661*, 14663-14669*, 14671-14682*; **UMMZ:** 88635-88639, 88640*, 91606, 91902-91903, 94586; **USNM**: 143604.
Sorex ventralis (19): **ENCB:** 1490*(**), 2344-2345*(**), 2389- 2391*(**), 5229*(**), *16479-16481*(**)*; **UAMI:** 14683-14685*(**), 15100-15105*;* **JRP***: 3979-3980*.*
Sorex veraecrucis altoensis (Nueva especie) (9): **CNMA:** 790, 9059, 38637; **UAMI:** 14616, 14645*(**), 14647*(**), 14656*(**), 14662*(**), 14670.
ORDER CHIROPTERA
Anoura geoffroyi lasiopyga (10): **CNMA:** 3921, 15478, 16965, 22570, 34646, 42768-42769; **USNM:** 559548-559550.

Choeronycteris mexicana (16): **CNMA:** 15485-15486, 16865, 42760-42767, 44063-44066; **ENCB:** 41209.
Glossophaga soricina handleyi (38): **CNMA:** 441-456, 2627-2632; **KU:** 27980-27992; **YPM:** 3771-3773
Leptonycteris (14): **YPM:** 4252-4256, 4262, 11021-11022, 10766-10767.
Leptonycteris nivalis (4): **CNMA:** 4201, 4729, 4731, 15471.
Leptonycteris yerbabuenae (44): **CNMA:** 457-472, 478-479, 8972, 27782-27783, 34647, 42770-42771; **ENCB:** 18868; **UAMI:** 5210; **KU:** 27995-28012.
Macrotus waterhousii mexicanus (3): **CNMA:** 27784-27785, 37258.
Artibeus lituratus palmarum (1): **UAMI:** 1862.
Mormoops megalophylla megalophylla (7): **CNMA:** 3410, 3928, 9886, 10621, 27780, 37259; **ENCB:** 472.
Pteronotus parnellii mexicanus (1): **CNMA:** 3409.
Natalus stramineus saturatus (1): **CNMA:** 27781.
Eumops perotis californicus (1): **CNMA:** 16866.
Molossus ruffus (1): **CNMA:** 5212.
Nyctinomops laticaudatus ferruginea (1): **UAMI:** 4858.
Nyctinomops macrotis (29): **CNMA:** 2626, 3919, 3925-3927, 4197, 8739, 8973, 9246, 11497-11498, 17035, 39349, 40871-40873, 44093; **ENCB:** 30502, 39122; **MADUG-MA:** 270, **MZFC:** 1339, 6684-6685; **UAMI:** 3160, 3832, 4858, 5260, 8361-8363;
Tadarida brasiliensis mexicana (64): **CNMA:** 477, 1054, 1747, 3922-3923, 4809, 7907, 9874, 10068, 11495, 13444-13445, 14878, 15562, 15580, 15642, 18531, 45935; **ENCB:** 4468-4470, 7601, 18845, 30501, 41844; **FMNH:** 8807-8808, 15124-15126; **INAH:** 6064; **KU:** 28298; **MZFC:** 3308, 5648; **USNM:** 50814-50819, 52454-52468; **UAMI:** 1622, 4857, 10410-10412, 12075.
Eptesicus fuscus miradorensis (28): **CNMA:** 3930; **ENCB:** 3730, 40672, 40695-40704, 41575-41586, 42208-42209; **UAMI:** 5234.
Lasiurus blossevillii teliotis (9): **CNMA:** 3924; **ENCB:** 40705-40708, 41587-41588, 41825-41826.
Lasiurus cinereus cinereus (19): **CNMA:** 493-494, 7679, 10682, 30150, 40426; **ENCB:** 1235, 2043, 5187, 27932, 36833, 40085, 41827-41829, 42196, 42210; **UAMI:** 11969; **UMMZ:** 91904.
Lasiurus ega panamensis (2): **CNMA** 45931; **UAMI:** 885.
Lasiurus intermedius intermedius (1): **CNMA:** 16061.
Corynorhinus mexicanus (27): **CNMA:** 7358, 9484, 9718, 10686, 28350; **CFB-FB**: 2381, 2397; **ENCB:** 136-138, 469-471, 494, 2484, 5188, 41589-41592, 42211-42216; **UAMI:** 2741.
Corynorhinus townsendii australis (1): **ENCB:** 1677.
Idionycterys phyllotis (1): **CNMA:** 6145.
Myotis (1): **CFB-FB**: 2397.
Myotis californicus mexicanus (18): **CNMA:** 12198; **ENCB:** 40646, 40656, 40668, 40671, 40687, 41210, 41533-41534, 41819-41823, 42197-42198; **UAMI:** 5531, 14850.

Myotis occultus (4): **CNMA:** 474, 11461; **ENCB:** 4238, 18861.
Myotis thysanodes aztecus (4): **ENCB:** 41211-41212, 41535, 42378.
Myotis velifer velifer (74): **CNMA:** 475-476, 1134, 5169, 8241, 9069, 16867, 18523, 28854-28863, 45807-45808; **ENCB:** 1680-1694, 2008, 2312, 3848, 7641, 39121, 41213-41214, 41216-41217, 41536-41551, 42201, 42207, 42377; **INAH:** 748-749; **KU:** 28020; **MZFC:** 1340, 3307; **UAMI:** 2733, 3107, 3824, 4221.
Myotis volans amotus (121): **CFB-FB**: 2356; **ENCB:** S/N, 40634-40645, 40647-40667, 40669-40670, 40673-40686, 40688-40694, 41215, 41218-41250, 41252-41274, 41699, 41824, 42203-42206.
ORDER CARNIVORA
Canis latrans cagotis (3): **CFB-FB**: 1563, 1679, 1964.
Lynx rufus escuinapae (8): **CNMA:** 1129-1130; **CFB-FB**: 1564, 1660, 1671, 1674, 1693, 1722.
Urocyon cinereoargenteus nigrirostris (2): **CNMA**: 15636, **MZFC** S/N
Mustela frenata frenata (18): **CNMA:** 5209, 7200, 9623, 16886, 27265, 28414, 38937, 40183, 45937; **CFB-FB**: 1682, 1727, 2400; **INAH:** 7247; **USNM** : 1060, 3009, 50826-50827.
Taxidea taxus berlandieri (2): **CNMA:** 3798; **CFB-FB**: 1557.
Conepatus leuconotus leuconotus (4): **CNMA:** 3872, 18307, 28410; **CFB-FB**: 1701.
Mephitis macroura macroura (12): **CNMA:** 487, 14592, 15634, 16516, 16749-16750, 16887, 17054, 28915; **CFB-FB**: 1558; **UAMI:** 139; **UMMZ:** 96281.
Spilogale putorius angustifrons (13): **CNMA:** 481, 16885, 42772, 44069, 44089; **ENCB:** 520, 5875-5876; **USNM:** 50821-50825
Bassariscus astutus astutus (12): **CNMA:** 42913, 42917, 43383, 44067-44068; **CFB-FB:** 1643; **KU:** 28021; **USNM:** 3007, 50058-50061.
Procyon lotor (4): **USNM**: 51151; **CFB-FB:** 1617, 1706, 1713.
Nasua narica (1): **CFB-FB**: 1579.
ORDER ARTIODACTYLA
Odocoileus virginianus mexicanus (9): **CNMA:** 1131, 32074, 38261, 45936; **CFB-FB:** 1646, 1686, 1694, 2973; **USNM:** 50184
ORDER RODENTIA
Sciurus (1): **CFB-FB:** 1582.
Sciurus aureogaster nigrescens (60): **CNMA:** 489-490, 1374, 39693, 42915, 43381-43382; **ENCB:** 17169, 42802-42829, 42903-42904; **MZFC:** 4392-4397; **USNM:** 50081-50083, 50085-50088, 51153, 234276, 189462-189464; **UMMZ:** 88207-88208, 88645, 92234.
Spermophilus adocetus adocetus (3): **CNMA:** 15585, 16872; **UAMI:** 5551.
Spermophilus mexicanus mexicanus (18): **CNMA:** 484-486, 16056; **ENCB:** 7661; **INAH**: 7246, **KU:** 28022; **USNM:** 50095-50098, 188762; **UAMI:** 8914; **UMMZ:** 88203-88206, 108274.
Spermophilus variegatus variegatus (40): **CNMA:** 488, 4370, 4428, 8303, 15588-15589, 15643-15644, 16006, 16487, 16746, 16873-16875, 17040, 26147, 27271, 27693, 34648-34649, 39694, 42914, 43378-43380, 44070, 45809; **ENCB:** 34, 41, 787, 2016-2018, 10250; **KU:** 28024; **MZFC:** 189, 461-462, 5640; **UAMI:** 5552.
Dipodomys phillipsii phillipsii (29): **CNMA:** 522; **USNM:** 50297-50323, 50704.

Liomys irroratus alleni (63): **CNMA:** 515-517, 846, 4584, 11549; **ENCB:** 968; **FMNH:** 55791-55792; **KU:** 28052, 28056, 32027; **USNM:** 50357-50358, 51233**; UAMI:** 2781-2782, 5598, 12142-12144, 13391; **UMMZ:** 88729-88742, 88748-88774.
Perognathus flavus mexicanus (16): **FMNH:** 56002; **USNM:** 50706-507018; **UAMI:** 12169-12170
Cratogeomys merriami merriami (182): **CNMA:** 495, 498-511, 800, 3799, 5493, 5835, 7054, 8245, 10089-10090, 11513-11515, 14582, 15118, 16007, 16747-16748, 16870-16871, 27251, 27260, 27280-27287, 27290-27292, 34149-34179, 34700-34713, 38905, 45810; **ENCB:** 99, 323, 473, 2019-2022, 2125-2133, 16059, 22590-22597, 36422, 41596; **INAH:** 477; **KU:** 28035-28044; **MCZ:** 32150, 32403, 59211**; USNM**: 1S/#, 50111-50116, 59211, 115610, 143605, 148174, 148176-148178, 188763, 188765-188769, 189459, 203562; **UAMI:** 163, 937-938, 1932, 2768, 3834, 4249, 8364-8369, 10413-10416; **UMMZ:** 91710, 104630-104638.
Cratogeomys tylorhinus tylorhinus (19): **KU:** 66163; **UMMZ**: 88218-88228, 88649, 94628; **USNM**: 115611-115612, 143606, 148179, 204251.
Thomomys umbrinus peregrinus (4): **CNMA:** 39675; **KU:** 38367-38369.
Microtus mexicanus mexicanus (456): **CNMA:** 1S/#, 849-870, 872-875, 911, 3235, 3404, 4336, 8258-8259, 10744, 11532, 16045, 24248, 30948-30965, 31692, 39734, 45811-45889; **ENCB:** 16, 210, 212-225, 528-532, 534-541, 615-620, 2364, 2402, 2409, 16508-16520, 22663-22670; **FMNH:** 55810-55815, 56114-56116; **INAH:** 670; **KU:** 28266-28271, 28382, 35380-35386, 38767-38779; **MVZ:** 43232-43239, 100634-100635, 100695; **UAMI:** 111, 5667-5668, 10408-10409, 13395-13456, 14899-14918, 16523-16524, 16658-16701; **UMMZ:** 88484-88485, 89277-89309, 89311-89314, 92226-92228, 95529-95531, 107251-107257, 107376-107379; **USNM:** 50751-50756, 115613-115617, 148173, 148175, 188764, 188770-188776, 189458, 189460-189461.
Baiomys taylori analogus (219): **CNMA:** 200, 586-591, 594-619, 842-844, 11678-11686; **ENBC: 1S/#,** 27, 198-199, 201-202, 692, 831-832, 1161-1163, 1179, 22652-22662; **FMNH:** 55928-55941, 55969-55970, 56125-56131; **INAH:** 283; **KU:** 28075-28101, 66961-66968; **MVZ:** 100329-100330; **UAMI:** 10342, 12264-12275, 13563; **UMMZ:** 88909-88944, 88953-88954, 89405, 89625-89628**; USNM:** 50679-50703, 143550-143554, 143588, 204252.
Neotoma mexicana torquata (42): **CNMA:** 5471-5472, 8257, 12487, 15223, 16869, 30947, 31956, 34680-34682, 36475; **ENCB:** 22-25, 833, 5182, 10281-10285, 16505-16507; **MZFC:** 2378; **UAMI:** 5626, 12282-12283, 14945-14948, 16229-16234; **UMMZ:** 92225, 92408.
Neotomodon alstoni (512): **CNMA:** 801-805, 811-827, 830, 832-841, 8175-8177, 8386-8389, 11521-11525, 15402, 15610-15617, 16360, 19612-19645, 24238-24245, 30874-30919, 36476-36482, 45890-45900, 45902-45904; **BYU:** 15507-15510; **ENCB:** 37-38, 40, 1123-1128, 1497-1506, 4492-4496, 5212-5226, 5257-5270, 5344-5353, 10267-10280, 16497-16504, 22588-22589, 41292-41294, 41843; **KU:** 28256-28560, 28376, 41292-41294, 41843; **FMNH:** 55816-55821; **MCZ:** 59215**; MZFC:** 2369, 3382, 5159; **MSU:** 9699, 9698-9719, 9726-9732, 9737-9740; **TTU:** 35391-35393, 37957-37961, 41165. **UAMI:** 971, 5627-5628, 13589-13672, 13680-13708, 16235-16237, 17252-17255; **UMMZ:** 88999-89000, 89006, 89009, 89011-89013, 89015, 89018, 89041-89043, 89047, 89173-89177, 92219-92223, 92370, 95430-95442, 97669, 111934-111942; **USNM:** 50641, 50655-50662, 50665-50666, 143589-143592, 143596-143599, 143603, 143607-143610; **WNMU:** 6325
Oryzomys couesi crinitus (3): **USNM:** 50181-50183
Peromyscus sp. (22): **USNM**: 143199-143201, 204254-204461, 204463-204471, 249850.

Peromyscus difficilis felipensis (354): **CNMA:** 766, 768, 787-789, 24326-24350, 27818-27819, 31930-31932, 45901, 45933-45934; **BYU:** 15514-15526; **ENCB:** 449, 2393, 4491, 5255, 5256, 10265, 16496; **KU:** 28242-28243; **USNM:** 50663-50664, 50671, 148013-148014, 148158, 148172; **UAMI:** 12398-12416, 13673-13679, 13709-13712, 14204, 14979-13989, 16257-16258, 17050-17251; **UMMZ:** 89026-89034, 89036-89038, 89045-89046, 89182, 92194-92203, 92364-92369, 95405-95412, 96360-96361.
Peromyscus gratus gratus (645): **CNMA:** 686-687, 689-735, 737-749, 752-754, 756-760, 797-798, 845, 3804, 9479, 10113-10122, 11556-11565, 11567-11677, 14863-14864, 15123-15124, 15208-15209, 15608, 15650, 28098, 33517-33525, 33591, 34650-34676, 42916, 44071-44082, 44086-44088, 45905-45908; **ENCB:** 26, 29, 182-196, 204-205, 574-580, 694, 1140-1160, 1236, 16492, 16495, 22622-22650; **FMNH:** 55833-55848, 55861-55865, 55968, 56034-56043, 56050, 56084, 56144; **INAH:** 224, 282; **KU:** 28169-28186, 28188-28241, 32028, 66925-66934; **MVZ:** 100510-100541; **MZFC:** 184-186; **TTU:** 40745-40746; **UAMI:** 2788-2789; **UMMZ:** 89093-89117, 89119-89132, 89135, 89138-89155, 89157-89165, 89407, 89494-89496, 90714, 93442-93443; **USNM:** 50602-50621, 50626-506227, 50629-50630, 50635-50636, 50638, 50640, 51178, 307646-307647, 143202-143209, 143549, 143555-143561, 143563-143564, 143574-143583, 143585, 188761.
Peromyscus hylocetes (6): **CNMA:** 1118-1123.
Peromyscus levipes levipes (28): **CNMA:** 688, 750, 755, 761, 3013, 10720-10725, 10727-10730, 11566, 15648-15649; **ENCB:** 2406-2408, 16369, 16411; **FMNH:** 56045; **UAMI:** 2787; **UMMZ:** 89025, 89039, 89054; **USNM**: 143562.
Peromyscus maniculatus fulvus (97): **CNMA:** 684, 1108, 1110, 1112, 1127, 19646-19657, 39719-39727; **ENCB:** 545, 557-568, 1094, 1111-1115, 1117-1119, 5209-5211, 5248-5249; **TTU:** 39850-39852, 40901-40903; **UAMI:** 99-101; **UMMZ:** 89048-89053, 89055-89063; **USNM:** 13888, 115618-115619, 148180-148181, 172247-172247, 174848-174849, 188751-188760.
Peromyscus maniculatus labecula (238): **CNMA:** 629, 632-635, 637-661, 663, 685, 736, 751, 1125-1126, 15422-15429, 15601, 15604-15607, 15622-15624, 15647, 15651-15652, 16864, 24278-24322, 24325; **FMNH:** 55831-55832, 55857-55860, 55892, 55967, 55971, 56044, 56046-56049, 56075-56083, 56111; **KU:** 28116-28143, 28232, 28348, 66799; **MZFC:** 2925, 5160; **USNM**: 50622-50625, 50631-50634, 50642-50643, 50647, 189465-189466, 204253; **UMMZ:** 89001-89002, 89019, 89021-89022, 89035, 89040, 89044, 89118, 89133-89134, 89136-89137, 89156, 89166, 89169, 89179-89181, 89183-89188, 89190-89218, 89408, 89491, 90713, 92356-92358, 93644-93645.
Peromyscus melanophrys melanophrys (7): **CNMA:** 32015, 44510-44512, 45932; **ENCB:** 22651; **UMMZ:** 89189.
Peromyscus melanotis (1176): **CNMA:** 631, 636, 786, 1106-1107, 1109, 1111, 1113-1117, 10704-10706, 10708-10719, 10726, 10732-10741, 10743, 15621, 24249-24277, 30927-30946, 31934-31940, 45909-45918; **BYU:** 15533-15569. **ENCB:** 17-20, 35, 36, 39, 116, 167, 2346-2354, 2394-2401, 4235, 4489, 5236-5247, 5250-5254, 5331-5343, 12395, 16335-16336, 16338-16368, 16370-16410, 16412-16478, 16493-16494. **FMNH:** 55871-55782. **KU:** 28156-28157, 28361-28362; **UAMI:** 97-98, 2853-2855, 5657-5659, 14065-14408, 15034-15067, 16288-16295, 16702-17049; **UMMZ** 88994-88998, 89003-89005, 89007-89008, 89010, 89014, 89016-89017, 89020, 89023, 89024, 89167-89168, 89170-89172, 89178, 89492-89493, 92073-92078, 92359-92360, 95015-95027, 96234-96238, 111931-111933; **USNM:** 50644, 50646, 50648-50654, 50667-50670, 50672, 50820, 148159-148161, 204462, 270510.

Reithrodontomys chrysopsis chrysopsis (74): **CNMA:** 557-561, 13740, 24246-24247, 30966-30967, 30969-30971, 30974, 30976-30979; **BYU:** 15571-15574, 15582; **ENCB:** 16487-16488; **FMNH:** 56133; **USNM:** 50747-50750; **UAMI:** 14501-14518, 15068, 15071, 15080-15088, 16312; **UMMZ:** 88808, 89629, 91812-91814, 91816, 94193-94200.
Reithrodontomys fulvescens toltecus (60): **CNMA:** 541-542, 546, 580, 693, 5941, 11550-11555, 19658, 34677-34679, 44083-44084; **ENCB:** 21, 28, 351, 1137-1139; **FMNH:** 55917; **KU:** 28067-28069; **UAMI:** 5664, 13086-13093, 15069; **UMMZ:** 88807, 88810-88811, 88813-88814, 88821, 88827-88829, 88833, 88843, 88846-88847, 88849, 88857, 92311, 95924-95926; **USNM:** 50745-50746, 143586-143587.
Reithrodontomys megalotis saturatus (202): **CNMA:** 524-529, 531-540, 543-545, 547, 563-571, 576-579, 8173, 8305, 10707, 10731, 10742, 11520, 15593-15599, 30968, 30972-30973, 30975, 39348, 39728-39733, 45919-45921; **BYU:** 15593-15595; **ENCB:** 1491-1493, 2355, 2392, 2404-2405, 4233-4234, 4485-4488, 5206-5208, 5230-5235, 5317-5330, 16482-16486, 16489-16491, 22598-22617; **FMNH:** 55918-55927, 55942-55947, 61829-61831; **KU:** 28058-28066, 28317-28318, 66667; **MVZ:** 100270-100272; **UAMI:** 1167, 15070, 15072, 16313; **UMMZ:** 88809, 88812, 88822-88826, 88830-88832, 88834-88842, 88844-88845, 88848, 88850-88856, 88858, 89401, 92309-92310, 94144; **USNM**: 146899-146900; **YPM:** 4737.
Reithrodontomys microdon wagneri (3): **UMMZ:** 91815, 94186, 95923.
Reithrodontomys sumichrasti sumichrasti (7): **ENCB:** 2403; **KU:** 35393; **UAMI:** 2896; **UMMZ:** 92312-92313, 93643, 94201.
Sigmodon hispidus (4): **CNMA:** 3425-3426; **ENCB:** 5873-5874.
Sigmodon leucotis (12): **CNMA:** 18303, 19659-19662, 30920-30926.

Table 1. Museum specimens that document the presence of mammals in Mexico City and other localities in The Distrito Federal. The total number of specimens per species is shown in parenthesis; the other numbers correspond to catalogue numbers. CNMA = Colección Nacional de Mamíferos, Instituto de Biología, UNAM; UAMI = Universidad Autónoma Metropolitana, Iztapalapa; ENCB = Colección de Cordados, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. MZFC = Museo de Zoología "Alfonso L. Herrera", Facultad de Ciencias, UNAM; CFB = Colección de Fotocolectas Biológicas del Instituto de Biología, UNAM; INAH = Instituto Nacional de Antropología e Historia; MADUG-MA = Museo Dugès, Universidad de Guanajuato, Gto, Mexico; UMMZ = Museum of Zoology, University of Michigan; USNM = Smithsonian Institution National Museum of Natural History; KU = Natural History Museum, University of Kansas; FMNH = Field Museum of Natural History; BYU = Brigham Young University, Monte L. Bean Life Science Museum; MVZ = Museum of Vertebrate Zoology, University of California, Berkeley; MSU = Michigan State University Museum; YPM= Yale University Peabody Museum; MCZ=Harvard University Provider and TTU = Texas Tech University, Museum of Texas Tech University.

3.2 Updated list and taxonomic composition of wild mammals in The Distrito Federal

The updated list (Table 2) only registers the species that were supported by voucher specimens stored in biological collections, except *Canis latrans cagotis*, and *Nasua narica* which present footprints and photographs as evidence which are deposited at the Colección de Fotocolectas Biológicas del Instituto de Biología, UNAM. The records of probable occurrence were not mentioned in this study. The historical records were not included. Only wild species were

considered, the domestic species like cats and dogs *(Felis silvestres* and *Canis familiaris*) were eliminated, as well as the exotic as rats and mice (the gray rat and the black rat; *Rattus norvegicus, Rattus rattus;* and the domestic mouse, *Mus musculus).*

Species	Condi-tion	Distribu-tion	NOM-059	UICN	CITES
Class Mammalia					
Subclass Theriformes					
Infraclass Metatheria					
ORDER DIDELPHIMORPHIA Guill, 1872					
Family Didelphidae Gray, 1821					
Subfamily Didelphinae Gray, 1821					
Didelphis virginiana californica Bennett, 1833				LC	
ORDER CINGULATA Illiger, 1811					
Family Dasypodidae Gray, 1821					
Subfamily Dasypodinae Gray, 1821					
Dasypus novemcinctus mexicanus Peters, 1864				LC	
ORDER LAGOMORPHA Brandt, 1855					
Family Leporidae Fisher de Waldheim, 1817					
Romerolagus diazi (Ferrari-Pérez, 1893)	Mo	E	D	EN	I
Sylvilagus cunicularius cunicularius (Waterhouse, 1848)				LC	
Sylvilagus floridanus orizabae (Merriam, 1893)				LC	
ORDER SORICOMORPHA Gregory, 1910					
Family Soricidae G. Fisher, 1814					
Subfamily Soricinae G. Fisher, 1814					
Tribe Blarinini Kretzoi, 1965					
Cryptotis alticola (Merriam, 1895)	Mo	E*	SP	DD	
Cryptotis parva soricina (Merriam, 1895)		E*	SP	LC	
Tribe Soricini G. Ficher, 1814					
Sorex oreopolus Merriam, 1892	Mo	E*		LC	
Sorex orizabae Merriam, 1895	Mo	E*			
Sorex saussurei saussurei, Merriam, 1892				LC	
Sorex ventralis Merriam, 1895	Mo			LC	
Sorex veraecrucis altoensis Carraway, 2007	Mo				
ORDER CHIROPTERA Blumenbach, 1779					
Family Phyllostomidae Gray, 1825					
Subfamily Glossophaginae Bonaparte, 1845					
Tribe Glossophagini Bonaparte, 1845					
Anoura geoffroyi lasiopyga (Peters, 1868)				LC	
Choeronycteris mexicana Tschudi, 1844	Mo		E	NT	
Glossophaga soricina handleyi Webster and jones, 1982	Mo			LC	
Leptonycteris nivalis (de Saussure, 1860)	Mo		E	EN	
Leptonycteris yerbabuenae Martínez y Villa-R., 1940	Mo**		E	V	

Species	Condi-tion	Distribu-tion	NOM-059	UICN	CITES
Subfamily Phyllostominae Gray, 1825					
Macrotus waterhousii mexicanus de Saussure, 1860				LC	
Subfamily Stenodermatinae Gervais, 1856					
Tribe Stenodermatini Gervais, 1856					
Artibeus lituratus palmarum J. A. Allen and Chapman, 1897				LC	
Family Mormoopidae de Saussure, 1860					
Mormoops megalophylla megalophylla (Peters, 1864)				LC	
Pteronotus parnellii mexicanus (Miller, 1902)				LC	
Family Natalidae Gray, 1866					
Natalus stramineus saturatus Dalquest and Hall, 1949				LC	
Family Molossidae Gervais, 1856					
Subfamily Molossinae Gervais, 1855					
Eumops perotis californicus (Merriam, 1890)				LC	
Molossus rufus E. Geoffroyi St-Hilaire, 1805	Mo			LC	
Nyctinomops laticaudatus ferruginea (Goodwin, 1954)				LC	
Nyctinomops macrotis (Gray, 1839)	Mo			LC	
Tadarida brasiliensis mexicana (de Saussure, 1860)				LC	
Family Vespertilionidae Gray, 1821					
Subfamily Vespertilioninae Miller, 1897					
Tribe Eptesicini Volleth y Heller, 1994					
Eptesicus fuscus miradorensis (H. Allen, 1866)				LC	
Tribe Lasiurini Tate, 1842					
Lasiurus blossevillii teliotis (H. Allen, 1891)				LC	
Lasiurus cinereus cinereus (Palisot de Beauvois, 1796)				LC	
Lasiurus ega panamensis (Thomas, 1901)				LC	
Lasiurus intermedius intermedius H. Allen, 1862				LC	
Tribe Plecotini Gray, 1866					
Corynorhinus mexicanus G. M. Allen, 1916	Mo	E*		NT	
Corynorhinus townsendii australis Handley, 1955				LC	
Idionycterys phyllotis (G. M. Allen, 1916)					
Subfamily Myotinae Tate, 1942					
Myotis californicus mexicanus (de Saussure, 1860)				LC	
Myotis occultus Hollister, 1909	Mo			LC	
Myotis thysanodes aztecus Millar and G.M. Allen, 1928				LC	
Myotis velifer velifer (J.A. Allen, 1890)				LC	
Myotis volans amotus Miller, 1914)				LC	
ORDER CARNIVORA Bowdish, 1821					
SUBORDER FELIFORMIA Kretzoi, 1945					
Family Felidae Fischer von Waldheim, 1817					
Subfamily Felinae Fischer von Waldheim, 1817					

Species	Condition	Distribution	NOM-059	UICN	CITES
Lynx rufus escuinapae J. A. Allen, 1903				LC	
SUBORDER CANIFORMIA Kretzoi, 1943					
Family Canidae Fischer von Waldheim, 1817					
Canis latrans cagotis C. E. H. Smith, 1839					
Urocyon cinereoargenteus nigrirostris (Lichtenstein, 1850)				LC	
Family Mustelidae Fischer von Waldheim, 1817					
Subfamily Mustelinae Fischer, 1817					
Mustela frenata frenata Lichtenstein, 1831				LC	
Taxidea taxus berlandieri Baird, 1858			E	LC	
Family Mephitidae Drago and Honeycutt, 1997					
Conepatus leuconotus leuconotus (Lichtenstein, 1832)				LC	
Mephitis macroura macroura Lichtenstein, 1832				LC	
Spilogale putorius angustifrons Howell, 1902				LC	
Family Procyonidae Gray, 1825					
Bassariscus astutus astutus (Lichtenstein, 1830)				LC	
Nasua narica (Linnaeus, 1766)					
Procyon lotor hernadezii Wagler, 1831					
ORDER ARTIODACTYLA Owen, 1848					
Family Cervidae Goldfuss, 1820					
Subfamily Capreolinae Brookes, 1828					
Odocoileus virginianus mexicanus (Gmeil, 1788)				LC	
ORDER RODENTIA Bowdich, 1821					
SUBORDER SCIUROMORPHA Brandt, 1855					
Family Sciuridae Fischer de Waldheim, 1817					
Subfamily Sciurinae Fischer de Waldheim, 1817					
Tribe Sciurini Fischer de Waldheim, 1817					
Sciurus aureogaster nigrescens Bennett, 1833				LC	
Subfamily Xerinae Osborn, 1910					
Tribe Marmotini Pocock, 1923					
Spermophilus adocetus adocetus (Merriam, 1903)				LC	
Spermophilus mexicanus mexicanus (Erxleben, 1777)				LC	
Spermophilus variegatus variegatus (Erxleben, 1777)				LC	
SUBORDER CASTORIMORPHA A.E. Word, 1955					
Family Heteromyidae Gray, 1868					
Subfamily Dipodomyinae Gervais, 1853					
Dipodomys phillipsii phillipsii Gray, 1841		E	E	LC	
Subfamily Heteromyinae Gray, 1868					
Liomys irroratus alleni (Coues, 1881)				LC	
Subfamily Perognathinae Coues, 1875					
Perognathus flavus mexicanus Merriam, 1894				LC	
Family Geomyidae Bonaparte, 1845					

Species	Condition	Distribution	NOM-059	UICN	CITES
Cratogeomys merriami merriami (Thomas, 1893)				LC	
Cratogeomys tylorhinus tylorhinus (Merriam, 1895)					
Thomomys umbrinus peregrinus (Richardson, 1829)				LC	
SUBORDER MYOMORPHA Brandt, 1855					
SUPERFAMILY DIPODOIDEA Fischer, 1817					
Family Cricetidae Fischer, 1817					
Microtus mexicanus mexicanus (de Saussure, 1861)				LC	
Subfamily Neotominae Merriam, 1894					
Baiomys taylori analogus (Osgood, 1909)				LC	
Neotoma mexicana torquata Ward, 1891				LC	
Neotomodon alstoni Merriam, 1898	Mo	E*		LC	
Oryzomys couesi crinitus					
Peromyscus difficilis felipensis Merriam, 1898				LC	
Peromyscus gratus gratus Merriam, 1898				LC	
Peromyscus hylocetes Merriam, 1898	Mo	E*		LC	
Peromyscus levipes levipes Merriam, 1898				LC	
Peromyscus maniculatus fulvus Osgood, 1904				LC	
Peromyscus maniculatus labecula Elliot, 1903				LC	
Peromyscus melanophrys melanophrys (Coues, 1874)				LC	
Peromyscus melanotis J. A. Allen and Chapman, 1897	Mo	E*		LC	
Reithrodontomys chrysopsis chrysopsis Merriam, 1900				LC	
Reithrodontomys megalotis saturatus J. A. Allen and Chapman, 1897				LC	
Reithrodontomys microdon wagneri Hooper, 1950			E	LC	
Reithrodontomys sumichrasti sumichrasti (de Saussure, 1861)				LC	
Subfamily Sigmodontinae Wagner, 1843					
Sigmodon hispidus Say y Ord, 1825				LC	
Sigmodon leucotis Bailey, 1902		E*		LC	

Table 2. Taxonomic list of the mammals of Mexico City and other localities of The Distrito Federal following the nomenclature and classification of Wilson & Reeder (2005) and Ramírez-Pulido (2005). Condition: Mo= Monotypic. Distribution: E= endemic taxon. * Endemic condition follows Carraway (2007) and Wilson & Reeder (2005). The category of extinction risk and the range of geographical distribution follow the Norma Oficial Mexicana 059 (SEMARNAT, 2010). D = Danger of extintion, E= endangered and SP= special protection. The categories used by the International Union for Conservation of Nature and Natural Resources (IUCN, 2004) are: LC= least concern, NT= near threatened, Vu= vulnerable, EN= endangered, DD= deficient data. According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2009), Appendix I= Endangered species that are or could be affected by commerce. It is also indicated when the taxa is monotypic. The mammals recorded by first time with in the political boundaries of Mexico City are marked with two asterisks.

Our data show that the taxonomic composition of the mammals of Mexico City and other localities of The Distrito Federal is 8 orders, 19 families, 51 genera, 80 species and 81 subspecies (Tables 2 and 3). The best documented orders were Chiroptera (35% = 28 species) and Rodentia (35% = 28 species) followed by Carnivora (13.75% = 11species), Soricomorpha (8.75% = 7 species), and Lagomorpha (3.70% = 3 species). The orders Didelphimorphia, Cingulata and Artiodactyla are represented with one species (1.23%) each.

Order	**Family**	**Genus**	**Species**
DIDELPHIMORPHIA	1	1	1
CINGULATA	1	1	1
LAGOMORPHA	1	2	3
SORICOMORPHA	1	2	7
CHIROPTERA	5	18	28
CARNIVORA	5	11	11
ARTIODACTYLA	1	1	1
RODENTIA	4	15	28
TOTAL	**19**	**51**	**80**

Table 3. Taxonomic composition of mammals from Mexico City and other localities in Distrito Federal.

3.3 Type specimens

The literature reports are 13 holotype specimens for Mexico City corresponding to 5 type localities in The Distrito Federal (Álvarez *et al*., 1997). The taxa are *Mustela frenata* (*Mustela frenata frenata*), *B*[*assaris*] *astuta* (*Bassariscus astutus astutus*), *Cratogeomys tylorhinus arvalis* (*Cratogeomys tylorhinus tylorhinus*), *Mephitis macroura* (*Mephitis macroura macroura*), *Nyctinomus drepressus* (*Nyctinomops macrotis*), *S. a. angustifrons* (*Spilogale putorius angustifrons*), *Blarina soricina* (*Cryptotis parva soricina*), *Pr*[*ocyon*] *hernandezii* (*Procyon lotor hernandezi*), *Oryzomys crinitus* (*Oryzomys couesi crinitus*), *Perognathus flavus mexicanus, Peromyscus gratus* (*Peromyscus gratus gratus*), *Reithrodontomys levipes toltecus* (*Reithrodontomys fulvescens toltecus*), *Liomys irroratus pullus (Liomys irroratus alleni).* One of these type specimens *N. macrotis,* is deposited at CNMA.

3.4 Endemic species

There are no species endemic to México City or The Distrito Federal. However, 11 species of mammals endemic to Mexico ocurr within The Distrito Federal: a rabbit (*Romerolagus diazi*), 4 shrews (*Cryptotis alticola, Cryptotis parva soricina, Sorex oreolopus* and *Sorex orizabae)*, 1 bat (*Corynorhinus mexicanus)*, and 5 rodents (*Dipodomys phillipsi, Neotomodon alstoni, Peromyscus hylocetes, Peromyscus melanotis* and *Sigmodon leucotis* (Table 3).

3.5 Monotypic species

Our database showed that 17 species found in México City and other localities in The Distrito Federal are monotypic. They are conformed by a rabbit (*Romerolagus diazi*), 4 shrews (*Cryptotis alticola, Sorex oreolopus, Sorex orizabae,* and *Sorex ventralis*), 9 bats (*Choeronycteris mexicana, Glosophaga soricina Leptonycteris nivalis, Leptonycteris yerbabuenae, Molossus aztecus,*

Molossus rufus, Nyctinomops macrotis, Corynorhinus mexicanus and Myotis occultus), and 3 rodents *(Neotomodon alstoni, Peromyscus hylocetes and Peromyscus melanotis,* Table 1).

3.6 Species protected by the Mexican government

We found out that 8 mammal species ocurring in México City and other localities of The Distrito Federal are within a category of extinction risk as defined by the Mexican government (SEMARNAT, 2010, Table 1). The zacatuche rabbit (*Romerolagus diazi*) is an endangered species; three species of phyllostomid bats (*Choeronycteris mexicana, Leptonycteris yerbabuenae,* cited in NOM-059, 2010 as *L. curasoae),* and *Leptonycteris nivalis,* one carnivore (*Taxidea taxus berlandieri*), two rodents, the kangaroo rat (*Dipodomys phillipsii phillipsii*), and *Reithrodontomys microdon wagneri,* and two species of shrews (*Cyptotis alticola* and *Cryptotis parva)* are listed under the category of special protection status (Table 2). The kangaroo rat ocurred throughout the Valley of México.

3.7 Species protected by international regulations

According to the International Union for the Conservation of Nature and Natural Resources (IUCN, 2004) there are several mammals ocurring in México City and other localities of The Distrito Federal that are included in the Red List of Threatened Species. Two bat species are in the category of near threatened (*Choeronycteris mexicana* and *Corynorhinus mexicanus)*, one bat as vulnerable (*Leptonycteris yerbabuenae),* and two species are listed as endangered (*Romerolagus diazi* and *Leptonycteris nivalis)*. According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2009) our list only contains one species: *Romerolagus diazi,* this rabbit is listed in appendix I (Fig. 4).

Fig. 4. *Romerolagus diazi* is monotipic and endemic specie from Mexico, endangered by NOM-059, (2010) and IUCN, and in Appendix 1 in CITES (2009)

3.8 Representation by counties in Distrito Federal

The representation by political delegations varies from 1 to 55 species and the number of specimens goes from 3 to 2,035. The Tlalpan delegation had the highest diversity with 55 species, 67.9% (2035 specimens, 35.55%), Coyoacan in second place with 46 species, 56.79% (821 specimens, 14.34%). Xochimilco and Milpa Alta with 29 species each, 35.80% (304 specimens, 5.31%, 461 specimens, 8.05% respectely). Other delegations with a high diversity were La Magdalena Contreras and Álvaro Obregón with 28 species, 34.56% (547 specimens, 9.56% and 856 specimens 14.94% respectely) and those that followed were Cuajimalpa de Morelos, with 25 species, 30.86% (312 specimens, 5.45%). There are other delegations with very low diversity: Iztapalapa, with 18 species, 22.22% (128 specimens, 2.23%), Miguel Hidalgo with 17 species, 20.99% (117 specimens, 2.04%), Cuauhtémoc with 14 species, 17.28% (39 specimens, 0.68%), Tláhuac with 12 species, 14.81% (28 specimens , 0.49%), Benito Juárez with 11 species, 13.58% (30 specimens, 0.50%), Gustavo A. Madero with 9 species, 11.11% (21 specimens, 0.37%). The delegations with the lowest diversity and the lowest number of specimens collected are Venustiano Carranza with 3 species, 3.70% (16 specimens, 0.28%) and Iztacalco with 3 species, 3.70% (5 specimens, 0.09%). Azcapoptzalco did not have any specimen or species collected.

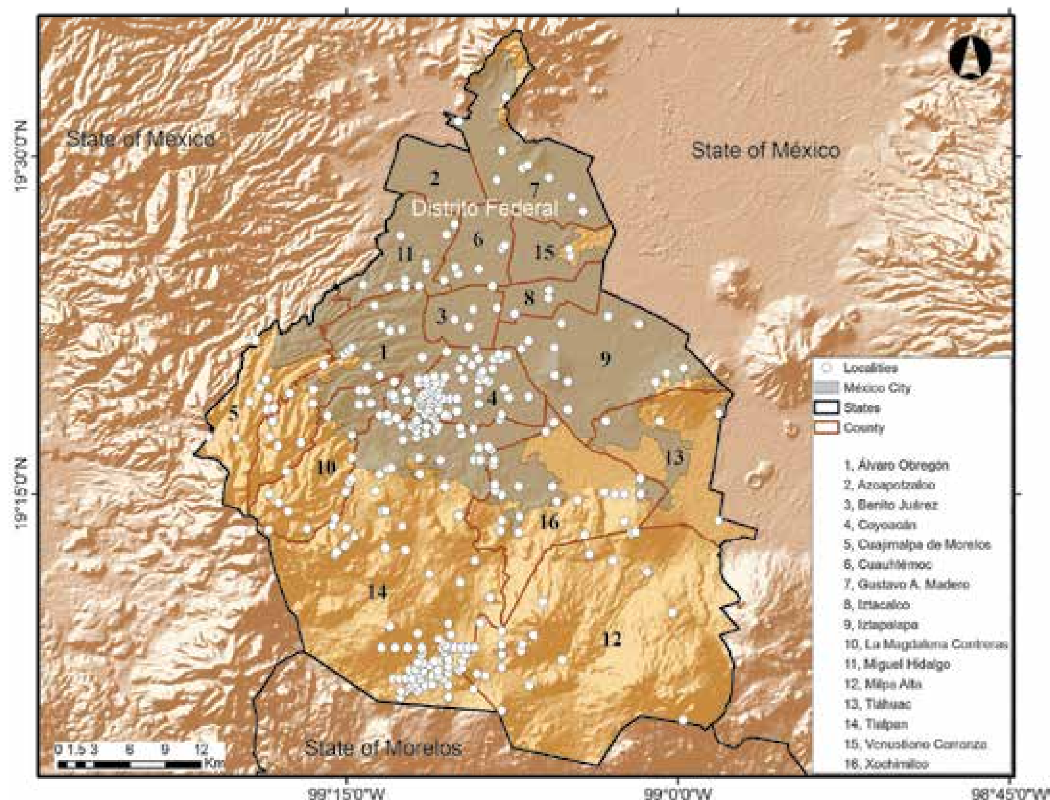

Fig. 5. Collecting localities of mammals in Mexico City and other localities of Distrito.

The political delegations showing the highest diversity were those within the conservation area such as Tlalpan, Xochimilco, Milpa Alta, La Magdalena Contreras, Álvaro Obregón and Cuajimalpa de Morelos. The exception is Coyoacán, which is found inside the limits of Mexico City. The explanation for Coyoacán is that the Ecological Reserve of Pedregal de San Ángel is found within this political delegation, and it is one of the last relicts of natural vegetation inside Mexico City (Hortelano-Moncada *et al.*, 2009). The delegations with the lowest diversity were Iztapalapa, Gustavo A. Madero, Iztacalco and Venustiano Carranza, which are found inside the limits of Mexico City and which also happen to be the driest part of the Distrito Federal.

3.9 Mammal distribution in different vegetation types

Mammal distribution in different vegetation types (CONABIO, 1999) shows that 32 % of the species are found in Oyamel coniferous forests (*Abies religiosa*). The species found in these forests are: opossum, rabbit *Sylvilagus cunicularius*, almost all species of shrews except *C. parva*, three bats, *Tadarida brasiliensis*, *Corynorhinus mexicana* and *Myotis velifer*, deer *Odocoileus virginianus* and several species of rodents. In Pine coniferous forests (*Pinus*) we find 43% of the mammal species among which we have one opossum, four species of shrews, three species of rabbits, 8 species of bats, one deer, two species of squirrels and 16 species of mice. A smaller percentage (13%) of mammals is found in Oak (*Quercus*) forests: one rabbit, *Sylvilagus cunicularius* one shrew, one weasel, one skunk and 7 species of rodents. In Sarcocrassicaule (Xerophitic) thickets we have: *Liomys irroratus*, *Neotoma mexicana torquata*, *Neotomodon asltoni*, *Peromyscus difficilis* and *Reithrodontomys fulvescens*. In halophytic and gypsophytic vegetation there are 3 species of rodents: *Liomys irroratus*, *Microtus mexicanus* and *B. taylori*. An analysis of records also showed that 76% of mammal species from the Distrito Federal is distributed within areas used for agriculture, cattle ranching and forest management, a fact that is not surprising if one considers that these types of area comprises much of the conservation area. A high percentage (71%) of the mammals are distributed within the urban area that comprises most of Mexico City. The Ecological Reserve of El Pedregal de San Ángel, with 33 described species, is found inside this same urban area.

We compared the updated list obtained from our research with the previous published lists of wild mammals from the Distrito Federal (Table 4), including 20 regional, state, national and North American list. From the North American reports we carefully selected only those records showing that the collection site was within the boundaries of the entity. However , we did not considered publications with records for the Distrito Federal with only one or few records although they are analyzed and mentioned in our research. This paper describes almost two century of mammal records; all the specimens are housed in Mexican and North American scientific collections and the oldest records are probably hold in Europe.The taxonomic composition of wild mammals from the Distrito Federal included 80 species. The published lists vary from having 39 up to 77 species. Hall (1981) reported 77, Ceballos & Galindo (1984), and Villa & Cervantes (2003), reported 74, Ramírez-Pulido *et al.* (1986) reported 64 and Villa-R, 1952 reported 39. Four species were heretofore unrecognized such as the hare, *Lepus callotis callotis* (Hall, 1981; Ramírez-Pulido *et al.*, 1986; López-Forment, 1989 and Villa & Cervantes, 2003), two bats *Dermanura azteca* (Hall,1981, for the Basin of Mexico (Sánchez *et al.*, 1989; Monroy-Vilchis *et al.*, 1999), *Eumops u. underwoodi* (Ceballos & Galindo,

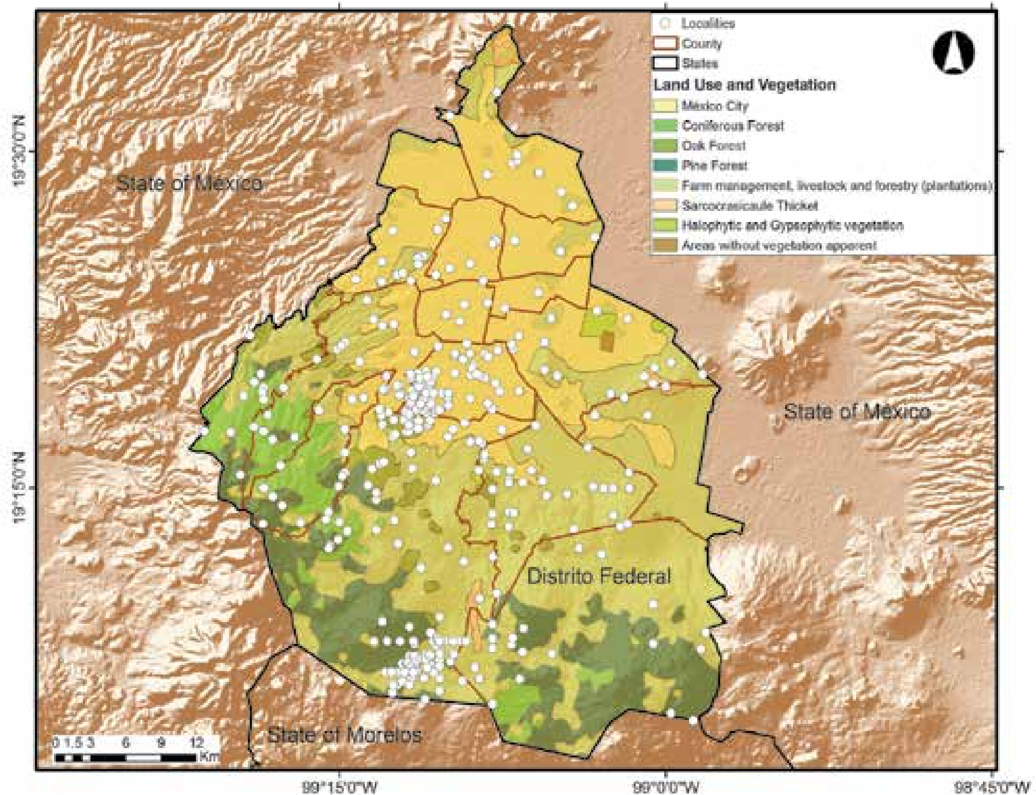

Fig. 6. Geographical distribution of mammals by vegetation type and land use.

1984; López-Forment, 1989; Álvarez *et al.*, 1997), this last record had not been recognized in previous publications (Sánchez-H *et al.,* 1989; Hortelano-Moncada *et al.*, 2009). One squirrel, *Sciurus occulatus* (Villa-R, 1952; Hall, 1981; Ceballos & Galindo, 1984; Ramírez-Pulido *et al.*, 1986; Villa & Cervantes, 2003) which is a specimen from Parres, Tlalpan, was wrongly identified, it is *S. aureogaster* indeed. Two shrews, *Sorex orizabae* and *S, veraecrucis altoensis,* are reported for the Distrito Federal in a relatively recent study (Carraway, 2007), *S. veraecrucis altoensis* is recorded as a new species. On the other hand, one more bat record was incorporated, *Nyctinomops laticaudatus ferruginea* is mentioned in previous publications as a record of probable occurrence (Hall, 1981; Polaco *et al.*, 1992, Villa & Cervantes, 2003; Bárcenas & Medellín, 2007).

4. Discussion

Most mammal records from the Distrito Federal are found in two Mexican collections: CNMA and UAMI, nevertheless, the type specimens and some of the oldest records are found in collections outside the country, which makes their study difficult, although access through electronic means has helped with this problem. Although there are several studies by regions and by groups, some taxa still need to be studied as a result of which there are still some poorly represented species. Another problem is that some areas have been visited only sporadically. The species accumulation graphs support this problem: in general, those periods with the higher number of collections were also the best represented regarding number of species. Some of the oldest records of the Distrito Federal belong to 4 specimens of *Oryzomys couesi*, collected in 1892 (*Oryzomys crinitus*, Merriam, 1901, NMNH: 50181), nevertheless, there is one paper reporting specimens of this species collected in Xochimilco, Distrito Federal, but it does not specify where this biological material is found theefore a verification is not possible (González-Romero, 1980). Other old records correspond to *Dipodomys phillipsii* whose first records date from 1892 and the last ones to 1944. On the other hand the Distrito Federal has 14 species with only one record, and some are very old; some of these correspond to *Molossus rufus* 1960, *Idionycteris pyllotis* 1962, *Corynorhinus townsendii* 1965, *Sorex orizabae* 1971, *Eumops perotis* 1976, *Lasiurus intermedius* 1977, *Nyctinomops laticaudatus* in 1983 and *Natalus stramineus*, 1985. Records not older than 25 years correspond to the rabbit *Sylvilagus cunicularius*, last collected in 1986; the shrew *Cryptotis parva* is represented by only 5 specimens in collections and the last record dates from 1987, whereas the one for *C. alticola* corresponds to 1998, the last record in scientific collections of the skunk *Mephitis macroura* dates from 1990, and *Perognathus flavus*, *Baiomys taylori* and *Spermophilus mexicanus* are from 1991.There are other species with old records, but recent revisions document their presence in certain areas of the Distrito Federal, a fact that underlines the importance of periodical revisions of the lists. As an example, we have the weasel *Mustela frenata* whose last record dates from 1991, but there is, nevertheless, a photographic record from 2009, from Tlalpan; and there is also one specimen, of an individual hit by a car in 2011, in Milpa Alta. The last record of *Liomys irroratus* dates from that same year but there is a photographic record from 2009 (Guevara *et al.*2010) that indicates its presence in this entity. The last record for *Spilogale putorius* dates from 1979, nevertheless, it was found in collections from 2004-2006; two squirrels, *Sciurus aureogaster* and *Spermophilus variegatus*, also have recent records (2006). Many species were recently documented for the Distrito Federal; these are: one opossum *Didelphis virginiana*, five species of shrews: *Cryptotis alticola*, *Sorex oreopolus*, *S. ventralis*, *S. veraecrucis* and *S. saussurei*; 13 bats *Anoura geoffroyi*, *Choeronycteris mexicana*, *Leptonycteris yerbabuenae*, *Corynorhinus mexicanus*, *Tadarida brasiliensis*, *Eptesicus fuscus*, *Lasiurus cinereus*, *L. ega*, *L. blossevillii*, *Myotis californicus*, *M. thysanodes*, *M. velifer* and *M. volans*; 15 rodents *Sciurus aureogaster*, *Spermophilus variegatus*, *Cratogeomys merriamii*, *Thomomys umbrinus*, *Perognathus flavus*, *Neotomodon alstoni*, *Microtus mexicanus*, *Baiomys taylori*, *Neotoma mexicana*, *Peromyscus difficilis*, *P. Melanophrys*, *P. melanotis*, *P. maniculatus*,*Reithrodontomys chrysopsis*, *R. fulvescens* and finally one deer, *Odocoileus virginianus* and three carnivores, *Spilogale putorius*, *Bassariscus astutus* and *Mustela frenata*.

Non-invasive tools, such as photographs or prints have also contributed to species´ records (Aranda, 2010, Bárcenas & Medellín, 2007, Farías, 2010, Guevara-López, *et al* 2010, Ortega, 2010). This is especially useful regarding medium- and large-sized species. One of these records belongs to *Procyon lotor*, one of the first species recorded in the Distrito Federal in 1830, another specimen was collected 52 years later (NMNH 51151) and its presence was recently documented through prints. Several authors mention in their papers having heard *Canis latrans* howling, but have not provided any records, and no record of their presence has been found in biological collections. Their presence has been recently documented through prints. Recent records, prints, include those of *Lynx rufus*, *Mustela frenata*, *Taxidea taxus*, *Conepatus leuconotus*, *Mephitis macroura*, *Bassariscus astutus*, *Nasua narica* (Aranda, 2010), *Odocoileus virginianus* (Aranda, 2010, Guevara-López, 2010) in sites belonging to Álvaro Obregón, Benito Juárez, Cuajimalpa de Morelos, La Magdalena Contreras, Milpa Alta and Tlalpan.

There were 80 wild mammals in the Distrito Federal which represent 17 % of the national biodiversity of land mammals (Ramírez-Pulido *et al*., 2005) and the highest number of species reported for Mexico´s basin (79 species, Ceballos & Galindo, 1984) and southern part of Mexico´s basin (59 species, Monroy-Vilchis *et al*., 1999). The number reported in this paper is high when compared with less diverse states in the country, such as Aguascalientes (61 species, Alvarez-Castañeda et al., 2008) and Querétaro, 67 species, and it is low compared with the most diverse states which are Oaxaca (190 species, Briones et al., 2004) and Chiapas (204 species, Lorenzo et al., 2008). Nevertheless, the surface of Mexico´s basin must be compared with that of these last states and, also, the fact that the Distrito Federal has, within its borders, one of the cities with the largest population in the planet, not only today but historically speaking: Cuicuilco was one of the first cities in the basin with a large population (Pérez-Campa, 2007).

In this paper we also record the highest number of species for the Distrito Federal compared with previous studies of the entity: 40 species in Villa (1952), 74 species in Ceballos & Galindo (1984), 62 species in Ramírez-Pulido (1986), 78 species in Hall (1981) and 74 in Villa & Cervantes (2003). These last authors only have 29 and 44 species with their locality recorded within the borders of the Distrito Federal, the rest are potential records.

On the other hand, the highest distribution of mammals in the Distrito Federal concentrated in two delegations: Tlalpan and Coyoacán, this last one found within the border of Mexico City. Species with the largest distribution were *Microtus mexicanus* and *Lasiurus cinereus*, the first one with numerous records/delegation and the second one with one or five records/delegation. Other species with a wide distribution were: one opossum *Didelphis virginiana*, one rabbit *Sylvilagus floridanus*, seven rodents *Cratogeomys merriami*, *Peromyscus maniculatus*, *Peromyscus difficilis*, *Neotomodon alstoni*, *Peromyscus gratus*, *Peromyscus melanotis* and *Reithrodontomys fulvescens*, and six bats: *Tadarida brasiliensis*, *Eptesicus fuscus*, *Myotis velifer*, *Nyctinomops macrotis*, *Leptonycteris yerbabuenae* and *Corynorhinus mexicanus*. The latter analysis shows that the Distrito Federal is an entity the preserves a large mastofaunistic diversity and that in order to preserve it, it is necessary to preserve the natural habitats that still exist. Another fact to be considered is that the species with more restricted habitat requirements are also the most vulnerable to human actions such as agriculture, fires and poaching.

Species	Synonymous	RS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Didelphis virginiana californica	*D. virginiana* [7, 5,18]	R(63)	R		P	R	X		X	X	X			X		R	R		X	X
Dasypus novemcinctus mexicanus	*D. novemcinctus* [7,5, 18]	R(5)	R		P	R	X		X						R	R	R	X		X
***Lepus callotis callotis*					P		X		X						P					
Romerolagus diazi		R(159)	R		R	R	X		X						P	R		X		
Sylvilagus cunicularius cunicularius	*S. cunicularius*[5]	R(17)	R		P	R	X		X		X				P			X		X
Sylvilagus floridanus orizabae	*S. orizabaeL, S.floridanus*[12,5, 14]	R (67)	R		P	R	X			X	X			X	P	R	R	X		X
Cryptotis alticola	*C. goldmani*[5] ,*C. g. aticola*[3,4,15];	R (12)			R	R	X								R		R			X
Cryptotis parva soricina	*C. parva* [7,5]	R (3)			R	R	X		X						R	R				
Sorex oreopolus	*S. o. ventralis* [4]	R (6)			P	R			X						R				X	
Sorex orizabae		R (1)																		
Sorex saussurei saussurei	*S. vagrans* [7]; *S. saussurei* [7,5,18]	R (102)	R		P	R	X		X	X		X		X	R		R		X	X
Sorex ventralis	*S.vagrans* [7], *S. oreopolus ventralis* [10]	R (19)			P	R				X					R	R				
Sorex veraecrucis altoensis	*S. oreopulus* [6]	R (9)																		
Anoura geoffroyi lasiopyga	*A. geoffroyi* [5,18]	R (7)		R	R	R	X	R	X	X				X	P				X	X
Choeronycteris mexicana	*C. mexicana* [5]	R (16)			P	R	X	R	X	X				X	P	R				X
Glossophaga soricina handleyi	*G. soricina* [7,5]; *G. s. morenoi* [1], *G. s. leachii* [2], *G. morenoi* [2]	R (35)	R	R		R	X	R	X						R	R				
Leptonycteris nivalis		R (4)		R	P	R	X	R	X	X				X	R					
Leptonycteris yerbabuenae	*L. yerbabuenae* [4,12,3,15]; *L. sanborni* [6, 5],	R (44)			P	R	X	R	X	X				X		R				
Macrotus waterhousii mexicanus		R (3)			P										P				X	
***Artibeus aztecus*	*Dermanura azteca* [18]				P			P												X
Artibeus lituratus palmarum	*A. l. intermedius* [3, 6]	R (1)			P			R												
Mormoops megalophylla megalophylla	*M. megalophylla*[5], *Aello m. megalophyla*[3]	R (7)			P	R	X	R		X				X	P					
Pteronotus parnellii mexicanus	*P. parnellii* [5], *P. p. mexicana* [12]	R (1)			R	R	X	R	X					X	R				X	
Natalus stramineus saturates	*N. stramineus* [5, 15]	R(1)			P	R	X	P							P					
Eumops perotis californicus		R (1)						R							P					
***Eumops underwoodi underwoodi*						R			X					X						
Molossus rufus	*M. ater*[*5], *M. a. nigricans*[2,6]	R(1)		R	P	R	X	R	X						P					
Nyctinomops laticaudatus ferruginea		R(1)																		
Nyctinomops macrotis	*Tadarida molossa*[1,2] *T. macrotis*[12,4]	R (28)	R	R	P	R	X	R	X	X				X	R	R				
Tadarida brasiliensis Mexicana	*T. mexicana*[1], *T. brasiliensis*[7,5]	R (39)	R	R	P	R	X	R	X	X	R			X	R	R	R			
Eptesicus fuscus miradorensis	*E. fuscus* [5, 14, 7]	R (28)		P	P	R	X	R	X	X					P	R	R			
Lasiurus blossevillii teliotis	*L. borealis teliotis*[2, 6], *Nycteris borealis teliotis* [3]	R (9)		P	P			R							P	R				

Table 4. (Continued)

Species	Synonymous	RS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Lasiurus cinereus cinereus	*L. cinereus*[5, 18]; *Nycteris cinerea cinerea* [3]	R (19)	R	X	P	R	X	R	X	X				X	P	R				X
Lasiurus ega panamensis	*Nycteris ega panamensis*[3], *L. e. xantiurus* [2, 4,6], *L.e.xanti* [3]	R(2)		P	P	R		R	X											
Lasiurus intermedius intermedius	*Nycteris i. intermedia* [3]	R(1)						R												
Corynorhinus mexicanus	*Plecotus mexicanus*[2, 12, 8, 11, 4, 6, 7, 5]; *P. rafinesqui, C. rafinesqui mexicanus*[1]	R (25)	R	R	P	R	X	R	X	X				X	R	R	R			X
Corynorhinus townsendii australis	*Plecotus townsedi*[5], *P. t. australis*[2,3,4,6]	R (1)		R	P	R	X	R	X						P	R				
Idionycterys phyllotis	*Plecotus phyllotis*[2]	R (1)		R	R	R	X	R	X	X					R					
Myotis californicus mexicanus	*M. californica mexicana* [13]; *M. californica*[18]	R (18)		P	P	X		R	X						P	R				X
Myotis keaysi pilosatibialis		R(1)																		
Myotis occultus	*M. lucifugus* [5, 7]; *M. l. occultus*[15, 6, 12]; *M. lucifuga occulta* [13], *M. auriculus* [5]	R (4)			R	R	X	R	X					X	R					
Myotis thysanodes aztecus		R (4)		P	P	X			X						P					
Myotis velifer velifer	*M. velifer* [5, 14], *M. v. velifera* [13]	R (75)	R		P	R	X	R	X	X				X	P	R	R			
Myotis volans amotus		R (120)		P	P	X		P							P	R				
Myotis yumanensis lutosus					P			P	X						P					
Lynx rufus escuinapae	*L. rufus*[1, 16, 18]	R (2)	R		P	R	X		X						R	R	R	X		X
Canis latrans cagottis		R	P		P	X	X		X						P	R	R	X		X
Urocyon cinereoargenteus nigrirostris	*U. cinereoargenteus*[7, 18]	R (1)			P	R			X	X				X	P	R				X
Mustela frenata frenata	*M. f. perotae*[8], *M. frenata* [7, 16, 18]	R (10)	R		R	R	X		X	X				X	R		R	X		X
Taxidea taxus berlandieri		R (1)			P	R			X						R					
Conepatus leuconotus leuconotus	*C. m. mesoleucus* [3, 4,15]	R (3)			P	X			X							R		X		
Mephitis macroura macroura	*M. macroura* [5, 7, 16, 18]	R (12)	R		P	R	X		X	X	R			X	P	R	R	X		X
Spilogale putorius angustifrons	*S. angustifrons*[12], *S. putorius,*[7]; *S, a. angustifrons*[1]	R (9)	R		R	R	X		X	X				X	R	R	R			
Bassariscus astutus astutus	*B. astutus*[5,12, 18]	R (6)	R		R	R	X			X				X	R		R			X
Procyon lotor hernandezii	*P. lotor*[18]	R			P	R	X		X						R			X		X
Nasua narica	*N. n. molaris* [13]	R							X						R					
Odocoileus virginianus mexicanus	*Dama virginiana mexicana* [3], *O. virginianus* [5, 7, 16, 18]	R (4)	R		R	R	X		X						R	R	R	X		X
Sciurus aureogaster nigrescens	*S. aureogaster* [4, 5, 7, 14, 18]; *S. n. nelsoni*[1]	R (51)	R		P	R	X		X		X				P	R				X
**Sciurus occulatus tolucae*	*S. occulatus* [5]		R		R	R	X								R					

Table 4. (Continued)

Species	Synonymous	RS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Spermophilus adocetus adocetus		R (3)													R					
Spermophilus mexicanus mexicanus	*S. mexicanus*[7], *Citellus m. mexicanus*[1]	R (3)	R		R	R	X		X	X				X	R	R				
Spermophilus variegatus variegates	*S. variegatus*[5,14,18]; *Citellus v. variegatus*[1]	R (40)	R		P	R	X		X	X	X	X	X	X		R		X		X
Dipodomys phillipsii phillipsii	*D. phillipsii* [5,7]	R (1)	R		R	R	X		X						R					
Liomys irroratus alleni	*L. irroratus* [5,7, 18;] *L. i. pullus*[1]	R (60)	R	R	R	R	X		X	X	R				R					X
Perognathus flavus mexicanus	*P. flavus*[5,7]	R (3)	R		P	R	X		X		R				R					
Cratogeomys merriami merriami	*Pappogeomys merriami* [5, 7, 8, 10, 12]; *P. m. irolonis*[4], *P. m.merriami; C. merriami* [18]	R (161)	R		R	R	X		X	X	R			X	R	R	R			X
Cratogeomys tylorhinus tylorhinus	*Pappogeomys tylorhinus* [5,] *P.t.tylorhinus* [4], *C, t. arvalis*[1]	R (14)	R		R	R	X		X						R					
Thomomys umbrinus peregrinus	*T. umbrinus*[5, 7, 18;] *T. u. peregrinus*[3,4,]	R(5)	R		R	R	X		X						R	R				X
Microtus mexicanus mexicanus	*M. mexicanus*[5, 18]	R (433)	R		P	R	X		X	X				X	P			X		X
Baiomys taylori analogus	*B. taylori*[5,7]	R (195)	R		R	R	X		X	X	R			X	R	R				
Neotoma mexicana torquata	*N. mexicana* [5,7, 16, 18]	R (42)			P	R	X		X	X	R	X	X	X	P	R		X		X
Neotomodon alstoni	*N. a. alstoni* [3, 4]	R (500)	R		R	R	X		X						P	R	R	X		X
Oryzomus couesi crinitus	*O. palustris crinitus* [3]	*R***			R	R	X		X						R					
Peromyscus difficilis felipensis	*Peromyscus difficilis*[5,7,18]	R (347)	R		R	R	X		X		R				R	R				X
Peromyscus gratus gratus	*P. truei gratus* [1, 3, 4, 12]; *P. gratus*[6, 11;] *P. truei* [5]	R (588)	R		R	R	X		X	X		X	X	X	R	R				
Peromyscus hylocetes	*P. aztecus* [5, 7]	R (6)	R		P	R	X		X						P					
Peromyscus levipes levipes	*P.levipes*[9,18],*P. boylii levipes*[3,;] *P. boylii*[5,7]	R (29)			P	R	X		X		R				R	R				X
Peromyscus maniculatus fulvus	*P. maniculatus*[5, 18]	R (80)	R		R	R	X		X						R	R	R			X
Peromyscus maniculatus labecula		R (222)	R		R	R				X	R			X	R	R	R			
Peromyscus melanophrys melanophrys	*P. melanophrys* [5]	R (7)			R	R	X		X						R		R			
Peromyscus melanotis		R(1163)	R		RP	R	X		X	X					P	R	R			X
Reithrodontomys chrysopsis chrysopsis	*R. chrysopsis*[5,7, 18]	R(70)			R	R	X		X						R	R	R			X
Reithrodontomys fulvescens toltecus	*R. fulvescens* [5, 7, 18]	R(46)	R		R	R	X		X	X		X	X	X	R		R			X
Reithrodontomys megalotis saturatus	*R. megalotis* [5, 18]	R (208)	R		P	R	X			X	R			X	R	R	R			X
Reithrodontomys microdon wagneri	*R. microdon*[5,7, 18]	R (3)			R	R	X		X						R					X
Reithrodontomys sumichrasti sumichrasti	*R.sumichrasti*[5, 6, 18]	R (19)			P	R	X		X						P	R				X
Sigmodon hispidus	*S. h. berladieri* [3,4,8,10,13,] *S. h. obvelatus*[3]	R (4)			P	R			X	X					R					
Sigmodon leucotis	*S. l. leucotis* [4]	R (12)			P	R			X											

Table 4. Updated list and previous records of wild mammals in The Distrito Federal. One asterisk indicates that is a new record and two asterisks that are not recognized by the

authors of this study, because they were not documented. Synonyms used in previous works are mentioned, and a superindex indicates in which publication appeared RS= recent study, number of specimes in parentheses, 1. Villa-R, 1952; 2. Villa-R, 1966; 3. Aranda *et al.*, 1980; 4. González-Romero, 1980; 5. Hall, 1981; 6. Ceballos & Galindo, 1984;
7. Ramírez-Pulido, 1986; 8. López-Forment, 1989; 9. Sánchez *et al.*, 1989; 10. Negrete, 1991; Negrete & Soberón, 1994; 11. Castro-Campillo, 1992; 12. Chávez & Ceballos, 1992,1994;
13. Chávez, 1993a, b; 14. Álvarez *et al.*, 1997; 15. Monroy-Vilchis *et al.*, 1999; 16. Villa & Cervantes, 2003; 17. CONANP-SEMARNAT, 2006; 18. Bárcenas & Medellín, 2007;
19. Navarro *et al.*, 2007; 20. Gómez-Jiménez, 2009. R = documented record, P = records indicating expected (or presumable) occurrence, X= record cited in the literature.

5. Conclusions

The Distrito Federal has a great biological diversity. The best represented groups of mammals are bats and rodents and, in smaller numbers there are also groups of opossums, shrews, rabbits, armadillos, carnivores and deer. Some of these mammals are protected by International and Mexican legislation. The type localities of 13 species of Mexican mammals are found in Mexico. Species found in almost all of the vegetation types were: opossum *Didelphis viginiana*, shrew *S. saussurei*, rodents *Liomys irroratus*, *Microtus mexicanus*, *Neotoma mexicana*, *Neotomodon alstoni*, *Peromyscus difficilis*, *P. maniculatus labecula* and *R. fulvescens*. Records obtained in this database show that the species restricted to only one type of vegetation are *Romerolagus diazi* and *Sorex veraecrucis*.
With this study we have found species that have adapted to the new conditions of the city, and they now live in parks, buildings and green areas; some of these species are bats, opossums (*Didelphis*) and squirrels (*Sciurus* and *Spermophilus*), while other species, such as rodents, shrews (*Sorex* and *Cryptotis*), one rabbit (*Romerolagus*) and deer (*Odocoileus*), carnivores like (*Lynx*, *Canis*, *Urocyon*, *Mustela*, *Procyon* and *Nasua*) can only be found in protected areas. On the other hand we have found many historical records of mammals but, in this research, we did not find any evidence of their distribution in Mexico City. This study underlines the importance of updating inventories, of having them well documented and verifiable. It is of the utmost relevance to update the nomenclature. The study also demonstrates the value of the information obtained from biological collections, of the way it contributes to knowledge regarding past and present distribution of species in a region. Our study demonstrates that, for some taxa, collections are the only source of information, and this is especially useful regarding areas that have undergone drastic vegetation changes. Inventories are, undoubtedly, basic tools in the studies, monitoring, and management and conservation plans of wild fauna and, in this case, when used in Mexico City. Conservation of biodiversity is strongly linked to society´s welfare.

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16

Microbial Biodiversity and Biogeography on the Deep Seafloor

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1. Introduction

Microbes are widely distributed on and within the Earth (Gold, 1992; Whitman et al., 1998). They have co-evolved with the Earth through their history and have acquired their diversity. Although most of microbes (estimated more than 99% of the total species numbers present on Earth) are still uncultivated (Amann et al., 1995), vigorous surveys in natural environments, from cold poles to hot deep-sea vents, by microbiologists revealed the wide distribution of microbes. The accumulation of 16S rRNA gene sequence data and the development of useful bioinformatics tools allow us to image a big picture of microbial biodiversity and biogeography in natural environments (Martiny et al., 2006). This will help us to address some fundamental questions about microbial community: where they are; what species are present there; how they constitute communities; what are the factors that control the diversity and distribution pattern of the communities; and how they have evolved from the past to present and will evolve in future. Considering the wide distribution and powerful metabolic function of microbes, they are likely to contribute to the maintenance of the whole ecosystem on Earth and to global climate change.

1.1 Biodiversity and biogeography

"Microbial biodiversity" (or simply "biodiversity", "microbial diversity") includes phylogenetic (genotypic) and physiologic (phenotypic) diversity of microbial communities. In many previous papers (and also in this chapter), microbial biodiversity indicated the phylogenetic diversity that can be measured based on variation in nucleotide sequences of genes (16S rRNA gene have been used frequently). The biodiversity measures can be distinguished into the measure of diversity within a single community (α-diversity) and that of the partitioning of diversity among two or more communities (β-diversity). The biodiversity can be measured qualitatively (based on the presence/absence of each taxon) and quantitatively (taken account for the abundance of each taxon). Furthermore, species-based (treated all taxa as equally) and divergence-based (taken account for the phylogenetic distance between each taxon) measurements are used. The above classification and methods for biodiversity measurements have been well summarized (Lozupone & Knight, 2008). To avoid misinterpretation of results, it is needed to understand the principles of these diverse measurements for microbial biodiversity. Because the microbial diversity potentially affects

on the ecosystem functioning (Duffy & Stachowicz, 2006; Prosser et al., 2007), it is important to measure and interpret correctly the microbial diversity in natural environments.
Microbial biogeography is the descriptive and explanatory study of microbial biodiversity over space and time. It aims to reveal where microbes live, and what kinds of microbes are present there and how many they exist. The scope of biogeography extends to understand the underlying mechanism of generating and maintaining the distribution pattern of microbial communities in natural environments. Traditional biogeography has focused on large eukaryotes, such as plants and animals. Recent development of molecular biological techniques enabled us to approach the biogeography for microbes, including protists and prokaryotes in gene sequence level (Darling et al., 2000; Whitaker et al., 2003). This offers a challenge to the famous classical Baas Becking hypothesis *'Everything is everywhere*, but, *the environment selects'* (Baas Becking, 1934). To assess how the environmental similarity (i.e., contemporary physicochemical condition) and geographic distance (i.e., historical event) affect on the distribution patterns of biodiversity of microbial communities or population, β-diversity are associated with physicochemical and geographic differences among each environment. A variety of habitats in natural environments have been targeted for microbial biogeography, such as soil (Cho & Tiedje, 2000; Fierer & Jackson, 2006), lake sediments (Yannarell & Triplett, 2005), ocean (García-Martínez & Rodríguez-Valera, 2000), deep-sea sediments (Schauer et al., 2009) and deep-sea hydrothermal fields (Kato et al., 2010). These studies have suggested that the distribution pattern of microbes in habitats is controlled by not only environmental factors (e.g., pH, salinity and oxygen concentration) but also geographic isolation. However, further data collection and reliable explanation are needed to propose and evaluate theories regarding the generation and evolution of distribution pattern of microbes in natural environments. While the study of microbial biodiversity and biogeography seems to be descriptive, it is the first step and the essential base for theory construction in microbial ecology (Prosser et al., 2007).

1.2 Seafloor microbial communities

Deep seafloor is seemingly an unrelieved, monotonous and poor environment, like a desert where organisms are scarcely present. Actually, this notion is not always correct. There are variable environments on the deep seafloor, such as hydrothermal vents, cold seeps, iron-rich mats, out crops of young crustal rocks and aged ferromanganese crusts (hereafter, Mn crusts). Furthermore, phylogenetically and physiologically diverse microbes (especially prokaryotes, i.e., the domain *Bacteria* and *Archaea*) thrive in these environments (e.g., Takai & Horikoshi, 1999; Inagaki et al., 2002; Santelli et al., 2008; Kato et al., 2009a; Nitahara et al., 2011). Following the Baas Becking hypothesis, these microbes may adapt to each environment and should form a unique community structure. However, the hypothesis has not been tested well for the microbial communities on the deep seafloor, especially non-hydrothermal and unsedimented areas far from land where organic inputs derived from surface photosynthetic ecosystems are not significant. Such deep seafloor accounts for a large part of the surface area of Earth (Smith & Sandwell, 1997). Microbes on and within the seafloor are thought to play a role in geochemical cycling between oceans and Earth crusts (Edwards et al., 2005). Hence, understanding the microbial diversity and biogeography on the deep seafloor is important for modeling the global relationship between microbes and Earth at present and can be applied for in past and future. Furthermore, recently, massive sulfide deposits and Mn crusts on the deep seafloor have been focused on as mineral

resources (Rona, 2003; Hoagland et al., 2010). There are diverse microbes on/in these seafloor minerals (Kato et al., 2010; Nitahara et al., 2011). Microbial biogeography on the deep seafloor will contribute ultimately to develop deep-sea mining techniques utilizing microbes in future.

2. Analytical methods

The study of microbial biodiversity and biogeography starts upon collecting data of microbial communities in the environments. In this chapter, we introduce the analysis methods of microbial communities based on nucleotide sequences of genes, especially the small subunit ribosomal RNA gene (called 16S rRNA gene for prokaryotes) which is generally used for such analysis of biodiversity and biogeography. 16S rRNA genes have some merit for the analysis: 1) all prokaryotes have this gene; 2) its sequence length (approximately 1500 bases) is moderate and adequate for analysis; 3) there are some conserved regions that allow to design PCR primers; 4) there are some variable regions that allow to affiliate the sequences in species-level; 5) enormous sequences have been deposited in public database and can be used conveniently; and 6) useful bioinformatics tools specialized for this gene are available.

Recent rapid development of molecular biological techniques including gene amplification and nucleotide sequencing enabled us to approach unexpected biodiversity of microbes in natural environments. Especially, next-generation sequencing techniques (e.g., pyrosequencing) open new windows for approaching microbial biodiversity (Sogin et al., 2006). However, it is hard to gain biological meanings of the biodiversity resulted from short sequences (~400 bases) that are produced by the next-generation sequencing. At least, nearly full-length sequences of 16S rRNA genes are needed to connect biodiversity to ecology. It should be noted that information on the gene sequences cannot be related to ecology directly. To connect biodiversity to ecology, the determination of the whole-genome sequence is not enough and information on function of microbes derived from the gene sequences must be obtained by culture-dependent analysis. However, cultivation of all microbes in an environment is impossible by now. Actually, most prokaryotes on Earth are still uncultivated (Amann et al., 1995).

There are several steps to measure and compare the biodiversity of microbial communities based on 16S rRNA gene analysis (Figure 1). The target environmental samples are collected, genomic DNA is extracted from the samples, and then 16S rRNA gene sequences in the genome DNA extracts are determined by PCR-cloning-sequencing analysis. In some cases, analyses of electrophoresis patterns, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), without sequencing process are also used for biodiversity measurement; however these analyses mask valuable information on the biodiversity in contrast to sequencing analysis (Nocker et al., 2007). It should be noted that PCR-cloning-sequencing analysis alone is insufficient for the determination of biodiversity because of the presence of methodological biases (Wintzingerode et al., 1997) in addition to relatively high cost performance regarding time and money. For example, even if the same DNA extract was used, the diversity and composition of microbial communities determined using different primer sets were dramatically different from each other (Kato et al., 2011). Hence, for reliable assessment of the biodiversity and distribution pattern of microbial communities, 16S rRNA gene sequences used for comparative analysis should be obtained by the same method.

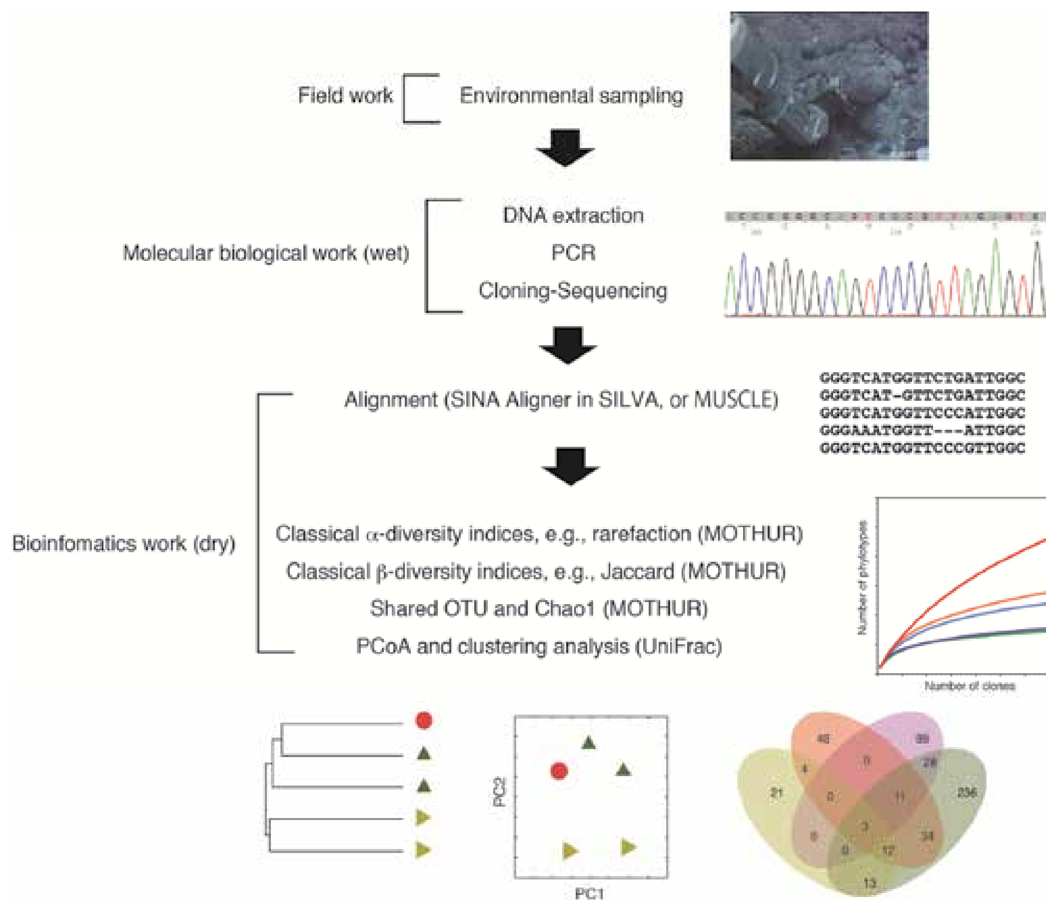

Fig. 1. Flow chart of the recommended analysis steps for microbial diversity and biogeography.

All of the 16S rRNA gene sequences collected are aligned into an alignment dataset. The alignment process is very important to assess more accurate biodiversity and biogeography because the wrong alignment dataset cause overestimation of biodiversity and of difference among communities. Alignments have often been performed by multiple sequence alignment tools such as ClustalW (Larkin et al., 2007) and MUSCLE (Edgar, 2004). However, against the vast 16S rRNA gene datasets including one thousand sequences or more, it takes an immense amount of time. Recently, improved alignment methods incorporating the secondary structure of 16S rRNA genes and using a reference alignment have been provided from several 16S rRNA gene database projects, such as RDP using Infernal (Cole et al., 2009), Greengenes using NAST aligner (Desantis et al., 2006), and SILVA using SINA aligner (Pruesse et al., 2007). However, these methods, in particular NAST, have predicted higher diversity as compared with the results from the pair-wise and multiple alignment methods (Schloss, 2010). The sequences with long insertions seem to be better aligned by Infernal built in RDP than the other methods from our experience. It is known that several prokaryotes, especially *Archaea*, have long insertions (including introns) in their 16S rRNA gene (Burggraf et al., 1993; Itoh et al., 1998; Itoh et al., 2003). Chimera sequences have often

observed in datasets. Such chimera sequences must be removed from the datasets before the following analysis by using chimera-check tools such as Mallard (Ashelford et al., 2006) and Bellerophon (Huber et al., 2004). NAST automatically checks chimera sequences in the datasets and remove the chimeric part of the sequences. However, the sequence with relatively longer insertion seems to be also recognized as a chimera sequence by NAST. Until more improvement of the alignment method and chimera check function, we recommend not using NAST for the following phylogenetic analysis. Overall, for alignment of 16S rRNA gene sequences, SINA aligner built in SILVA, or MUSCLE in the case of lower sequence numbers, is recommended for alignment. Finally, accuracy of the alignment dataset should be confirmed by the naked eyes.

After the construction of a 16S rRNA gene alignment dataset, the sequences are assigned as operational taxonomic units (OTUs) or phylotypes for each habitat. An OTU is a group of similar sequences each other, which is defined based on the genetic distance thresholds. In general, 97% (0.03 cut-off), 95% (0.05 cut-off) or 80% (0.20 cut-off) similarity threshold are used as species-, genus- and family-level taxonomic definition, respectively (Ludwig et al., 1998). For comparative analysis of communities, the same definition level of OTUs must be used. Assessment of sequences to OTUs can be performed using DOTUR (Schloss & Handelsman, 2005) and its current version mothur (Schloss et al., 2009). A distance matrix generated from the alignment dataset using ARB (Ludwig et al., 2004) or DNADIST in PHYLIP package (Felsenstein, 1989) is needed for calculation using DOTUR. The matrix can be generated by mothur itself.

α-diversity measures can be calculated using the distance matrix generated from the alignment dataset: Chao1 species richness estimates, abundance-based coverage estimator of species richness (ACE) and rarefaction curves (species-based, qualitative), Shannon and Simpson indices (species-based, quantitative), Phylogenetic Diversity (PD; divergence-based, qualitative) and θ (divergence-based, quantitative), and so on (Lozupone & Knight, 2008). Species-based measurements of α-diversity can be performed using mothur at once. PD and θ can be calculated by PHYLOCOM (Webb et al., 2008) and ARLEQUIN (Excoffier et al., 2005), respectively.

β-diversity can be also measured using the distance matrix generated from the alignment dataset: Sørensen and Jaccard indices (species-based), UniFrac and F_{ST} (divergence-based), and so on (Lozupone & Knight, 2008). These β-diversity values provide measures of distance between pairs of communities. Furthermore, the measured distance matrix can be used for multivariate statistical techniques such as clustering [e.g., unweighted pair group method using arithmetic average (UPGMA)] and ordination [e.g., principal coordinate analysis (PCoA)]. Several species-based measures of β-diversity can be calculated using SONS (Schloss & Handelsman, 2006), which has been incorporated into mothur. UniFrac (Lozupone & Knight, 2005; Lozupone et al., 2006), and its current version Fast UniFrac (Hamady et al., 2009), is an effective divergence-based method for β-diversity (Lozupone et al., 2010) and can easily perform clustering and ordination analyses. For UniFrac analysis, a phylogenetic tree and definition data for OTUs and habitats are needed as input data. This tree can be constructed from the alignment dataset by neighbor-joining (NJ) or maximum-likelihood (ML) method. NJ tree can be constructed using ARB or ClearCut (Sheneman et al., 2006). ML tree can be constructed using FASTTREE (Price et al., 2010) or PHYML (Guindon et al., 2010). Such clustering and ordination analyses can also be performed using R (R Development Core Team, 2011). In addition, the shared OTU numbers and the shared

Chao1 richness among communities can be calculated and viewed automatically in Venn diagrams using mothur. Overall, our recommendation of the analytical steps is shown in Figure 1.

3. Microbial biodiversity and biogeography of microbial communities on deep seafloor

3.1 Data collection

In this chapter, we try to assess microbial biodiversity and biogeography on deep seafloor using the recent useful bioinformatics tools as described above, though few data from non-hydrothermal and unsedimented deep-seafloor in open sea are available for biogeographical analysis. To investigate the distribution pattern of microbial communities on the seafloor in open sea, several data were collected (Table 1) and used for the following analysis. The locations where the samples were collected are shown in Figure 2. The samples of the collected data are basaltic rocks, Mn crusts, sulfide deposits called as dead chimney which were collected from hydrothermally inactive vents, sandy sediments that were not organic-rich, and overlying bottom seawater (Figure 2). Although the samples were mainly collected on spreading ridges, they were collected far from hydrothermal vents and may not be significantly influenced by hydrothermal activity. We analyzed and compared the communities as described above using the 16S rRNA gene sequences collected.

3.2 α-diversity

α-diversity for a microbial community is often indicated using Chao1 species richness estimates, rarefaction curves and/or Shannon's index value. However, we should not simply compare these indicators of α-diversity provided by the investigators because these indicators are biased by the PCR primers, alignment software and OTU or phylotype clustering methods used in the analyses. As is often the case, the sequences deposited into public databases do not contain all clones in the libraries but only the representative OTUs. Furthermore, the definition levels of OTUs are not always consistent; $OTU_{0.03}$ (i.e., 97% similarity level) are usually used, but $OTU_{0.01}$ or others are also used in some cases. In general, $OTU_{0.03}$ or $OTU_{0.05}$ is used as species or genus level definition, respectively. In this chapter, to compare α-diversity for the communities for the collected data as impartially as possible, the percentage of the number of $OTU_{0.05}$ in total clone numbers, $N_{0.05}/N_t$, were used (Table 1). This comparison can roughly address the difference in the α-diversity, even if only representative sequences were deposited and several definition levels of OTU (<0.05 cut-off) were used.

The $N_{0.05}/N_t$ are summarized in Figure 3. The $N_{0.05}/N_t$ for the samples of dead chimneys and seawater, except Asp, were <40%. In contrast, those for the samples of Mn crusts, basaltic rocks, except Rh3, and sediments were 50% or higher. Noted that the $N_{0.05}/N_t$ for Re5 and Rj were very high (>90%) likely due to the small total clone numbers compared with the other rock or sediment samples (Table 1). The relatively high $N_{0.05}/N_t$ of Asp may be due to contamination from seafloor sediment at the sampling. In fact, some phylotypes recovered from Asp were closely related to those from seafloor rocks and sediments (Kato et al., 2009b). The $N_{0.05}/N_t$ did not correlate with the total clone numbers analyzed (r^2 = 0.223) when the data of Asp, Re5 and Rj were excluded (the plot is not shown). Thus, the difference in the α-diversity associated with each habitat type is potentially meaningful for microbial

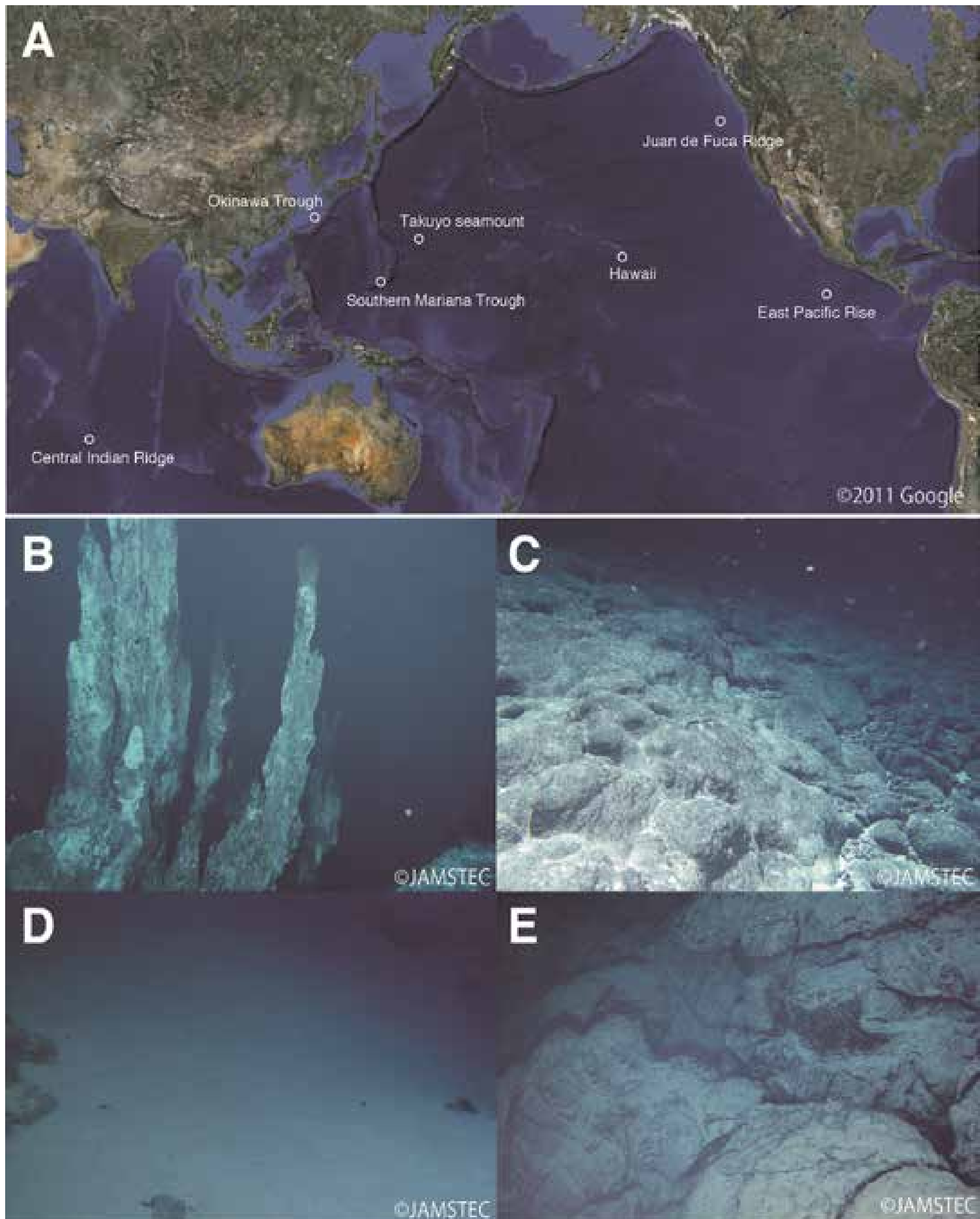

Fig. 2. (A) Location of the samples of which data were used in this chapter, and photographs of on-site observation of (B) sulfide chimney, (C) Mn crust, (D) sandy sediment and (E) basaltic rocks. These photographs (B-E) were taken at the Southern Mariana Trough and Takuyo-Daigo Seamount.

ID	Sample type	Location	Longitude	Latitude	Library name	Total clone number	$OTU_{0.05}$ number	$OTU_{0.05}$ number /Total clone number	Ref
Ae	Ambient seawater	East Pacific Rise	9º28.89'N	104º14.78'W	4055-N3	62	11	17.7	(Santelli et al., 2008)
Asf	Ambient seawater	Southern Mariana Trough	12º57.22'N	143º37.16'E	Fasw	45	17	37.8	(Kato et al., 2009b)
Asp	Ambient seawater	Southern Mariana Trough	12º55.15'N	143º36.96'E	Pasw	44	28	63.6	(Kato et al., 2009b)
At	Ambient seawater	Takuyo seamount	22º40.98'N	153º14.62'E	953sw	100	37	37.0	(Nitahara et al., in press)
Dc	Dead chimney	Central Indian Ridge	25º19.17'S	70º02.43'E	Ind	242	18	7.4	(Suzuki et al., 2004)
Do	Dead chimney	Okinawa Trough	27º47.48'N	126º53.78'E	Ihe	174	10	5.7	(Suzuki et al., 2004)
Dsp	Dead chimney	Southern Mariana Trough	12º55.15'N	143º36.96'E	Ipltc	271	73	26.9	(Kato et al., 2010)
Dsy	Dead chimney	Southern Mariana Trough	12º56.60'N	143º36.80'E	Iydc	144	43	29.9	(Kato et al., 2010)
Me	Mn crust	East Pacific Rise	9º30.36'N	104º13.48'W	3970-MO1A(MnO)	84	58	69.0	(Santelli et al., 2008)
Mt	Mn crust	Takuyo seamount	22º40.98'N	153º14.62'E	953Mn	86	65	75.6	(Nitahara et al., in press)
Re1	Basaltic rock	East Pacific Rise	9º28.48'N	104º15.0'W	3968-O8a(FeO2)	81	53	65.4	(Santelli et al., 2008)
Re2	Basaltic rock	East Pacific Rise	9º50.38'N	104º17.86'W	4059-B2(AlO)	74	40	54.1	(Santelli et al., 2008)
Re3	Basaltic rock	East Pacific Rise	9º49.41'N	104º16.32'W	3965-I2(FeO1)	52	46	88.5	(Santelli et al., 2008)
Re4	Basaltic rock	East Pacific Rise	9º49.42'N	104º16.32'W	3967-O2(FG)	79	43	54.4	(Santelli et al., 2008)
Re5	Basaltic rock	East Pacific Rise	9º50.78'N	104º17.58'W	9NBGBact	26	25	96.2	(Mason et al., 2007)
Rh1	Basaltic rock	Hawaii	18º58.31'N	155º53.42'W	PV550-X3(SP2)	91	46	50.5	(Santelli et al., 2008)
Rh2	Basaltic rock	Hawaii	18º58.31'N	155º53.42'W	PV550-X3(SP1)	64	44	68.8	(Santelli et al., 2008)
Rh3	Basaltic rock	Hawaii	18º52.17'N	155º14.53'W	PV547-X3(LSR)	71	25	35.2	(Santelli et al., 2008)
Rh4	Basaltic rock	Hawaii	18º55.02'N	155º15.53'W	PV549-X2(LPP)	246	148	60.2	(Santelli et al., 2008)
Rj	Basaltic rock	Juan de Fuca Ridge	46º41.95'N	130º55.94'W	JdFBGBact	21	21	100	(Mason et al., 2007)
Sdi	Sediment	Izu-Bonin Trench	30º55.00'N	141º49.00'E	BD5	18	9	50.0	(Li et al., 1999)
Sdt	Sediment	Takuyo seamount	22º40.98'N	153º14.62'E	953Sed	61	46	75.4	(Nitahara et al., in press)

Table 1. List of the clone libraries used in this study.

ecology. It is difficult to answer questions, like why the diversity of dead chimneys and seawater are lower than that of Mn crusts, basaltic rocks and sediments. Further data collection and experimental investigations are needed to answer.

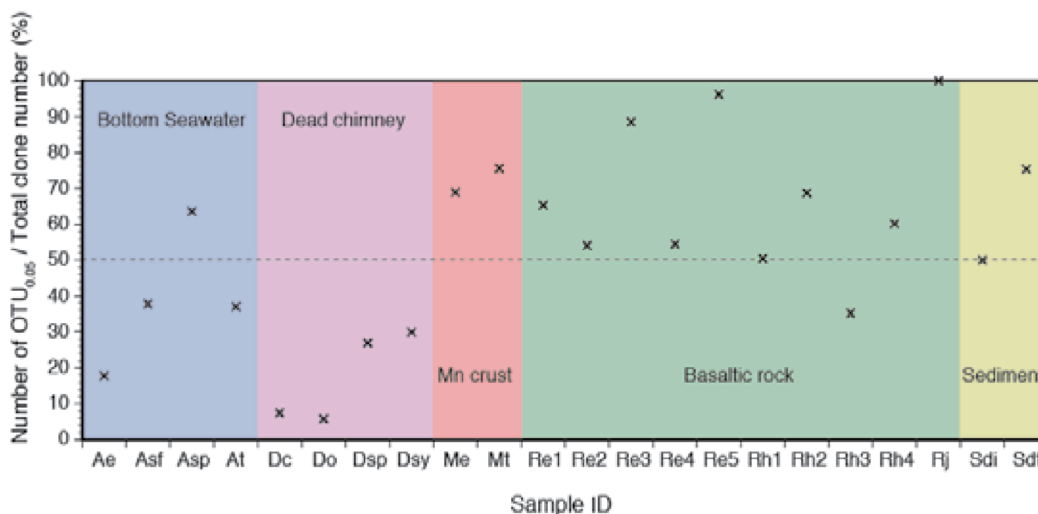

Fig. 3. The percentage of the number of $OTU_{0.05}$ in the total clone number for each sample.

Measurement and comparison of α-diversity is the first step in the study of biogeography. Investigating what kinds of habitats represent high or low diversity within a community is important for understanding the mechanism how microbial communities acquire the diversity. It should be noted that unification of the methodological process including DNA extraction and PCR primer sets in sequencing process (Wintzingerode et al., 1997), and alignment software, used region in the alignment dataset and distance calculation methods in phylogenetic and statistic processes (Schloss, 2010) is important to compare fairly the diversity among communities.

3.3 β-diversity

To compare the microbial communities for each sample based on the β-diversity measures, UniFrac was used here. The result of clustering analysis is shown in Figure 4A. In UniFrac analysis, the jackknifing method can be used to assess confidence in the nodes of the UPGMA tree. In the present case, all nodes, except the root, in the tree were not strongly supported by jackknifing (<50%). The PCoA results are shown in Figures 4B to E. Figure 4B is a three-dimensional image representing the first, second and third principal coordinate axes. Based on the results of clustering analysis and PCoA and taken each habitat-type into account, the samples were affiliated to six groups as shown in Figure 4. Group1 to Group3 represent the communities of basaltic rocks and Mn crusts and of each one of the sediment and chimney, respectively. Group4 and Group5 represent communities of dead chimneys and those of ambient bottom seawater, respectively. Group6 including As and Sdi is the most far from the other communities. Re5 was excluded from any groups due to the unambiguous behavior that may be caused by the small size of the clone libraries.

Using UniFrac, we can easily compare the samples and see the difference in community-level (Figure 4). The results indicated that seawater communities Group5 were clearly

distinguished from the other communities. However, the difference between Group4 (representing dead chimney communities) and Group5 were not shown by the two-dimensional image by the first and second axes (describing 10.81% and 7.79% of the variation, respectively; Figure 4C). On the other hand, these communities were separated along the third axis describing 6.93% of the variation. This means that the third most influential factor for the community similarity, not the first and second factors, is the factor separating the seawater and dead chimney communities. If certain environmental characteristics in habitats (e.g., pH and availability of energy sources such as iron, sulfide and ammonium as electron donors and oxygen, nitrate and sulfate as electron acceptors) and physiological characteristics of microbes in the communities (e.g., life styles of free-living and attachment) were correlated with the third axis, this traits would be the factor causing the difference between seawater and dead chimney communities. Likewise, details

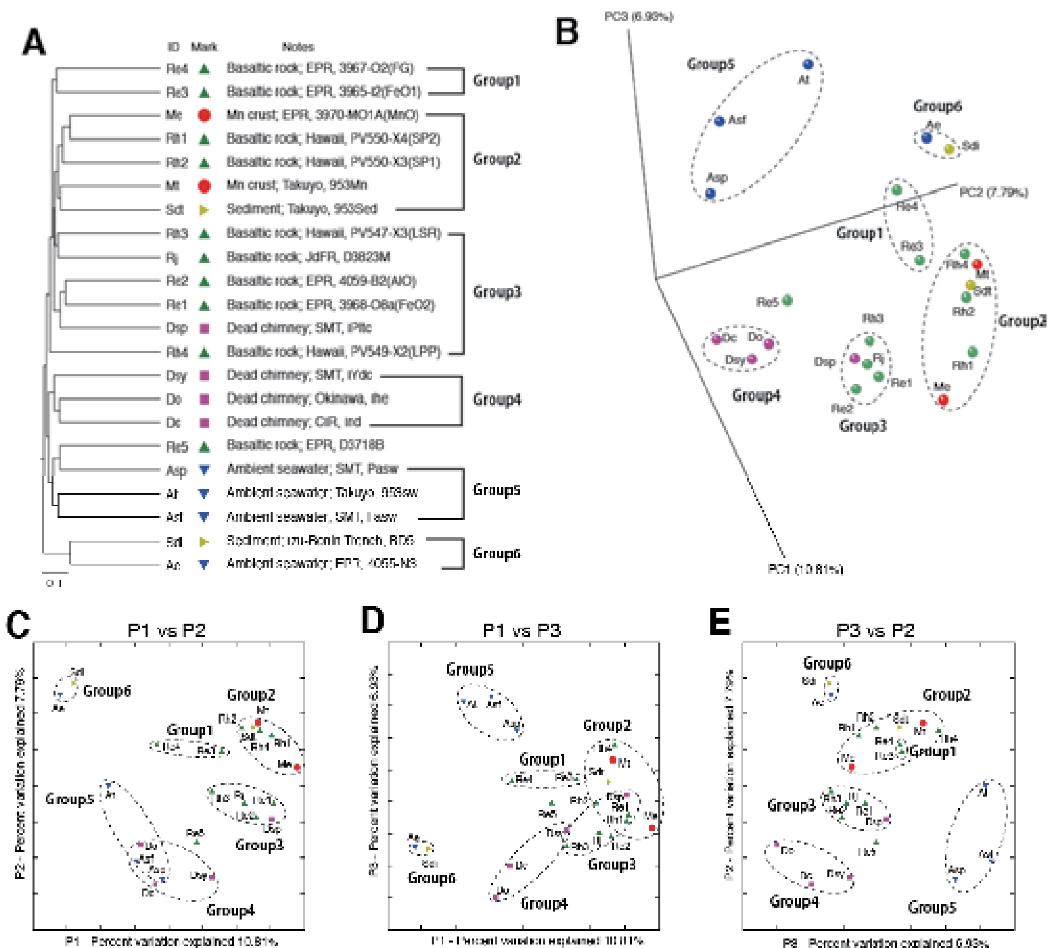

Fig. 4. The comparative results from (A) cluster analysis and (B-E) principal coordinate analysis using UniFrac. (B) The 3D image based on the first to third principal coordinates and (C-E) 2D images for the first vs. second, first vs. third, and second vs. third principal coordinates are shown, respectively.

of geographical and physicochemical characterization of the environments and physiological characterization of the communities in the habitats will help us to address the factors, for example, causing the difference among Group1 to Group4, causing the grouping of Group2 that contains Rh1, Mt and Sdt despite of the different habitat types (Mn crust, basaltic rock and sediment), and causing the difference between Group6 and the other groups.

Geographic distance may be one of the factors affecting the biogeography of the microbial communities. For example, the genetic distance between pairs of populations of *Synechococcus* or *Sulfolobus* in hot springs is related to the geographic distance despite the similar environmental characteristics of each habitat, which can be interpreted due to genetic drift caused by geographical isolation or to adaptation to the fluctuating environment in the past time (Papke et al., 2003; Whitaker et al., 2003). For the deep seafloor communities, the relationships between the community similarity and the geographic distance are shown in Figure 5. For all habitat types (i.e., basaltic rocks, Mn crusts, dead chimneys, sediments and bottom seawater), the similarity between the communities seems not to be related to the geographic distance (Figure 5A). For basaltic rocks and dead chimneys (Figure 5B and C), positive correlation between the community similarity and geographic distance is also not observed. In such solid habitats, environmental characteristics can be varied; for example, the gradient of oxygen concentration may occur due to chemical and biological consumption. The environmental varieties will affect on the microbial community diversity and composition in these habitats. This means that the community traits would be dramatically biased by its sampling position (e.g., interior or exterior parts of a sample). Hence, it is difficult to show the clear relationship between the community similarity and the geographic distance for solid habitats. For overlying bottom seawater (Figure 5D), our result implies that the community similarity is related to the geographic distance. Such correlation for marine microbial communities has been already reported (García-Martínez & Rodríguez-Valera, 2000). Further data sampling from various depths and locations in global oceans will provide a more clear view of oceanic biogeography.

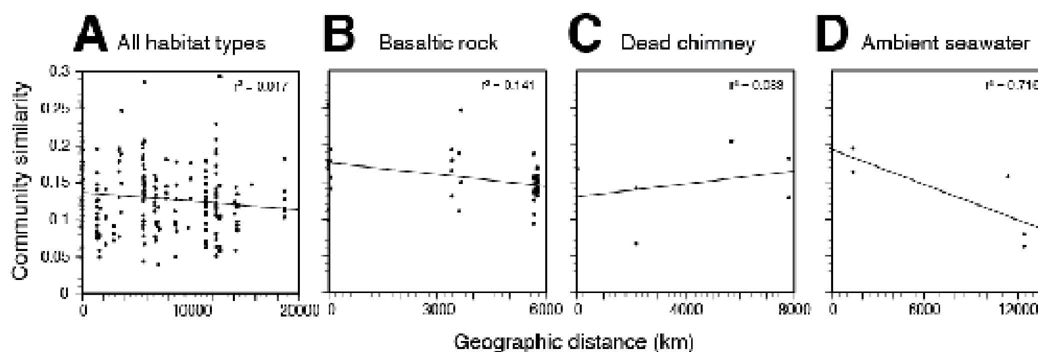

Fig. 5. The relationship between the community similarity and the geographic distance. The results of (A) all habitat types integrated, (B) basaltic rocks, (C) dead chimneys, and (D) ambient seawater are shown, respectively. The similarities between pairs of communities are calculated using UniFrac: the value 1 means that the two communities are the same. The geographic distance between pairs of habitats is calculated using spDistN1 in sp package of R software. Fitted line and coefficient regression value are shown in each figure.

To address the relationship between the community similarity and the phylogeny of the OTUs in each community, the OTUs in each community must be compared at nucleotide-sequence level, neither the band pattern of DGGE nor the fragment sizes of T-RFLP. As shown by the results of UniFrac (Figure 4), the community similarity on the deep seafloor has positively related to the habitat types. How many OTUs were shared among each community in genus- and family-levels (i.e., 0.05 and 0.20 cutoff) are shown in Venn diagrams (Figure 5) depicted by mothur.

Among the solid samples (i.e., basaltic rocks, Mn crusts, dead chimneys and sediments), the integrated community for the dead chimneys and that for the basaltic rocks contain many unique $OTUs_{0.05}$ (67.7% and 70.0%, respectively) in genus-level in contrast to that for sediments or Mn crusts (39.6% and 41.9%, respectively) (Figure 6A). In family-level, over 80% of $OTUs_{0.20}$ of each community were shared with others (Figure 6B). These unique $OTUs_{0.05}$ potentially contain indigenous members for each habitat; for example, the unique clusters for basaltic rocks, Ocean Crust Clades I to VII defined by Mason et al. (2007), and for sulfide chimney, Cluster A to C defined by Kato et al. (2010). Unfortunately, it is unclear whether and how these potential indigenous members play a role in the microbial ecosystem and elemental cycling because they are not phylogenetically close to known cultured species and their physiological characteristics are unknown (Mason et al., 2007; Kato et al., 2010). Further cultivation effort and characterization is important to link the phylogeny of the OTUs to their function and significance in the environments.

Over 50% of the $OTUs_{0.05}$ in the Mn crusts were shared with those in the basaltic rocks (Figure 6A). Furthermore, all of the $OTUs_{0.20}$ in the Mn crusts were shared with those in the basaltic rocks (Figure 6B). These results indicate that the bacterial members in the communities of the Mn crusts and basaltic rocks are phylogenetically close to each other, which is consistent with the UniFrac (divergence-based) result that the Mn crust communities clustered with some basaltic rock communities (Group2 in Figure 4). The phylogeny of the shared and unique OTUs can be confirmed by phylogenetic analysis (such as homology search against public databases and phylogenetic tree construction), although this is not shown in this chapter. Although the physiology of OTUs (e.g., metabolic function, growth rate and optimal growth temperature and pH) cannot be directly determined by their phylogeny, the physiological characteristics of OTUs may not be so different from those of certain cultured species that are closely related to the OTUs. The physiology of OTUs inferred from their phylogeny will provide basal information for constructing working hypothesis of the microbial ecosystem modeling and for preparing culture media targeting these uncultured members.

Such comparative analysis using nucleotide sequences are also used to check cross-contamination among each habitat. The shared OTUs between the bottom seawater community and others are shown in Figure 6C and D. In genus-level, approximately 6-8% of the total $OTUs_{0.05}$ of the Mn crusts, dead chimneys or basaltic rocks were shared with the seawater community (Figure 6C). In family-level, 83%-100% of $OTUs_{0.20}$ of each community were shared with others (Figure 6D), similar to the comparison among the solid samples (Figure 6B). Given that all of the $OTUs_{0.05}$ detected in the seawater are indigenous in the seawater, these shared $OTUs_{0.05}$ observed in the solid samples are potentially contaminants from the seawater community. However, it is also possible that these shared $OTUs_{0.05}$ are different from each other in higher-similarity level (e.g., 97% or 99% similarity). Microdiverse clusters at the level of >99% similarity have been reported for marine prokaryotes such as *Pelagibacter* (SAR11 cluster) (Acinas et al., 2004), Marine Group I

Crenarchaeota (Durbin & Teske, 2010), and for *Halomonas* and *Marinobacter* (Kaye et al., 2011). Hence, we need to be careful in concluding the shared OTUs between target and reference environments to the contaminants from the reference environment.

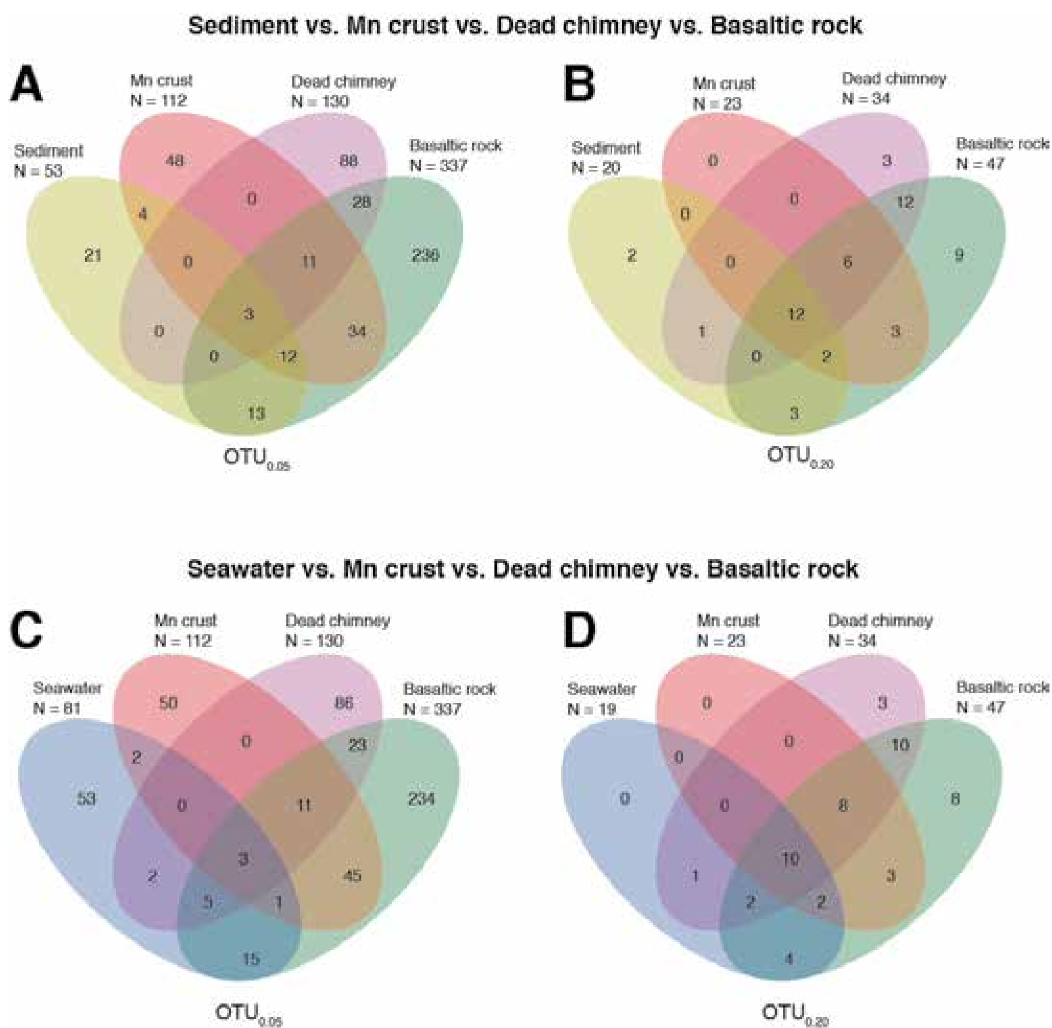

Fig. 6. Venn diagrams comparing the $OTU_{0.05}$ or $OTU_{0.20}$ memberships found in the sandy sediment, Mn crusts, dead chimneys, basaltic rocks and seawater samples. The comparative results among the sandy sediment, Mn crusts, dead chimneys and basaltic rocks are shown in (A) $OTU_{0.05}$ or (B) $OTU_{0.20}$ levels. The comparative results among the seawater, Mn crusts, dead chimneys and basaltic rocks are shown in (C) $OTU_{0.05}$ or (D) $OTU_{0.20}$ levels.

4. Concluding remarks

In this chapter, we introduce the recent bioinformatics tools for assessing the microbial diversity and biogeography. These useful tools allow us to analyze vast sequence data fast and correctly and to get the entire view of the biogeography of microbial communities in natural environments. We should use these tools for analysis of microbial biodiversity and biogeography effectively. Furthermore, both nucleotide sequencing technology and bioinformatics are developing steadily. Microbiologists, especially who study not only biogeography and biodiversity, but also evolution, ecology and biogeosciences, should always try not to overlook these advancing techniques and to apply to their studies. We applied the recent bioinformatics tools for actual data collected from deep seafloor environments. Our results provide insight into the microbial diversity and biogeography of the global deep seafloor in open oceans: for example, relationship between the community similarity and habitat types or geographic distance, commonality and difference among the communities in community- and OTU- levels. For providing persuasive explanation regarding the biogeography in the global deep seafloor, carefull collection of more molecular biological and environmental data from more seafloor habitats in various locations are needed.

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17

Natural Selection: Finding Specimens in a Natural History Collection

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1. Introduction

The natural history domain is rich in information. For hundreds of years, biodiversity researchers have collected specimens and samples, and meticulously recorded the how, what, and where of these objects of research. To retrace this information, however, deep knowledge of the collection and patience is necessary. Whereas traditional access methods (e.g., analysing paper logs of specimen finds) can be used for smaller collections, the sheer size of most current natural history collections prohibits this. At the same time, information technology has advanced to the point where it is able to capture the intricacies of biodiversity collection information and provide the first steps towards full digital access.

The need for collection information access is dire, as lack of access impairs our ability to answer questions about species biodiversity, diversity and change through time (Scoble, 2010). Examples from the young field of biodiversity informatics stress that in order to assess and tackle problems such as predicting a species' reaction to changing environment or prioritisation of preservation policies, digitisation of and access to (large) collection databases is imperative (Guralnick & Hill, 2009; Johnson, 2007; Raes, 2009; Soberón & Peterson, 2004). Although much progress has been made, for example with the Global Biodiversity Data Portal[1] (Berendsohn et al., 2010), many collections have not yet been (fully) digitised.

In this contribution, we first present a new approach to collection digitisation, as well as a novel collection registration management system (CRS) as implemented at the Netherlands Centre for Biodiversity (NCB Naturalis). The new approach to digitisation at NCB Naturalis implements a cascaded digitisation approach: in parts of the collection that have not yet been digitised, first a shelf or drawer is assigned a unique ID in the CRS, along with a description of the specimens contained within it. Whenever the shelf or drawer is revisited, the new policy dictates that specimens that are taken and used from this set be recorded in the CRS. Furthermore, the CRS is linked to taxonomic resources, which enable integration with reference sources. We present two use cases that illustrate the benefits for smarter collection

[1] http://data.gbif.org

information management systems, employing natural language processing techniques. The first use case focuses on data cleaning (Section 5), the second on data retrieval (Section 6). Prior to the use cases, we first explain the background of the NCB Naturalis (Section 2), followed by an overview of the key features of the collection registration system (Section 3) and the collection data used in our studies (Section 4).

2. NCB Naturalis and its collections

The Netherlands Centre for Biodiversity Naturalis[2] is a collaboration between the University of Amsterdam[3], Leiden University[4], Wageningen University[5], and the Dutch National Museum of Natural History Naturalis[6]. They form the combined institute that collaborates with the academic partners to foster the expertise in biodiversity in the Netherlands. The institute will harbour the largest natural history collection in the Netherlands, consisting of over 37 million objects, currently the fifth largest collection worldwide.

NCB Naturalis collections contain fossils, vertebrates, invertebrates, insects, botanical and geological specimens. The majority of the specimens are collected in former colonies of the Netherlands in tropical America, South-East Asia, and Africa, but the collection also provides a broad account of Dutch biodiversity.

In order to manage such a collection properly and make parts of it available to researchers, for example via the Internet, a sound management system is needed. Like those of many other natural history institutions in the world, the collections at NCB Naturalis go back a long way in history. Part of the collection dates back to the 18th century and ranges from specimens collected during the voyages of Captain Cook in the South Pacific and Von Siebold and Bürger in Japan to recent marine and terrestrial collections from expeditions to South-East Asia. With the growth of the collections, curation and collection management practices evolved, but only in the past two decades has technology come into play in collection management systems. As with any innovation, use and best practices have needed time to develop and take root. Over the past few years, NCB Naturalis has been taking stock of the various ways each department have organised their collection information and have started to develop an institution-wide collection management system, taking into account the lessons learnt from each department. In the following Section we detail how this has influenced our design choices for the new NCB Naturalis Collection Registration System (CRS).

3. NCB Naturalis collection registration system

The collection registration system (CRS) at NCB Naturalis is novel in the sense that it is specifically designed for natural history collections, by researchers and collection managers at NCB Naturalis in collaboration with a database company. The CRS differs from other collection management systems in that it is not only a collection management tool for a wide range of users that allows retrieving objects in the collection, and inspect what is their condition or whether they are on loan, but also a tool for researchers. Most systems currently used in natural history institutions are developed for only one of these goals. In the CRS, different user roles are defined, that give users rights to see only general data, or or all data.

[2] http://www.ncbnaturalis.nl/
[3] http://www.uva.nl/
[4] http://www.leidenuniv.nl
[5] http://www.wur.nl
[6] http://www.naturalis.nl

Some data fields are restricted (such as the monetary value of an object), and are not made public.
Although the CRS employs its own, custom-made underlying data model, it is based on the Access to Biological Collection Data (ABCD) standard, 'Extension for GeoSciences' (EFG)[7]. It is furthermore compatible with existing protocols as CIDOC-CRM[8], Spectrum[9], and various technical standards.
To overcome the overwhelming backlog in collection registration, the CRS implements a cascaded registration approach; first the drawers containing boxes filled with specimens are registered, then the boxes contained in the drawers, and finally the individual specimens. This ensures that at least series of specimens are registered and can be located, which is an important consideration in a collection of 37 million objects. In particular, the entomology collection contains millions of specimens; the cascaded approach moves the recording of individual specimens to the future. In other sub-collections with relatively fewer specimens, for instance those of birds and mammals, each specimen will be recorded.
Whereas in the past users of the collections has the choice to enter a specimen they inspected into the database, new policy enforces that the specimen be entered in the CRS if it does not have an individual record yet. The most basic set of metadata information that can be entered about a specimen or collection unit is the information that is on the labels attached to it. This information can further be enriched by records from existing registration or acquisition books, some of which may already have been digitised and are available as databases in the CRS, or from research data such as field books or scientific publications on the unit. Objects are to be registered by copying information 'as is' from the label or paper register. It is considered important to retain the raw information to avoid information loss that may occur when some of the original paper record is incorrectly deemed unimportant. This is in line with the growing awareness of the importance of always keeping the original data and as much of its provenance information, such as a trace of the permutations on the data (i.e., who did what to the data) (Chapman, 2005).

4. Data used

Reptiles and amphibians

The Reptiles and Amphibians (R&A) database is a resource compiled from a manually created database containing 16,870 records (used in Section 5 and Section 6) and an additional automatically populated database containing 39,688 records (used in Section 6). Together, the manual and automatically created databases cover the entire reptiles and amphibians collection at NCB Naturalis.
Each record describes where, when and under what circumstances a reptile or amphibian specimen in the NCB Naturalis collection was found and how it is preserved. The manually created database was compiled by researchers at the institution. It contains 37 columns, of which twelve contain taxonomic information, and eight contain geographic information. The remaining columns describe additional features of the specimen and administrative information. The automatically populated database was created by automatically segmenting and labelling the field notes and registers (this process is described in (Lendvai & Hunt, 2008)). The database is mostly composed in Dutch and English, but also contains some information in German and Portuguese.

[7] `http://www.geocase.eu/efg.asp`
[8] `http://www.cidoc.ics.forth.gr`
[9] `http://www.mda.org.uk/schema`

Taxonomic resources

For the amphibians, the Frost taxonomy is used, as published online (Frost, 2009). The version used in this work (version 5.3) contains descriptions of 6,433 amphibian specimens with references to the literature and synonyms.
For the reptiles, the TIGR Reptile Database (Uetz et al., 2008) is used. It is compiled from books, checklists, monographs, journals, and other peer-reviewed publications from the domain of reptile taxonomy. It is currently maintained by the Systematics working group of the German Herpetological Society (DGHT). It lists all species and their position in the taxonomy. 8,600 reptile species are described.

GeoNames

GeoNames[10] is an aggregated geographical data base that is available through a Creative Commons attribution license and accessible through various Web services. The GeoNames database is compiled from a collection of smaller geographic resources. In June 2009, GeoNames contained over eight million geographical names, of which 6.5 million unique entities. It is an attractive resource to pair our taxonomic data with, as it contains alternative names for geographic entities in numerous languages. In Section 5, we describe the utilisation of GeoNames for the automated detection of inconsistencies in geographical fields in collection databases. In Section 6, we show how GeoNames can be employed to increase recall while querying a multilingual database.

5. Knowledge-driven data cleaning

While data typists and curators do their utmost to create database records meticulously, errors are impossible to avoid. It is estimated that about 5% or more of all data entered by humans contains errors (Maletic & Marcus, 2000; Orr, 1998; Redman, 1997). Most errors that are reported in natural history data occur in the taxonomic, geographic and person name columns (Chapman, 2005). Errors in the taxonomic information regarding a specimen can be caused by an incorrect determination of the specimen. It can, for example, be the case that a specimen was determined quickly and imprecisely in the field, straight after collection. Sometimes errors in the taxonomic fields can be detected automatically as they are misspellings or inconsistencies with an accepted taxonomic resource. Some errors can only be detected through double-checking or revisiting the determination decision as part of collection maintenance.
Geographic errors are mostly induced by imprecise or circumscript recordings of a location in the field (e.g., 'Meyer's farm, 5km South of Sipaliwini'). There are geographic inconsistencies that can be detected automatically in a database, particularly those that pertain to changes in naming of locations (e.g., Ceylon vs. Sri Lanka, Bombay vs. Mumbai) or inconsistencies in the geographic hierarchy (e.g., 'Alaska, Canada'). Modern technology such as GPS units have made it easier for collectors to record the precise locations of their findings.
Errors in person names are less frequent than errors in the taxonomic or geographic information about a specimen. The main error encountered here is inconsistent formatting. Person names are, for instance, given with or without initials and if given, initials are found before and after the last name. Citations are often incomplete, e.g., only an author is given (e.g., 'Kopstein') and the author is sometimes even abbreviated (e.g., 'L'. for 'Linnaeus, 1758'). One could argue that experts know to which publication such an abbreviation refers but for laypersons it is unintelligible and, due to its random nature, automatic indexing and linking to these publications is hampered.

[10] `http://www.geonames.org`, Last queried 15 July, 2009

As it is unfeasible to manually correct all records, there is a need for the automatic checking of information in databases, so that experts can be guided towards prioritised lists of potential errors. Another argument for developing a computer-supported means of data correction for taxonomic databases, is that the information in these databases is subject to change as the taxonomy continues to be debated, revised, and expanded. We therefore developed an automatic approach that uses knowledge from existing taxonomic and geographic resources, as well as a set of rules to decide which database values are suspicious.

5.1 Name and date normalisation

Any first step in data cleaning should consist of making sure all data fields are formatted consistently. To show that this is a non-trivial step, we have normalised (1) diacritics in person names (e.g., removal of umlauts), (2) date formatting (i.e., converting dates to yyyy-mm-dd), and (3) name formatting (i.e., converting person names to *lastname, firstname* or *lastname, initials*) in the reptiles and amphibians database. Table 1 illustrates the amount of data affected by these three types of inconsistencies.

Type of Normalisation	Column	# Filled (%)	# Corr. (%)
Diacritics			
	Author	15,043 (89.17)	1,342 (8.92)
	Collector	14,954 (88.64)	449 (3.00)
	Determinator	10,036 (59.49)	4 (0.04)
	Donator	4,395 (26.05)	50 (0.11)
Date			
	Collection date	14,288 (84.69)	4,789 (33.52)
	Determination date	2,432 (14.42)	1,150 (47.28)
	Entry date	9,144 (54.20)	497 (5.44)
Names			
	Collector	14,954 (88.64)	1,674 (11.19)
	Determinator	10,036 (59.49)	10 (0.10)
	Donator	4,395 (26.05)	578 (13.15)

Table 1. Statistics on corrections provided by normalisation. The table shows the number and percentage of filled cells per database column (Filled) and how many of these were affected by the normalisation process (#Corr., given in numbers and percentages)

The amount of formatting consistency varies greatly; for some person name columns such as *Determinator* and *Donator*, only 0.10% of the cell values do not comply with the preferred format, whereas for others, such as the *Determination Date*, almost half (47.28%) of the cells need to be reformatted to fit the preferred format. This strengthens the claim that normalisation is a necessary step in data cleanup.

5.2 Content cleaning

Our knowledge-based database cleaning approach utilises knowledge about the domain from taxonomies and other resources to infer whether a value is correct or suspicious. It works by combining pieces of information from the collection information system and an external resource or rule, to decide whether a value is correct or not. We give a schematic example in Figure 1. The fictitious domain ontology with main concepts A, B, and C is represented on the left-hand side. In this figure, the operators $>$, $<$, $==$, and $!=$ are used to express possible relations in the domain. According to the ontology there should be a $>$ relation between

co-occurring values of concepts A and B, and an == relation between co-occurring values of concepts B and C. These relations are imposed on the database, which is represented on the right-hand side of the figure. The classes are translated to the database columns and the ontological relations as relations between the database columns. If values in the database do not comply with the relations or rules that hold between the database columns, such as those between *a*2 and *b*2 and between *b*1 and *c*1, they are flagged as possibly erroneous and returned to the user to validate the system's decision.

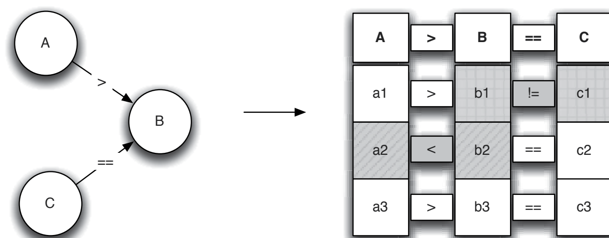

Fig. 1. Schematic overview of the ontology-based error correction approach

In the remainder of this section, we present three types of error detection experiments. The first experiment shows how knowledge about the collection process captured in rules can aid in identifying incorrect database values. In the second and third experiments we link the database to external knowledge sources to find such inconsistencies. In each experiment, we take a database record and compare the values of two different database columns against the knowledge resource (rules or external resource). If one of the values is not consistent with the rule or the resource, it is considered suspicious, and flagged to be checked by a user. In some cases the resource can suggest a correction, but as it is not possible to always know what caused the inconsistency, we choose to keep the human in the loop to determine whether the flagged value is indeed an error, and if so, if the correction is the right value that should replace the incorrect one.

Cleanup of temporal data

To identify inconsistencies in the temporally related information in the database, the database columns containing date information were selected for inspection. The temporal information regarding *Collection* is described by column 'collection date'. The temporal information pertaining to *Entry in Collection* is described by 'Entry date'. For *Determination* the temporal information is described by 'Determination date' and for *Creation of Database Record* the temporal information is described by 'Recorder date'. The four columns, 'collection date', 'entry date', 'determination date', and 'recorder date', are interrelated by *Occurs before* relations. The chronological order of the events related to these dates are summarised in Table 2. Inferred relations are also listed; such a relation, for instance, is present between the *Collection* and *Creation of Database Record* concepts. In the chronological course of the animal collection and registration process the *Collection* occurs first, after which the *Entry in Collection* takes place. The *Entry in Collection* is followed by the *Determination* event and then the *Creation of Database Record* takes place. The *Occurs before* relation is transitive: if A occurs before B, and B occurs before C, then A occurs before C, and thus it can be inferred that the *Collection* takes place before all other events, up to the *Creation of Database Record* event. This is indicated by the inferred relations in Table 2.

Event	Relation (→)	Event
Collection	Occurs before	Entry in Collection
Entry in Collection	Occurs before	Determination
Determination	Occurs before	Creation of Database Record
Inferred		
Collection	Occurs before	Determination
Collection	Occurs before	Creation of Database Record
Entry in Collection	Occurs before	Creation of Database Record

Table 2. Summary of chronological relations present in specimen data

The results of the consistency check on dates are presented in Table 3. When a constraint violation is detected by the rule, it is not possible to determine which date is the one containing an erroneous value: the error can be in either value, or in both.

Columns	Flagged by VALIDATO
Collection - Entry	64
Entry - Determination	7
Determination - Recorder	26
Collection - Determination	5
Collection - Recorder	5
Entry - Recorder	2

Table 3. Results of ontology-based error detection experiments on temporal data

The approach has two important limitations. First, the approach cannot suggest a correct date if a constraint violation is encountered, as the domain offers no rules about how much time there should be between the different events. Second, only cases are flagged in which a value is violating constraints, not when the value is incorrect while no constraints are violated, such as when a recorded collection date is off by a few days, but all other dates pertaining to the database record are much later. Information that would be needed to detect and correct errors of this type could come from resources that describe the expedition, such as a logbook, or from employment records at the deteminator's lab. In certain cases this type of detective work may be warranted, and automatic techniques may assist in this type of expert work, but this lies beyond the scope of the current contribution.

Cleanup of geographical data

To detect inconsistencies in the geographical information, such as a record that contains a value for a city and an incorrect country, e.g., city: Paris, country: Italy, the *Falls within* relations are translated to rules that flag pairs of database cells that do not comply with this restriction. In order to do so, the values from the different cells are looked up in the GeoNames database; if there is no containment relation found in the returned records the database entry is flagged as containing possibly inconsistent geographic information. The relations that hold between selected geographic classes in the specimen database are summarised in Table 4.

Due to the multilingual nature of the data the rules need to leave room for considerable variation. If for instance the city-country pair 'swamp ca . 10 km E . of Parga'-'Griekenland' is encountered, the value in the city cell is first stripped of all non-capitalised and numeric tokens and tokens shorter than 2 letters. This results in the value 'Parga', which is then queried against the GeoNames database, returning twenty records. Along with every returned result all possible alternatives for the country name in the languages present in the R&A database are

Class	Relation	Class
City	Falls within	Province
Province	Falls within	Country
Inferred		
City	Falls within	Country

Table 4. Summary of relations holding between the different geographic classes in the specimen data

looked up and compared to the original country value 'Griekenland'. In this case, a positive match occurs between a GeoNames match of 'Parga' – 'Greece', 'Griekenland' being the Dutch word for 'Greece'; thus, the record is not flagged as containing inconsistent geographic information.
In cases where no match between the city and country values is found in GeoNames, the database entry is flagged as containing a potential inconsistency, and the country name of the country for which most hits were found is returned as suggestion. A similar process is carried out for all 'province' - 'country' and 'city' - 'province' value pairs.
The results of the experiments are presented per pair of columns in Table 5. The disagreements flagged by the data cleaning system were analysed and classified as either being cases in which one of the terms could not be found in the geographic resource (NF), cases in which the value was correct but in the wrong column (wrong column errors, denoted by 'WC' in the table), cases in which a content error is detected (CE) and cases in which the database uses a synonym that is not found in GeoNames (SYN). The numbers in brackets indicate how many of the cases were unique errors.

Columns	# Flagged	NF	WC	CE	SYN
city - province	51	30 (6)	1	20 (4)	0
province - country	1	0	1	0	0
Inferred relations					
city - country	55	8 (6)	15 (4)	1	31 (4)

Table 5. Results of ontology-based error detection experiments on geographical information

The most prevalent cause for the system to flag a possible error is the non-standard usage of the 'city', 'province' and 'country' columns. In the 'city' column, values are found such as '4 km W. of airstrip Tafelberg' and 'Right kabalebo river, kamp keyzer, voet K. valle'. It is indeed a dilemma for the person entering the nearest city name, as specimens are often found well outside habited areas, and the nearest city may not at all be the most obvious anchor point to describe the geographical coordinates of the finding. Yet, entering circumscript phrases such as '5km NW of' or 'near' only obfuscates the precise location. Modern technology can aid in such cases as a location could unambiguously defined by the usage of a GPS device. For older data it would be better to redefine the column as 'city or nearest city', and a separate column 'other localisation information' could be devised in which additional information such as '4km W. of airstrip' could be entered.
Most errors in the city-province test are a systematic mix-up of the two Surinam districts *Nickerie* and *Sipaliwini*, which is a frequency effect of the many expeditions that took place in these districts, and the erroneous values proliferating. In cases where the value in the 'city' field could not be matched properly there was often a very common city name involved (such as *St. Jean*) and a province value that could not be matched (*Dep. Guyane*) because it was, for

example, abbreviated in non-standard way. This particular case illustrates the limitations of GeoNames as *Departement Guyane*[11] would have matched.
The fact that there is only one error found for the province-country combination is that the 'country' field is fairly often empty. The entry that is flagged as erroneous contains the continent value 'Zuid-Amerika' (South America) in the country field and the value 'South America' in the province field. The majority of the errors in the 'city' - 'country' experiments are caused by the fact that a term is used for the country name that is not present in GeoNames (e.g., *U.S.A.* for *United States*). In nine cases, the name from the city cannot be disambiguated properly by GeoNames, for instance because of a typo. It occurs that the database contains *La Rochette - Luxemburg*, and the system suggests *La Rochette - Belgium*, whereas the value could also be *Larochette - Luxemburg*. For such cases, it is of vital importance that an expert checks the suggestions of the system.

Cleanup of taxonomic data

Taxonomic inconsistencies in the data are detected through a process similar to the detection of geographical inconsistencies. The taxonomic hierarchy can be defined through a *Has broader term* relation. This transitive relation applies to 'species', 'genus', 'order', 'family' and 'class' consecutively as shown in Table 6. The 'subspecies' level could not be queried as the data formatting of the resources prevented reliable identification of the 'subspecies' values.

Taxonomic Level	Relation	Taxonomic Level
Species	Has broader term	Genus
Genus	Has broader term	Family
Family	Has broader term	Order
Order	Has broader term	Class
Inferred		
Species	Has broader term	Family
Species	Has broader term	Order
Species	Has broader term	Class
Genus	Has broader term	Order
Genus	Has broader term	Class
Family	Has broader term	Class

Table 6. Summary of hierarchical taxonomic relations holding between the different taxonomic levels

To investigate why the system flagged an instances, the flagged instances were analysed and classified as either being cases in which one of the terms could not be found in the taxonomic resources (NF), cases in which the information was correct but did not belong in that column (LE), and cases in which the system identified a content error (CE). The results are presented in Table 7.
The most peculiar result from the taxonomic data cleaning experiments is the extraordinary number of wrong column errors found for the order column. Some 5,600 of these cases can be ascribed to the value *Sauria* being present in the 'order' column, whereas it denotes a suborder of reptiles of the *Squamata* order.
Incompleteness of the resources accounts for the majority of the cases in which the taxonomic name could not be found in the resource (e.g., the genus *Astylosternidae* is not described in Frost 2009, but it is listed in, for example, the Encyclopeadia of Life[12] and the Global

[11] The full official name is Département de la Guyane.
[12] `http://www.eol.org/`

Columns	# Flagged	NF	LE	CE
Species - Genus	4,122	3,035 (300)	0	1,087 (142)
Genus - Family	3,341	514 (81)	14 (1)	2,813 (124)
Family - Order	8,641	1,017 (23)		7,624 (66)
Order - Class	8,460	2,643 (6)	213 (6)	5,604 (2)
Inferred relations				
Species - Family	4,311	2,890 (215)	0	1,421 (91)
Species - Order	6,097	2,909 (202)	0	3,188 (362)
Species - Class	251	64 (7)	0	187 (3)
Genus - Order	8,583	515 (84)	0	8,068 (440)
Genus - Class	562	518 (81)	14 (1)	30 (11)
Family - Class	675	645 (21)	0	30 (9)

Table 7. Results of ontology-based error detection experiments on taxonomic information

Biodiversity Information Facility[13]). In a few cases, a value cannot be matched because of a spelling error such as *Alligatoridaer* instead of *Alligatoridae* or abbreviations such as *sp.* in the species field to indicate that the species has not been identified and that it could be any species in the genus indicated (in this case genus *Typhlops*). In some cases, the ontology driven cleanup uncovers an update to the taxonomy such as for the genus-family pair *Dendrobatidae-Mannophryne*. Here the approach suggests *Aromobatidae* as value for family which can be explained by a change in the taxonomy, as in 2006 *Aromobates* were removed from the *Dendrobatidae* family to form its own family, *Aromobatidae* (Grant et al., 2006).
Overall, the approach detects a variety of error types, and except for the cases in which the term is not present in the resource, all cases it flags are genuine errors. As the suborder vs. order error is overly frequent, the addition of a suborder column in the database might be considered.
The same types of knowledge that helped clean up the database can also help increase access to it. Due to the complexity of the data, simple queries are often not enough. As we want to preserve as much information as possible, it is important that for example synonyms of taxons are linked, so that a single query can retrieve all specimens of a species, regardless of the name they are registered by. To show the benefits of this, we carried out experiments with and without simultaneous synonym search.

6. Knowledge-driven specimen access

To improve the accessibility of specimen information in natural history data collections through search engines, we developed a knowledge-driven database access method that utilises domain knowledge at three different stages in the retrieval process. The domain knowledge is employed to (1) aid query formulation, (2) expand queries with relevant synonyms, and (3) rank results. We compared the knowledge-driven access method with the original collection database system. Our results show that the domain knowledge markedly improves recall results on the reptiles and amphibians domain that we tested the approach on: from 32% to 86%.

6.1 Queries

External researchers often request access to NCB Naturalis' extensive specimen collection or to the meta-data that is found in the databases describing the collections. As the databases are

[13] http://www.gbif.org/

not (yet) publicly available, these questions are usually directed to the collection managers at NCB Naturalis. To test the system, collection managers have saved these questions they received regarding the reptiles and amphibians collection. These queries give a good idea of the type of information researchers are looking for.
The questions were extracted from longer (often email) messages. The questions have been summarised into only the information request and not the introduction for why the information is requested. For each of the queries the relevant records in the databases were identified manually to create a gold standard.

Reptile and amphibian queries

The 100 reptile and amphibians queries were gathered from requests to the reptile and amphibian collection managers and researchers at NCB Naturalis that were received between September 2003 and December 2008.
Some example queries are:

- What type specimens of New Guinee skink do you have in your collection?
- Do you have male specimens of *Hypsilurus godeffroyi*?
- Are there Dipsas species other than *D. catesbyi* and *D. variegata* from the Guianas and Venezuela in the collection?
- How many species of *Rana palmipes* as defined by Spix in 1824 are in the collection?

12% of the questions enquire after a genus, 86% after a genus and a species, and in 41% the request poses a restriction on the geographical location of where the specimen was collected. Additionally, in 15% of the questions a registration number is given, which should make it easier to retrieve correct database record, but as registration numbers are not unique this is not always the case.
For 16 queries no relevant records were present in the database. For the remaining 84 queries the number of returned records varies greatly. For example, for 21 queries only 1 relevant result is present in the database whereas there are 4 queries for which over 500 relevant results are present in the database.

6.2 System architecture

In this section, the system setup is presented. An overview of the system is presented in Figure 2. The domain knowledge comes from taxonomic and geographic resources (see Subsections 4 and 4), a domain ontology, domain-specific rules and analysis of typical queries in the domain. Below, each of the system modules is described.

6.2.1 Query interpretation

Most of the queries in the test sets require more precise formulation than queries using the operators ‘and’ and ’or’. Consider for example the query *Are there Dipsas species other than D. catesbyi and D. variegata from the Guianas and Venezuela in the collection?*. Here, the user is looking for database records that describe specimens of genus Dipsas, but not those records of species *Dipsas catesbyi* and *Dipsas variegata*. The second constraint is that the user wants the relevant records about specimens collected in the Guianas or Venezuela.
To be able to handle such queries, we devised a query language that can encode that for part of the query any query term should match and for part of the query all query terms should match. The query language can also exclude terms on the basis of a negation. The query terms that we extract from the example query are: *dipsas*, *-catesbyi*, *-variegata*, *guianas* and *venezuela*. To express that specimens of genus *Dipsas* found in the *Guianas* or in

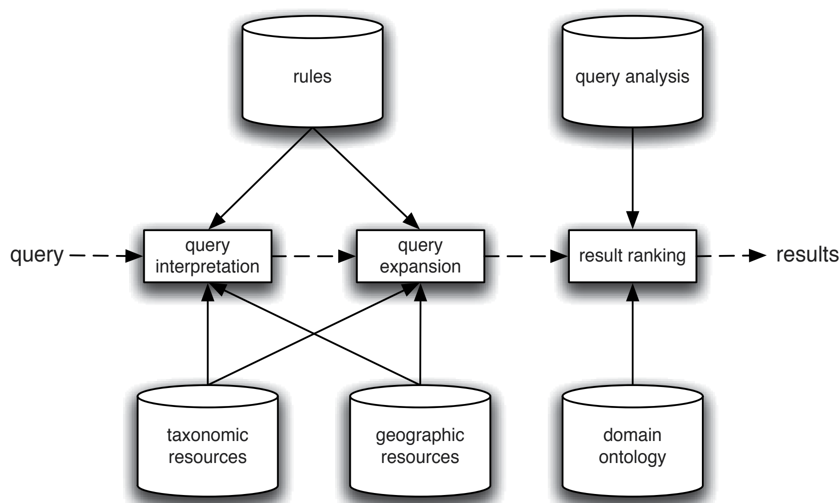

Fig. 2. Overview of the knowledge-driven specimen information retrieval system

Venezuela are to be retrieved, the query is rewritten to *all(dipsas,any(guianas,venezuela))*. To exclude the records on specimens of species *catesbyi* and *variegata* the query is written as *all(dipsas,-catesbyi,-variegata),any(guianas,venezuela))*.
Users can be taught this query format, but due to the availability of taxonomic resources, the system can also automatically translate basic query term enumerations such as *dipsas*, *-catesbyi*, *-variegata*, *guianas*, and *venezuela* into the desired complex query for the reptiles and amphibians. In order to do so, it looks up each query term in the taxonomic and geographic resources to classify it as either a genus, species or geographic name. The module can also recognise registration numbers as terms that contain two or three capital letters and 3 to 6 numbers. After each term is classified, the module constructs the query according to rules that restrict possible combinations of types of terms.
The automatic translation module is checked against a gold standard of manual rewriting of each query. For the reptiles and amphibians, it translates 77% of the questions correctly. The cause for the translation module to fail is, in all cases, due to a term not matching in the resource.

6.2.2 Query expansion

The query expansion modules in the system are aimed at increasing the recall by providing additional keywords or to remedy the influence of language variation on the retrieval of relevant results.

Taxonomic term expansion

As a consequence of changes in species classifications due to new insights, accepted taxonomic lists that describe the classification of a taxonomic class contain many synonyms and outdated names for each taxon. For example, if one wants to retrieve all snakes present in the collection, one could query for all records describing a specimen of suborder 'Serpentes', but this suborder is also known as 'Ophidiae'. An additional problem with this query is that as we noted earlier, the reptiles and amphibians database does not contain a suborder column (although sometimes the suborder value is entered in the order field), hence in order to retrieve all snakes in the collection one would have to query the database for all 18 snake

families, which each may be known by synonyms as well. To relieve users from having to formulate a query that contains each of the 18 snake families with their possible synonyms, the system applies a knowledge-based query expansion approach that expands query terms with their taxonomic synonyms.

Geographic term expansion

Similar to the taxonomic term expansion, but slightly different in operation is the enrichment with a geographic resource. If we reconsider the example given in Subsection 6.2.1, *Are there Dipsas species other than D. catesbyi and D. variegata from the Guianas and Venezuela in the collection?*, we notice that *the Guianas* does not denote one country, instead it denotes Guyana (formerly British Guiana), Suriname (formerly Dutch Guiana) and French Guiana. Furthermore, for each of these names alternate spellings exist, and the fact that our database contains data in several languages may also impair relevant records from being retrieved. Fortunately, GeoNames contains many of the synonyms to automatically expand our query with.

Several flavours of a geographical expansion module were investigated, such as in addition to expanding to synonymous terms (for example in different languages), to expand to hypernyms or hyponyms, following the idea of (Voorhees, 1994). Hypernym expansion operates in such a way that if the query contains the term 'Nebraska', the query is expanded to 'United States of America', to remedy the negative influence of missing values in the 'province/state' column. Hyponym expansion works the other way around; a broad term such as 'United States of America' is expanded to all of its known hyponyms in the next level of the geographical ontology. Although hypernym and hyponym expansion are popular approaches that have been known to work for other systems (see Navigli & Velardi, 2003 for an overview) it does not aid object retrieval for the herpetological collection in these experiments. Therefore the geographical expansion was limited to expanding only to synonymous terms and location names in different languages.

6.2.3 Ranking

In order to present the user with the more relevant records first, two ranking methods were investigated.

RecordRank

RecordRank is a simplified version of the basic PageRank algorithm developed by the founders of Google in 1998 to rank results by relevancy (Brin & Page, 1998). The main assumption behind PageRank is that some webpages are more authoritative than others and those should rank higher than pages that are deemed less authoritative. The idea to rank the retrieval results by some measure of authority is given by the hypothesis that researchers might pose more questions about the specimens or species NCB Naturalis is known for (e.g., the reptiles and amphibians collections contain many specimens from the Amazon, therefore researchers might ask more about that part of the collection than about specimens collected in Africa as there are fewer of those).

Authority in PageRank is measured by the number of incoming links to a page. Also, links from pages with a higher PageRank are considered more important than links from pages with a lower PageRank.

The PageRank algorithm has sparked interest in applications other than search engines as ranking results for entity relation graphs (Chakrabarti, 2007) and Word Sense Disambiguation (Agirre & Soroa, 2009). Similar to our aim, the PageRank algorithm has also been translated to a relational database setting in (Balmin et al., 2004). In this work, databases

are translated to modelled graphs in which objects are nodes and their semantic connections the edges. Although the database we used was originally a flat table, the domain ontology that was developed for the natural history domain can enrich the databases with the necessary structure to consider them as a relational data resource.
In order to go from a ranking of objects in the domain to a ranking of records in the database the scores of all objects that occur in a database record are added up and normalised over the number of objects present in the database record (as database cells can be empty). For every database record the scores of every value are added up resulting in a RecordRank score by which the database records can be ranked.
A drawback of RecordRank is that for broader queries in a smaller domain the same set of database entries is always ranked on top. It may therefore be more useful to present a ranking of importance relative to a query. This idea was explored by Haveliwala in 2002, who presents a topic-sensitive PageRank approach. The idea of only computing the rank over the retrieved result is also used in the HITS algorithm, another link analysis algorithm that is used to rank web pages according to authority (Kleinberg, 1999). In Haveliwala (2002)'s approach, a set of topic-specific PageRank vectors is computed only from pages relevant to the query, which are then used to retrieve results for a query on a particular subject. Since the reptiles and amphibians database provides a smaller domain that cannot be easily broken up in more subdomains, the query-sensitive RecordRank module does not use precomputed vectors. Instead, for each query the RecordRank scores are computed at run-time, but only for the retrieved results. We distinguish the two flavours of RecordRank as Global RecordRank, in which database records are ranked by authority regardless of the query, and Local RecordRank, in which database records are ranked after records are retrieved.

Column order by importance

Analysis of the queries has shown that queries do not usually pertain to information in some of the longer database columns such as special remarks. Hence, when giving each column equal importance a query such as *Bufo marinus* will return results such as:

> *RMNH 34003* ***Bufo marinus*** *Lely Range, airstrip, distr. Marowijne, Surinam, 11-05-1975, 15.50h, on airstrip, near tall forest, 650m, l + d. X.X. Xxxxxxxxx. RMNH 34003*

as well as:

> *RMNH 20761 TANK NO Slide 1980-10- 37 (fell) Paleosuchus trigonatus 1 ex. km 110, 19-09-1980, 20.45 h, in swamp, flooded part of forest with many dead trees and low bushes, near jeep trail through tall forest, 100 m. length 1.445 m, skin and carcass to create skeleton. Stomach contents kept separately: crab +* ***Bufo marinus*** *+ grit. Observed this specimen already on 16-09-1980 (see p.89).*

After analysis of the queries it was clear that a large majority of the queries pertain to the request for information from the genus and species columns and never from the special remarks column in which one might find information on a specimen's stomach contents. Records with matches found in these columns, as well as in the registration number column are thus presented before records with matches found in other columns.

6.3 Experiments and results

In this section, the results of the experiments of the retrieval of records from the reptiles and amphibians database are presented. Only the first 5000 results returned for each query are evaluated using the evaluation script used in the Text REtrieval Conferences (TREC)[14]. In

[14] http://trec.nist.gov/ Last visited: 27 April 2011

ALL	UnExp	TaxExp	GeoExp	TaxGeoExp
Precision	33.07	**22.84** ▽	**20.92** ▽	32.88 ▽
Recall	31.67	**68.66** ▲	**83.30** ▲	**61.82** ▲
MAP	30.04	**41.45** ▲	**47.61** ▲	**44.78** ▲
ANY	UnExp	TaxExp	GeoExp	TaxGeoExp
Precision	21.62	**15.88** ▽	21.56 ▽	21.62 •
Recall	84.37	84.37 •	84.37 •	84.37 •
MAP	28.28	28.87 ▲	28.87 ▲	28.87 ▲
COMPLEX	UnExp	TaxExp	GeoExp	TaxGeoExp
Precision	40.13	**22.86** ▽	**20.95** ▽	30.38 ▽
Recall	37.59	**69.18** ▲	**85.85** ▲	**54.18** ▲
MAP	35.87	**44.29** ▲	**51.61** ▲	41.14 ▲

Table 8. Precision, recall and mean average precision scores for baseline and expansion modules

each of the tables presented below, the bold face results are significant with respect to the baseline results that the module is compared to. All significance scores are computed at the p=0.05 level using a paired t-test. The ALL query mode denotes a simple keyword search in which only records should be retrieved in which all query terms match. The ANY query mode is another simple query mode in which records should be retrieved in which any of the query terms match. The interpreted query mode (as described in Subsection 6.2.1) is denoted by COMPLEX in the tables.

The precision, recall and mean average precision (MAP) for the interpretation and expansion modules are presented in Table 8. As the results in Table 8 show, the ALL query mode benefits more than the ANY query mode of the query expansion. This is due to the fact that the ANY query mode already achieves high recall, simply because it retrieves records in which at least one of the query terms match. Separately, the expansion modules perform best (denoted by TAXEXP for taxonomic expansion and GEOEXP for geographic expansion). When combined, and thus when they expand both the geographic and the taxonomic queries (TAXGEOEXP), the achieved results are mixed. For the ALL query mode, the precision does not deteriorate significantly (whereas it does for the separate expansion modules), but recall does not improve as much as expected, therefore this module is not further investigated. This is probably due to an explosion of expanded terms for each query term and the subsequent retrieval of too many records.

The experiments carried out with the query interpretation module are found in the lower part of Table 8. The precision and mean average precision scores for the interpreted query mode are significantly higher than for the simple query modes. On its own, the COMPLEX query mode improves the mean average precision with 5.83% over the ALL query mode, and with 7.59% for the ANY query mode. Together with the query expansion modules, the COMPLEX query mode helps improve the scores even more, in particular the geographic expansion module. The difference in recall between the unexpanded ALL query mode experiments and the geographically expanded COMPLEX query mode experiments is even more than 50% (from 31.67% to 85.85%). Also the ALL query mode benefits from query expansion.

In Table 9 the mean average precision scores for the ranking modules are presented. Our assumption that the RecordRank modules would aid performance because the more authoritative records are presented first proved wrong. For the unexpanded queries, the mean average precision improves, but not significantly. For the expanded queries, the RecordRank modules even harm performance.

ALL	UnExp	TaxExp	GeoExp
GlobalRecordRank	30.27 ▲	**23.81** ▽	**18.25** ▽
LocalRecordRank	30.24 ▲	**27.79** ▽	**19.51** ▽
GenSpec	30.40 ▲	39.77 ▽	41.68 ▽
Unranked	30.04	41.45	47.61
ANY	UnExp	TaxExp	GeoExp
GlobalRecordRank	29.47 ▲	**23.81** ▽	**18.98** ▽
LocalRecordRank	29.17 ▲	23.42 ▽	**19.51** ▽
GenSpec	**42.38** ▲	**39.89** ▲	**39.86** ▲
Unranked	28.28	28.87	28.87
COMPLEX	UnExp	TaxExp	GeoExp
GlobalRecordRank	36.15 ▲	**23.83** ▽	**18.25** ▽
LocalRecordRank	36.11 ▲	**27.80** ▽	**19.49** ▽
GenSpec	36.23 ▲	39.75 ▽	**41.60** ▽
Unranked	35.87	44.29	51.61

Table 9. Mean average precision results expanded ranked reptile and amphibian queries

	UnExp	TaxExp	GeoExp	TaxGeoExp
ALL	32	66	78	63
ANY	78	78	78	78
COMPLEX	38	66	78	54

Table 10. Number of reptiles and amphibians queries for which one or more relevant results are retrieved

Due to the precise manner of querying provided by the COMPLEX query mode and the limitations imposed by the ALL query mode, result ranking only significantly aids the ANY query mode.
The GENSPEC module, that ranks records in which a match is found in the genus and species columns higher than the records in which a match is found in other columns does significantly improve results for the ALL and ANY query modes. For the interpreted query mode, results were already better and thus the ranking does not significantly aid performance.
If we look at the results in Tables 10, we see that, even though the precision drops when query expansion is used, the number of queries for which at least one relevant record is retrieved more than doubles. Thereby, it must also be noted that there are 16 queries for the reptiles and amphibians, for which there are no relevant records present in the databases. This means that for only six queries for which a relevant record should have been retrieved remain unanswered.

7. Conclusions

In this contribution, we first presented a new approach to collection digitisation, and a novel collection registration system (CRS) as implemented at NCB Naturalis. The new CRS enables the researcher to search in unfiltered collection unit metadata, allowing for new interpretations. Previously, searching in collection databases produced filtered, interpreted data, further constrained by the fact that databases only covered specialised sub-collections, making it hard for the expert, and impossible for the non-expert, to assess the value of the search results. The new system, operating on enriched data, promises to not only aid

the expert better, but also provide means to visualise search results in ways suitable to the layperson, such as plotting findings on maps and timelines.
Our contribution then focused on two systems aimed at improving the accessibility of data in the CRS; the systems are semi-automatic, in the sense that they perform automated steps in the process of data cleaning and data retrieval, with the aim of supporting experts by saving time (as manual cleaning of all data is simply infeasible) and finding relevant information faster.
The computer-supported data cleaning system presented uses logical rules to detect clear violations of constraints in pairs of dates (a collection of a specimen always precedes all other actions), in geographical names (a Brazilian city needs to be located in Brazil), and in taxonomic names (a species name has to fit a path in the taxonomic tree). The fact that 'hard' domain constraints are used does not constrain the applicability of the system (although all kinds of variations and changes through time can cause certain relations be softer than the rule-based method assumes).
The knowledge-driven data retrieval system indeed boosted the usability of digital information considerably. We observed significantly better retrieval of specimen cases from the CRS when the queries were automatically improved, either by expansion of taxonomic or geographical names (e.g., by their synonyms), or by guiding the matching function to match on particular database fields rather than all fields. Authority-based re-ranking as used in web search engines did not prove to be useful, indicating that the collection database, when viewed as a graph (a relational database) does not have the typical small-world network properties with 'authority' nodes that the web has.

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As everybody knows, the dynamic interactions between biotic and abiotic factors, as well as the anthropic ones, considerably affect global climate changes and consequently biology, ecology and distribution of life forms of our planet. These important natural events affect all ecosystems, causing important changes on biodiversity. Systematic and phylogenetic studies, biogeographic distribution analysis and evaluations of diversity richness are focal topics of this book written by international experts, some even considering economical effects and future perspectives on the managing and conservation plans.

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