Pedro M. Galetti Jr. *Editor*

# Conservation Genetics in the Neotropics

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*To the best of my genes, Catarina, André and Fernando*

# Preface

*Conservation Genetics in the Neotropics* explores how genetics and the new technologies in genomics have been used for the conservation of plants and animals in the Neotropics and presents the new perspective for conservation genetics beyond the use of theory and methods in genetics for saving species at risk of extinction. In this new perspective, conservation genetics and genomics can assess communities through phylogenetic diversity, comparative phylogeography and environmental and invertebrate-derived DNA. Conservation genetics and genomics are presented as a helpful tool for solving taxonomic uncertainties and uncovering hidden biodiversity, in addition to assessing populations and their extinction risks, performing genetic management, conducting wildlife forensic analyses, assessing biology and molecular ecology, promoting the conservation of plant and animal communities and, finally, using conservation biology and genetics in science learning, exploring and illustrating these issues in the context of neotropical biodiversity. Focusing in the Neotropics, the book is organized into 23 chapters distributed across 7 sections – Introduction; Species and Conservation; Assessing and Managing Populations; Wildlife Forensic Genetics, Ecotoxicology and Conservation; Assessing Molecular Ecology and Communities; Conservation Genomics; Science Learning and Conservation. From DNA barcoding to gene trees and phylogenomics, and from chromosomes to molecular markers and genomics, the readers will be presented an overview of how genetics and genomics have been applied in the Neotropics for the conservation of species and populations. Expanding from the species level to the community or ecosystem levels, the book highlights the use of population genomics, phylogeography and environmental and invertebrate-derived DNA and how they can promote new paradigms in conservation genetics. The book also shows how both genetics and conservation can provide motivational tools for science learning and environmental education. Applications of conservation genetics for policy and decision making, as well as for the planning and implementation of conservation practices in the Neotropics, are addressed across the chapters. This

book might be useful for researchers and students in conservation genetics and biological conservation who are interested in Neotropics. It is expected that stakeholders and decision makers in conservation biology will also find this book useful.

São Carlos, Brazil Pedro M. Galetti Jr.

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# Part I
# Introduction

# Chapter 1
# A Fresh Look at Conservation Genetics in the Neotropics


**Pedro M. Galetti Jr.**

## 1.1 Introduction

It has been more than a century since genetics was first used to interpret an ecological response, when a mutant gene was reported to have been selectively eliminated by predation in caterpillars (Gerould 1921), thus founding what would later become known as ecological genetics. This discipline is defined by the study of the genetic bases of an organism's adaptation, i.e., the adaptations of wild populations to their environment (Ford 1964). Since then, genetics, evolution, ecology and conservation have been continuously intertwined, motivating many researchers to think about new challenges and propose new disciplines. Thus, the last ten decades have been a period of great transformation of our knowledge about all kinds of living organisms and their persistence on Earth. However, human activities have led to severe changes on our planet, resulting in a rapid loss of species and populations, and characterizing a true global biodiversity crisis (Bellard et al. 2012; Dirzo et al. 2014; Haddad et al. 2015). Many efforts have been made to mitigate the impacts of human activities on biodiversity, as it is a well-established fact that genetic diversity plays a crucial role in the long-term persistence of species and populations (Hoban et al. 2023). Therefore, the use of genetics can provide important information and emerge as a powerful tool for conservation and effective decision-making (Torres-Florez et al. 2018).

In this scenario, conservation genetics emerged as an application of genetics in the scope of biological conservation, which later became a multidisciplinary field of study marked by the perception that the disorderly growth of human activities has a huge impact and jeopardizes local and global biodiversity. The birth of conservation

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genetics dates back to the early 1970s, with the papers of Sir Otto Frankel (Frankel 1970; Frankel 1974), a plant geneticist who first recognized the genetic importance for conservation. In the emblematic book *Conservation and Evolution*, Frankel and Soulé (1981) presented genetic problems associated with conservation, explored the meaning of genetic diversity for the maintenance of populations and ecosystems, and highlighted principles and practices of conservation genetics for the long-term conservation of nature. This established the foundations of conservation genetics. Later, Frankham et al. (2002) defined conservation genetics as a discipline that uses theoretical and methodological approaches of genetics to save species and populations facing the risk of extinction. A central idea in conservation genetics is that small, isolated populations can be threatened by the occurrence of random genetic drift and inbreeding (Ouborg et al. 2010). Genetic drift, defined as the random fluctuation of allele frequencies across generations, can lead to the random loss or fixation of alleles when it occurs in small populations. This can result in the loss of adaptive alleles, or in the fixation of deleterious alleles. Inbreeding, on the other hand, increases the frequency of homozygotes, which can expose deleterious alleles and lead to inbreeding depression, i.e., a reduction in individual fitness on average. Although both genetic drift and inbreeding can occur in large populations, their effects are much more pronounced in small populations, and at least three important consequences can result from these effects. In small, inbred populations, the reduction of individual fitness can decrease their viability in the short term. In addition, the loss of genetic variants in a small population can compromise its evolutionary adaptive potential, and reduce its long-term viability in a changing environment. Finally, genetic drift, independently occurring in small and isolated populations, may promote genetic divergence between them, compromising the genetic unity of the species. This may also lead to outbreeding depression, if the gene flow between the groups is restored (Frankham et al. 2017; Frankham et al. 2002).

The increasing development of molecular technologies has allowed researchers to evaluate these theoretical effects on small populations and confirm conservation genetics as a well-established, empirical discipline. However, despite the great expansion of conservation genetics worldwide, it remains disproportionately understudied in the Neotropics. The Neotropics harbor a huge biodiversity, with endemic species which have been increasingly threatened by habitat degradation and wild population decline, which could lead to high rates of extinction in the future (Dirzo et al. 2014). Despite all this, between 1992 and 2014, publications on conservation genetics from Latin America featured almost ten times less frequently in indexed journals than those from European countries (Torres-Florez et al. 2018), and there is no reason to believe that this scenario has changed in recent years. Many factors may be causing this disproportionality, but it is clear that conservation genetics in the Neotropics has much to contribute to the conservation of this important biodiversity.

## 1.2 Conservation Genetics at the Species Level

Traditionally, conservation genetics has been focused on biological diversity at the species level, and, while its main goal is to save endangered species from the risk of extinction (Frankham et al. 2002), many studies published worldwide in the field of genetics applied to conservation have been targeting on non-threatened species (Torres-Florez et al. 2018). Genetics – applied to a species or its populations – has been utilized for a range of general topics in biodiversity conservation, from resolving taxonomic uncertainties to the long-term monitoring of populations. Genetics has been employed around the world to assist in various aspects of conservation, such as defining evolutionary significant units (ESUs) and/or management units, minimizing inbreeding and loss of genetic diversity in populations, managing captive populations for reintroduction, assessing invasive species and their impacts on threatened species, estimating sex ratio, population size and demographic history, contributing to management plans and forensic actions, and predicting extinction risk and responses to environmental changes (Frankham et al. 2017; Torres-Florez et al. 2018); more recently, the use of genetics in conservation has been increasing in the Neotropics as well.

Fish, for instance, are among the most diverse groups of neotropical vertebrates, and present several taxonomic challenges. Morphologically similar species often form what is known as a species complex, in which species are virtually indistinguishable through their morphology or color patterns. In these cases, genetic tools, such as DNA barcoding (using the COI gene) or other molecular gene identification techniques, have revealed a significant hidden biodiversity (Pires et al. 2017; Ramírez et al. 2017a; Silva-Santos et al. 2018). Molecular analyses have been integrated with chromosome and morphology studies to describe new species (Garavello et al. 2021) or even entirely new genera (e.g., *Megaleporinus*, Ramírez et al. 2017b).

Indeed, there is no major living taxon that has not received some contribution from genetic investigations for the identification of hidden biodiversity, either by chromosomal analyses, molecular methods, or both. Metagenomics has revealed to science numerous new bacteria and archaebacteria, most of which are known only as Operational Taxonomic Units (OTUs) and have been deposited in public databases such as GenBank, but still require further biological characterization. Plants and animals have also benefited from the power of molecular tools to reveal their hidden biodiversity. Molecular phylogenies and species delimitation methods, as well as DNA barcoding, can be included in a growing body of methodologies which have proven to be useful in revealing hidden biodiversity in plants (Vijayan and Tsou 2010; Lima et al. 2018) and in most animal groups (Ahmed 2022; Fišer and Buzan 2014) all over the world, including the hyperdiverse Neotropics.

The number of genetic population studies that have contributed to the conservation of threatened species in the Neotropics has increased significantly. However, for various reasons, most studies are conducted on species of low conservation concern. In a world increasingly devastated and fragmented by human actions, reduced gene flow and the loss of genetic variation have been described in several

populations of neotropical plants (Vitorino et al. 2020), fish (Machado et al. 2022), birds (Banhos et al. 2016), and mammals (Ayala-Burbano et al. 2017). Inbreeding in small populations has also been reported in fish (Langen et al. 2011; Coimbra et al. 2020), frogs (Nali et al. 2020), and mammals (Collevatti et al. 2007). It is worth noting that, until the last review in 2014, around 40% of the conservation genetic studies in Latin America had focused on population structure (Torres-Florez et al. 2018), highlighting the concern of researchers in clarifying the genetic consequences of habitat fragmentation. There is no reason to believe that this concern will diminish in the near future. While ESUs have been suggested in fish species such as *Pseudoplatystoma corruscans*, which has a wide distribution in various hydrographic basins (Carvalho et al. 2012), genetic analyses have recently been used to evaluate the translocation and reintroduction of a small endangered primate (Moraes et al. 2017), *Leontopithecus rosalia*, which was successfully done a few decades ago. Several other genetic studies have focused on *ex situ* populations, which can serve as an insurance policy for an endangered species by producing new individuals that can help in recovering wild populations at risk of extinction. However, *ex situ* populations are often comprised of a reduced number of individuals, and are prone to showing loss of genetic variation, inbreeding, and inbreeding depression, which have been investigated in primates (Ayala-Burbano et al. 2017, 2020), for instance, or hybridization, which has been investigated in birds (Costa et al. 2017).

Genetics applied to conservation has already made meaningful contributions to the knowledge of species and their populations, and it has shown great potential to help monitor and manage the *in situ* and *ex situ* populations of threatened species. Expanding these studies could lead to significant progress, particularly for the persistence of biodiversity in the hyperdiverse Neotropics.

## 1.3 Genetics for Studying Communities and Ecosystems

Biodiversity has long been recognized as encompassing not only the diversity of species, but also genetic and ecosystem diversity (Wilson 1988). While the primary goal of conservation genetics is to preserve endangered species and their genetic diversity, it is noteworthy how genetic and molecular tools can also aid in the conservation of communities and ecosystems, such as in identifying priority areas for community conservation. At least three important approaches – phylogenetic diversity, comparative phylogeography, and environmental DNA – can illustrate the powerful use of genetics and molecular information to contribute for a better understanding of structures and functions of the biological diversity present in diverse ecosystems.

### *1.3.1 Phylogenetic Diversity*

Phylogenetic diversity (PD) was introduced by Faith (1992) to address the goal of conserving biodiversity at the environmental or community levels, rather than just assessing biodiversity at the species level. There are various metrics for estimating phylogenetic diversity, all of which are most frequently based on molecular data, making genetic information essential for these studies. The most commonly used metric, Faith's phylogenetic diversity ($PD_{Faith}$) (Faith 1992), is based on cladistic information, i.e., it is the sum of the lengths of all branches in a molecular phylogenetic tree containing a set of taxa from the entire community. PD is strongly correlated with species richness (Tucker et al. 2017), whereas other metrics based on the average pairwise genetic distance of all species (MPD, Webb 2000), or on the average pairwise genetic distance between closely related species (MNTD, Webb et al. 2002), are less dependent on species richness. Higher MPD (Mean Pairwise Distance) values indicate that the assessed set of species in the community are from a wide range of clades, whereas a high MNTD (Mean Nearest Taxon Distance) suggests that closely related species do not co-occur in the community. Regardless of the metric used, a more complex and less redundant local community will show higher phylogenetic diversity, suggesting higher priority for the conservation of a broader biodiversity. In contrast, a local community showing lower phylogenetic diversity might indicate a local loss of species, and might be a measure of the impact of habitat loss.

Phylogenetic diversity has been assessed in several taxa in the Neotropics, such as in plants (Perea et al. 2022), bees (Antonini et al. 2017), birds (Hanz et al. 2019), and mammals (Gómez-Ortiz et al. 2017), but few studies have primarily focused on conservation. For example, PD was used to evaluate the impacts of habitat loss on the evolutionary diversity of snakes (Fenker et al. 2014), the effects of oil palm management on bird communities (Prescott et al. 2016), spatial variation in communities of Atlantic Forest opiliones (Nogueira et al. 2019), loss of phylogenetic diversity of bats across a habitat gradient in the Amazon (Aninta et al. 2019), the identification of areas of high mammalian phylogenetic diversity in order to suggest priority areas for conservation (Aguillar-Tomasini et al. 2021), and to guide the conservation of crop wild relatives (González-Orozco et al. 2021).

Indeed, by assessing biological diversity at the community level (Faith 1992), phylogenetic diversity can provide a good picture of the evolutionary history of communities, and how they might respond temporally and spatially to a range of stressors, such as habitat loss and fragmentation. Phylogenetic diversity can also be used to guide large-scale conservation approaches, particularly for protecting the megadiversity of the Neotropics.

### *1.3.2 Comparative Phylogeography*

Phylogeography can be a powerful tool for conservation. This field of study, which aims at understanding the geographic arrangement of genotypes, was first proposed by Avise et al. (1987), and was rapidly recognized as an important approach for inferring population evolutionary history. Phylogeography focuses heavily on describing population relationships within a single species, and has been widely used to study almost all living groups. In plants and animals, phylogeography has revealed several cases of spatial genetic differentiation among populations, potentially contributing to the conservation of these populations. More information on this topic can be found in Chaps. 6 and 21.

With the expansion of these studies, comparative phylogeography (CP) emerged with the aim of understanding the evolutionary and biogeographical history of species that are co-distributed in space. In an integrative work, comparative phylogeography among resident vertebrates in the wet tropical rainforests identified genetically divergent areas important for conservation (Moritz and Faith 1998). The authors concluded that combining comparative phylogeography (population level) with phylogenetic diversity (species level) could improve biodiversity conservation planning. It is impressive to observe how much comparative phylogeography applied to conservation studies has advanced in the few decades since its birth. A quick search on Web of Science, using the terms "comparative phylogenetic*" AND "conservation", revealed almost four hundred papers published from 1997 to 2023. In general, these works combined the phylogeographies of two or more co-distributed species to infer areas of highest priority for conservation. For instance, comparative phylogeography within a crustacean group (*Excirolana*) highlighted the importance of this molecular approach in supporting conservation actions on sandy beaches, an ecosystem highly impacted by anthropogenic stressors (von der Heyden et al. 2020). Similarly, a study involving tree species was used to investigate large-scale conservation corridors in subtropical shrublands, and to support planning decisions for their conservation (Potts et al. 2013).

Comparative phylogeography is still in its infancy in the Neotropics, and has primarily been used to investigate the association between the evolutionary histories of two or more species, and to understand the dynamics of their evolution in different habitats or biomes. For instance, CP has been used in birds and bats to evaluate whether the presence of barriers can promote different phylogeographic patterns among ecologically diverse species (Matos et al. 2016; Loureiro et al. 2020, respectively), to test biogeographic hypotheses in river otters (Ruiz-García et al. 2018), and to investigate the impact of the climate change which occurred in the Pleistocene on orchid bees (López-Uribe et al. 2014). To our knowledge, there are still no comparative phylogenetic studies primarily designed to answer questions on conservation in the Neotropics. Considering the aforementioned potential, the use of CP to identify priority areas in the Neotropics could be valuable for the conservation of its megadiversity.

### *1.3.3 Environmental DNA and Invertebrate-Derived DNA and Conservation*

The use of environmental DNA (eDNA) and invertebrate-derived DNA or ingested DNA (iDNA), in association with modern sequencing technologies, has been increasingly recognized as a powerful tool for biodiversity assessment and conservation (Carvalho et al. 2022; see Chap. 18 for more details). Human-promoted habitat loss and climate change have led to a true global biodiversity crisis (Bellard et al. 2012; Dirzo et al. 2014; Haddad et al. 2015), and a more comprehensive understanding of biodiversity is critical for nature conservation. Traditional methods for surveying species are generally limited to sampling at a local scale and with a substantial effort. New technologies, such as metabarcoding using eDNA and iDNA, can be powerful tools for biodiversity surveys, and for supporting the conservation of natural ecosystems (Carvalho et al. 2022).

Environmental DNA obtained from water, soil, or air can provide more accurate and less time-consuming biodiversity surveys, as it is capable of assessing the species diversity – including rare and elusive species – from a large number of samples and in large-scale surveys, notably reducing labor costs (Bohmann et al. 2014; Rees et al. 2014). In addition, the community of vertebrates can also be assessed through the iDNA obtained from the guts of invertebrates such as flies, mosquitoes, leeches, and beetles (Calvignac-Spencer et al. 2013; Schnell et al. 2015; Kocher et al. 2017a), as easily and efficiently as with eDNA.

According to Taberlet et al. (2012), the term "environmental DNA" first appeared at the beginning of the 2000s, coinciding with the emergence of the earliest metagenomic studies (Rondon et al. 2000; Gillespie et al. 2002). However, the first reference to an eDNA extraction method is credited to Ogram et al. (1987), who described a method for extracting microbial DNA from sediments. Since then, the application of metagenomics to conservation has become a feasible and convenient task. For example, a combination of metagenomics, microscopy, microbe cultivation, and water chemistry, was used to characterize microbial communities in coral atolls, furthering the scientific understanding of the association of microbes with the degradation of coral reef ecosystems across the globe (Dinsdale et al. 2008). Soon after, eDNA began to be used to assess eukaryote communities on a global scale (e.g., Bhadury et al. 2006), mainly through next-generation sequencing and metabarcoding for taxon identification (e.g., Chariton et al. 2010).

Few studies have utilized eDNA or iDNA to evaluate eukaryotic communities in the Neotropics. The first study using eDNA in the Neotropics assessed amphibian communities in Brazilian Atlantic Forest streams, and compared the results with conventional field surveys (Sasso et al. 2017). Of the ten species that had been previously identified – over a five-year period – through visual-acoustic methods, being thus linked with the streams at least during one of their life stages (i.e., egg, tadpole or post-metamorphic), the authors were able to detect nine of them through eDNA metabarcoding from water samples collected over 4 days. This result illustrates how the eDNA method can be beneficial in supporting the conservation of neotropical

amphibians. In the same year, Kocher et al. (2017b) reported short mitochondrial sequences for the identification of Amazon mammals through metabarcoding.

Subsequently, eDNA from water samples and metabarcoding were successfully employed to assess the mammalian communities in two highly biodiverse regions of Brazil, the Amazon and the Atlantic Forest (Sales et al. 2020). To our knowledge, this was the first study aimed at detecting neotropical mammals using DNA extracted from water, an effort in which the potential and challenges of eDNA monitoring for mammals were highlighted. Indeed, eDNA from water bodies has predominantly been used to detect fish communities around the world (for a review, see Carvalho et al. 2022), and this has also been observed in the Neotropics (Cantera et al. 2019; Milan et al. 2020; Sales et al. 2021; Santana et al. 2021; Carvalho and Leal 2023).

On the other hand, iDNA obtained from the guts of insects (either hematophagous, saprophagous, or coprophagous ones) has been predominantly used to assess mammal communities (Calvignac-Spencer et al. 2013; Schnell et al. 2015; Rodgers et al. 2017; Saranholi et al. 2023), although other vertebrates have also been identified (Calvignac-Spencer et al. 2013; Saranholi et al. 2023). iDNA has also been used in ecological investigations, such as dietary studies focused on disease transmission by hematophagous insects (Bitome-Essono et al. 2017), pathogen and virome assessment (Bass et al. 2023), trophic interactions (Paula et al. 2016), and biological control (Paula and Andow 2022). In the Neotropics, the use of iDNA to assess animal communities is still very incipient, and it is mostly dedicated to testing and comparing different insect groups, mainly for surveys focused on mammals (Massey et al. 2022; Saranholi et al. 2023).

An important limitation in the use of eDNA/iDNA and metabarcoding is the availability (or rather, the lack thereof) of reference barcoding sequences, especially when working in the Neotropics. Further efforts are still required to obtain good sets of these sequences. However, due to the relative ease of collecting insects or environmental samples from different biomes, as well as the cost-effectiveness and time-saving benefits of eDNA/iDNA analyses, and their potential for future technological and methodological advancements, these approaches may still become the primary tools for conducting easy and efficient biodiversity surveys worldwide, particularly in the hyperdiverse Neotropics.

## 1.4 Conservation Genomics in Neotropics

Conservation genomics can be defined similarly to conservation genetics, with the difference being the amount of molecular information available from genomic studies (Avise 2010). Thus, conservation genomics refers to the use of genomic techniques to address problems in conservation biology (Allendorf et al. 2010). The number of genomes sequenced is rapidly increasing and, while the first reported eukaryotic genome sequences were from model species (e.g., *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Drosophila melanogaster*), most of the currently sequenced species are non-model organisms, indicating a growing availability of

genomic information from an ever-expanding number of plants and animals (Ellegren 2014; for more on this, see Chaps. 19, 20, and 21).

Genomic techniques can be categorized into three basic classes (Allendorf et al. 2010). The first one consists of SNP (Single Nucleotide Polymorphism) genotyping microarrays, which are used to detect single-base polymorphisms across the DNA of a population. However, using SNP microarrays or SNP chips suffers from an important limitation. Because an SNP chip is built to be species-specific, its use is often limited to the target species. Thus, considering the high costs associated with their development and construction, SNP microarrays are most commonly designed for either model species or species of great economic interest. For instance, SNP microarrays are used in human genetics, from detecting single-gene mutations (Bruno et al. 2011) to forensic investigations (Voskoboinik et al. 2015). They have also been used for genotyping in animal breeding, such as in alpacas (*Vicugna pacos*) (Calderon et al. 2021). The second class of genomic techniques is reduced-genome representation sequencing – or GBS (Genotyping-by-Sequencing), with methods such as RADseq (Restriction-site Associated DNA sequencing) and ddRADseq (Double-digested Restriction-site Associated DNA sequencing), which employ next-generation sequencing technology to target orthologous regions across the genome of different individuals. For example, ddRADseq has been used to develop a panel of SNPs to investigate population polymorphisms in migratory birds (Larison et al. 2021). A similar approach has been used to investigate diversification within a neotropical toad species, and to test a set of hypotheses concerning reduced gene flow among populations (Thomé et al. 2021). Finally, whole-genome sequencing, which was initially used for describing the genomes of various organisms, from viruses and bacteria to plants and animals, has now been increasingly used in population genomic studies. Public databases (e.g., GenBank) already make a considerable number of genomes available, which have been studied in order to answer a wide variety of questions. It is noteworthy that all these genomic techniques produce vast amounts of data, requiring the critical use of bioinformatics for their analyses (Allendorf et al. 2010).

Population genomics focuses on the variations between genomes and populations (Luikart et al. 2003), and the large-scale resequencing of genomes from various populations could lead to the identification of genes and genomic regions linked to fitness-related traits (Ellegren 2014). Conservation genomics may take advantage of this approach to study the genetic bases of local adaptations, or inbreeding depression (Allendorf et al. 2010). On the other hand, with the use of neutral markers, conservation genomics can also estimate population parameters such as genetic diversity, gene flow, and effective population size, which may be employed to support species management and conservation. Thus, genomic tools have great potential to improve the management of populations for conservation, from estimating the genetic parameters of populations with basis on a large number of neutral markers, to identifying loci linked to local adaptations (Allendorf et al. 2010).

In the Neotropics, genomic tools have been increasingly used for the conservation of plants and animals. For example, landscape genomic analyses have been used to produce insights on the negative consequences of habitat loss, and to

recommend gene flow restoration among populations of endangered turtles (Gallego-García et al. 2019). Fish are among the groups that have been most extensively assessed by genomic approaches in the Neotropics, likely due to the expansion of aquaculture of native species, and to the importance of conserving these resources. Indeed, genomic tools have greatly enhanced our understanding of neotropical fish, and can aid in their conservation. For example, the development of SNP panels for population genetics (Martínez et al. 2016, 2017; Mastrochirico-Filho et al. 2016; Delord et al. 2018), the assessment of genetic diversity in breeding species (Mastrochirico-Filho et al. 2019), and the investigation of hybrid zones in an annual fish genus (García et al. 2019) have provided valuable insights. In addition, the production of linkage maps and the utilization of genome-wide association studies to investigate pathogen resistance (Mastrochirico-Filho et al. 2020; Ariede et al. 2022) and genes linked with the absence of intermuscular bones (Nunes et al. 2020) demonstrate the potential of genomic tools for supporting both aquaculture and conservation efforts.

The Neotropics are primarily composed of low- and middle-income countries that lack the infrastructure to manufacture equipment and chemicals for next-generation sequencing, which has made genomic research in the region difficult due to the high costs involved. Nevertheless, the lowering costs of these technologies have made them more accessible globally, which should have a significant impact on future biodiversity conservation studies in the Neotropics. (For more information, see Chaps. 19, 20, and 21).

## 1.5 Final Considerations

Conservation genetics is a well-established field of study around the world, including in the hyperdiverse Neotropics. From DNA barcoding to genomics, conservation genetic approaches have been widely used to address a variety of conservation questions in plants and animals, and to offer management actions for target species (Torres-Florez et al. 2018). Still, despite the many achievements of the past decades, several important questions related to selectively important genetic variation, fitness and adaptation, as well as genetic and environmental interactions, continue to challenge conservation geneticists. Most inferences on conservation genetics are obtained from microsatellite-based population studies, but the true value of neutral genetic diversity for species conservation is still under debate (García-Dorado & Caballero 2021; Teixeira & Huber 2021; Hoban et al. 2023). In this context, conservation genomics can offer new opportunities for monitoring changes in allele frequency (both neutral and non-neutral), and for evaluating the effects of genetic drift and natural selection within and between populations (Allendorf et al. 2010), in addition to identifying genes and genomic regions involved in adaptation (Ellegren 2014).

Expanding from the species level to the community or ecosystem levels can help address broader conservation concerns, and using techniques such as phylogenetic

diversity, comparative phylogeography, and eDNA/iDNA can promote new paradigms in conservation genetics. Regardless of the questions being asked, it appears that the importance of conservation genetics and genomics is still poorly understood by decision makers. In their review, Torres-Florez et al. (2018) found few cases of improvements in species protection resulting from published research on conservation genetics. Applying conservation genetics and genomics information to policy and decision making, as well as to the planning and implementation of conservation practices, remains a significant challenge. This is particularly true in the Neotropics, where biodiversity is vast and includes strategic biomes such as the Amazon Forest, which is crucial for global sustainability.

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# Part II
# Species and Conservation

# Chapter 2
# DNA Barcoding for Assessing Biodiversity


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## 2.1 Introduction

Hebert et al. (2003a) proposed a method to identify species by using a standardized DNA sequence that would serve as *taxon* barcode, and thus be able to discriminate species, even without being an expert taxonomist. Using a fragment of the COI mitochondrial gene (cytochrome c oxidase subunit 1), they evaluated the divergence between closely related species showing that this marker is powerful enough to distinguish species in all animal phyla, except Cnidaria (Hebert et al. 2003a, b). This identification system would allow species to be identified even from a specimen fragment, eggs or immature stages, since the DNA barcode of these biological materials will correspond to that of taxonomically identifiable adult specimens (Stockle and Hebert 2008; Handy et al. 2011; Steinke et al. 2016). On the other hand, this system would also allow the identification of potential new species or cryptic species (Hebert et al. 2004a; Clare et al. 2011).

After that, BOLD Systems (https://www.boldsystems.org/), a repository for these barcodes, in which the COI barcode sequences and descriptive metadata and images of the voucher specimens can be uploaded, was developed (Ratnasingham and Hebert 2007). The challenge was to have a reference database to help everyone to identify species (Vernooy et al. 2010; Janzen 2010), "democratizing taxonomy" (Holloway 2006). Certainly, it is of great help in this time of biodiversity crisis in which the destruction of habitats is taking place at a very accelerated rate, with the consequent disappearance of species not yet known (Vernooy et al. 2010), and aid to face the "taxonomic impediment", i.e. the scarcity of trained taxonomist (Jörger

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and Schrödl 2013; Engel et al. 2021). Nevertheless, the DNA barcode is a complementary methodology and the training of students in the taxonomy of various groups of biodiversity should also be encouraged (De Carvalho et al. 2007; Engel et al. 2021).

## 2.2 DNA Barcoding, a Tool for Biological Identification

### *2.2.1 Molecular Markers*

The use of molecular markers has been showing aspects of nature previously interpreted differently by using only morphological characters (Woese and Fox 1977), and Avise et al. (1987) opened the doors to the interior of the species with the use of mitochondrial DNA (mtDNA). Due to its characteristics of no recombination, maternal inheritance and evolutionary rates faster than nuclear markers, mtDNA allows an accurate analysis of intraspecific and interspecific variation, and therefore a higher taxonomic resolution (Avise et al. 1987; Leray et al. 2019). Different mtDNA markers were already used to identify species, including 16S rRNA (Douris et al. 1998), COI (Wilding et al. 2000), cytochrome B (Harris et al. 2000; Rodríguez et al. 2009), control region (Eizirik et al. 1998; Dutton et al. 1999; Zink et al. 2001), even when revealing cryptic species (Holland et al. 2004; Beheregaray and Caccone 2007).

However, there was no unifying vision to identify the species of the great biodiversity of the planet, until Hebert et al. (2003a) propose the use of a 658 bp fragment from the 5′ end of the mitochondrial COI gene for animal identification, which, unlike the ribosomal genes (16S, 12S), presents no indels (insertions and deletions) that tend to hinder the alignment of the sequences. Being a standardized segment for all animal species, it should be possible to amplify with universal primers, and those of Folmer et al. (1994) were available. This segment is now known as "COI barcode region" (Barr et al. 2012) or "COI Folmer region" (Pentinsaari et al. 2016; Elbrecht and Leese 2017). Subsequently, other primers were designed for better COI amplification of particular groups (Ivanova et al. 2007), to amplify smaller fragments (mini barcodes) useful for working with degraded samples (Hajibabaei et al. 2006b; Meusnier et al. 2008; Rodrigues et al. 2020; Rodríguez-Castro et al. 2020) and also for metabarcoding (Elbrecht and Leese 2017).

Unfortunately, the COI gene is not useful as a barcode for plants and fungi. The experts searched for possible DNA markers and more than one region were suitable in plants, determining the use of plastidial genes, as rbcL (Ribulose-bisphosphate carboxylase) and matK (Maturase K) (Hollingsworth et al. 2011). In the case of fungi, the ITS (Internal Transcribed Spacer Region) marker was chosen as the universal DNA barcode (Schoch et al. 2012).

### 2.2.2 The Distance Approach and the DNA Barcoding Gap

For evaluating the intraspecific and interspecific genetic distance between COI sequences in animals and validate the DNA barcoding method as a molecular tool for species identification, Hebert et al. (2003a, b) used the Kimura-two-parameter nucleotide substitution model. In their evaluation of 13,320 pairs of congeneric species, they found more than 98% with a divergence greater than 2%, varying up to 53.7%; those species pairs with smaller divergences suggested recent origins (Hebert et al. 2003b). This variation can be visualized in a scatter plot (Fig. 2.1), showing intraspecific variation *versus* interspecific divergences of each species, where the threshold can be marked as 2%, for example (Hebert et al. 2004b). An alternative way is a histogram (Fig. 2.2), and in this case when we have morphologically and genetically differentiated species, the intraspecific variation is separated from the interspecific divergences, forming the "barcoding gap' '(Meyer and Paulay 2005). In the Automatic Barcode Gap Discovery (ABGD) method this barcoding gap analysis was automated (Puillandre et al. 2012). But there are cases in which the intra- and interspecific variations overlap, making it necessary to verify the morphological identification of the species, since they may be misidentifications or represent cryptic species (Hebert et al. 2004b; Meyer and Paulay 2005). Developed on

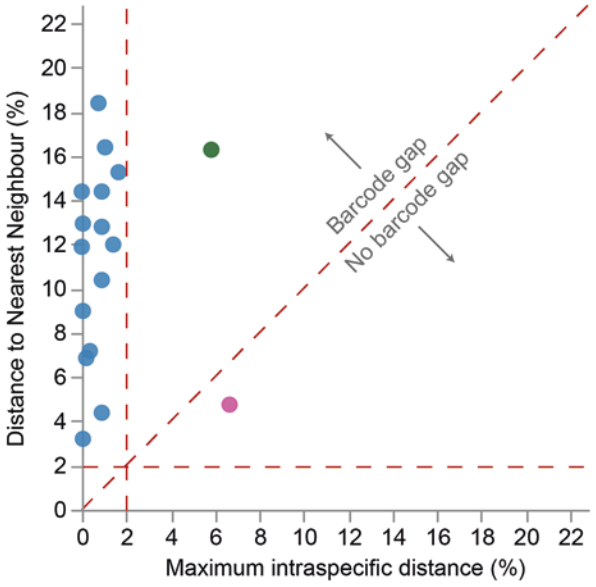


**Fig. 2.1** Scatter plot showing DNA barcode gap by each species in the analysis. The graph plots maximum intraspecific distance vs distance to nearest neighbour. The diagonal red line represents where both distances are equal. The 2% threshold is plotted as an example. The green circle represents a species with high intraspecific distance (higher than the threshold used), hiding a potential cryptic species. The red circle represents a species without barcode gap where the intraspecific distance is higher than the interspecific

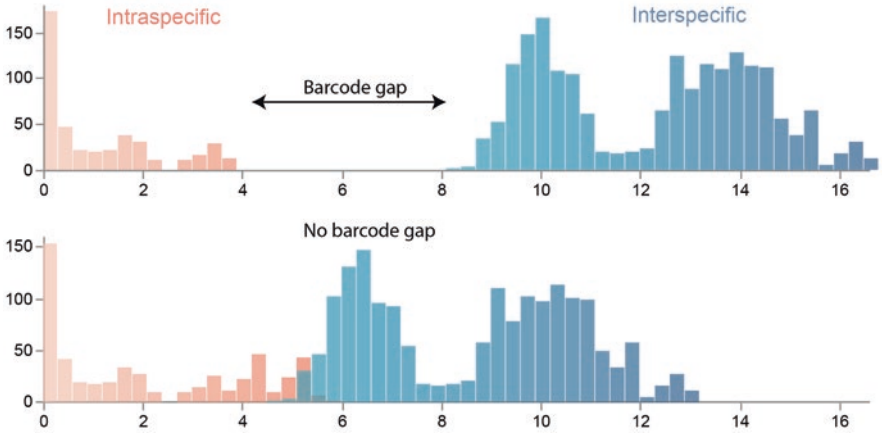


**Fig. 2.2** Histogram of intraspecific (red) and interspecific (blue) genetic distances. The figure illustrates the difference between a dataset with barcode gap versus a case with superposition of intraspecific and interspecific distances (no barcode gap)

the BOLD platform, the Barcode Index Number System (BIN) is a system that identifies unique clades in a Neighbour-Joining tree, which can represent either taxonomically well-identified species or putative species (Ratnasingham and Hebert 2013).

### *2.2.3 Worldwide Initiatives*

Soon this technology became known worldwide, the first DNA barcoding initiatives were promoted for the generation of official DNA barcodes, which could serve as a reference for the identification of species in various taxonomic groups. For example, in birds (All Birds Barcoding Initiative, ABBI) (Hebert et al. 2004b), fishes (Fish Barcode of Life Initiative, FISH-BOL) (Ward et al. 2009), lepidoptera (All Lepidoptera Barcode of Life) (Wilson 2011), and others. But how to get DNA barcodes from all the species on the planet, where there are still many species to be discovered and for which we still have no DNA barcodes? Several other initiatives were organized around the world with which the process has been accelerated (Miller et al. 2016), such as the Marine Barcode of Life (MarBOL) (Trivedi et al. 2016), or at the national level such as those of Canada (Hebert and Gregory 2005) and Germany (Hausmann et al. 2020), or more regional as the Área de Conservación Guanacaste (ACG) in Costa Rica (Miller et al. 2016), or by a particular group, such as disease-transmitting species, the Mosquito Barcoding Initiative (Madeira et al. 2021). In addition, COI sequences have also been generated by independent projects, not necessarily related to any registered initiative (Kwong et al. 2012).

It is important to note that all these initiatives are linked to the International Barcode of Life (iBOL), which achieved the goal of having 500,000 species barcoded in December 2015. A new project was recently launched, BIOSCAN, with more than 1000 researchers from 40 countries involved (IBOL 2021), that "will not only expand the barcode reference library to more than two million species, but will also deliver rich data on community composition from thousands of ecosystems around the planet and explore the biotic associations of hundreds of thousands of individual organisms by metabarcoding these specimens along with their cohort of symbionts, parasitoids, and other associated organisms, including host plants or prey species" (Hobern 2021).

## 2.3 DNA Barcoding Methodologies

### *2.3.1 BOLD System*

The BOLD Systems is a large bioinformatics platform launched in 2005, developed by the Center for Genomic Biodiversity in Canada. It emerged as a support to researchers in order to build a large reference library of DNA barcodes for eukaryotes (https://biodiversitygenomics.net/projects/bold/). However, BOLD has grown to become a powerful online workbench and central computing hub for the DNA barcode community and a resource for the general public (Milton et al. 2013). This platform allows storing, sharing, and analyzing DNA barcoding data (Hajibabaei et al. 2006a; Ratnasingham and Hebert 2007; Paz et al. 2011). It houses all phases of analytics, from data collection to validation of the registered library (Ratnasingham and Hebert 2007).

The platform features four main portals (i) data (over 1.7 M public records for sequence search and comparison), (ii) education (teachers and students can explore and contribute records), (iii) BIN registry (putative species) and (iv) a workbench of data collection and analyses (work area for researchers). It also has an updated record of the number of barcodes, BINs, species of animals, plants and fungi among others deposited in the platform. In addition, it makes available a collection of scientific articles, as well as the sequences of the primers used.

### *2.3.2 Voucher and Collection Data*

Molecular data have transformed the systematics and taxonomy of all organism groups. To publish this type of data, most journals require that sequences are deposited in accessible repositories, like GenBank (Pleijel et al. 2008). The main problems of these platforms are the non-request of vouchers, and the rapid increase in the number of sequences, inducing taxonomic uncertainties (Funk et al. 2005;

Pleijel et al. 2008). Currently, there are a large number of sequences deposited in GenBank that are incorrectly labeled, keeping *taxa* incorrectly associated with the deposited sequences (Pleijel et al. 2008).

To avoid these problems, vouchers represent an essential connection between the molecular data and the *taxon* and allow verifying and updating the taxonomic identity of the sequenced species (Lue et al. 2021). A DNA sequence will always be linked to a *taxon* voucher. Without the voucher, a molecular study cannot be fully confirmed, having to trust the person making the identification (Lue et al. 2021). Vouchers in biology are specimens, preparations, pictures or any part of the specimen that allow other researchers to examine the taxonomic identity assigned to a specimen by the author of a study (Astrin et al. 2013). The BOLD system uses vouchers that are associated with single-locus DNA sequences (Hebert et al. 2003a).

The deposit of vouchers in scientific collections is a prerequisite for the DNA barcoding (Peterson et al. 2007; Taylor and Harris 2012), as this voucher will be linked to a *taxon*, which will receive a molecular and taxonomic identification. For DNA barcoding, the voucher must contain the following information: (1) institution name and catalog number for each voucher sample, (2) geographic location, and (3) nature of voucher (e.g., entire specimen, only photograph, skeleton) (Peterson et al. 2007).

These *taxa* correctly identified, and their stored DNA can greatly encourage taxonomy and other fields of application, such as genome sequencing, Next-Generation Sequencing (NGS), data management, among others (Wong et al. 2012). It would also reduce costs as it would avoid repeating the processes of collecting and identifying samples, issuing vouchers and DNA sequencing (Taylor and Harris 2012).

Sample collection methods are different according to the taxonomic group. Before collection, it is necessary to gather several information on the target species, as intraspecific morphological variation, breeding season, ecological specialization, and distribution pattern (Eymann et al. 2010; Gemeinholzer et al. 2010). In the latter case, it is important to make assumptions about gene flow patterns and the number of individuals and populations that should be sampled, since animals with a fragmented distribution are indicators of genetic differentiation in isolated populations (Eymann et al. 2010; Gemeinholzer et al. 2010).

All voucher specimens must fill out the collection form before being deposited in the collection, this form must include general data about the location and especially georeferencing data (GPS) (Eymann et al. 2010; Gemeinholzer et al. 2010).

Sampled species are usually identified by morphological characters and should be photographed prior to tissue sampling and voucher preparation. In general, sampling at least five specimens of each different species is ideal (Ward et al. 2005), but more individuals would be sampled if the morphological diversity within the population is large (Eymann et al. 2010; Gemeinholzer et al. 2010).

Some large specimens due to their dimensions could represent a challenge for voucher storage. In these cases, an e-voucher would be an option, which can be summed up in a good photograph of the specimen combined with the registration of its tissue (DNA) (Eymann et al. 2010; Gemeinholzer et al. 2010). The collection of organisms in general (plants, animals, or fungi) must be in accordance with the

national and international legal aspects of the collection region, and if it is an unauthorized collection, this could harm the populations of endemic or native species, and also have serious legal consequences (Peterson et al. 2007; Eymann et al. 2010). The primary purpose of collection should always be to preserve DNA integrity and try to maintain a high-quality sample, because one without the other loses its value (Weigt et al. 2012).

### *2.3.3 Biological Preservation Methods*

There are several biological fixing methods considering the preservation of the specimen but not the integrity of DNA. Traditionally, most vertebrates use 10–20% formaldehyde as the main method of fixation, and depending on the size of the specimens, it is also necessary to inject it into their abdominal cavity to accelerate the fixation process inside their bodies, and later to preserve tissues, specimens are transferred to 70% ethanol (Uieda and Castro 1999; Ceríaco et al. 2014). That process could damage the DNA integrity, being difficult to downstream molecular protocols. In most of cases it is preferred to extract a piece of tissue and preserved it in 96–99% ethanol prior to specimen fixation.

With the advent of molecular tools, many fixation methods were modified specifically for DNA preservation. Most invertebrates and vertebrates are fixed in 96–99% ethanol for correct DNA preservation (Ward et al. 2005; Bergsten et al. 2012; Jeong et al. 2013), although some mofications occur. In zooplankton for example, after a short time of fixation with 96% ethanol, more than 50% of the initial volume is exchanged for a new 96% ethanol and refrigerated from the moment of collection in the field (Elías-Gutiérrez et al. 2018).

### *2.3.4 DNA Extraction Methods*

There are different methods of DNA extraction, those produced in each laboratory "in house", often based on a previously described protocol, and commercial kits (different brands) that follow the manufacturer's recommendations. Among the most common "in house" methods, (i) cetyl trimethylammonium bromide (CTAB) method for plants (Doyle and Doyle 1990), and modified for use in different animals (Chen et al. 2010; Chakraborty et al. 2020); (ii) saline solution (Aljanabi and Martinez 1997) and (iii) phenol-chloroform and isoamyl alcohol (PCI) (Sambrook et al. 1989), both frequently used for animal tissues; (iv) heating in NaOH inside a boiling water bath (H2 method) (Asadzadeh et al. 2010) for zooplankton; and glass fiber (GF) (Ivanova et al. 2006), for animal tissues, generally worked in plates of 96 samples.

In turn, many commercial kits have been developed for different *taxa* (e.g., insects, mollusks, plants), and protocol and/or components vary slightly according

to the type of the sample (blood, muscle, bone, hair and feces, in case of animals). For DNA extraction of a new taxonomic group it is always recommended to test different protocols, since each method has different strengths and weaknesses dependent of the *taxa* and presence of PCR inhibitors.

### 2.3.5 PCR and Primers Commonly Used

The DNA amplification is carried out through the polymerase chain reaction (PCR) method using a thermal cycler. For PCR to occur, in general, is necessary ultrapure water, PCR buffer 10x, 50 mM $Cl_2Mg$, 10 μM of each primer (forward and reverse), 10 mM dNTP mix, *Taq* polymerase (1–5 U/μL) and DNA template. Concentration of each chemical can change according to the *Taq* polymerase used. In some cases of complex samples, it is necessary to use additives such as 10% Trehalose (insects) (Wilson 2012), 15% Trehalose + Bovine serum albumin (invertebrates) (Evans and Paulay 2012).

The choice of primers will depend on the *taxa* under study (Table 2.1, for review Kress and Erickson 2012). No consensus has emerged for a universal barcode for plants, and different orders have been barcoded with different primers of different genes.

The programming in the thermal cycler will follow the steps of a conventional PCR (denaturation, annealing and extension), however, the annealing temperature will change according to the selected primer (40–55 °C).

### 2.3.6 Uploading Data to BOLD

To upload the DNA barcode sequences to BOLD, it is necessary to create an account and then a project (code and title), informing molecular marker used, initiative in which the collection is associated, if any, a project description, and users with different level of permission to perform various actions on the uploaded information (upload, edit, or both). Subsequently, to register a sample, an ID of the sample and the taxonomic level assigned (it can be at the phylum level) are required, besides the name of the *taxon*, voucher data (stored institution and catalog number), collection report (collector, date and coordinates), specimen identifier, COI sequence (animals) with at least 500 bp, primers used in the PCR and finally the trace files (.ab1 format).

The records can be uploaded individually (by filling in the requested fields and moving forward) or in groups (using the Excel tables and then uploading them to the system). Additionally, the trace files, the photographs of the specimens, and the edited sequences, can be uploaded individually or in groups. The advantage of uploaded individually is that you can have the information immediately while for a

**Table 2.1** Main DNA barcoding primers used and the target taxonomic groups

| Primers | Sequence 5′-3′ | Sense | *Taxa* | References |
|---|---|---|---|---|
| **Animals** | | | | |
| LCO1490 | GGTCAACAAATCATAAAGATATTGG | Forward | Metazoa (Universal) | Folmer et al. (1994) |
| HCO2198 | TAAACTTCAGGGTGACCAAAAAATCA | Reverse | | |
| dgLCO | GGTCAACAAATCATAAAGAYATYGG | Forward | (Degenerate Universal) | Meyer (2003) |
| dgHCO | TAAACTTCAGGGTGACCAAARAAYCA | Reverse | | |
| LepF1 | ATTCAACCAATCATAAAGATATTGG | Forward | Mammalian | Hebert et al. (2004a) |
| LepR1 | TAAACTTCTGGAGTCCAAAAAATCA | Reverse | | |
| CrustDF1 | GGTCWACAAAYCATAAAGAYATTGG | Forward | Crustacea | Radulovici et al. (2009) |
| CrustDR1 | TAAACYTCAGGRTGACCRAARAAYCA | Reverse | | |
| Fish F1 | TCAACCAACCACAAAGACATTGGCAC | Forward | Fish | Ward et al. (2005) |
| Fish R1 | TAGACTTCTGGGTGGCCAAAGAATCA | Reverse | | |
| ZplankF1 | TCTASWAATCATAARGATATTGG | Forward | Zooplankton | Prosser et al. (2013) |
| ZplankR1 | TTCAGGRTGRCCRAARAATCA | Reverse | | |
| BirdF1 | TTCTCCAACCACAAAGACATTGGCAC | Forward | Bird | Hebert et al. (2004b) |
| BirdR1 | ACGTGGGAGATAATTCCAAATCCTG | Reverse | | |
| LepF1 | ATTCAACCAATCATAAAGATATTGG | Forward | Butterfly | Hebert et al. (2004a) |
| LepR1 | TAAACTTCTGGATGTCCAAAAAATCA | Reverse | | |
| **Plants** | | | | |
| 390F | CGATCTATTCATTCAATATTC | Forward | Several *taxa* | Cuénoud et al. (2002) |
| 1326R | TCTAGCACACGAAAGTCGAT | Reverse | | |
| XF | TAATTTACGATCAATTCATTC | Forward | Several *taxa* | Ford et al. (2009) |
| 5R | GTTCTAGCACAAGAAAGTCG | Reverse | | |
| 3F_KIM | CGTACAGTACTTTTGTGTTTACGAG | Forward | Several *taxa* | Jeanson et al. (2011) |
| 1R_KIM | ACCCAGTCCATCTGGAAATCTTGGTTC | Reverse | | |
| rbcLbF | AGACCTWTTTGAAGAAGGTTCWGT | Forward | Several *taxa* | Dong et al. (2015) |
| rbcLbR | TCGGTYAGAGCRGGCATRTGCCA | Reverse | | |
| **Fungi** | | | | |
| ITS1 | TCCGTAGGTGAACCTGCGG | Forward | Several *taxa* | White et al. (1990) |
| ITS4 | TCCTCCGCTTATTGATATGC | Reverse | | |
| ITS1 | TCCGTAGGTGAACCTGCGG | Forward | | |
| ITS2 | GCTGCGTTCTTCATCGATGC | Reverse | | |
| ITS3 | GCATCGATGAAGAACGACGC | Forward | | |
| ITS4 | TCCTCCGCTTATTGATATGC | Reverse | | |

group upload it usually takes 3–7 days for the information to be available, although is easier to upload a lot of data in a single submission.

BOLD performs a screening of the uploaded data, taking the following considerations: the sequences are translated into amino acids and through the Hidden Markov Model is detected if they came from nucleotide sequences of the COI gene, and the presence of stop codons and possible sequence contamination are evaluated. If an error is detected, the researcher is informed, and the data is labeled (a barcode is not generated until the error is corrected and re-evaluated). Finally, BOLD

evaluates the trace files and determines the PHRED score (Ewing et al. 1998) for each nucleotide and for the entire sequence, using four categories for a quality classification: failed (no sequence), low quality (mean PHRED <30), medium quality (mean PHRED = 30–40) and high quality (mean > 40) (Ratnasingham and Hebert 2007).

The information uploaded into a BOLD project is the responsibility of the researchers. There are no major reviews. If there is any sequence that diverges by more than 2% from another reported as the same species, it is possible that the BOLD alerts the users, indicating that more studies are necessary, perhaps leading to the description of a new species (Ratnasingham and Hebert 2007).

Additional information on BOLD Systems can be found in the BOLD Print Handbook for BOLD v4 (https://www.boldsystems.org/index.php/Resources), where it is indicated how to upload the data step by step and it is suggested to review this information.

## 2.4 Single-Gene Species Delimitation Methods

Centuries after the Linnaean classification system, the biologists are still debating how to define a biological species, and it has been difficult to define a species concept that can be applied to all forms of life and different purposes. However, from a conservation perspective, a concept designed to be universal, such as the General Lineage Concept (De Queiroz 2007), in which species are fundamental units that represent lineages evolving separately, can be useful.

For that, we need precise methodologies to delimit and identify species, as it is fundamental for biodiversity studies. Species delimitation may involve morphological, ecological, or molecular data, among others. However, integrative taxonomic studies, including multiple data sources, are expensive, require multiple types of expertise, and time-consuming to complete. In addition, the current shortage of trained taxonomists makes the art of delimiting species more complicated. At this rate, describing all the unknown animal diversity will take centuries and cost billions of dollars (Carbayo and Marques 2011).

Since the beginning of DNA barcoding, the process of species identification is intrinsically linked to the need to delimit species. We can identify a species based on similarity with a taxonomically well identified specimen, but we can find specimens that are not related to any known species or are different to the species that we believe they belong. So, we need to delimit before identifying. Several methodologies have been developed using DNA barcoding data (or molecular data) to delimit species or, more precisely, Molecular Operational Taxonomic Units (MOTUs), allowing a rapid and comprehensive assessment of biodiversity.

### 2.4.1 Molecular Operational Taxonomic Units

The molecular data could be clustered on sets of orthologous sequences, called MOTUs. This term was introduced for the first time by Floyd et al. (2002) as a simile of Operational Taxonomic Unit (OTU), commonly used in taxonomic studies. We can define MOTU as a cluster of sequences delimited by an explicit algorithm (Jones et al. 2011). The advantage of this concept is that no formal correlation to a recognized species or morphological description is required, eliminating the need for explicit identification at the species level (Floyd et al. 2002). Clusters of sequences based exclusively on molecular data from individuals without any prior taxonomic information can be obtained, and the MOTU can be easily compared to morphological, ecological, and other type of biological data. We need to be aware that MOTUs may not represent 'true' species; however, delimiting MOTUs has been shown to be a good approach to estimating species-level diversity (Roxo et al. 2015; Kekkonen et al. 2015; Ramirez et al. 2017, 2021).

With the advances of DNA barcoding initiatives, with more than ten million records available and a well-standardized methodology, these data are ideal for applying species delimitation methods. Delimiting MOTUs together with DNA barcode studies offer a tool for early species discovery (Kekkonen et al. 2015; Lukhtanov 2019). Although we have several tools to facilitate MOTUs delimitation from COI data (or other single gene), the results obtained may not be correctly interpreted without a deep taxonomic knowledge of the target groups. A species delimitation analysis can reveal several new MOTUs but confirmation that they represent true species will need more taxonomic effort to include other information (e.g., geographic distribution, morphology, etc.), to finally make a taxonomic decision and even describe new species (Lukhtanov 2019).

### 2.4.2 Distance vs Coalescent Species Delimitation Approaches

Genetic distance is one of the first methodological approaches used to DNA barcoding, and it is expected that genetic distance between specimens of the same species will be lower than between specimens of different species. This basic concept led Hebert et al. (2003a) to propose the use of a standard genetic distance COI threshold, which needs to be established for each group studied, to guide species diagnosis. Later, Hebert et al. (2004b) proposed the use of a standard COI sequence threshold equal to 10 times the mean intraspecific variation value of the group studied, but also stated 2.0% as a good general threshold, based on the fact that 98% of sister species were observed to have K2P mtDNA genetic distance greater than 2% (Johns and Avise 1998). Several studies have been using the 2% genetic distance value as a universal threshold in different *taxa* (e.g., Smith et al. 2005; Pereira et al. 2013; Roxo et al. 2015; Ge et al. 2021), although other studies have already shown that this threshold can be lower in several animal groups (Hubert et al. 2008),

especially when closely related species with recent divergence are analyzed (Ramirez and Galetti 2015). Indeed, the genetic distance threshold value for DNA barcoding within a group depends on the natural history of the species (e.g., generation length, geodispersal pattern, population structure) (Hebert et al. 2003a), as well as methodological aspects, such as incomplete taxonomic sampling (Wong et al. 2009). Several tools based on the genetic distance calculation were designed for DNA barcoding analyses using threshold values defined by the user, as in jMOTU (Jones et al. 2011), or automatically determined, as in the widely used ABGD (Puillandre et al. 2012) or the recently developed assemble species by automatic partitioning (ASAP) method (Puillandre et al. 2021). All these methods have the advantage of low computational cost, enabling the analysis of large data sets. However, these methods have a weak connection to evolutionary theory, failing to identify variation in interspecific and intraspecific genetic distance and variation in substitution rate between lineages (Fujisawa and Barraclough 2013).

To overcome the weakness of the genetic distance approach, several probabilistic models have been developed to work with single-locus data (Pons et al. 2006; Zhang et al. 2013; Kapli et al. 2017). In general, these methods underlie an evolutionary model and coalescent process to modeling speciation events. One of the most popular species delimitation approach based on coalescence is the generalized mixed Yule-coalescent (GMYC) model (Pons et al. 2006; Fujisawa and Barraclough 2013). This method requires a time-calibrated ultrametric tree (a rooted tree with all the leaves equidistant to the root and with branch length representing time) to model branching events. The species delimitation methods based on tree data rely on the prediction that distinct genetic clusters are separated by internal branches longer than branches separating intra-cluster individuals (Fujisawa and Barraclough 2013). Thus, GMYC classifies branches as inter- and intraspecific and identifies the transition point between them by maximizing the likelihood score of the Yule model (Pons et al. 2006; Fujisawa and Barraclough 2013). The main disadvantage of GMYC is the accuracy and difficulty to obtain a time-calibrated ultrametric tree, being a process computationally intensive that require Bayesian framework with MCMC (Markov Chain Monte Carlo) methods. One of the most used software to obtain the ultrametric tree is the BEAST package (Bouckaert et al. 2019), requiring expertise to set up the Bayesian priors and obtain MCMC convergence. To overcome this limitation, Zhang et al. (2013) developed the Poisson tree processes (PTP) to delimit species without requiring ultrametricity. In this case, the PTP explicitly models the branching processes based on the number of substitutions and determines the transition point between inter- and intraspecific branches that best fit the data (Zhang et al. 2013; Kapli et al. 2017). This model was then modified to include Bayesian support (bPTP) and the potential divergence in intraspecific diversity (mPTP) (Zhang et al. 2013; Kapli et al. 2017). All these coalescent single-gene species delimitation methods have been proved to be effective on simulations and empirical data (Talavera et al. 2013; Tang et al. 2014), being widely used to assess biodiversity (Pons et al. 2006; Machado et al. 2017; Hofmann et al. 2019; Ramirez et al. 2020b, 2021; Cañedo-Apolaya et al. 2021).

### 2.4.3 Barcode Index Number

A species delimitation method that is closely related to DNA barcoding is the refined single linkage (RESL) analysis. This method was developed by Ratnasingham and Hebert (2013) to delimit MOTUs, as an interim taxonomic nomenclature to accelerate species discovery. These authors created the already mentioned BIN, an alphanumeric system structured on BOLD, to avoid confusion with the Linnaean nomenclature. The BIN system consists of a register of all MOTUs identified by RESL, providing additional data available in BOLD for each BIN. The RESL algorithm was designed to process large amounts of data with low computational cost. By early 2022, the RESL algorithm had described nearly 800,000 BINs based on ~240,000 species. Interestingly, this method is based on distance, but uses no a simple cut-off rule, instead it uses a single linkage cluster analysis followed by Markov clustering. This allows RESL to use 'training data' based on all data deposited on BOLD, allowing the BIN system to gain power as species coverage increases. In the end, BIN system implies a collaborative effort to describe biodiversity.

### 2.4.4 Comparing Methods

Every species delimitation method has advantages and disadvantages and can be used together to support the delimitation hypothesis. The resulting scenarios can be compared with the taxonomic information (previous information based on morphological diagnosis) to determine the degree of concordance. The MOTUs obtained can match the previous information or can show conflicts. Some authors have created classification systems according to the pattern of the match found (Costa-Silva et al. 2015; Rossini et al. 2016) or created consensus MOTUs based on the majority rule (Ramirez et al. 2020b). The use of different single-gene species delimitation methods is recommended and offers a powerful tool to understand biodiversity.

## 2.5 DNA Barcoding in Neotropics

### 2.5.1 Using DNA Barcoding to Assess Biodiversity in Birds

Birds were one of the first groups to undergo DNA barcoding studies, with the ABBI (All Birds Barcode Initiative) being created in 2005 with the aim of sequencing 10,000 existing bird species. Among the first important studies on bird barcoding is the one by Hebert et al. (2003a), in which 260 species representing 40% of the bird diversity of North America were analyzed. By 2016, more than 4200 species had been sequenced, representing 41% of the known bird species in the world (Barreira et al. 2016). Currently, the avifauna barcode continues to grow worldwide,

with important records in the Eastern Palearctic, Neotropics, Scandinavia, the Netherlands, Japan, and Turkey (Hebert et al. 2003a; Kerr et al. 2007; Johnsen et al. 2010; Tavares et al. 2011; Saitoh et al. 2015; Bilgin et al. 2016). This enormous availability of sequences made it possible to study the divergence of species through geographical barriers and gene flow between continents. For example, it has been shown greater genetic distances between bird species of Argentina than between species of the Nearctic, suggesting that the diversity of the Neotropics is due to a lower rate of extinction and not to recent speciation events (Kerr et al. 2009; Barreira et al. 2016).

Among the main limitations for DNA barcoding studies in birds is the cost of collecting birds, which can be overcome with the use of museum collections. The most common sample used in DNA barcoding of birds is obtained from the pectoral muscle or cardiac muscle. Other tissues are also collected, despite their low yield because, for example, they have high enzyme activity, such as liver samples, or have fewer mitochondria, such as blood cells (Kerr et al. 2009). Eggs, frequently deposited in scientific collections, are another important source of DNA, and DNA barcoding can be very helpful for precise identification of these eggs. In recent works, tissues from eggshells deposited in Natural History Museums were identified using the 12S rRNA gene, thus allowing at least half of the identities of the eggs to be unraveled (Grealy et al. 2021).

### *2.5.2 Using DNA Barcoding to Assess Fish Biodiversity*

Currently, actinopterygians (bony fish) have 284,129 COI standard segment sequences in the BOLD database, representing 22,094 BINs (http://www.boldsystems.org/). Besides its recognized importance for fish taxonomy, DNA barcoding has shown important aspects on the fish biogeography (Machado et al. 2017; Ramirez et al. 2020b) and genetic diversity within fish populations that are exploited for commercial purposes (Ardura et al. 2013). In addition, DNA barcoding has been increasingly used for species authentication and trade control, validating derived products and guaranteeing consumers of an authenticated product (Bhattacharya et al. 2016).

The use of DNA barcoding on neotropical fish has revealed countless cases of food fraud or mislabeling, where a fishery product of higher value is substituted in the market for one of lower value without the consumer knowing it. In South America, cases of fraud in frozen Atlantic cod fillets have been reported (Calegari et al. 2020), detection of threatened species such as the Atlantic bluefin tuna *Thunnus thynnus* sold in restaurants (Velez-Zuazo et al. 2021) or the substitution of the Common snook *Centropomus undecimalis* in 98% of the frozen fillets sold in Colombia (Lea-Charris et al. 2021).

The importance of DNA barcoding for fish taxonomy has been increasingly demonstrated in the Neotropics, a region known for its enormous diversity of fish fauna, housing approximately a quarter of the world's richness of freshwater fish

(Reis et al. 2003). In the Alto Rio Paraná basin (Brazil), for example, where more than 300 species of fish have been recorded, it was possible to identify 252 species using molecular barcodes (Pereira et al. 2013).

Other interesting application of DNA barcode in fish is the recognition of spawning sites, a fundamental factor for fisheries management. The identification of fish eggs and larvae at the species level is extremely difficult due to the lack of characters in the early stages of development, and DNA barcode can be fundamental for such identification (Kerr et al. 2020).

Fortunately, the standardization of DNA barcoding methodology has been successful in a variety of aquatic environments, allowing its application in freshwater, marine, estuarine and rocky reef ecosystems (Fazzi-Gomes et al. 2017; Ramirez et al. 2020a; Shan et al. 2021), which makes it a valuable tool for the knowledge and conservation of neotropical ichthyofauna.

### *2.5.3 Using DNA Barcoding to Assess Biodiversity in Marine Realm*

Compared to terrestrial or even freshwater aquatic ecosystems, marine environments harbor an enormous diversity of macro and microfauna. However, DNA barcoding effort in the neotropical region is still insufficient to understand marine biodiversity (Ramirez et al. 2020a). A variety of marine communities can be assessed by DNA barcoding, such as seagrasses, mangroves, and marine phytoplankton. The biodiversity evaluation of these ecosystems is very important ecologically and economically, since the ecosystem services provided by mangroves, for example, are equivalent to more than one and a half trillion dollars/year worldwide (Costanza et al. 1998).

Using molecular markers and the DNA barcoding technique, it is possible to assess this diversity even in larval stages, once reference sequences obtained from correctly identified adults are available. The efficiency of larval identification was recently tested in echinoids, using a COI and 16SrRNA sequence database previously obtained for this taxonomic group (Collin et al. 2020).

The molecular identification of marine organisms through mitochondrial genes such as COI and 16SrRNA in mollusks and cnidarians, or the combination of nuclear genes such as rbcL-3P and ITS2 in the case of diatoms, has benefited not only studies of biological diversity and biogeography, but it has also played an important role in food security and the detection of invasive species (Gong et al. 2018).

### 2.5.4 Using DNA Barcoding to Assess Biodiversity in Insects

As it happens in many highly diverse groups, the application of DNA barcoding in insects has been accelerating the identification of adult individuals and unraveling the identification of early stages. In lepidoptera, most diagnostic characters are based on morphology of the adult genitalia, however most individuals are captured as larvae. Among them, Heliothines contain some of the most important agricultural pests whose larvae are frequently observed in inspections of commodities shipment worldwide, and DNA barcoding was proven to be very powerful for identifying species within this group (Gilligan et al. 2019). Likewise, in beetles, whose distribution spans almost all continents and are of great importance as biological indicators and drug development, the presence of sexual dimorphism and interspecific variations make morphological identification difficult, and DNA barcoding has been successfully implemented to identify the species (Jung et al. 2016; Sire et al. 2019).

The application of the barcode in insects has been implemented in the border control (Harwood et al. 2009; Vänninen et al. 2011; Moslonka-Lefebvre et al. 2011; Whittle et al. 2013) in order to enrich pest control and prevent the entry of invasive species, avoiding damage that translates into billions of dollars worldwide (Madden et al. 2019).

Due to the high abundance of many insect groups, capture for monitoring studies is less challenging than vertebrates. However, just like taxonomic identification, molecular identification faces some limitations when it comes to insects, such as bacterial infections that affect the quality of the results obtained (Smith et al. 2012).

Because Neotropics own a great diversity of insects the use of DNA barcoding for taxonomy and conservation purposes are strongly encouraged.

### 2.5.5 Using DNA Barcoding to Assess Biodiversity in Plants

DNA barcoding has been applied to identify plant species and build inventories of biodiversity, in addition to conducting phylogenetic studies, detecting illegal trafficking of protected species and fraud in commercial products.

Phylogeny studies based on DNA barcode carried out in different habitats and environmental conditions have informed, for example, which abiotic factors can exert dominance through microhabitats or within the same forest (Muscarella et al. 2014). By allowing identification at the species level, it is possible to broaden the knowledge about plant – parasite and plant – plant interactions (Smith et al. 2011).

Due to their great economic value, some plant species are also subject to trafficking of protected species and DNA barcoding can be a tool against this type of crime. This methodology was reported to be efficient to identify threatened commercial woody Angiosperms, mainly of the Lauraceae family, inhabiting the humid forests of Araucaria, in Brazil (Bolson et al. 2015). DNA-based authentication is also very powerful for fraud identification of commercial plant products, such as traditional

medicines, herbs and teas. In a recent global review, Ichim (2019) reported that approximately 30% of the herbal products commercialized in the marketplace worldwide are adulterated when their content was tested against their labeled species, and South America was among the continents where fraudulent substitutions were frequent.

Many limitations for an extensive use of DNA barcodes must be overcome in the databases where several species have no reference sequences or vouchers have completely lost the diagnostic characteristics and the cases examined are often placed within species complexes as, for example, the situation of some medicinal roots (De Boer et al. 2014).

## 2.6 Final Considerations

Because from the huge global eukaryotic diversity only a small portion has been described and recorded (Scheffers et al. 2012) together with that we are going through the sixth mass extinction (Raven and Wilson 1992), DNA barcoding can assume a crucial role for speeding up the species identification worldwide. Although identification based on morphological characters is quite informative, its application is complicated in cases of cryptic species (Hebert et al. 2003a), and DNA barcoding has proved very helpful, particularly in the Neotropics where recent speciation seems a common event within different *taxa* (Pereira et al. 2013; Ramirez et al. 2017). In addition, DNA barcoding has been solving a variety of problems such as pest control, fishing inspection, detection of food fraud, identification of endangered groups, hunting and illegal trafficking of fauna and flora (e.g. Baker and Palumbi 1994; Calegari et al. 2020; Velez-Zuazo et al. 2021). In control of infectious diseases, for example, where it is essential to identify both the vector and the host, it is necessary to have markers capable of identifying vertebrates among mammals, birds, reptiles and amphibians, as well as invertebrates such as arthropods and arachnids (Alcaide et al. 2009).

Neotropical watersheds and forests have an ecological and economic importance as a livelihood for local communities, and globally due to the ecosystem services provided, including access to water, flood control, purification of air and climate regulation (Hall et al. 2015). To ensure adequate management of these ecosystems, the scientific community must be able to expand and accelerate the biodiversity studies. Particularly in South America, where most countries are of low or medium income, together with the existing knowledge gap, the scarcity of economic financing for research, challenge our capability to assess the huge biodiversity. DNA barcoding may represent a shortcut to overcome this problem and promote knowledge of biodiversity in the hyperdiverse Neotropics.

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# Chapter 3
# Genetic Tools for the Conservation of Bats

**Camila F. Gonçalves, Carolina da Silva Carvalho, Valéria da Cunha Tavares, Ludmilla M S Aguiar, Ana Paula Carmignotto, and Pedro M. Galetti Jr.**

## 3.1 Introduction

The order Chiroptera is the second most diverse group of mammals, representing 22% of all living mammals and displaying great physiological and ecological diversity (Hutson et al. 2001; Mammal Diversity Database 2022). Bats can be found in every region of the world, except the Arctic and Antarctic, and certain isolated oceanic islands (Simmons 2005a, b; Fenton and Simmons 2015). Over 52 million years of evolutionary history bats have diversified into almost 1450 species (Simmons et al. 2008; Kunz et al. 2011; Mammal Diversity Database 2022). Their weight ranges from as little as two grams, in the case of the bumblebee bat (*Craseonycteris*

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*thonglongyai*), and three to four grams for the neotropical *Furipterus horrens*, to over 1 kg in the golden-capped flying fox (*Acerodon jubatus*) (Safi et al. 2013; Fenton and Simmons 2015). Some species of bats may form one of the largest aggregations of nonhuman mammals when measured in terms of individual numbers (e.g. Moratelli and Calisher 2015). They can be classified into several feeding strategies, including aerial insectivores (which capture insect in flight), sanguivores (blood), nectarivores (flowers, nectar, and pollen), frugivores (fruits), omnivores (arthropods, flowers, fruits, and vertebrates), and animalivores (arthropods and small vertebrates) (Fauth et al. 1996; Meyer and Kalko 2008; Kunz et al. 2011).

Frugivorous bats play a vital role in habitat restoration because they can carry the seeds of various plant species away from the mother plants, increasing the likelihood that these seeds will germinate and establish themselves in a new area (Lobova et al. 2009). In the Neotropics, neotropical fruit bats (Phyllostomidae) alone disperse seeds from at least 549 plant species belonging to 191 genera and 62 plant families (Ramírez-Fráncel et al. 2022). Similarly, old-world bats disperse seeds of around 300 plant species, which produce at least 448 economically valuable products (Fujita and Tuttle 1991). Despite the significance of bats in seed dispersals, the impacts of land use changes on bats and seed dispersal services are still poorly understood (e.g. Regolin et al. 2020).

Pollination is another essential ecosystem service provided by bats, which play a vital role for plant ecology and food production (Kunz et al. 2011). The neotropical subfamilies Glossophaginae and Lonchophyllinae are specialized nectar-feeding bats (Tschapka et al. 2015). The few information available for nectarivorous bats movement include travel distances up to 3.8 km each night, combining short-range flights of up to 500 m with longer flights of 2 to 3 km that take them away from their core areas (Aguiar et al. 2014). In the Neotropics, bats pollinate approximately 549 plant species distributed in 191 genera and 62 families, and old-world Pteropodid bats pollinate around 168 plant species in 100 genera and 41 families (Kunz et al. 2011). Bat-pollinated plants encompass significant sources of biomass in their habitats; columnar cacti, for example, are the dominant vegetation in neotropical xeric and semi-arid habitats (Zamora-Gutierrez et al. 2021).

In Brazil, *Glossophaga soricina* and *Lonchophylla sp*. are known to pollinate *Pilosocereus tuberculatus*, a relict tree-like cactus distributed in a few localities of the Caatinga in the states of Pernambuco, Bahia, Sergipe and Piauí (Rocha et al. 2007). The flower-visiting niche of bats in the Neotropics is defined by variations in the combination of shape, time, and space (Diniz et al. 2022). Bat-pollinated plants are more dependent on their respective vertebrate pollinators than bird-pollinated plants; thus, the significance of bats as pollinators must be addressed, also due to the economic value of certain bat-pollinated crops (Ratto et al. 2018).

Bats have a diverse range of roosting sites, including foliage, caves, rock crevices, hollows in trees under peeling bark, and various man-made structures (Jones et al. 2009a; Garbino and Tavares 2018). In the Neotropics, guano produced by fruit-eating, hematophagous, and insect-eating bats is a key resource for cave-dwelling arthropods and contributes largely to the foundation of the food web in caves (Ferreira and Martins 1999). The diversity of guano types makes the food

webs in neotropical caves more species-rich than those found elsewhere (Ferreira and Martins 1999; Fontanetti et al. 2002). In Thailand and Cambodia, guano is collected from bat shelters and used as a fertilizer for plants (Kunz et al. 2011), contributing to the local economy (Leelapaibul et al. 2005; Chhay 2012). In the early 2000s, guano extracted from the super bat populated Bracken Cave in Texas was sold for a price of $2.86 to $12.10 per kg (Tuttle and Moreno 2005).

In addition to the guano in caves, natural and urban shelters are crucial for preserving insectivorous species that provide insect population regulation services. In the U.S., the species *Tadarida brasiliensis* saves US$741,000 annually by avoiding insecticide costs and preventing loss of cotton production (Cleveland et al. 2006). In Thailand, *Mops plicatus* prevents the loss of tons of rice due to arthropod infestations that damage crops (Wanger et al. 2014). Even artificial shelters in urban areas that house insectivorous bats contribute to the provision of pest control services. In Brazil, bats save US$94 per hectare of corn plantation, resulting in annual savings of US$390.6 million per harvest (Aguiar et al. 2021).

On the other side, bats are targeted by farmers because of their reputation as vectors of rabies. Current management practices aimed at controlling vampire bats have a devastating impact on bat populations, including both vampire bats and other species that coexist with vampire bat colonies, by destroying important roosts and indirectly affecting populations (Aguiar et al. 2010). The neotropical region has been greatly impacted by human-induced changes in land use and land cover, with some of the highest deforestation rates in the world. Brasileiro et al. (2022) recently found that, except for nectarivores, larger bats are generally more impacted by habitat loss and are disappearing faster than smaller bats, following a pattern of downsizing and its functional consequences that has been documented for other taxonomic groups (Dirzo et al. 2014; Galetti et al. 2015; Donoso et al. 2020). Furthermore, the lack of awareness, and negative perception of bats continue to threaten bat populations (Rocha et al. 2020).

Due to the great diversity of species and habits, including the fact that bats are primarily nocturnal and therefore difficult to observe directly (Kalko et al. 1996), many questions about their taxonomy, genetics, evolution, ecology, and behavior remain unanswered. This is especially true in the Neotropics, where there is a high diversity of bats and their ecosystem services, but also numerous gaps still exist in our understanding of their ecology, behavior, acoustics, and conservation status (Bernard et al. 2011). Molecular tools can be crucial in helping to answer questions about historical (e.g., range expansion, colonization events), contemporary (e.g., gene flow), and ecological processes by providing important genetic data (Dool 2020).

In this chapter, we provide an overview of studies that have utilized genetic data to enhance our understanding of the Chiroptera order based on an ongoing comprehensive systematic review. The Neotropics, which harbor the greatest diversity and abundance of bats in the world (Burgin et al. 2018), were highlighted. Additionally, we discussed the significance of these data for the conservation of these iconic animals.

## 3.2 Literature Review of Genetic Techniques Applied in Bat Conservation

We conducted a literature survey of papers that used genetic data to study the Chiroptera group in the following databases: Web of Science (http://www.webofscience.com) and Scopus (https://www.scopus.com). The survey was limited to English-written, peer-reviewed journal articles available until October 2020, and used search terms related to higher-level traditional bat taxa, including: Chiroptera* OR Megachiroptera OR Microchiroptera OR Yangochiroptera OR Yinpterochiroptera OR Craseonycteridae OR Emballonuridae OR Furipteridae OR Hipposideridae OR Megadermatidae OR Miniopteridae OR Molossidae OR Mormoopidae OR Mystacinidae OR Myzopodidae OR Natalidae OR Noctilionidae OR Nycteridae OR Phyllostomidae OR Pteropodidae OR Rhinolophidae OR Rhinopomatidae OR Thyropteridae OR Vespertilionidae OR Rhinonycteridae OR Cistugidae AND genetic*. A total of 2041 papers were retrieved and screened, and papers that either did not focus on conservation issues for Chiroptera or were not focused on the bats (e.g., papers focusing on diseases and health) were removed. After this screening process, 475 articles remained and were processed, selected, and screened using the software StArt (State of the Art through Systematic Review) v. 2.3.4 Beta (Zamboni et al. 2010), following the methodology of Berger-Tal et al. (2019). Information was manually extracted from each article, including: focal family and species; molecular markers used (e.g., mtDNA, microsatellites, SNPs, complete genome, mtDNA and nuDNA combined); biogeographic region according to the Slater-Wallace classification, as well as countries where the samples were collected; origin of animal samples (wild, captive, museum, online database); number of samples, capture method, and main conclusions.

The papers were categorized into 13 different topics based on their keywords and objectives. If the objectives were unclear, the paper was assigned to the topic that best reflected its analysis and conclusions. Papers under the category "Systematic Questions" aimed to assess taxonomy and species delimitation. The category "Effects of Fragmentation on Genetic Diversity" included works that analyzed fragmented landscapes and the resulting effects on genetic diversity distribution and population structure. "Impacts of Barriers on Gene Flow" covered studies that evaluated the impact of specific barriers, such as the open sea, on gene flow between populations. "Bat Adaptations" referred to works that investigated the genetic adaptations of the group. "Impacts of Geographic Distance and/or Topology on Populations" encompassed studies that assessed, in general, how species populations distributed nearby are genetically affected by distance and topology. "Genetic Divergence among Populations" compared genetic diversity between two or more populations under similar ecological conditions. "Understanding Demographic History and Biogeography" included studies that aimed to comprehend the historical processes responsible for the geographical distribution of individuals. "Individual Identification" included papers whose goal was to identify the species of a sample under different conditions or in a new location, and methodological studies that described new molecular markers for a specific species (e.g., developing microsatellites). "Influence of Behavior on Genetic Diversity Distribution" covered studies that evaluated the impact of mating systems or other social behaviors on genetic

diversity. "Local Population Structure" referred to papers that evaluated or described the genetic diversity of a local population. "Genome Description" comprised the publication of complete mitogenomes and nuclear genomes. "Phylogeographic Hypotheses" included studies that tested pre-defined phylogeographic hypotheses. "Unclear" was a category for papers that did not fit into any of the above topics.

## 3.3 Issues and Timeline Overview

Overall, the main targets of studies within the Chiroptera group are systematics, and understanding demographic history and biogeography (Fig. 3.1), and there have been several advances in the methodologies applied to uncover phylogenetic relationships, detect significant evolutionary units, delimit species and their geographic ranges, and define taxonomic status and classification. These advances aid in the understanding of the diversity, evolution, and biogeography of the group, which are essential for their conservation. Out of the reviewed papers, only three were published in the 1980s, and they utilized chromosomal data and electrophoresis to infer genetic differentiation between species. For example, at the end of the decade, Bennett et al. (1988) used mtDNA markers to resolve an important phylogenetic

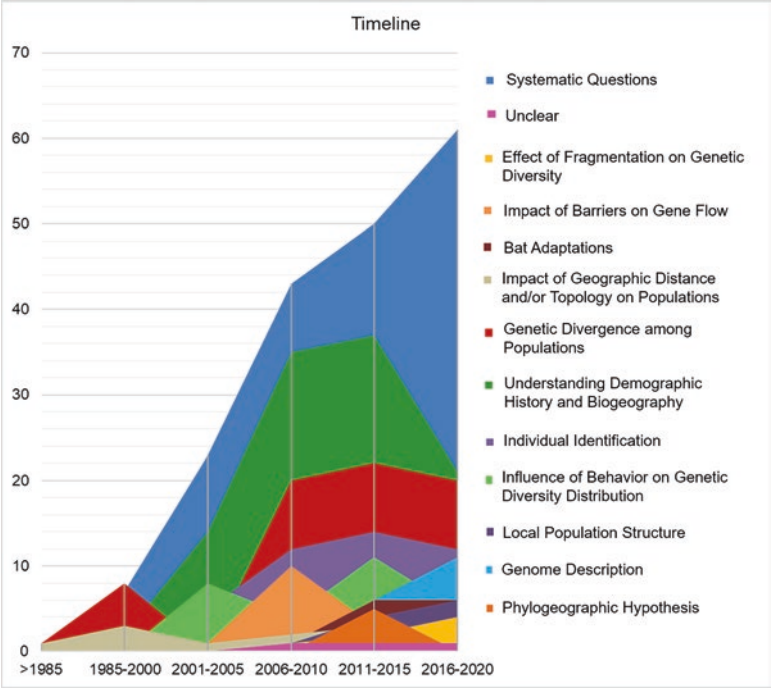


**Fig. 3.1** Timeline of studied topics based on a literature survey from 1982 to 2020

question – that megabats (Pteropodidae family) are indeed bats, not "flying primates", as previously suggested by Pettigrew (1986).

In the next decade (1990–1999), the number of papers recovered almost quintupled, totaling 14. The topics of systematics, and demographic history and biogeography accounted for 64% of the published studies, and the main methods used included mtDNA, microsatellites, and cytogenetics. Interestingly, the first paper using genomic data, specifically cosmid libraries, was produced during this decade, aiming to infer taxonomic levels from the new-world leaf-nosed bat, *Macrotus waterhousii* (Baker et al. 1997).

In the first decade of the twenty-first century (2000–2009), there was a significant increase in the number of publications about the Chiroptera group, with over 150 papers appearing in our survey. Systematics, and demographic history and biogeography accounted for 71.8% of the published studies. The increase can be attributed to the advancements in genetic technologies, which made it possible to employ mtDNA studied either through direct sequencing or electrophoresis fragment analysis, as well as microsatellites, for population genetic studies. Concerning the most recent decade (2010–2020), we found over 300 papers within our scope, repeating the trend of increased topics and publications observed in the previous decade. Of these papers, 68.1% (215) were about systematics, and demographic history and biogeography. There was also an increase in studies involving genomic data or complete genome descriptions, which likely reflects advances in molecular technologies, the development of several statistical approaches, and increased computing power (Beaumont and Rannala 2004; Pearse and Crandall 2004).

Neotropical studies followed the global trend, with less publications until the early 2000s, subsequently increasing in number and methods to support the resolution of various questions, particularly in terms of phylogenetic relationships. In the most recent year reviewed, the description of a new species, *Lasiurus arequipae*, endemic to Peru, was published (Málaga et al. 2020). Garbino et al. (2020) conducted a comprehensive revision of the genus *Chiroderma* (Phyllostomidae) using mtDNA combined with morphology. Additionally, systematic and taxonomy studies at the species level, using both mtDNA and nuDNA, were conducted for *Platyrrhinus chocoensis* (Palacios-Mosquera et al. 2020), *Tonatia saurophila* (Basantes et al. 2020), *Anoura caudifer*, and *Anoura geoffroyi* (Vargas-Arboleda et al. 2020). Also, next-generation sequencing was combined with morphological data to enhance knowledge of the phylogeny of the genus *Molossus* in the Neotropics (Loureiro et al. 2020).

Overall, we reviewed 475 articles on both the Yinpterochiroptera and Yangochiroptera suborders, from 1982 to October 2020. The studies were dominated (95%) by the five largest bat families, as reported by Mammal Diversity Database (2022), with the Vespertilionidae (199) being the most studied, followed by Phyllostomidae (77), Rhinolophidae (66), Pteropodidae (62), and Molossidae (44). Most of the studies include the use of samples from wild animals (345), followed by those using museum specimen samples (125) and sequences from public databases (61), with only a few using samples from captive animals (3). DNA was extracted from wing membranes or non-specified muscle/tissues.

The articles reviewed (as seen in Fig. 3.2) covered a range of subjects, and the impact of these studies on the field of neotropical bats will be emphasized in the following sections.

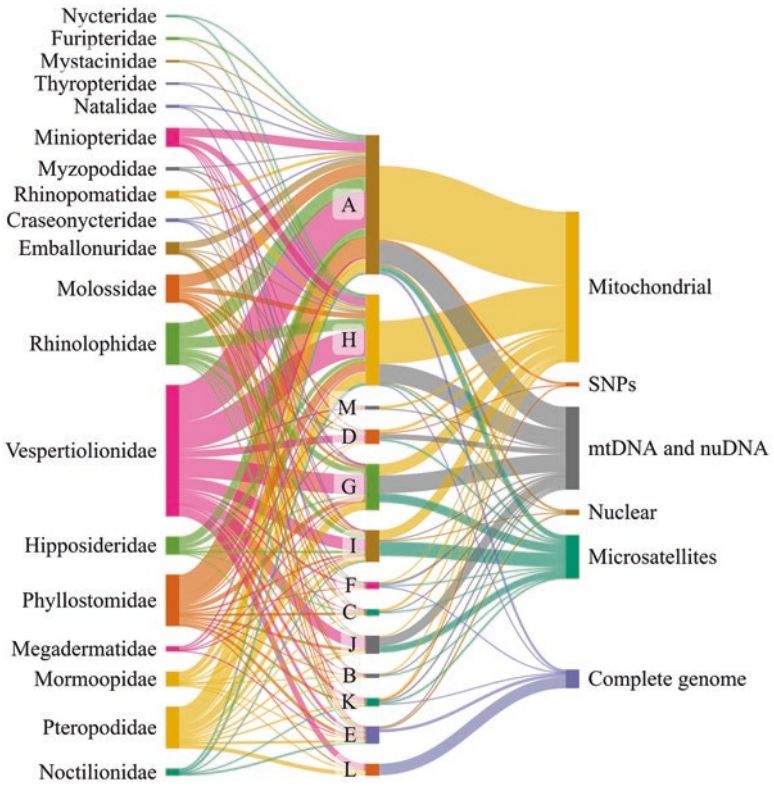


**Fig. 3.2** Flow of information from bat families, through the key research themes identified, to the molecular markers used in 475 studies on Chiroptera from 1982 to October 2020. The size of the bars and the thickness of the lines are proportional to the number of interactions for each theme. The themes include: [A] Systematic Questions; [B] Unclear themes; [C] Effects of Fragmentation on Genetic Diversity; [D] Impacts of Barriers on Gene Flow; [E] Bat Adaptations; [F] Impacts of Geographic Distance and/or Topology on Populations; [G] Genetic Divergence among Populations; [H] Understanding Demographic History and Biogeography; [I] Individual Identification; [J] Influence of Behavior on Genetic Diversity Distribution; [K] Local Population Structure; [L] Genome Description; [M] Phylogeographic Hypotheses

## 3.4 Molecular Identification and Taxonomy

Speciation events in bats have been documented to occur without significant morphological changes, leading to the identification of cryptic species across the entire order (e.g. Baker 1984; Barrat et al. 1995; Hoffmann and Baker 2003; Hulva et al. 2004). To address this challenge, the use of modern molecular techniques in conjunction with ecological and morphological data provides valuable information for species delimitation. The most frequently studied topic within the group of reviewed works was the use of genetic data on revisionary systematics (Fig. 3.2) in order to clarify phylogenetic relationships and identify cryptic species and new taxa. The most widely used methods for this purpose employed mitochondrial markers or a

combination of mitochondrial and nuclear markers (including microsatellites) with or without the combination of morphological data. The advent of next-generation sequencing (NGS) opened up new opportunities to improve species delimitation and evolutionary inferences (Pinto et al. 2019).

Thanks to NGS, the number of genetic markers available (e.g., Single Nucleotide Polymorphisms, or SNPs) increased, offering a greater amount of genetic information enhancing phylogenetic resolution (Wagner et al. 2013). In Northeastern India, SNP data uncovered distinct geographic lineages within *Cynopterus sphinx*, as well as a previously undiscovered, cryptic cynopterine lineage that coexists with *C. sphinx* (Chattopadhyay et al. 2016).

In the Neotropics, studies aimed at supporting taxonomy accounted for over 51%, reflecting the ongoing challenge on species delimitation in this region due to its large geographical extent and high diversity of species, including areas with over 100 sympatric species (e.g. Ingala et al. 2021). As an example, Loureiro et al. (2020) utilized SNPs to address a taxonomic challenging complex contained in the *Molossus* genus (belonging to the Molossidae family), which is widely distributed in the Neotropics. With this approach, the number of species increased from 11 (Loureiro et al. 2019) to 14, with the discovery of two cryptic species within *M. rufus*, leading to the division of the group into two species (*M. currentium* and *M. bondae*).

Five of the reviewed papers focused on species identification using guano as a source of genetic material, a non-invasive sample that does not require animal handling or sacrifice. These papers often resulted in the identification of new records in caves, which typically expanded the known distributional range of species (Walker et al. 2016). Two mitochondrial DNA (mtDNA) genes, Cytochrome b (Cyt-b) and Cytochrome c Oxidase subunit 1 (COI), have been commonly utilized for bat species identification (Ahmad et al. 2019; Kundu et al. 2019).

## 3.5 Demographic History, Biogeography, Life History and Genetic Diversity

Contemporary biodiversity patterns are the outcome of a prolonged and intricate evolutionary history, influenced by ecological processes and shaped by external environmental forces such as climatic changes, mountain uplifts, and sea level fluctuations (Rull 2011). To gain a deeper understanding of the evolution of species, it is important to examine the biogeographical regions and the historical processes that have acted within each region.

The late Pleistocene period was crucial for the population dynamics of vertebrates in America, affected by both climate oscillations and the formation of geographical barriers. These processes led to population expansion in reptiles (Castoe et al. 2009), avians (García-Moreno et al. 2004; Barber and Klicka 2010), and mammals (Guevara-Chumacero et al. 2010; Hurtado and D'Elía 2022), including bats.

The *Artibeus jamaicensis* complex is a significant group of bats in South, Central, and Middle America, whose mainland populations present higher genetic diversity compared to other *Artibeus* species and other bat species (as seen in Ruiz et al. 2013). Demographic history analysis suggests that there were at least two population expansion events during the Middle Pleistocene to the Late Pleistocene (Ruiz et al. 2013). The contraction and expansion of the tropical forest likely facilitated the northward movement of *Artibeus* lineages through corridors, enabling them to explore and colonize new habitats in South and Central America (Phillips et al. 1991).

Another important neotropical bat complex, the *Pteronotus* complex, which includes *Pteronotus personatus*, *Pteronotus parnellii*, and *Pteronotus davyi* (Rull 2011), had its demographic history outlined in the Early Pleistocene during a glacial event. Different lineages of *P. personatus* have been identified with the use of mtDNA markers, in localities such as: (i) the Gulf of Mexico, the Mexican Pacific coastal plain, and the Isthmus of Tehuantepec, (ii) Southeastern Mexico, (iii) Guatemala, (iv) Guyana and Suriname (using COI), and Guyana and Venezuela (using Cytb), and (v) Guyana, Suriname, French Guiana, and Brazil. Their vicariant events, which occurred roughly 1.6 million to 2.4 million years ago, had Central America as the center of two separate diversification processes – one towards Mexico and the other in South America (Pavan and Marroig 2016; Zárate-Martínez et al. 2018).

The combined analysis of large genetic and morphological evidence from fossil and extant Short-faced bats, which comprise a substantial part of the radiation of the fruit-eating phyllostomid bats (Stenodermatina), showed that between five to 2.5 million years ago, Caribbean Island bats move from the islands to the South American mainlands and diversified thereafter (Tavares et al. 2018).

In addition to environmental factors, aspects of behavior such as migration and philopatry can greatly impact gene flow patterns (Rodrigues et al. 2010). For example, the social system can result in asymmetrical gene flow, as seen in certain bat species (Kerth et al. 2002). This can have significant effects on population dynamics and dispersal, including the threats associated with such behaviors. Our review shows that the many bat species display male-biased dispersal and female philopatry, as observed in species such as: *Desmodus rotundus* in the Neotropics (Huguin et al. 2018); *Eptesicus nilssonii, Miniopterus schreibersii, Nyctalus leisleri*, and *Hipposideros armiger* in the Palearctic (Xu et al. 2010; Naďo et al. 2017; Gürün et al. 2019; Smirnov et al. 2016); *Chaerephon pumilus*, three species of *Scotonycteris*, and *Casinycteris argynnis* in the Afrotropics (Naidoo et al. 2016; Hassanin et al. 2015); and *Myotis vivesi* and *Antrozous pallidus* in the Nearctic (Floyd et al. 2010; Arnold and Wilkinson 2015).

However, the male-biased dispersal seen in the mainland population of *A. jamaicensis* (Ortega and Arita 1999) may not hold true in the population on Cozumel Island, where individuals move among localities regardless of sex, indicating that – despite the species' polygynous nature – both males and females disperse across the island (Vázquez-Domínguez et al. 2013). It's worth noting that the common mating system in bats, particularly the case of female philopatry, is often cited as a cause for their high genetic structuring, substantial differentiation between colonies, and

potential population bottlenecks (Xu et al. 2010; O'Donnell et al. 2016), but different patterns may be found.

Bats can be highly gregarious, forming large colonies and interacting with each other (Veith et al. 2004). In temperate zones, certain bat species visit underground sites for short periods during the transition from their summer colony to winter hibernation, a behavior known as "swarming". These sites are also used for mating and sperm transfer (Fenton 1969; Thomas et al. 1979; von Helversen 1989), allowing for gene flow among colonies and the consequent relaxation of their genetic borders (Veith et al. 2004). There are no known records of swarming sites in neotropical bats, but they often form groups year-round (McCracken and Wilkinson 2000; Kunz and Lumsden 2003), which may represent an important mechanism to explain their genetic diversity in the region. Studies of genetic structure and cryptic diversity in the Neotropics can aid in bat conservation by assessing species diversity and movements at a landscape level (e.g. Pavan and Marroig 2016; Moras et al. 2016; Ito et al. 2022).

## 3.6 Lessons from Population Genetics

### *3.6.1 Impacts of Geographical Barriers and Distance on Gene Flow*

Topographical barriers can impact the genetic structure of animal populations (Andriollo et al. 2018). For bats, landscape features such as mountain ranges and large bodies of open water can act as major barriers to dispersal (Castella et al. 2000; Davalos 2004). However, the effect of these barriers may vary among bat species, with varying degrees of impact.

Studies have shown that bats have a significant capacity for dispersal, but ocean straits can act as barriers for some species, such as the *Myotis nattereri* complex in the Strait of Gibraltar (García-Mudarra et al. 2009), and *T. brasiliensis* in the Northwest and Northeast Providence Channels (Speer et al. 2017). However, this is not always the case, and the ocean may not pose a strong barrier for other species, such as *Ardops nichollsi*, *Brachyphylla cavernarum*, and *A. jamaicensis* in the Lesser Antilles, in the Caribbean (Carstens et al. 2004). The Azorean bat (*Nyctalus azoreum)* (Thomas 1901), the only endemic mammal in the Azores Archipelago, was separated from its continental ancestor, the Leisler's bat (*N. leisleri)*, during the late Pleistocene or early Holocene.

Microsatellite analysis by Salgueiro et al. (2010) showed a lack of contemporary gene flow between the Azorean bats and Leisler's bats (which is expected between different species), but also limited gene flow among the *N. azoreum* populations inhabiting all six Azorean islands, suggesting that open sea can act as an important barrier in isolating the Azorean bats. This highlights the need for attention to the conservation of this species (Salgueiro et al. 2008).

Other barriers have been identified between two frugivorous phyllostomid species, *Sturnira parvidens* and *Sturnira hondurensis*. Both are distributed throughout the Mesoamerican region and are separated by intervening highlands or lowland

forests. The estimated divergence time between the two species is thought to have occurred during the early Pleistocene (circa 1.84 Ma) and Pliocene (circa 2.5 Ma), respectively. Despite both species having high genetic diversity, mitochondrial data and modeling indicate that isolation by landscape resistance (IBR) has played a role in separating *S. hondurensis* populations. The highland habitat heterogeneity may have limited gene flow in regions such as the Sierra Madre Oriental, Sierra de Los Tuxtlas, Chiapas, and Guatemala (Torres-Morales et al. 2019).

Besides geographic barriers, distance also affects genetic diversity distribution. Although bats are capable of flight, increased geographic distances can result in decreased gene flow in various bat species (Rodrigues et al. 2010; Laine et al. 2013; Ripperger et al. 2014; Real-Monroy and Ortega 2017) due to isolation by distance (IBD). For example, in the mountainous region of the Gulf of Mexico, isolation by distance is a more significant factor than landscape features in the structuring of *S. parvidens* populations (Torres-Morales et al. 2019).

In bats, the level of IBD can vary based on the species' dispersal ability, and species with higher dispersal rates exhibit less genetic structuring compared to those with lower mobility (Meyer et al. 2009). For example, mitochondrial and microsatellite data have shown that the distances between the islands of the Mariana Islands, ranging from 5 to 100 km, do not serve as barriers for *Pteropus mariannus* (Brown et al. 2011), as gene flow was maintained. The gene flow, verified with mtDNA, was also retained among *A. jamaicensis* populations in the Lesser Antilles, where the distances between islands exceed 100 km (Carstens et al. 2004). However, a much shorter distance of 14 km in the Strait of Gibraltar represents a barrier to the dispersal of *Myotis myotis*, even though high levels of gene flow were observed among continental colonies over large distances (up to 770 km) (Castella et al. 2000).

Therefore, the studies revised here showed that factors such as mobility and dispersal abilities, open water between islands (or between mainlands, or between island and the mainland), philopatry, and whether a species is migrant or non-migrant, impact the degree of isolation of a bat species (Rodrigues et al. 2010; Frankham et al. 2002). It is important to note that a significant challenge for population genetic studies in bats, particularly in the Neotropics, is the lack of available molecular markers (microsatellites and SNPs) for effective population analysis. Currently, only 22 papers have aimed to develop microsatellites for bat species globally, four of which focusing specifically on neotropical species. The availability of more molecular markers should help improve our understanding of gene flow between populations (Loureiro et al. 2020), and the use of SNPs is expected to enhance our understanding of how neotropical bat populations are structured (Loureiro et al. 2020).

### *3.6.2 Genetic Structure of Populations*

Understanding and delineating population structure is a crucial step in comprehending the ecology and behavior of species, as well as in devising conservation management recommendations (Anderson et al. 2018). Both nuclear and mitochondrial markers are commonly used to achieve these goals, as demonstrated in a study of

the endangered New Zealand long-tailed bat, *Chalinolobus tuberculatus*, which occurs in two valleys of Fiordland, New Zealand (O'Donnell et al. 2016). In this study, all nine colonies were analyzed and found to have high genetic diversity, with moderate signs of genetic bottlenecks and, although all colonies were still connected by gene flow, small-scale genetic divergence was detected within a valley, across distances of 1.5–30.0 km (O'Donnell et al. 2016).

In the Neotropics, Ferreira et al. (2014) used the Cytb gene to show that a South American endemic bat species, *Artibeus obscurus* (Fig. 3.3), shows a deep divergence between monophyletic clusters representing populations in different biomes (Atlantic Forest and Amazon). This divergence appears in the form of a clear division between east and west within South America, separated by the "diagonal dry belt" of the Cerrado biome in Brazil.

Population genetic structuring was revealed in the Peruvian populations of the vampire bat (*D. rotundus*) through a DNA metabarcoding approach. Bohmann et al. (2018) found that populations of bats from the western coast of the Andes were distinct from those in the east, indicating that the Andes act as a barrier to dispersal for this species.

Ito et al. (2022) conducted a study of large colonies of *Pteronotus gymnonotus* in northeastern Brazil, ranging from 5,365 to 98,986 bats. They showed that the genetic distances among colonies of *P. gymnonotus* did not correlate with their geographical distances. This is a relatively mobile species with the broadest latitudinal range among mormoopid bats, and it is found in various habitats. Populations of *P. gymnonotus* had low inbreeding coefficients indicating no genetic differentiation between colonies and a weak pattern of isolation by distance.

### *3.6.3 Bats in Islands*

Bats are frequently the only native mammals on several isolated oceanic islands. Whereas about 60% of all bat species in the world live both on islands and mainland, 25% are endemic to islands (Jones et al. 2009), such as the endangered *P. mariannus* in the Mariana Islands, a US territory of Guam (Brown et al. 2011). A review conducted by Welch and Leppanen (2017) indicated that most bat extinctions occurred on islands, and the impact of invasive species – such as domestic cats – is worse for island populations than mainland populations. Island populations tend to display reduced genetic variation, as reported for some insular mammals (Paetkau and Strobeck 1994; Eldridge et al. 1999; Hinten et al. 2003; Wang et al. 2005) and bats. For instance, Salgueiro et al. (2010) found that the genetic diversity of *N. azoreum*, a bat endemic to the Azores Archipelago, was lower than that of the mainland *N. leisleri*.

Endemic island populations are more vulnerable to stochastic events, such as typhoons and volcanic eruptions. Such events can represent a significant threat to the isolated populations of *P. mariannus* on the Mariana Islands (Wiles et al. 1989; Wiles and Johnson 2004), and result in a drastic reduction in population size, thus increasing inbreeding and genetic drift, and potentially leading to a local extinction (Wiles et al. 1989; Wiles and Johnson 2004; Soulè 1983).

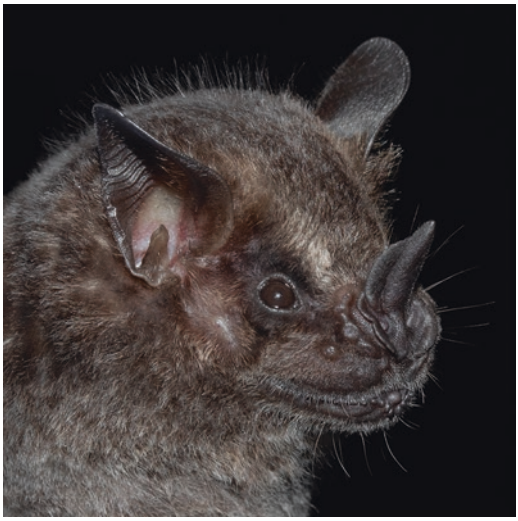

**Fig. 3.3** *Artibeus obscurus*. Photo: Roberto Leonan M. Novaes

Despite the threats faced by some populations, endemism levels on islands are high, particularly among the fox bats of the genus *Pteropus* from the Indian Ocean. Despite being able to fly over 50 km in one night (not limited by the absence of forested areas), and to traverse open water as well (Tidemann and Nelson 2004; Larsen et al. 2014), endemism remains high among these bats.

In the Neotropics, the Caribbean archipelago comprises numerous islands that differ in age, size, habitat, and level of isolation from other islands and from the mainland (Loureiro et al. 2020). The archipelago is divided into two main regions: the Lesser Antilles, located on the eastern margin of the Caribbean tectonic plate, and the Greater Antilles. Over 60 bat species inhabit these islands, including several endemic species (Davalos 2004; Loureiro et al. 2018; Tavares et al. 2018). Loureiro et al. (2020) used the genotyping-by-sequencing (GBS) method to identify distinct patterns of population differentiation between *Molossus molossus* and *Molossus milleri* in the Caribbean, finding evidence that oceanic straits were acting as barriers to gene flow within *M. milleri* from the Greater Antilles, but not within *M. molossus* from the Lesser Antilles.

### *3.6.4 Effect of Fragmentation and Other Anthropogenic Effects on Genetic Diversity*

Bats, in spite of being relatively mobile compared to other animal groups, also suffer from the impacts of habitat fragmentation (Meyer et al. 2009). Bat populations can be negatively impacted by: Urbanization (lighting, traffic noise), deterioration of water quality (eutrophication), and increased agricultural activity (with the loss of habitat boundaries, and use of pesticides), among other changes (e.g. Jones et al. 2009). Many species of bats are forest dwellers, and the loss of forest cover, as well

as the reduction in availability of foraging and roosting habitats, can lead to significant impacts (Hutson et al. 2001; Racey and Entwistle 2003). Thus, habitat fragmentation and destruction may contribute to substantial loss of genetic variation in bat populations.

Less mobile species, such as *Carollia perspicillata* in the Neotropics (Meyer et al. 2009), and the endemic and endangered New Zealand long-tailed bat, *Chalinolobus tuberculatus* (O'Donnell et al. 2016), are likely to be more severely impacted by human-driven landscape modification. On the other hand, bats with higher mobility may be less affected by habitat fragmentation. For example, *Carollia castanea* is relatively more resistant to habitat fragmentation than other low-mobility phyllostomid bat species, and its genetic diversity might take longer to respond to the effects of fragmentation (Ripperger et al. 2014). The authors emphasize the importance of considering not just mobility, but also the landscape composition and life history traits of a species, when assessing their response to habitat fragmentation.

In addition to habitat fragmentation, predation by domestic cats has been documented in several bat species, including *M. vivesi* (Vázquez-Domínguez et al. 2004), *Plecotus auritus* (Woods et al. 2003), and *Pipistrellus coromandra* (Virkar and Shrotriya 2013). Scrimgeour et al. (2012) used DNA samples to identify a single male cat as being responsible for killing 102 individuals of *Mystacina tuberculata* in just 7 days, emphasizing the impact that domestic animals can have on bat populations.

Bats of the Pteropodidae family, as well as a few other species, face the threat of being hunted, which is mostly illegal and exacerbated in certain cases, such as *P. mariannus* in the Mariana Islands (Brown et al. 2011), and *Pteropus rodricensis* on the Rodrigues Island, in the Indian Ocean (O'Brien et al. 2007). This hunting can be driven by cultural traditions where the bats are considered a delicacy food, or for commercial purposes (Wiles et al. 2010). Despite this threat, the genetic consequences of hunting on bat populations are still not well understood.

### *3.6.5 Bat Adaptations*

With around 70% of fossil data missing (Eiting and Gunnell 2009), the evolutionary history of bats is still a topic of debate, with many aspects of their phylogenetic reconstruction and unique adaptations yet to be understood (Teeling et al. 2018). This history likely involves introgressions, hybridizations, adaptations, and the incorporation of inherited genetic elements in different ways, such as through behavioral changes, which can then lead to rapid diversification and the evolution of associated mechanical and physical adaptations (Duckworth 2009; Mayr 1963). For feeding, animals must use a combination of foraging behavior and sensory perception, and changes in behavior can sometimes be mediated by the remodeling of sensory systems (Goldman-Huertas et al. 2015; Karageorgi et al. 2017). In a recent study, Davies et al. (2020) used transcriptome assembly to detect contrasting levels

of positive selection in genes associated with the development, maintenance, and scope of visual function in bats. They traced these changes back to the origins of the superfamily noctilionoids, and of the family with the most dietary diversity, the neotropical leaf-nosed bats (Phyllostomidae), finding evidence of positive selection in vision genes during subsequent shifts to either nectarivory or frugivory. These changes likely reflect an effective preadaptation to the use of visual cues for identifying food and roosts, as well as for orientation.

An interesting case of feeding adaptation can be found in the greater bulldog bat (*Noctilio leporinus*). This species, which is relatively large, feeds on a variety of food sources including fish, flying insects, and aquatic invertebrates. The development of the piscivory habit in this genus has been a recent occurrence (Pavan et al. 2013), and the most likely explanation is that it arose after the radiation of existing *Noctilio* species, without any significant modifications in morphology or physiology (Khan et al. 2014; Liem 1973). Overall, our understanding of the genetic basis of bat adaptations is still limited, but recent advancements in genomics hold promise for improving our knowledge in the future.

## 3.7 Final Considerations

Due to the high diversity of bats and our limited understanding of many aspects of their biology and species, we emphasize the importance of continued genetic research as to further our comprehension of their population dynamics, and of the impacts of human activities on their conservation. This is particularly crucial for species that are considered vulnerable and located in regions with high levels of diversity, such as the Neotropics, where endemic genera and species have been more recently detected and described (Gregorin and Ditchfield 2005; Tejedor et al. 2005; Nogueira et al. 2012; Tavares et al. 2014, 2022; Novaes et al. 2022).

The continued improvement of genetic techniques to obtain DNA, such as non-invasive (guano) and minimally-invasive (wing tissue, buccal samples) sampling methods, makes it possible to monitor individual bats and study their spatial and temporal patterns of dispersal and habitat use (Carroll et al. 2018). Furthermore, the collection of DNA from fecal samples and subsequent use of DNA metabarcoding to assess the diets of multitrophic assemblages has allowed for inferences of population structure (Bohmann et al. 2018; Ingala et al. 2021), making it a valuable tool for improving the understanding and conservation of bats.

The study of bats and their genomes holds the potential to impact a variety of scientific fields, such as healthy aging, disease resistance, ecosystem functioning, and the evolution of sensory perception (Teeling et al. 2018). Our review recovered 19 bat genome descriptions, including complete mitogenomes, transcriptomes, and cosmid libraries. So far, the complete mitochondrial genomes of the following bat species are available: *Myotis frater* (Chung et al. 2018), *Myotis rufoniger* (Bhak et al. 2017), *Myotis brandtii* (Jiang et al. 2016), *Myotis davidii* (Wang et al. 2016), *M. myotis* (Jebb et al. 2018; Huang et al. 2016), *Myotis muricola* (Yoon and Park

2015a), *P. personatus* (López-Wilchis et al. 2017), *Pteropus alecto* (Gao et al. 2016), *Pteropus vampyrus* (Lu et al. 2016), *Hypsugo alaschanicus* (Kim and Park 2015), *Murina ussuriensis* (Yoon and Park 2015b), *Rousettus leschenaultia* (Szcześniak et al. 2014), *Rhinolophus luctus* and *Hipposideros armiger* (Xu et al. 2012), *Pteropus scapulatus*, and *Pteropus poliocephalus* (Barragán et al. 2002). Additionally, the transcriptomes of *M. myotis* (Huang et al. 2016) and *Rousettus aegyptiacus* (Lee et al. 2015) have been determined, as well as a cosmid library of *M. waterhousii* (Baker et al. 1997). Despite the large number of bat species worldwide, the number of available genomes is still small, a trend also seen in other groups due to the high cost and specialized labor required.

Despite the increase in the number of genetic studies involving bats in the early 2000s, several families (such as Nycteridae, Furipteridae, Mystacinidae, Thyropteridae, and Natalidae) still require further investigation. There is also a need for a deeper understanding of the impact of barriers, fragmentation, and geographic distance on bat populations (Fig. 3.2). The continual advancement of new technologies has shown the potential of these tools in a wide range of genetic studies, and their contribution to the management and conservation of bats.

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# Chapter 4
# Status Quo and Orchid Conservation Challenges in the Neotropical Region


**Wagner Franco Molina, Fábio de Almeida Vieira, and Cristiane Gouvêa Fajardo**

## 4.1 Introduction

Conservation genetics since its conception has focused on the genetic consequences of small populations, which can limit the survival of species (Frankel 1974). It also provides tools to guide conservation and restoration efforts, facilitating the evaluation of these projects by quantifying the flow of genes or demographic changes in the target populations (Mijangos et al. 2015).

Genetic diversity is associated with individual fitness and population persistence in the ecosystem (Reynolds et al. 2012). Its evaluation by genetic markers helps decisively in genetic conservation programs, in the monitoring of rare and threatened species and allows to the identification of resilience potential, evolutionary capacity and survival rate of future generations (Kramer and Havens 2009).

The increasing interference of anthropic action in natural environments has promoted a rapid erosion of biodiversity and constitutes enormous current challenges for biological preservation. At the same time, great efforts are being made to quantify these changes, particularly in terrestrial systems (Tydecks et al. 2018), to understand the consequences for the ecosystem and its effects on human well-being. For some plant groups, ecological requirements are critical, as they present low population turnover and distribution in areas under economic exploitation. This is the case of several groups of the diverse Orchidaceae family that are particularly fragile and deserve specific conservation programs.

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## 4.2 Genetic Conservation in Orchidaceae

Orchidaceae is the second largest family in the number of flowering plant species after Asteraceae (Niu et al. 2017; Antonelli et al. 2020). Despite being more abundant and diversified in tropical forests, its distribution is cosmopolitan (Rao 2020). According to the updated classification of the Angiosperm Phylogeny Group – APG IV (Chase et al. 2016), it belongs to the order Asparagales, with approximately 28,484 species (Govaerts et al. 2020), of which more than half are epiphytes (Zotz 2013). These plants present a great diversity of habits, including terrestrial, rupicolous, perennial and saprophytes species (Gravendeel et al. 2004; Hu et al. 2015).

Although it is a large family, it is also one of the most endangered, partly due to its complex life-history strategies. Its high diversity includes many unique species with specialized floral structures, specific pollinators, coevolution processes, among other ecological requirements. All orchids depend on fungi for germination and carbon capture at the protocorm stage, and in many taxa they are associated with mycorrhizal in a mandatory way for survival. Some orchids of the mycoheterotrophic lineage have lost all photosynthetic capacity and rely entirely on fungi to obtain nutrients. Finally, orchids exhibit extraordinary floral diversity, with striking adaptations for different pollinators among close relatives, providing an extreme convergence and divergence between no related taxa (Givnish et al. 2015). All these aspects make this a group with challenging models for conservation biology (Pillon and Chase 2007; Hinsley et al. 2018).

The diversity of endangered orchids has become alarmingly increased, over which conservation programs around the world reveal low success rates in species translocation (Phillips et al. 2020). According to the IUCN, 1641 orchid species are on the Global Red List, of which 51.5% are classified into the threatened categories (IUCN 2020). Therefore, like most plants, orchids are particularly endangered by environmental degradation and loss of habitats, especially terrestrial species that are sensitive to soil pollution and the epiphytes that predominantly inhabit primary forests (Pillon and Chase 2007; Gale et al. 2018).

Another issue of concern with natural orchid populations is the indiscriminate collection for commercial purposes of ornamental plants, food or medicinal use. Predatory harvest causes a break in ecological connections, changes in abiotic conditions, and favors the spread of disease-causing pests, leading several species to the threat of extinction (Swarts and Dixon 2009b). They are often also affected by deforestation, derived from logging, fires, road construction and expansion of forest plantations and agriculture (Laurance et al. 2012). Additionally, they still suffer from the impacts of climate change that have negative consequences for many species in their degraded habitat (Pereira et al. 2010).

Despite the many advances made in the last decades to effectively conserve biodiversity, it is a priority to initially know the existing diversity, allowing the identification of high priority places for conservation (Fay 2018).

Unfortunately, Orchidaceae is a group with a complex taxonomy, permeated by excessive taxonomic errors due to morphological variability and complex

life-history (Pillon and Chase 2007). In tropical regions, many groups of orchids are still poorly understood, and phylogenetic studies are needed to detect phylogenetically divergent species that are isolated in specific groups and thus have high conservation value (Fay 2018). Groups with high diversity of taxa, phylogenetically isolated, derived from rapid evolutionary irradiation should be given priority in terms of actions to support their conservation (Crozier 1997).

Molecular techniques have been increasingly used to well-established practices aimed at the conservation of orchids, including with applications at botanical gardens (van den Berg et al. 2009). The development of molecular markers is no longer an obstacle, as the dissemination of faster and lower-cost laboratory practices has facilitated this task. The use of genetics results in qualitatively broader and more informative data, which gives better possibilities for planning and conservation actions (Fay 2018).

The molecular data provide an empirical framework through which emerges information necessary to set priorities, reduce costs and improve management decisions. In particular, molecular data allows to address issues of genetic variation between populations, species delimitation and the maintenance of evolutionary processes (van den Berg et al. 2009). At the population level, genetic studies can be used to identify regions or populations that should be considered as a high priority for conservation (Fay 2018). Regarding these aspects, orchids present several particularly complex genetic issues, related in part to their high diversity and, for many taxa, to the recent evolutionary radiation (Swarts and Dixon 2009b).

## 4.3 Neotropical Orchids

The unique biodiversity found in the neotropical region contains several of the Earth's hotspots (Myers et al. 2000), and it has attracted the attention of naturalists for centuries. Our knowledge on the origin of this enormous biodiversity is often limited by the scarcity of available model organisms that could be employed to address key issues of evolutionary diversification processes in the region (Pinheiro and Cozzolino 2013). Despite significant advances in recent years in understanding the origin and diversification of various taxa in this biogeographic region, several questions still need to be answered (Antonelli et al. 2018).

Essentially, all temperate orchids are terrestrial, but most orchids inhabit tropical forests and more than 80% of them are epiphytes (Givnish et al. 2015). It is known that the epiphytic plants are major contributors to the overall biodiversity in the Neotropics (Küper et al. 2004; Ozanne et al. 2003). Specifically, neotropical epiphytic orchids are generally characterized by restricted populations with a small number of individuals (Crain and Tremblay 2012; Pandey et al. 2013). Understanding the factors that affect and limit orchid colonization in the environments provides valuable insights on the establishment processes of these plants and has important conservation implications for neotropical biodiversity (Trapnell et al. 2013).

As documented for many taxa, the diversification of orchids in the neotropical region was strongly impacted by the rise of the northern Andes (Chazot et al. 2016; Diazgranados and Barber 2017; Bacon et al. 2018). The majority of Andean orchids originated in the last 20–15 million years, correlated with orogeny processes, and multiple dispersion and recolonization events over the Andes of lineages derived from ancestors of the Amazon plain, with additional contributions from Central America and the Antilles (Pérez-Escobar et al. 2017).

## 4.4 Phylogenetic Relationships

Recent phylogenetic analyses based on the *rbcL* plastid DNA sequences have identified the monophyly of the five subfamilies (Apostasioideae, Vanillioideae, Cyripedioideae, Orchidoideae, Epidendroideae) currently recognized in the Orchidaceae family. Among these, Epidendroideae and Orchidoideae represent the most diverse groups (Chase et al. 2015; Freudenstein and Chase 2015).

Rapidly evolving markers, such as the nuclear internal transcribed spacer (ITS) of ribosomal RNA genes, and both plastidic DNA regions, the intergenic spacer *trn*LF and maturaseK gene (*matK*), have been extensively used in studies at the level of genera and species (van Den Berg et al. 2000; van Den Berg 2014; da Silva 2013). Based on genetic profiles, the taxonomic validation and phylogenetic relationships can assist in defining conservation priorities (Swarts and Dixon 2009a) and decisively support conservation projects in Orchidaceae (Gale et al. 2018). More precise floristic inventories are necessary to reveal cryptic species and complexes of rare species, which deserve recognition and protection, and allow us to understand the evolutionary processes related to diversification.

According to Givnish et al. (2015), orchids emerged about 112 million years ago, with the divergence of their largest subfamilies, Orchidoideae and Epidendroideae, dating from the end of the Cretaceous. Probably, the orchid species diversification is correlated with the evolution of several features, such as pollinia, epiphytic habit, Crassulacean acid metabolism (CAM photosynthesis), a photosynthetic pathway in which atmospheric $CO_2$ is absorbed during night hours, tropical distribution, and pollination by lepidopterans or Euglossine bees. It is believed that deceptive pollination (i.e. plants exhibit false signals to the insects, imitating rewarding conditions, regarding food, shelter, and sex) has doubled the number of orchid species (Gaskett 2011).

Some species of orchids have complex life histories and are still under intense scrutiny. Several knowledge gaps on the interspecific relationships can be essential to define appropriate strategies for conservation of specific groups or taxa. Reconstructing the phylogeny of these groups can contribute to the recognition of their enormous diversity of species. One of these groups is the *Vanilla* genus, which houses more than 100 species distributed around the world. Phylogenetic trees have been used to recognize synapomorphic traits, which have medicinal and economic value within this group, in which aromatic fruit species belong to the neotropical

species clade (Cameron et al. 1999; Cameron 2005). Within this clade, only *Vanilla planifolia*, probably endemic to the tropical forests in eastern Mexico, has significant economic value as a natural source of flavor and vanilla fragrance (Bory et al. 2008).

There is an increasing amount of new molecular information aimed at elucidating about the phylogenetic relationships within *Vanilla* (Ramírez et al. 2007; Bory et al. 2008; Gallage and Møller 2018; Kim et al. 2020). The knowledge on the degree of relationship between species of economic potential has applicability in the coordination of crossings aiming genetic improvement of target traits. Two other species of this genus, *Vanilla tahitensis* and *Vanilla pompona*, although on a smaller scale, are already cultivated as aromatic plants. Although *ex situ* conservation actions have helped to protect wild individuals of this genus, doubts remain about the extent of the different species to be protected. In this sense, there is a great international effort to protect and study the species of this genus in their areas of origin, as well as in areas where the culture was introduced (Bory et al. 2008).

It is important to note that there are a considerable number of orchid species with no direct economic interest, but their knowledge can provide important clues about the evolution, ecology, or biogeographic patterns of the family. For example, within the Vanilloideae subfamily, the relationship between both *Clematepistephium* and *Eriaxis*, from New Caledonia, a Pacific archipelago, with species of the *Epistephium* genus, from South America, points to an ancient common origin (90 Ma), related to its distribution in the Gondwana supercontinent (Cameron 2010). These age estimates are relatively concordant with Ramírez et al. (2007), in which a cladistic analysis, calibrated with the orchid fossil *Meliorchis caribea*, discovered preserved along with the extinct stingless bee, *Problebeia dominica*, in a Miocene amber in the Dominican Republic, pointed out that the origin of all extant Orchidaceae occurred in the Late Cretaceous (76–84 Ma).

Phylogenetic analyses are thus a powerful tool to date historical events and contribute to information on the evolution of ecosystems (Bytebier et al. 2011). The subfamily Cypripedioideae, with a disjunct distribution in temperate and tropical regions, is composed of five genera. Popularly known as "slipper" orchids, this group is at high risk, with 90% of the evaluated species falling into one of the threatened categories (Fay and Rankou 2017). The attractive flowers of these orchids make them have high ornamental and economical values (Guo et al. 2012). The detailed analysis of its phylogeny clarified the diversification of this group. Guo et al. (2012) revealed that the *Cypripedium* genus, widely distributed in both temperate and subtropical regions, diverged first in the group, followed by *Selenipedium*, which is endemic in South America. It is suggested that the high species diversity and present wide distribution of the *Cypripedium* genus were developed to adapt to new niches created by climatic oscillations in the late Cenozoic, while *Selenipedium* appeared later in the Paleocene. The *Mexipedium* and *Phragmipedium* genera, occurring in the Neotropics, are closely related to *Paphiopedilum* from tropical Asia. The reconstruction of ancestral areas indicated vicariance processes as responsible for the disjunct distribution of slipper orchids in neotropical and palaeotropical regions (Guo et al. 2012).

New molecular tools have expanded the means of identifying and assigning plant taxa. Molecular techniques like amplified fragment length polymorphism (AFLP), conserved DNA-derived polymorphism (CDDP), diversity arrays technology (DArT), inter-primer binding site (iPBS), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), inter simple sequence repeats (ISSR), start codon targeted (SCoT), single nucleotide polymorphisms (SNP), simple-sequence repeats (SSR), sequence-related amplified polymorphism (SRAP), DNA barcoding, plastidial genome, among others, have been used for studies of plant species diversity (Arif et al. 2010; Amom and Nongdam 2017; Farooq et al. 2020; Kim et al. 2020). Many evolutionary and taxonomic questions can now be answered based on these markers, which would not be possible only with phenotypic morphological methods.

## 4.5 Population Genetics

At the population level, molecular tools clarify the action of different evolutionary factors involved in intraspecific genetic variability patterns. Molecular approaches have characterized the impacts of habitat fragmentation on gene flow and orchid population structure (Juárez et al. 2011; Trapnell et al. 2013; Minasiewicz et al. 2018), the evolutionary potential of populations (Kisel et al. 2012; Li et al. 2020), inbreeding (Blambert et al. 2016; Brys and Jacquemyn 2016; Picolo et al. 2016), genetic consequences of pollination syndromes (Gaskett 2012; Papadopulos et al. 2013; Phillips et al. 2020), apomixis (Campacci et al. 2017; Firetti 2018), genetic drift (Pinheiro et al. 2014; Jaros et al. 2016; Yun et al. 2020), and spatial genetic structure of populations on a fine-scale (Ilves et al. 2015; Cortés-Palomec et al. 2019; Hedrén and Lorenz 2019). Other studies have focused on elucidating evolutionary processes involved in the diversification of species and lineages, such as hybridization and polyploidization (Marques et al. 2014), and demographic factors originated by the historical dynamics of climatic fluctuations (Kolanowska et al. 2020).

Many orchids can have a long-life cycle allowing numerous crosses and seed dispersal over a long distance. These characteristics are favorable to the formation of genetically diverse populations with little differentiation between them. Hamrick and Godt (1996) found a mean genetic differentiation ($G_{ST}$) equal to 0.087 within Orchidaceae. That is considered relatively low, yet within the genetic pattern for herbaceous species (Jesus et al. 2001; Pereira et al. 2007). However, current data indicate that there are no clear trends in population genetic diversity in Orchidaceae, which makes it difficult to standardize methodologies for orchid conservation programs. It appears that for each species, an appropriate management plan based on genetic data is necessary. Hence, the molecular analysis of populations and species represents an essential tool for delimiting the origin, sampling and propagation of seeds, as well as to improve taxonomic precision, identification of clones and selection of suitable genotypes for long-term storage and reintroduction programs.

Complementarity, it is also necessary to add information about the natural landscapes where species occur (Storfer et al. 2007, 2010; Balkenhol et al. 2015) and identification of their most likely niches at different time scales (Carstens and Richards 2007; Alvarado-Serrano and Knowles 2014).

The knowledge of genetic diversity improves conservation strategies since the long-term persistence of species depends on the level of genetic variation. Species showing high levels of genetic diversity have more advantage for supporting the environmental changes, because their populations are better able to adapt to these alterations. Higher genetic diversity increases fitness and decreases the risk of extinction (Brzosko et al. 2011).

Low levels of genetic variation are usually associated with species rarity (Frankham 2003). Theoretical predictions and empirical data show that low genetic variation is often a consequence of small population size (Nazareno and Jump 2012; Nazareno et al. 2017). Small and isolated populations are often generated from continuous fragmentation or habitat loss due to anthropic action (Frankham 2003; Desalle and Amato 2017; Cheeseman et al. 2019). Human impacts reduce population size and increase the distance between remaining populations, reducing gene flow between them. Under these conditions, small fragmented and isolated populations generally have a low genetic variation. So, they are more vulnerable to demographic, environmental and genetic stochastic fluctuations. This will increase the genetic drift effects and the probability of inbreeding depression in these populations and, consequently, the risk of extinction (Brzosko et al. 2011).

The Orchidaceae family has numerous examples of species that have become extinct due to anthropic actions in several regions around the world (Koopowitz 2001). The destruction, modification and fragmentation of natural forests, as well as the illegal extraction of orchids from natural populations, have had a strong influence on the extinction of many species. Thus, the intensification of genetic diversity studies is decisive for elaboration of conservation plans in the family (Ávila-Díaz and Oyama 2007).

Some orchid species are suitable models for a better understanding of the evolutionary processes in plants, including possible reproductive isolation by post-zygotic barriers. For example, the extensive morphological and chromosomal variation found among different populations of *Epidendrum* make it an excellent model to test the influence of historical demographic events on the diversification and speciation of strains in the neotropical region (Pinheiro and Cozzolino 2013). Pinheiro et al. (2010) tested the hypothesis of gene flow between *Epidendrum fulgens* and *Epidendrum puniceoluteum*, distributed on the Brazilian coast, which present different levels of polyploidy. Molecular data, obtained by the use of plastidial and nuclear microsatellites from allopatric and sympatric wild populations, revealed the presence of hybrid zones, indicating that hybridization and introgression played an essential evolutionary role in the genus diversification. In another example, 13 allozyme polymorphic loci were used to assess the genetic diversity of six populations of *Oncidium hookeri*, a neotropical species threatened with extinction, found in remnants of the Brazilian Atlantic Forest (Alcantara et al. 2006). Several rare alleles were found in all populations and three populations had private alleles. There was

low genetic differentiation between populations, not correlated with geographic distance. Two populations showed signs of recent bottlenecks. According to the authors, the deficiency of heterozygotes in the populations was probably associated with the behavior of pollinators and the low frequencies of several alleles of different loci maintained due to the clonal propagation. Despite the stochastic nature of wind dispersion of the seeds of this species over long distances, the process seems to promote a sufficient gene flow between populations, and consequently avoid genetic differentiation.

Epiphytic orchids generally have a different pattern of genetic structure when compared to those with other habits. Most epiphytic orchids reveal a high genetic diversity and some level of population structuring. Others like *Laelia speciosa*, an endemic and endangered Mexican orchid, exhibits populations with low levels of genetic diversity throughout its geographical distribution. Given the low genetic diversity among populations, the restoration strategies indicated to maintain the original genetic structure of the species, and reintroduction of individuals obtained from the *in vitro* propagation of seeds of populations inhabiting near to the reintroduction area. In addition, multidisciplinary support for the species has been recommended, including biological studies and environmental education programs in local communities (Ávila-Díaz and Oyama 2007).

Increasingly threatened epiphyte orchids are affected by several factors, including anthropogenic habitat disturbance. As specific host tree recolonises deforested areas, the establishment and composition of epiphyte orchid populations often occur as a function of their colonization patterns. One of the most common orchids in neotropical habitats is *Brassavola nodosa*. Trapnell et al. (2013) demonstrated that very few individuals founded several populations of *B. nodosa*. Subsequent population growth resulted mainly from the recruitment of progeny produced by the founder individuals. A low genetic diversity observed was due to these populations being in the early stages of population expansion after the founding effect (Trapnell et al. 2013).

*Cattleya elongata*, a rupicolous orchid endemic to northeastern Brazil, occurs on rocky outcrop islands in a rocky field of Chapada Diamantina vegetation. Morphological and genetic variability were analyzed in nine wild populations, covering the entire area of distribution of the species. Genetic variability was high and the populations present moderate structuring (allozymes, $\phi_{PT} = 0.14$; ISSR, $\phi_{PT} = 0.18$) and low inbreeding (allozymes, $F_{IS} = 0.06$). Individuals exhibit high morphological variability with moderate differentiation among populations, however no evidence of a correlation between genetic, morphological and geographic distances was found (Cruz et al. 2011). The parameters evaluated highlighted their importance for the conservation, planning or management of the variability found within and among the different populations.

Habitat loss and intense collection pressure have shown a substantial impact on the conservation status of one of the most emblematic Brazilian orchids, *Cattleya labiata*. Many of its populations are not located in protected areas, which is an essential factor for the conservation priority of the species. Initial data on the genetic diversity and structure of six wild populations in remnants of the Atlantic Forest

located in northeastern Brazil studied by RAPD and ISSR markers indicated high levels of genetic diversity within populations, compared to other studies of Orchidaceae species. Moreover, an intricate genetic structure amongst populations was observed. The genetic similarity between individuals from different regions indicates the dispersion of anemochory seeds over long distances (Pinheiro et al. 2012).

Numerous neotropical orchids of high ornamental values are currently threatened with extinction as a result of intense collection pressure. It makes the remaining genetic diversity crucial to the maintenance of these species in the long term in natural environments. For example, in populations of *Cattleya granulosa* (Fig. 4.1), a vulnerable orchid (Martinelli and Moraes 2013) of the Atlantic Forest of northeast Brazil, a high genetic differentiation of populations ($F_{ST}$ = 0.391; P < 0.0001) was found (Fig. 4.2). The positive correlation between the geographical and genetic distances among populations (r = 0.794; P = 0.017) suggests an isolation by distance pattern within this species (Fig. 4.3). There was evidence of genetic bottleneck in most populations of *C. granulosa* (Fajardo et al. 2017).

There is a consensus that the *C. granulosa* populations are in drastic decline, and this is not only a consequence of the predatory collection actions but also due to the intense fragmentation of Atlantic Forest, in the northeast of Brazil. Even though the levels of genetic diversity along its geographical distribution appear to provide conditions for its ecological expansion (Fajardo et al. 2017), the recent history of the species in disturbed environments (i.e. intense urban growth in the coastal zone and increased agriculture) can quickly lead to loss of fitness and genetic variability.

A set of research for the conservation of *C. granulosa* (Fajardo et al. 2014, 2015, 2017) and collaboration with the Rio Grande do Norte Orchid Association (SORN), a non-governmental organization dedicated to the cultivation and conservation of native orchids, provided bases for actions that culminated in the State Law N°. 10,508, of May 16, 2019, that defined the orchid *C. granulosa* as a symbol flower of the State of Rio Grande do Norte. As a consequence, a week of state


**Fig. 4.1** Specimens of *Cattleya granulosa* (**a**, **b**) (Photo: Edson Mattos)

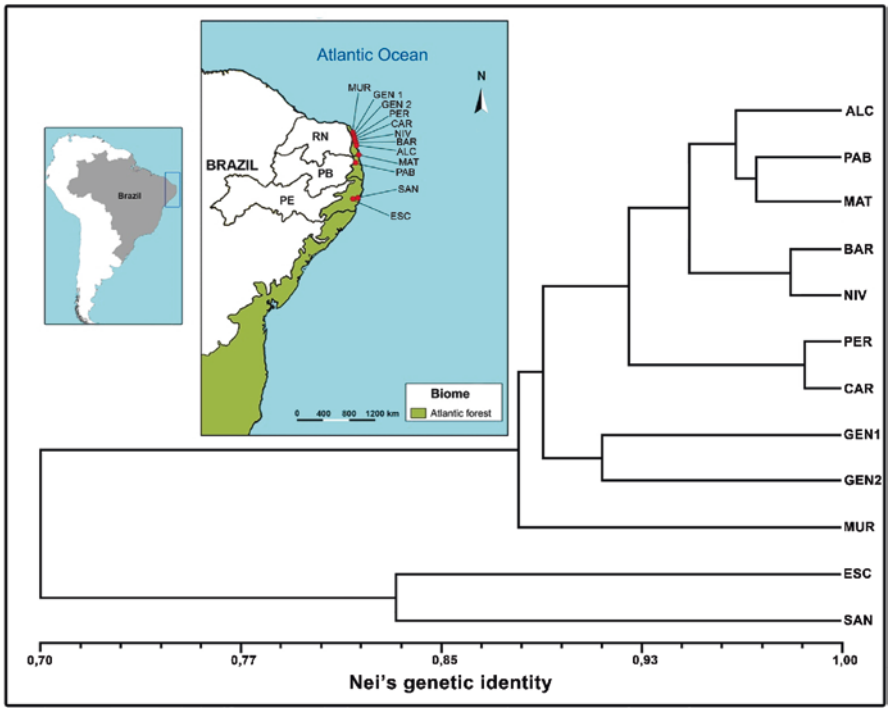


**Fig. 4.2** Geographical location of the sampling areas of *Cattleya granulosa*. A: map of Brazil with collection regions highlighted in gray. B: sampled populations in the states of Rio Grande do Norte/RN [Ceará-Mirim (MUR); Extremoz (GEN1; GEN2); Natal (PER; CAR; NIV); Parnamirim (BAR; ALC)], Paraíba/PB [(Mataraca (MAT); and Mamanguape (PAB)], and Pernambuco/PE [(Cabo de Santo Agostinho (SAN); Escada (ESC)] within the Atlantic Forest biome. Cluster analysis (UPGMA) based on Nei's genetic identity among populations (adapted from Fajardo et al. 2017)

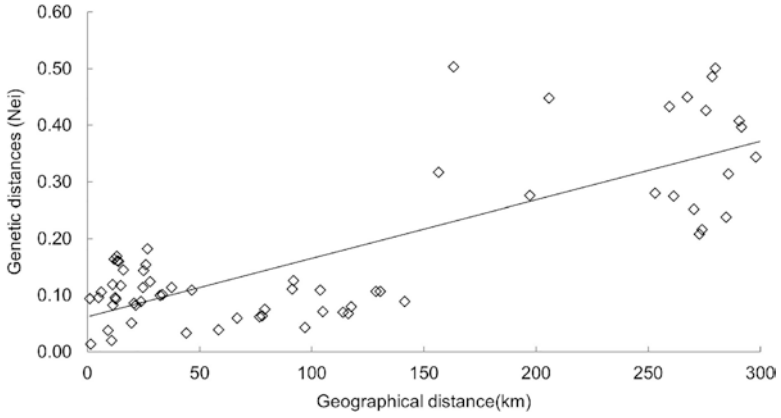


**Fig. 4.3** Relationship between genetic and geographic distances between populations of *Cattleya granulosa* (see Fajardo et al. 2017)

commemoration dedicated for conservation, appreciation and preservation of the orchid was established. The charismatic role of the symbolic species *C. granulosa* in attracting conservation actions indicates that orchids can be social mobilizers for biological conservation and important instruments in environmental awareness. Furthermore, environmental preservation and education create a synergy between social and governmental inspection to preserve other threatened species, as well as the remaining forest fragments (Fajardo and Vieira 2020).

Linnaeus and Darwin were great admirers of the exuberance of this family of plants. However, the size of the family has historically been an obstacle to their study (Fay and Chase 2009). In his "The origin of species" book, Darwin (1859) argued that the great diversity and incredible beauty of these plants would be driven by high levels of variability and complexity.

The remarkable diversification and specialization in orchids, the complex taxonomy of many groups and the growing global impacts on their species require detailed information on their genetic diversity. Tied to the environmental awareness of local communities and habitat preservation, the recognition of their biological interactions and environmental requirements have consolidated. There is a need for multidisciplinary conservation projects, encompassing their biological, ecological, biogeographic, taxonomic and phylogenetic levels.

## 4.6 Final Considerations

Unprecedented levels of extinction arising from human influence on climate change, habitat destruction, the introduction of invasive species, extractivism and occupation of natural areas, claim that genetic conservation priorities must be defined. Conservation programs are complex and require up-to-date knowledge about the taxonomic status, biology, ecology, phylogeny and phylogeography of species (Seaton et al. 2010). Working groups of orchid specialists are involved in a comprehensive worldwide portfolio of conservation projects. These include population monitoring, translocations, propagation, reinforcement, reintroduction, conservation genetics, endangered species listing, commercial research and studies of mycorrhizal associations and pollination (Fay and Rankou 2017).

Extinction risks are increasingly widespread among species mainly in the Neotropics. However, they become maximized in some susceptible groups of orchids as of the genus *Cattleya*. Its diversity of species, largely showing restricted distribution, and specialized ecological standards, suffers intense impact by predatory collection, habitat destruction and pollinator extinction. The high biological diversity in Orchidaceae is reflected in varied patterns of genetic differentiation of its populations that urgently need to be known. The protection of genetic diversity has been incorporated into conservation protocols. However, further progress is needed concerning knowledge and protection of genetic diversity, since conventional databases are still inadequate in planning land use and conservation strategies.

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# Chapter 5
# Population Differentiation with Introgression


**Edyane Moraes dos Santos and Reinaldo Alves de Brito**

## 5.1 Introduction

Species are considered by some to be the "currency" in biology (Agapow et al. 2004; Tobias et al. 2021) for several reasons, but chiefly for their importance in serving as a yardstick for measuring ecological and evolutionary parameters that have fundamental theoretical and practical consequences for several areas, especially for studies on biodiversity. Yet, despite its importance, there is still a great deal of controversy among biologists as to what constitutes a species, as can be inferred by the existence of dozens of different species concepts, which are based on different biological attributes and evolutionary forces (De Queiroz 2007). It is possibly because of this combination of relevance and dissent that several new methods have recently been proposed to help solve this riddle, some of which will be briefly mentioned in this chapter, not before we provide a historical context for this controversy. We will then contend that new methods and data, promising as they may be, have not assuaged these contentions. Rather, they have shown that there is much more complexity in nature to what constitutes a species than previously envisioned, and expanded our knowledge of the levels of introgression that exist among different species.

Charles Darwin's most famous book "On the Origin of Species" actually has the full title "On the Origin of Species by means of Natural Selection, or the Preservation of Favored Races in the Struggle for Life", which reflects how Darwin saw the process of species formation, having natural selection as the main driver for differentiation, though in his text he made sure to point out that it was not the only force involved with their differentiation. According to Darwin, as natural selection and

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adaptation proceeded, they would slowly drive populations apart. Darwin then saw differentiation as a continuum in which the effective separation of different "species" would be established more or less arbitrarily, so that species would have no reality in nature, contrary to the fixist view held at the time. Despite this continuity, the separation between populations promoted by their adaptation to different niches, and the extinction of ancestors and intermediaries, would be responsible for the gaps that we find among different species (Darwin 1859).

Even at Darwin's time, the suggestion that species would be relatively arbitrary constructs was met with skepticism by researchers who believed that any natural historian was able to see the reality of species when investigating them in nature (Sharp 1871). As time went by, the view that species in nature would be real was philosophically reinforced by researchers who considered species no longer as having an essence, as originally proposed by Aristotle, but initially as a class and then as an individual (Hull 1978), and the question switched to the understanding of the forces involved in its formation.

Though the vast majority of researchers today recognize species as real evolutionary units, the understanding of what determines this reality and which evolutionary forces would be involved in their maintenance remains major points of contention. Some researchers, such as Mayr and Dobzhansky, suggested that species' reality would be maintained because its members would share a common genetic/ecological framework, establishing what Mayr dubbed "fields of recombination" (Mayr 1963). This interpretation expanded the view of the evolutionary synthesis that mostly considered genes individually in speciation (in what Mayr derogatorily referred to as "beanbag genetics") to reinforce the expression of variation in the ecological and phenotypic context of an individual. This emphasis on the potential for genetic variation to be shared and appropriately expressed in different individuals in an ecological context made Mayr emphasize reproductive isolation as an essential aspect for the recognition of distinct species, leading to the "Biological Species Concept" (BSC).

The underpinnings of the BSC intuitively made sense to several researchers, so much so that it was widely accepted, and disseminated to arguably become the most common species definition even outside the scientific community. However, even back then botanists were finding several instances in which hybrids were formed between different "species" that still retained their identities and failed to homogenize. These hybrids even led some to emphasize the important role recombination of interspecific variation might have in the adaptive process (Anderson and Stebbins Jr 1954). The chasm between botanists and zoologists culminated with Ehrlich and Raven (1969) proposing that the maintenance of species identity would be a consequence of adaptations to specific niches, more than to reproductive isolation. Species would remain isolated because natural selection could overcome even significant levels of gene flow and maintain their identity. According to this "ecological species concept," species were groups of individuals who shared adaptations that would allow them to occupy a certain ecological niche, even in the face of some gene flow. Species boundaries would be established then by the balance between gene flow and natural selection.

Despite the prevalence of Mayr's biological species concept, mostly in the 1970s, other important researchers, even at the heart of the evolutionary synthesis, had already suggested that the existence of evolutionary lineages would be instrumental for species characterization (Simpson 1951). At first, it was suggested that species were evolutionary lineages which maintain their identity from other lineages and retain their own evolutionary tendencies and historical fate. Such a view did not make explicit what attributes would allow for that, much less what it was meant by their "fate," leading Joel Cracraft (1983) to simplify the definition of species as being groups of organisms that share synapomorphies, or derived characters, in his phylogenetic concept of species.

Since its first modern definition, several other species concepts have been proposed, emphasizing different biological aspects, or their combinations. Despite this complexity, De Queiroz (2007) proposed that differences between species concepts only lead in fact to different diagnoses and to an incongruity in a gray area that encompasses species that diverged recently and that harbor certain attributes. According to him, in this gray zone, the processes that are considered determinant for each of the different species concepts can happen at different speeds. With the passing of time, though, these processes tend to converge, especially if there are no intense evolutionary forces acting to supersede them. A relevant aspect of this view is that if we consider speciation from a macroevolutionary point of view, in fact there would not seem to be many inconsistencies, because after a lot of time elapsed, most, if not all, species concepts would converge on the same conclusions. However, a more populational view of the process, facilitated today by the increase in the number of genetic markers that are increasingly capable of differentiating even groups of individuals in a population, can reveal patterns in the demographic history of populations such as population size contractions and expansions. Due to these advances, there is increased ability to recognize intraspecific genetic structure, and different levels of introgression throughout their distribution and history, which point to phylogenetic heterogeneity across individuals and across their genomes that would have a potential impact on their species status, depending on the species concept used (Edwards et al. 2016; Smith et al. 2020; Hibbins and Hahn 2021).

## 5.2 Population Differentiation and Speciation Genomics

What has become clear, as more species and more parts of their genomes are being studied, is that the process of speciation is much more complex and heterogeneous than previously considered. This perhaps justifies the plethora of different species concepts proposed, so much so that there are some researchers today who suggest that we should revive Darwin's view that questions the reality of species in nature (Mallet 2001; Mallet et al. 2007; Abbott et al. 2013). These findings are changing the paradigm that reproductive isolation is inexorable for divergence to occur, since we are finding different degrees of reproductive isolation, along both, the geographic distribution of the species, as well as its history (Kronforst et al. 2013;

Kronforst and Papa 2015; Zhang et al. 2016; Barrera-Guzmán et al. 2017). These findings weaken the biological species concept breadth (Mallet 2001) by suggesting that there may not be major discontinuities among species, contrary to what Mayr and other researchers have proposed, at least on their incept. Studies using genomic data that have found evidence of gene flow during speciation also suggest that some genes evolving under natural or sexual selection play key roles in lineage diversification (Lamichhaney et al. 2015; Meier et al. 2017b). Thus, when two populations diverge in the presence of gene flow, or when they experience secondary gene flow after their differentiation, it is the balance between gene flow, drift, and natural selection that will determine the outcome of the adaptive process, particularly in the case of divergent selection, but also with balancing selection. The presence of gene flow can facilitate or hinder the adaptive process, but also increase the likelihood that populations might go extinct.

The emergence of new genomic methodologies that have increased the understanding of the process of species differentiation and have demonstrated that interbreeding is much greater than previously anticipated, even in animals (Mallet 2007; Solís-Lemus et al. 2016; Jackson et al. 2017a; Schield et al. 2019; Congrains et al. 2021; Hirase and Yamasaki 2021; Banker et al. 2022; Suvorov et al. 2022), led to a reassessment of the role of underlying evolutionary forces in the process of species formation. With this, reproductive isolation does not seem to be a property that necessarily affects the entire genome at once, and may occur at different levels in different parts of the genome, or at different points in the species distribution. It is clear then that reproductive isolation does not seem to be an "all or nothing" attribute in which species would be completely isolated, or completely compatible. Furthermore, the suggestion that gene flow may even involve genes that are selectively important, such as, for example, genes that confer adaptations to different hosts (Powell et al. 2014), or to certain environmental cues, raises important questions regarding species' formation which impact core premises of different species concepts, such as the nature of reproductive isolation, the role of adaptation in speciation, or the very existence of well-defined evolutionary lineages. Different parts of the genome could have different histories, respond differently to selection pressures, and be differentially permeable to gene flow (Zhang et al. 2016). This would not only produce different phylogenetic relationships inferred from different parts of the genome, but also when inferred from individuals collected from different points of the species distribution, which might lead to incongruences at species identifications depending on the concept considered. Although this view that reproductive isolation would occur piecemeal had already been considered even at the time of Dobzhansky (1937) and Muller (1942), which is why researchers have looked for reproductive isolation genes since that time, the vast majority of researchers considered that speciation, represented by the isolation in the biological species concept, would not only happen by variation in a small number of genes. Rather, these would rapidly expand to coadapted gene complexes, and eventually be fixed, for example, in chromosomal inversions and balanced polymorphisms, according to Dobzhansky, so that, in general, there would be little introgression across the genome of different species (Rieseberg and Burke 2001).

## 5.3 Balance of Gene Flow and Drift Shape Population Structure

As previously mentioned, greater knowledge of population structure and its genetic framework across different groups has revealed a more widespread presence of past and current introgressions influencing the segregation of genetic variation and producing more complex phylogenetic patterns. It is possible that this pervasiveness of gene flow and introgression was not detected before due to the difficulty of identifying such processes using previously available genetic tools, which were based on evaluating allele frequency changes in one or a few loci, or even a set of several microsatellites, for which historical information is difficult, or even impossible, to reconstruct (Sirén et al. 2011; Putman and Carbone 2014).

When populations are structured, that is, when species are divided into populations that show differences in allele frequencies and in their segregation history, the allotment of variation within and between populations is controlled by yet another balance between two evolutionary forces, in this case between gene flow and genetic drift. Just as before, if this balance tilts towards gene flow, the species tends to remain as a single evolutionary unit that shares most genetic variation and its history across different individuals, and parts of their genomes. On the other hand, if the balance is controlled by drift, populations tend to become increasingly differentiated, but also tend to increase inbreeding within each of these subpopulations, since they have smaller sizes. This process may eventually lead to speciation, although it may take some time, or to their extinction, especially if population sizes remain small for a long time.

If we consider populations that have diverged recently, genetic drift may have had little time to operate, so these populations would still segregate ancient polymorphisms inherited from their ancestral populations. With time, drift and other evolutionary forces, like selection, would lead these polymorphisms to fixation, which would make different regions of the genome harbor different evolutionary histories, reflecting the stochastic process of polymorphism segregation and fixation. Because it is considered that most genetic variation that segregates in populations is neutral (Kimura 1991), this process is determined mostly by the balance of genetic drift and mutation rates, which is weighted by effective population sizes. Even though this process does not impact the fixation rate of neutral alleles, regardless of the population size, the way that this happens varies in populations with different sizes. Large populations segregate more polymorphisms, whereas small populations have a higher chance of allele fixation and loss. This way, larger populations have a higher chance of retaining ancestral polymorphisms, when compared to smaller populations. Furthermore, this process would be accelerated if there was natural selection favoring diversification in different populations, and would be reduced if there was balancing selection. The latter, in fact, can complicate the identification of segregating regions between different populations (Guerrero and Hahn 2017), because polymorphisms would still segregate even after complete lineage separation.

Although the fixation process of ancestral polymorphisms by drift is stochastic, it is still shaped by the underlying population history, which means that phylogenetic inferences from different regions of the genome (referred to as gene trees) may present distinct topologies from one another, and from the underlying species tree. This process would be exacerbated in recently diverged populations, especially if they have large effective sizes, and would be less important as time passes by, and in smaller populations (Carstens and Knowles 2007). The segregation of these ancestral polymorphisms may lead to potentially incongruent phylogenetic inferences derived from different markers. Therefore, there may be less confidence on phylogenetic inferences that are based on a small number of markers, since they may not adequately reveal the global evolutionary history. However, the phylogenetic information obtained from markers across the genome can be used to infer a single and more complete evolutionary history represented in a species tree (Pamilo and Nei 1988). To that effect, several methods have been proposed to infer the most likely underlying species tree, even in the presence of multiple gene trees that exhibit diverse topologies (Drummond and Rambaut 2007; Larget et al. 2010; Drummond et al. 2012; Corl and Ellegren 2013; Jones 2017).

Some approaches that are generically called species delimitation models seek to infer whether phylogenetic trees derived from different parts of the genome allow determining whether there is genetic structure in populations and restriction of gene flow processes (Sukumaran and Knowles 2017), which is often mistakenly taken to indicate that these identified units would be different species. Relatively independently evolving populations may be, perhaps, distinct species, especially using the phylogenetic species concept, but that inference should not be so straightforward. Strategies that are able to detect population substructure are getting more and more powerful, and even though they have a potential problem since most assume that eventual incongruities between gene trees and species trees would be exclusively due to gene drift stochasticity, some recently have incorporated the role of gene flow and introgression (Than et al. 2008; Jackson et al. 2017b; Solís-Lemus et al. 2017; Hibbins and Hahn 2021). The various methods that have been described, together with the improvement in strategies to produce genomic data, show that different taxa harbor different, but relevant, levels of hybridization throughout their history (Fontaine et al. 2015; Jones et al. 2018; Taylor et al. 2020; Congrains et al. 2021; Pfennig 2021) and there is significant introgression between different species. Just to give one example, a large study of 155 species of Drosophilid fruit flies separated them into nine main groups, eight of which showed evidence of introgression (Suvorov et al. 2022). It now appears that contrary to what was previously thought, phylogenies with no evidence of gene flow and introgression may be the exception rather than the rule (Edelman and Mallet 2021).

## 5.4 Population Structure and Secondary Contact

When two species exchange genetic material, we may anticipate three different outcomes: the exchange of genetic material between species can be so extensive that these species merge into a single one; species can experience introgression involving only parts of the genome, from a few genes to a broader array of genes; or they can produce hybrids that do not lead to introgression between the species, either because they are sterile or even inviable (Allendorf et al. 2001), and this may cause a permanent hybrid or tension zone where there is some overlapping of species' distributions. All of these possibilities have important consequences that will be considered here, but first we will investigate the conditions that help define which of these outcomes is more likely.

In general, we can define introgression as the persistence and fixation of some genes in a population when there is an allele exchange between two different genetic groups (Bohling 2016). Although henceforth we may refer to these groups as species, such a definition is not based on any particular species concept, but only reflects the indication that these populations have, in general, different histories and genetic variation. In some cases, introgression promotes phenotypic and genotypic variation, providing necessary tools for natural selection to act, favoring alleles that allow populations to adapt (Meier et al. 2017a). We refer to as adaptive introgression when, either through gene flow, hybridization, backcrossing, linkage or genetic recombination, selection provides the species or structured populations greater adaptation and range expansion. As the main limitation to the adaptive process is the availability of genetic variability, sharing a part of the genome of another species can allow the recovery of depleted genetic variation and offer positive responses to changes in the environment. This may enable faster adaptation to new environments by allowing the exploration of new niches, processes which are becoming very important in rapidly changing environments (Harrison and Larson 2014; Schumer et al. 2018; Suarez-Gonzalez et al. 2018; Taylor and Larson 2019; Jones et al. 2020; Pfennig 2021), for instance, as a result of anthropic impacts and global warming (Oziolor et al. 2019).

While passing through the sieve of natural selection, adaptive introgression may be in some instances an important evolutionary source of new genetic variation for species, especially if combined with the impact of pleiotropic and epistatic interactions with their own genetic background (Barton 2001; Rieseberg et al. 2003; Tigano and Friesen 2016; Suarez-Gonzalez et al. 2018). This is particularly the case because it is more likely that hybrids are not as adapted as their parental species to a specific niche, which would make selection more likely to be stronger on them (Mitchell et al. 2022). Considering this, introgression may drive ecological adaptation and have important conservation potential, because it may increase evolutionary resilience, but it is not likely to be universal across the species (Pfennig 2021), unless for a limited number of positively selected and additive genes for which the genetic architecture might remain similar across species. This would be because these processes more likely involve local populations and local interactions, which

may exhibit genetic variation distinct from other populations and show adaptation to local environmental. Several ecological interactions, such as coevolution and competition, are highly influenced by local variation, and this should be the case for the hybrid formation as well. Only very rarely species are panmictic across their distribution, which means that local hybrid formation occurs to a subset of the total genetic variation which may lead to biased introgression (Pfennig 2021), caused by an increase in heterospecific crosses locally (Taylor et al. 2015; Abbott 2017). This process influences patterns of adaptive introgression not only across space, but across time. For instance, there may be more introgression farther away from the center of the species distribution, or its ecological optimum, which may make certain individuals that may have specific backgrounds and traits, such as different behaviors, to be more prone to hybridizing (Rosenthal 2013; Taylor et al. 2015; Mitchell et al. 2022). That would also lead to a potentially biased hybridization across their genome, particularly if these traits are controlled by few loci, or if they are evolving as supergenes. One such example seems to be the case for hybrids produced from some species of *Helianthus* in Texas and Oklahoma that were more likely to replicate genomic combinations and some adaptive traits independently across their distribution (Mitchell et al. 2022). In that case, there is evidence that portions of the genomes of the species involved evolved as supergenes with large units of selection, limiting their recombination (Todesco et al. 2020).

## 5.5 Patterns of Introgression Across the Genome

In general, as different lineages diverge, genetic incompatibility tends to increase and, consequently, chances of hybridization and introgression tend to decrease. This is one of the very reasons why Darwin believed there are discontinuities in nature. This relationship varies greatly across different groups, though, not only because of intrinsic attributes of each group, but also to the non-additivity of the differentiation process, which is supposedly influenced by the evolution of epistatic interactions explained by the Bateson-Dobzhansky-Muller model (Orr and Turelli 2001). In this model, substitutions that may be neutral, or even beneficial, in the genetic background of one species, may show negative epistatic interactions in the context of the other species' genetic background. The number of independent differences between species tends to increase as they diverge, either by drift, new mutations, or driven by selection, driving the number of these negative interactions, which tend to increase quadratically, and potentially leading in time to what has been referred to as a snowball effect (Matute et al. 2010; Presgraves 2010). Species that diverged more recently in this model are more likely to share similar genetic backgrounds, making it more likely that they share the selective patterns of introgressed loci (Verta and Jones 2019), be them neutral, positively or negatively selected, which increases the chance that introgression may be more widespread across the genome. This introgression may increase total genetic variation across the genome and even though weakly deleterious non-dominant alleles may persist, it would tend to involve

neutral and weakly advantageous alleles, because the introgressed genes have already withstood the test of selection and survival on a different species, as opposed to new alleles that arose by mutation (Templeton 2021).

The levels of introgression and the position of introgressed genes across the genome should therefore be influenced by the time since divergence, but also by the selection forces acting on these introgressed genomic regions. As species divergence increases, therefore, it becomes less likely that variation across the genome would freely flow between these species, unless these introgressed genes are adaptive (Edmands 2007), which seems to be the case even for some iconic species such as the Darwinian Finches (Grant and Grant 2021). This process is hard to generalize, because the genetic architecture of adaptive genes varies greatly, from being determined by a single locus to a slew of several genes, in general with small effects, and that adaptation depends as well on the ecological and genetic (epistasis) environment where this variation is expressed, due to the very nature of the segregation of this variation. Nonetheless, as divergence increases, it makes it more likely that only very localized loci on the genome, with increasingly larger effect, would be involved in this introgression. That is the case for the introgression of insecticide resistance genes from the pest moth *Helicoverpa armigera* into South American populations of *Helicoverpa zea* (Cordeiro et al. 2020; Valencia-Montoya et al. 2020), though there are several examples of neutral or slightly deleterious alleles that still introgress across species boundaries (Wen and Nakhleh 2018; Cruzan et al. 2021; Nikolakis et al. 2022).

The existence of regions on the genome that are resistant to introgression may be one of the evolutionary drives for speciation that are more common for sympatric species, where biological barriers prevent the interbreeding of different species that coexist in the same geographic region. Recent data has shown that at least for some time these do not seem sufficient to completely limit introgression (Nosil et al. 2009; Wolf and Ellegren 2017). In this case, genome regions with higher recombination rates have a greater tendency to be permeable to introgression, because selection may be more effective on unlinked alleles and can recombine away from deleterious alleles (Roesti et al. 2012; Aeschbacher et al. 2017; Kim et al. 2018; Edelman and Mallet 2021). Rates of recombination in genomic regions should be viewed with caution, because they change across different species and chromosomes, but in general, they tend to be lower close to the centromere and higher in telomeres, though there are exceptions due to heterogeneous recombination in other regions of the chromosome. It is possible that deleterious loci are linked in regions of low recombination or harbor polymorphic, advantageous and adaptive alleles, and this impacts introgression, as well as reproductive isolation (Kirkpatrick and Barton 2006; Roesti et al. 2012; Charlesworth and Barton 2018). It is then considered that regions with high recombination rate would be more permeable to introgression (Schumer et al. 2018), whereas regions with less recombination would show higher differentiation across species, a pattern that was observed in different natural systems (Carneiro et al. 2014; Banker et al. 2022). There are many factors that can determine the evolutionary success or demise of different species, and it is

possible that introgression is one of the more important for its beneficial effects on species diversity and conservation.

## 5.6 Balance Between Gene Flow and Selection and Population Structure

Even though a long line of theoretical and empirical research has indicated that population viability is positively affected by genetic variation (Lande and Shannon 1996; Frankham and Ralls 1998; Hohenlohe et al. 2021; Kardos et al. 2021), it is still difficult to suggest a straight link between these variables (Doyle et al. 2015), though there are several indirect associations (Willi et al. 2022). Part of the problem stems from the inherent differences across different species, but some people claim that a larger problem is that we survey, in general, neutral genetic variation, and that is not what is used by natural selection in adaptation (Teixeira and Huber 2021). Because of this, there are several approaches that suggest that we should investigate and focus on functional genetic variation that directly impact fitness, rather than standard methods that aim to preserve genome-wide genetic variation as a surrogate for the former (Kyriazis et al. 2021). Though knowledge of variation impacting fitness-related traits for endangered species would clearly benefit their management, for several species and scenarios we may not know which attributes and traits would be selectively important. When we do, in general, these attributes tend to be species-specific and have a complex genetic architecture which makes their identification costly and time-consuming. This is time and resources that in general we cannot afford to spend. Furthermore, nowadays we have been able to survey larger and larger parts of the genome at increasingly easier and cheaper costs, which facilitates the standard use of the principles of population genetics and evolution to bringing important information not only for conservation, but also to shed light on parts of the genome involved (Hoelzel et al. 2019; Mable 2019). This may provide important pedigree and kinship information that can even be used to provide demographic information that is invaluable for *in situ* as well as for *ex situ* management of endangered populations (Galla et al. 2022).

Furthermore, the investigation of parts of the genome that introgress between different species, and their potential impact on adaptation may provide important information to help solve this riddle, because it would help to identify portions of the genome involved with adaptation to specific ecological attributes, which could have biased introgression across their genomes (Pfennig 2021). For instance, when there is introgression between different species, we may investigate whether this variation may facilitate the recipient species to adapt to new or changing environments (Taylor et al. 2020; Blanco-Pastor 2022), which would make them expand their ranges and occupy new niches and even prevent their local extinction (Pfennig et al. 2016; Suarez-Gonzalez et al. 2018; Oziolor et al. 2019; Hodel et al. 2022; Mitchell et al. 2022). This strategy has been used, for instance, in big cats, to reveal

not only that there is a great deal of introgression across different species, but also which genes introgressed, leading to the identification of some coat color and vision genes that may have been important in their differentiation (Figueiro et al. 2017; Ramirez et al. 2022).

As we discussed earlier, the likelihood of introgression, and its potential evolutionary impact, is modulated by population size, divergence time, as well as isolation from other populations. In general, large populations may withstand more readily the influx of deleterious, or mildly deleterious, alleles. In large populations, individuals bearing said alleles will have lower fitness which should not be much affected by epistasis, more likely than not causing this genetic load to be more readily eliminated, though recessive deleterious alleles may accumulate, unless these populations are experiencing inbreeding. On the other hand, introgression from large to small populations may be important because they may bring new genetic variation to the latter and counteract impact of drift on inbreeding depression and loss of genetic variation, especially on very small or isolated populations.

Strategies that foster gene flow to recover genetic variation in small or isolated populations have been commonly proposed, but generally that approach has been restricted to closely related populations, because it is suggested that the risk of outbreeding depression in some instances outweighs its potential advantages (Allendorf et al. 2001; Edmands 2007; Laikre 2010). Some of these studies, as well as breeding programs for endangered species in captivity, commonly use molecular marker technology on neutral genetic markers to evaluate kinship as a management plan to increase diversity in a population and reduce extinction risk (Pacioni et al. 2018). These management and recovery programs require several processing steps, such as reference genomes from the species of interest, as well as from other phylogenetically close species, like what was done with the black stilt and other related Charadriiformes birds (Galla et al. 2019). The problem is that for several endangered species, there may not be alternative source populations to explore, so it has been suggested that other populations should also be considered (Jahner et al. 2019).

Some recently have suggested that limiting gene flow only to intraspecific populations may be too conservative (Bohling 2016), especially because several cases have been found in nature lately with remarkable success (Schumer et al. 2018; Zecherle et al. 2021; Banker et al. 2022). Even though introgression may produce individuals with fewer deleterious alleles than what was originally present in these small, potentially dwindling, populations, it should be noted that this should be carefully monitored. This screening is important not only because of the risk of outbreeding depression, but also because large populations may have accumulated higher number of recessive or partially recessive deleterious alleles, which, because of their size, remain in very low frequencies. If transferred to smaller populations, these lethal and small effect recessive alleles are more likely to increase in frequency and be exposed by homozygosity leading to inbreeding depression. This is thought to have been the case that involved the Isle Royale wolf population, which crashed after an introduction that led to several offspring being genetically related and inbred (Kyriazis et al. 2021). On the other hand, new alleles in a different genetic background may generate a new combination of alleles hitherto unavailable

which could enable populations to cross fitness valleys, such as those presented in different niches. The transference of wing coloration genes involved with mimicry across different species providing protection against predation in several species of *Heliconius* butterflies is a great example of how new genetic variation may enable such crosses and foster ecological divergence (Zhang et al. 2016; Kozak et al. 2021; Meier et al. 2021).

Though we emphasize in this review the positive impact of introgression as a process that may facilitate adaptation in some species groups, this process can have negative consequences for biodiversity conservation and lead to extinction of many populations and species, especially among those which are more rare and less locally frequent (Rhymer and Simberloff 1996; Allendorf et al. 2001; Crispo et al. 2011). In this case, populations that experience non-adaptive introgression may suffer inbreeding depression or loss of habitat for hybrid populations (Allendorf et al. 2001). Even ancient introgression traits, as observed in some lemur species (*Cheirogaleus sp. cf. medius, Cheirogaleus major, Cheirogaleus crossleyi* and *Cheirogaleus sibreei*) may have had their genetic diversity reduced by the persistence of introgressed alleles that negatively influenced adaptation to some environments (Williams et al. 2020). In addition, inbreeding depression can also be a threat to large populations even with considerable genetic diversity, and using populations with high levels of diversity may not be effective in recovering small populations (Robinson et al. 2019; Kyriazis et al. 2021), as indicated in the case of the Isle Royale wolves. Since the goal may be to preserve phenotypes or genotypes for evolutionary or ecological purposes, decisions concerning environmental management involve political aspects that can be complex and time-consuming (Coates et al. 2015; vonHoldt et al. 2018; Waples and Lindley 2018). Perhaps for this reason, decision-making on the conservation and preservation of ecosystems must take into account data that evaluate the intrinsic status of each species with the aim of preserving their environments as well. One possibility would be to verify the status of so-called "umbrella" species, which historically need large territories of protection and with them, protect, by territorial extrapolation, a large part of the habitat and other species (Noss 1990).

## 5.7 Anthropogenic Impact on Introgression

Historical patterns of gene flow have been altered by anthropogenic effects that have brought about great ecological and environmental changes (Thomas et al. 2004). Factors such as the introduction of new species, non-natural dispersal, landscape fragmentation and habitat modification have contributed to changing species' distribution and have led to hybridization, introgression, and loss of intraspecific variation (Rhymer and Simberloff 1996). In addition, even evolutionarily well-established local species may now be subject to competition for niches and resources with invasive species, threatening biodiversity (Seehausen et al. 2008, Crispo et al. 2011). One of the classic examples is the cutthroat trout (*Oncorhynchus clarki lewisi*)

which lost its commercial value after undergoing hybridization with the introduced species rainbow trout (*Oncorhynchus mykiss*), in addition to the demographic threat to the parental population (Allendorf and Leary 1988). Another poignant effect of introgression is what has been referred to as "speciation reversal", characterized by the loss of phenotypic distinction between species that can affect reproductive processes and recognition of the sexual partner (Kearns et al. 2018), in addition to affecting ecological and genetic networks leading to serious demographic declines and local extinction (Vonlanthen et al. 2012; Kearns et al. 2018).

Some of the most relevant anthropogenic impacts, however, relate to global warming. Climate change may effect several changes across different taxa, for example, to their geographic distribution and density, which may promote important ecological processes in communities and ecosystems (Franks and Hoffmann 2012; Shaw and Etterson 2012). The most drastic climate change impacts may lead to the reduction or expansion of species' habitat, and impact migration patterns. These processes may change hybridization likelihood between species, change reproduction time, and ultimately affect inbreeding and introgression of genes that may not be adaptive (Vallejo-Marín and Hiscock 2016; Quilodrán et al. 2020). In rapidly changing environments, the species' ability to quickly adapt becomes a limiting factor, but it can be reinforced when adaptive introgression is involved in the process, which can facilitate the dispersal of gene combinations that show greater plasticity (Suarez-Gonzalez et al. 2018).

The ability to adapt to new environmental conditions and the speed with which this process has occurred makes it more likely that selection is more efficient on existing genetic variation, rather than on new mutations (Orr and Unckless 2014). Studies on the effects of climate change on introgression are still incipient, but already suggests it to be relevant. Such is the case, for example, of salt tolerance in the hybrid species *Helianthus paradoxus* (Welch and Rieseberg 2002), cold tolerance in trees conifers (*Picea sitchensis* and *Picea glauca*) (Hamilton et al. 2013), formation of butterfly hybrids between *Lycaeides anna* and *Lycaeides melissa*, which seem to be more adaptive to new environments (Nice et al. 2013), thermal tolerance in the copepod *Tigriopus californicus* (Pereira et al. 2014) and in species of corals of the genus *Acropora* (Mao et al. 2018). In all these examples, the hybrids show peaks of adaptive fitness, revealing that interspecific recombination increases genetic diversity to remain in extreme conditions. Due to increasingly accelerated climate changes, the proportional role of introgression may increase, since it may allow for the combination and dispersion of new genes and genotypes more conducive to adaptation in extreme conditions (Kremer et al. 2012).

In contrast, population fragmentation and climate changes potentially threaten gene flow that occurs between local populations (Aitken et al. 2008). This occurs especially in small populations, whose gene pools do not favor rapid adaptations to environmental changes, have limited migration ability, and/or have experienced the emergence of geographic barriers that prevent migration (Aitken et al. 2008; Wilczek et al. 2014). During population expansions, formation of new hybrid zones can facilitate introgression (Buggs 2007), which can be asymmetric for a wide range of demographic conditions, including when invasive species have a much

higher density than local species or when there is competition between the two species leading to the extinction of local species (Currat and Excoffier 2004; Currat et al. 2008). Eventually, introgression is expected to be greater on invasive species than on already established species, which may cause introgressed genes to increase in frequency in invaders and may impact the persistence of natural species (Klopfstein et al. 2006; Quilodrán et al. 2020). Furthermore, species whose rates of genetic change are lower than rates of climate change may experience a decline in fitness over time, leading to local extinctions (Atkins and Travis 2010; Wang et al. 2010). In the face of accelerated climate change, organisms need to respond quickly through migration processes, changes in genetic parameters and through phenotypic plasticity which facilitates the adaptive process (Hamilton and Miller 2016). Morphological changes such as in Tawny owls (*Strix aluco*) (Karell et al. 2011), body mass and wing span change in Amazonian (Jirinec et al. 2021) and in Old World (Dubiner and Meiri 2022) birds, and thermal selection in flat periwinkle snails (*Littorina obtusata*) (Phifer-Rixey et al. 2008) are a few examples of increased global temperature consequences. Populations whose responses are not fast enough to these climate changes are at greater risk of reduced fitness and extinction (Aitken et al. 2008).

Conservation programs have been using genetic rescue of threatened species using introgressive processes, considering adaptation timelines and how much of the evolutionary history can be rescued from extinct subspecies through backcrossing (Hamilton and Miller 2016). A still little-known concept of "desintrogression" (Amador et al. 2011) applies to this, in which through programmed backcrossing, lost lineages and genotypic units are recovered, a strategy used to recover extinct species of Galápagos tortoises (*Chelonoidus spp.*) (Garrick et al. 2012; Edwards et al. 2013; Miller et al. 2017) and Przewalski's horse (*Equus przewalskii*) (Amador et al. 2011). Although it is a valid strategy for conservation studies, especially to recover the original gene pool of threatened species, these may suffer from the potential effects of outbreeding and inbreeding depression. At the same time, this methodology requires a high level of knowledge about the genome of the original species and is difficult to use in wild species and small populations, having a higher chance of success when the exogenous ancestry is recent (Amador et al. 2013; Bech et al. 2022; vonHoldt et al. 2022).

## 5.8 Differentiation with Gene Flow in the Neotropics

The processes mentioned above suggest that species that diverged recently are more likely to introgress, showing different levels of phylogenetic congruence among different traits. This is either due to the segregation of ancestral polymorphisms, or because differential gene introgression may lead to different phylogenetic relationships in regions across the genome, especially because some of these genes are driven by selection, and reflect the process of a rapid expansion of an adaptation (Feder et al. 2005), even across species boundaries (Edelman et al. 2019; Nosil et al.

2021). This problem is especially complex in the Neotropics, since several species have gone through distinct cycles of habitat expansions and contractions throughout their history and differentiation (Avise et al. 1998; Knowles 2001; de Brito et al. 2002) and may be separated by fluctuating boundaries, such as meandering rivers (Leite and Rogers 2013; Naka and Brumfield 2018). As a consequence, several species have gone through periods in which some populations were temporarily isolated and interacted locally with other species. When these boundaries changed, either by climate change, or change in river barriers, the species with which they interact change, as well as their environmental conditions. In situations where ecological interactions are intense and important, as in the case of predation, parasitism and competition, this can foster specific lineages' differentiation in different parts of the distribution and promote differential introgression across their distribution. These interspecies adaptations, in a manner similar to what happens to interactions between different sexual partners, reinforce local solutions, because of their complex genetic interactions, which creates the potential for epistasis and non-additive processes (Templeton 2021).

Several specializations to different host plants and coevolutionary patterns have been reported for different species in the fruit fly family Tephritidae (Zucchi 1988; Aluja and Norrbom 2000), and most of them relate to genera that differentiate in the Neotropics. Most differentiation in Tephritidae is not very recent, but has been shown to be very complex, which is the case of the most diverse genus in the family for the New World, *Anastrepha*. This genus is widely distributed in South and Central America and harbors some of the most important pest species in the Americas (Zucchi 2000; Norrbom et al. 2012). Species in the genus are associated with a wide array of plant species, several that show species-specific associations, whereas others use a wide array of different host plants. An extensive analysis of *Anastrepha* using several nuclear genes revealed a pattern of rapid and recent divergence, with several short branches still unresolved, particularly for species that diverged recently (Mengual et al. 2017). Molecular data derived from genomic and transcriptomic data of different *Anastrepha* species with different degrees of phylogenetic relatedness revealed great phylogenetic incongruity across different parts of the genome, though, in general, their joint analysis provided great support for diverse phylogenetic relationships between species groups and species allotted to these groups (Congrains et al. 2021; Congrains et al. Submitted).

Perhaps as relevant as the phylogenetic relationships inferred by several studies (Vaníčková et al. 2015; Dias et al. 2016; Mengual et al. 2017; Congrains et al. Submitted) are the findings that suggest that there has been considerable gene flow among *Anastrepha* species across their differentiation (Scally et al. 2016; Díaz et al. 2018; Congrains et al. 2021). This gene flow seems to have been an integral part of their differentiation process, since there is evidence that it happened even between species that diverged a long time ago, but even more so for closely related species (Congrains et al. Submitted). This finding has important consequences, depending on which genes are being exchanged between species. Even if the introgressing genes are not involved in the adaptive process, they can at least complicate the process of identifying species-specific markers. Parts of the genome that are not

involved in differentiation may be shared, depending on the degree of reproductive isolation. This may help explain why certain markers differ between populations and do not serve as common markers for all populations of the species, as observed (Thawornwattana et al. 2022; Congrains et al. Submitted).

Considering that sexual selection and host adaptation are important for Tephritidae differentiation and these processes may be involved with behavioral and genetic changes, it is possible that these changes precede observable morphological changes which may help explain the existence of cryptic species in lineages that have diverged recently, as may be the case for species in the *fraterculus* complex (Dias et al. 2016). The existence of cryptic species can say something about the process of species differentiation, as it may indicate that morphological characters (or easily identifiable morphological traits) and genes that are being important for their differentiation are evolving at a different pace (Struck et al. 2018), thus complicating the correct species identification, even by specialists.

In turn, if gene flow encompasses parts of the genome that are involved with the adaptive or reproductive process, these can lead to the production of inviable or sterile hybrids, but can also lead to introgression in distinct lineages of alleles with adaptations to a given environmental context, as an adaptation to a new host. This may result in specific adaptations present in a small set of individuals being shared with a large portion of the population if it brings adaptive advantages such as, for example, the ability to exploit a new host plant. Such a process can facilitate the generation and expansion of polyphagous species and can play an important role in the adaptive process akin to what has happened in the introgression of Müllerian mimicry in *Heliconius* across species boundaries (Martin et al. 2013; Moest et al. 2020), which also, incidentally, has important consequences for pest control, because it may foster the emergence of superpests, but that is not our focus here.

It may appear as a stretch that knowledge from pest species, not exactly the ones that come to mind when one thinks about conservation, could contribute to the understanding of species evolution and conservation in the Neotropics. We claim though that the combination of recent divergence, potentially incomplete reproductive isolation, adaptations to different hosts, intense natural and sexual selection, and anthropogenic impacts make *Anastrepha* an appropriate model for the study of the incipient stages of speciation and its consequences on differentiation and adaptation. This combination of factors may actually be very common for neotropical taxa, and may be one of the reasons why an abundance of cryptic species in the tropics has been observed for several different groups (Vieites et al. 2009; Funk et al. 2012; Gill et al. 2016; Huntley et al. 2019), so the study of *Anastrepha* may help understand general patterns of diversity in the Neotropics.

The Neotropics are characterized by great diversity such as in climate, soil, and landscape, that creates a wide diversity of ecosystems (Mittermeier 1986) and have experienced many climatic oscillations since the Pleistocene (Vasconcellos and Colli 2009). These Pleistocene climatic changes have even been used by a controversial theory, called ‘forest refugia,’ to propose that forest contractions and expansions produced historically preserved areas that retained high diversity. These historical changes would favor reproductive isolation among species present in

different refugia, and hybridization between them subsequently, if the isolation was not complete, which would reflect on the genetic structure of the species (Haffer 1969; Vanzolini and Williams 1981). Regardless of the actual existence of these refugia and high diversity areas (Bush 1994; Colinvaux 1996), it is likely that forest waxing and waning during climate changes affected species distribution and might have fostered the appearance of hybrid zones (Pulido-Santacruz et al. 2018; Pulido-Santacruz et al. 2020) or allowed for short periods of introgression among species during temporary forest corridors in the Amazon region (Batalha-Filho et al. 2013). The Amazon region, in particular, have several features that may foster introgression, such as its extension and limited barriers to prevent gene flow to species from several different ecosystems (Dexter et al. 2017). Some of the most important barriers seem to be historical, such as arches (Silva et al. 2018), rather than rivers, that promote the diversification of bird species (Naka and Brumfield 2018), but characteristically have meandering courses that could facilitate introgression. Furthermore, the profusion of environments and ecosystems produces more complex 'landscape mosaics' that facilitates the emergence of hybrid zones (Rieseberg et al. 1999).

The complex landscape and history of the Neotropics, with these weak or semi-permeable geographic barriers, can promote the process of hybridization and introgression, especially if the new genetic information provides abilities to increase geographic distribution by adapting to new habitats which might also create opportunities for allopatric speciation and adaptive radiation (Marques et al. 2019). The larger number of more closely related species that are found close to one another in the Neotropics may enhance these semi permeable borders that facilitate introgression, in a process that could be the genetic equivalent of fluctuating ecological borders. As in species complexes, cryptic species can also arise from hybrid lineages that have gone through introgression processes, and this can be facilitated by the landscape-scale structure in neotropical biomes that we discussed above (Hebert et al. 2004; Elmer et al. 2007), as demonstrated, for instance, in cryptic species of *Epidendrum* orchids (Vega et al. 2013), introgression and hybridization in neotropical frogs (Sequeira et al. 2011; Vallinoto et al. 2017), and in neotropical species of fruit flies (Congrains et al. 2021). Despite its great importance, and vast distribution, we still know very little of the impact of introgression and hybridization on neotropical diversity and differentiation, and even pest species may bring important insights to help understand its evolution.

## 5.9 Final Remarks

Species recovery and conservation programs should not consider only genetic features as the sole formula to save species from extinction. Measurements of genetic diversity, for example, should not be the only way to quantify and qualify the health status of a population or species for maintaining it in its habitat (Spielman et al. 2004; Teixeira and Huber 2021). Even though introgression can have very negative impact on conservation efforts, we cannot disregard that it can also be beneficial to

natural populations, especially when it plays an important role in adaptive evolution, as exemplified above. In some cases, it provides adaptive advantages to species experiencing inbreeding depression that can be reversed, in addition to producing new species and lineages differentially adapted to the environment, especially because it provides means for faster environmental adaptation (Edelman and Mallet 2021). The impact of species introgression, as well as other evolutionary processes, is still not very well explored in neotropical species, though it is well documented in temperate regions. In part because of that, recently diverged species, such as species in the genus *Anastrepha* discussed here, can be important biological models to help elucidate complex evolutionary histories and provide more comprehensive models for neotropical species.

One of the biggest challenges brought by the recognition of the historical impact of introgression across several different evolutionary lineages, and its pervasiveness, is how this impacts the definition of what constitutes a species (Mallet 2008; Abbott et al. 2013; Shapiro et al. 2016). We started this chapter describing several different views on species concepts and indicating the importance of "species" in different fields of knowledge, but would like to finish by emphasizing that introgression has great consequence on conservation. Important policy aspects, and how this has been written into laws and protocols, use species definitions that make it difficult to recognize, or limit, the value of hybrids, even going to the point of removing the protective conservational status of some. This has been actually promoted by several conservation biologists through time, considering that in several instances, hybrids are evolutionary dead-ends and would endanger the survival of the original species (Rhymer and Simberloff 1996; Levin 2002), though this has been shown to be a rare cause of species extinction (Draper et al. 2021). If we consider that it seems as if most species in nature have experienced at least some level of introgression throughout their history, and this is more likely to increase with anthropogenic changes that are currently happening, maybe a reconsideration of these conclusions would be warranted in light of the idea proposed by Darwin that species would be somewhat arbitrary constructs that we use to characterize and define biodiversity. As we increase our knowledge of species relationships, and their historical contingencies, this knowledge should help us better establish criteria to preserve different groups of populations, individuals, or lineages, that would ensure their long-term survival, as well as of their environment.

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# Part III
# Assessing and Managing Populations

# Chapter 6
# Phylogeography for Neotropical Species Conservation: Lineages Through Time and Space

**Carolina B. Machado and Manolo F. Perez**

## 6.1 Introduction

Phylogeography is a term that was coined in 1987 by Avise and collaborators to refer to a discipline that investigates the principles and processes that explain the spatial distribution of genealogical lineages, especially those found within and among closely related species (Avise et al. 1987). In other words, space and time are the main components for understanding the patterns in the genetic structures of populations, and distributions of genetic diversity (Fig. 6.1). Phylogeographical studies involve an interaction between demographic aspects and the dynamics of physical processes (geological or climatic) through history, specifically focused on gene flow, historical changes in demography, colonization routes, and species boundaries (Avendaño et al. 2017; Buzatti et al. 2018; Mondin et al. 2018; Miranda et al. 2021). Because of that, the discipline was originally considered a bridge between population genetics (microevolutionary processes) and phylogenetic systematics (macroevolutionary patterns) (detailed in Sect. 6.3). Currently, this

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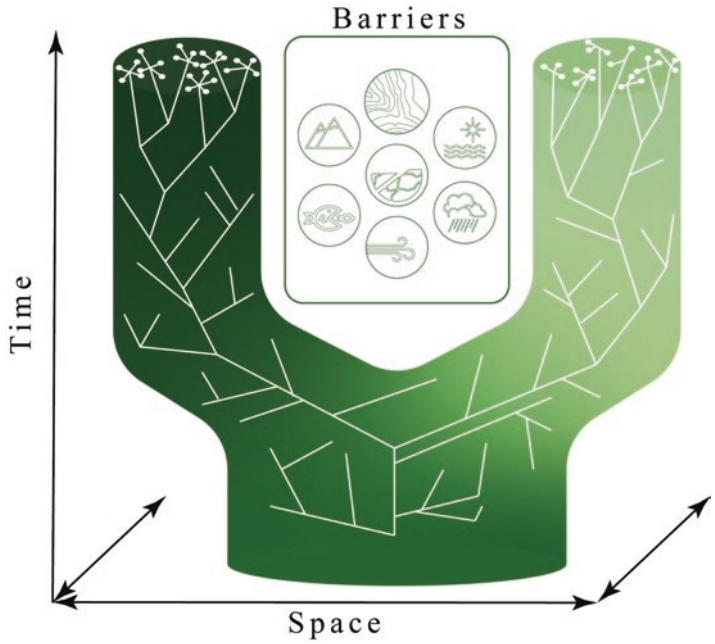


**Fig. 6.1** Hypothetical gene genealogy showing an ancestral population splitting into two descendant populations that evolve independently in the presence of abiotic (topographical, oceanic, tectonic, climatic, environmental, etc.) and biotic barrier (ecological interactions and behavior). This representation is based on Avise (2000). Image created by Helena Diaz

perspective has been expanded, and phylogeography is considered one of the most integrative disciplines in all of biology (Hickerson et al. 2010; Marske 2016).

Since its origins, phylogeography has been closely associated with analyses based on mitochondrial markers (Avise 2000), due to their lack of recombination, putative neutrality, extensive intraspecific variation, and smaller effective population size when compared to nuclear markers. This latter feature is important because it results in a shorter expected time for two lineages to reach reciprocal monophyly. All these characteristics illustrate the mtDNA's usefulness as a microevolutionary genetic marker. However, performing inferences based on a single locus is subject to stochastic processes which, in many cases, have been proven to be insufficient for the acquisition of parameter estimates; also, a single locus may be unrepresentative compared with other genomic regions, which explains the dramatic increase in the use of multi-locus data (Knowles 2009; Hickerson et al. 2010). A major criticism of sequence-based markers from the PCR era has been the scarcity of such markers and their low polymorphism, in addition to the trade-off between the number of individuals or populations sampled and the number of loci used due to high sequencing prices (Edwards et al. 2015). The advent of high-throughput sequencing (HTS) technologies showed a quantitative and qualitative gain in the field, improving the accuracy of statistical analyses and resulting in narrower confidence intervals in parameter estimates such as divergence times, effective population size, and migration rates between populations (Smith et al. 2014). It also facilitates the identification of loci under selection and the correlation of their alleles with environmental variables (Garrick et al. 2015).

Over 35 years of phylogeographic investigation, researchers have addressed relevant questions in the fields of evolutionary biology, ecology and conservation. Individual and comparative case studies have provided invaluable insights into landscape evolution (Capurucho et al. 2013), speciation processes (Machado et al. 2018), adaptive radiation (López-Fernández et al. 2012), biodiversity research and taxonomy (Soares et al. 2019), paleoecology (Vila et al. 2011), paleoclimatology (Carnaval et al. 2009; Mascarenhas et al. 2019), and conservation biology (Guillory et al. 2019). Concerning the latter, phylogeographic concepts have been increasingly applied to the biological conservation of species (Beheregaray 2008) because they provide a framework for developing insightful guidance for biodiversity protection policies, at multiple scales, aimed at preserving historical dimensions of biodiversity and their underlying evolutionary processes. Specifically, there are three ways in which phylogeographic studies can provide information for the conservation of biodiversity: (i) documentation of fine-scale genetic diversity of species/population in a region; (ii) identification of genetic lineages for the maintenance of evolutionary potential within species; and (iii) clarification of the historical processes driving changes in species range and abundance (Macqueen 2012).

In this chapter, we briefly explore the main mechanisms driving diversification in the Neotropics at different scales (from intraspecific to community levels) and the robust methods to improve the understanding of current genetic patterns. Beyond that, we also discuss the potential benefits and application of phylogeographic studies in conservation analyses.

## 6.2 The Origins and Drivers of Neotropical Diversity

The neotropical region comprises the tropics of the New World, extending from central Mexico to southern South America (Morrone 2017). The region is characterized by a diversity of biomes, each with a particular history of landscape and biotic evolution (Hughes et al. 2013; Fig. 6.2a). It is recognized as one of the world's most important biodiversity-rich regions, harboring approximately one-third of all global species (Raven et al. 2020; Fig. 6.2b–d). In addition to the high number of species, this region is also characterized by its high degree of endemism and threat, meaning that several of its biomes are global priority hotspots for conservation (Myers et al. 2000). Paradoxically, this region is one of the least studied (Beheregaray 2008; Turchetto-Zolet et al. 2013). Even with a significant increase in the number of phylogeographic studies, the mechanisms accounting for the origin, distribution and maintenance of biodiversity in the region remain elusive (Meseguer et al. 2021).

Due to the great complexity of phylogeographic patterns in the neotropical region, there are multiple explanations applicable to any biome or biological group. Studies point out that different environmental drivers acting at multiple spatiotemporal scales have led to the biotic and abiotic interactions that shaped the current biodiversity (Rull and Carnaval 2020). Specifically, geological and climatic historical events have played important roles in contemporary genetic diversity patterns

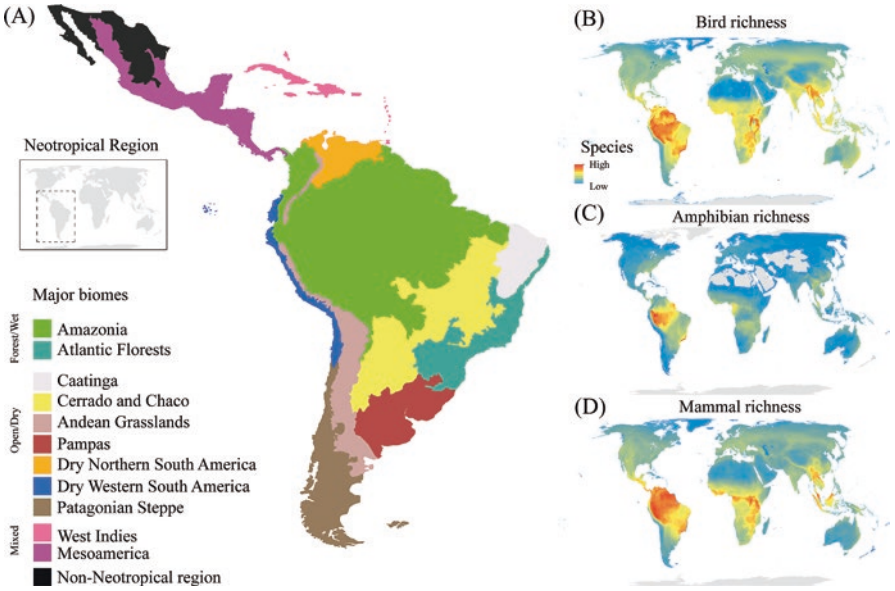


**Fig. 6.2** Map of the neotropical region (**A**) and its species richness (**B**-**D**). (**A**) Approximate natural distribution of major terrestrial neotropical biomes (adapted from Antonelli et al. (2018), Morrone (2014), and Olson et al. (2001)). Latitudinal distribution of species richness among birds (**B**), amphibians (**C**) and mammals (**D**) across the world based on Jenkins et al. (2013). Image created by Carolina Machado with the software ArcGIS v10.2 (https://www.arcgis.com). Free vector data from Löwenberg-Neto (2014) and BiodiversityMapping.org (https://biodiversitymapping.org/; Accessed September 10, 2022)

through demographic oscillation, population divergence, and species diversity in whole communities. Among the main historical events, we highlight (i) the West Gondwana breakup, (ii) the Andes uplift, (iii) the emergence of the Panama Isthmus, (iv) the marine incursions, and (v) Pleistocene climatic changes (Fig. 6.3). Because the phylogeographic patterns are better understood in light of these historical events, we will discuss them briefly below.

The West Gondwana started its breakup 130 Ma (*Megaannun*, time unit equivalent to a million years)[1] and culminated 90 Ma ago. This process resulted in the separation of Africa and South America, and in the opening of the Atlantic Ocean. Fossil records and phylogeographic studies show important diversification processes during this period in different biological groups, for example, mammals (Reguero and Goin 2021), birds (Feduccia 2003), amphibians (Frazão et al. 2015), fish (Cioffi et al. 2019; for details, see Chap. 21) and angiosperms (McLoughlin 2001).

The Andean orogenesis occurred as a consequence of the tectonic compression between the Nazca and South American plates. The process started 90 Ma ago and

[1] The geologic time scale used in this paper follows the International Commission on Stratigraphy (Cohen et al. 2013).

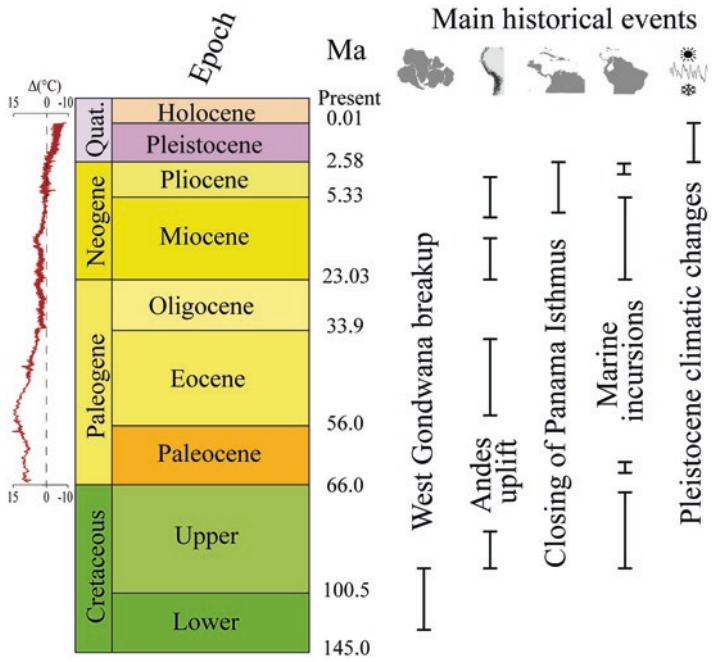


**Fig. 6.3** General chronology of main historical events responsible for biological diversification in the neotropical region (Lundberg et al. 1998; Albert and Reis 2011). Chronostratigraphic information is based on Cohen et al. (2013) and the left inset shows temperature fluctuations during these periods when compared to current conditions (Δ, in units of °C) according to Zachos et al. (2008) and Burke et al. (2018). (Adapted from Rull 2020)

throughout its history has undergone multiple phases of tectonic uplift which led to dramatic changes in drainage and climatic patterns, mainly in the northwest of South America (Hoorn and Wesselingh 2010). Due to the resulting landscape reshaping, the Andean uplift was considered a paramount diversification driver in this region (Hoorn et al. 2010). During the final stage of reorganization of the Coco, the Caribbean and South American plates culminated in the emergence of the Panama Isthmus during the Pliocene (~7 to 2.5 Ma), which led to the Great American Biotic Interchange, a migration wave of fauna and flora between the North and South American continents (Marshall et al. 1982). Marine incursions resulting from rises in sea levels inundated the continental land with oceanic waters. The Neotropics, specifically South America, experienced several marine incursion episodes since the West Gondwana breakup, resulting in the extinction and vicariance of terrestrial and freshwater populations mainly during the Miocene (Bloom and Lovejoy 2011).

Finally, climatic changes that happened during the Pleistocene were characterized by successive cycles of glacial and interglacial events, associated with abrupt and drastic changes in temperature and precipitation (Gates 1993). Although ice sheets did not cover the Neotropics, as they did in the northern hemisphere, there is strong evidence that these global climatic changes had profound effects on biomes

in the neotropical region – and promoted demographic oscillations, changes in gene flow patterns, range shifts and population divergences that have been decisive for the shaping of phylogeographic patterns (Thom et al. 2020).

Several hypotheses about the expected patterns of genetic architecture and population divergence models can be designed based on the geological and climatic history outlined above. These landscape transformations likely promoted large-scale vicariance, dispersal and extinction events that affected the whole of communities associated with the Neotropics, and therefore have a key role in the hypotheses discussed for the region (Posadas et al. 2006; Rull 2011). In the next section, we will briefly review the main hypotheses on neotropical diversification in terrestrial and freshwater organisms, as well as their predictions concerning phylogenetic and phylogeographic studies.

## *6.2.1 Terrestrial Organisms*

### 6.2.1.1 Refuge Hypothesis

Initially proposed by Haffer (1969) to explain the distribution patterns of birds in the Amazon Forest, the refuge hypothesis is based on climate-biome dynamics. This hypothesis is frequently associated with the Pleistocene period, in which glacial and interglacial events could generate a model of population dynamics that reflects habitat retraction and expansion (Connor 1986). Considered the most popular diversification theory, this model invokes vicariance as the main divergence process in tropical rainforests, since climate oscillations led to the fragmentation of forest patches during the cool and dry glacial episodes, which favored the expansion of savannas (Haffer 1969). Thus, the repeated isolation and re-expansion of rainforests have, together, been the major drivers of allopatric speciation. When we consider an intraspecific scale, the expected genetic signatures are high genetic diversity in populations occurring in stable areas (named refuges), and low genetic diversity in populations located in regions colonized after climatic shifts during the warm periods (unstable areas). Secondary contact and demographic changes are events that are also observed in this hypothesis (Hewitt 1996).

Many studies have identified similar patterns in many organisms (Mondin et al. 2018; Mascarenhas et al. 2019; Hamdan et al. 2020; Carvalho et al. 2021). Carnaval et al. (2009) proposed an emblematic study by implementing coalescent-based estimates, paleodistribution modeling analyses, and alternative demographic scenarios based on climatic models to assess the response of frog populations in the Atlantic Forest to the climatic changes of the Pleistocene. The authors detected that populations from the north/central Atlantic Forest had genetic signatures consistent with predictions made for stable areas, whereas the southern Atlantic Forest was climatically unstable. These results were important to establish new conservation priorities focusing on the previously neglected Atlantic Forest central corridor.

It is important to outline that the refuge hypothesis has also been used to explain diversification patterns in taxa associated with dry vegetation habitats. These habitats are in the Caatinga, Cerrado and Chaco biomes, which are distributed in a

southwest-northeast diagonal (termed the "dry diagonal") in the Neotropics. In this case, the theory predicts an expansion during colder and drier glacial periods, and a retraction to refugia during interglacial cycles. Based on the distribution patterns of several taxa associated with the seasonally dry tropical forests, which are nowadays fragmented, a more widespread distribution throughout the dry diagonal was proposed (Pleistocene arc hypothesis – Prado and Gibbs 1993). Phylogeographic studies on species associated with dry habitats show that their responses to climatic changes in the Pleistocene varied broadly (Turchetto-Zolet et al. 2013). Still, a somewhat general trend pointed towards range retraction during glacial periods for species inhabiting the savanna, while dry forests contained more cases of range expansion (Collevatti et al. 2020).

#### 6.2.1.2 Disturbance-Vicariance Hypothesis

Proposed by Bush (1994) to explain Amazon biodiversity, this hypothesis is also based on climate change during the Pleistocene (but not exclusive to this period; Lötters et al. 2010). However, unlike the refuge theory, it suggests that the temperature fluctuations would trigger the diversification processes rather than the aridification and physical fragmentation of forested regions. According to the author, warm and cool cycles affect vertical distribution ranges and fragment populations into suitable rainforest patches. Cool-adapted organisms, for example, would be restricted to montane habitats during interglacial periods, where they accumulate differentiation. This process would then be interrupted during glacial periods, which promoted downslope range shifts and a consequent secondary contact via lowlands (Colinvaux 1998). Because the peripheral Amazon regions are composed of high elevation areas (Andean forelands and Guiana highlands), greater genetic differentiation is expected to be found in this region than in the lowland core, which also showed lower genetic diversity (Leite and Rogers 2013). Such a scenario is commonly observed in frogs (Lötters et al. 2010; Fouquet et al. 2012; French et al. 2019).

#### 6.2.1.3 Riverine Barrier Hypothesis

This model postulates that major rivers act as geographic barriers to gene flow, and thus favor genetic differentiation between populations isolated on opposite banks (Wallace 1852). In general, an ancestral population suffers a split as the consequence of a river being formed, or due to a shift in a river's course, and so the separated populations evolve independently. The magnitude of the differentiation will depend on the width and flow rate of the river, as well as species-specific traits related to its dispersal capacity to cross the barrier (Leite and Rogers 2013). Riverine barrier effects have been used to explain the genetic pattern in several taxa across different drainages, such as Paraná-Paraguay (birds, Kopuchian et al. 2020), São Francisco (frogs, Bruschi et al. 2019) and the Amazon (primates, Boubli et al. 2015; trees, Nazareno et al. 2019).

#### 6.2.1.4 Marine-Incursions Hypothesis

As previously mentioned, marine incursions caused by sea level fluctuations were responsible for the formation of an interior seaway in the Amazon basin (Lundberg et al. 1998). This resulted in the isolation of three blocks of land: the eastern slope of the Andes, the Guianas, and the Brazilian Shields, which favored allopatric divergence (Aleixo 2004). The genetic predictions for the effect of marine incursion on terrestrial taxa are low genetic diversity in the Amazon lowland core, and high differentiation between populations located in highly elevated areas. This hypothesis is invoked to explain the genetic patterns found in *terra firme* birds (Aleixo 2004; Nores 2020), insects (Solomon et al. 2008; Sánchez-Herrera et al. 2020) and frogs (Santos et al. 2009; Castroviejo-Fisher et al. 2014).

#### 6.2.1.5 Gradient Hypothesis

Unlike previous hypotheses, the gradient model does not require allopatric isolation, and is essentially based on isolation by distance. The model assumes a contiguous population that differentiates along its distribution due to differential adaptive selection promoted by heterogeneous environments that may lead to parapatric divergence despite the presence of gene flow (Endler 1977; Smith et al. 1997). A high degree of genetic differentiation in geographically distant populations is expected, along with high genetic diversity towards the center of the taxon's distribution. Employing molecular markers and biological samples distributed in the Amazon-Cerrado ecological gradient, Machado et al. (2019) suggested a parapatric diversification of a rodent (*Hylaeamys megacephalus*) between the southern Amazonian and Cerrado. Due to the differentiation levels between lineages, the authors classified them as different Evolutionary Significant Units (ESUs), i.e., a lineage with a long-term evolutionary history that distinguishes itself from other populations or units (Moritz 1994).

According to Moritz et al. (2000), there are few studies available for the Neotropics that have either supported or refuted this pattern. For Leite and Rogers (2013), choosing an appropriate scenario and biological model to test this hypothesis can be difficult. Its original formulation predicts that population divergence is driven by contemporary ecogeography; thus, assessing the effects of past climate oscillations on the dynamics of environmental clines is a hard task. Another challenge cited by the authors is correlating the adapted traits and differentiation patterns among taxa with habitat attributes across the gradient. Moreover, distinct taxonomic groups might be more or less prone to isolation by distance as a result of their specific dispersal capacities (Perez et al. 2018). The advent of HTS technologies and their large resulting datasets may shed new light on testing the role of ecological gradients in promoting speciation in neotropical lineages.

### 6.2.2 *Freshwater Organisms*

For freshwater species, the diversification drivers are associated with the development of modern drainage systems (Lundberg et al. 1998). Studies support the idea that the dynamism of hydrographic systems, with ruptures and coalescent events resulting from tectonic rearrangements, shifting river courses, and repeated marine incursions and regressions, are the main promoters of allopatric diversification events (Albert and Reis 2011). Sympatric diversification processes may have also played an important role, but empirical studies are scarce and there are few convincing examples (Barluenga et al. 2006; Hubert et al. 2007).

Based on the hydrogeological landscape transformations, three main hypotheses – paleogeographic, hydrogeological and museum (Fig. 6.4) – are considered, to explain the allopatric processes responsible for the diversification of neotropical freshwater species. The paleogeographic hypothesis focuses on the emergence of impermeable barriers, particularly resulting from tectonic rearrangements (Albert et al. 2006; Hubert and Renno 2006). It represents a classic event of speciation by vicariance that, in general, involves multiple taxa and makes the phylogenetic prediction of reciprocal monophyly between lineages/species located in basins separated by a geological barrier (Fig. 6.4a). It is a pattern repeatedly observed in species located in the northwestern portion of South America, a region under the strong influence of the Andes (Machado et al. 2018; Melo et al. 2021; Ribolli et al. 2021).

The hydrogeological hypothesis emphasizes that geodispersal[2] events, promoted by headwater capture between adjacent basins, are responsible for diversification. The captures are commonly observed in unstable geological areas, covered by fractures and faults, which are continuously reactivated, promoting the rearrangement of the basins (Tagliacollo et al. 2015; Machado et al. 2018; Ramirez et al. 2020; Cardoso et al. 2021). The expected phylogenetic signature in this hypothesis is that

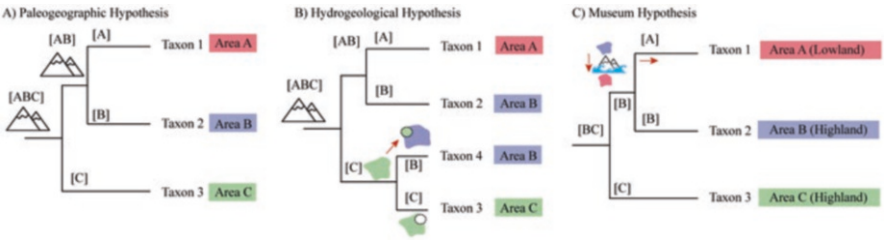


**Fig. 6.4** Graphical scheme of the main phylogenetic hypotheses for allopatric diversification of neotropical ichthyofauna. (**a**) Paleogeographic hypothesis (based on vicariance), (**b**) Hydrogeological hypothesis (based on geodispersal by headwater capture), (**c**) Museum hypothesis (based on geodispersal from highlands to low regions after marine regressions). The colors indicate different drainages, represented by letters. The arrow in the phylogeny represents geodispersal events

[2] A term introduced by Lieberman and Eldredge (1996), referring to the expansion of a species group's boundary due to the temporary removal of a barrier.

lineages/species are grouped in a clade containing individuals that occupy adjacent basins (Fig. 6.4b).

Finally, the museum hypothesis predicts that the widely distributed lineages/species have their ancestral lineages present in higher regions (Brazilian and Guiana crystalline shields – Hubert et al. 2007), given that marine incursions – which occurred in the Miocene (20–5 Ma) – fragmented the habitat and isolated populations in these areas, extinguishing populations in the lower portions. According to this hypothesis, lowland lineages emerged after the marine regressions (last 5 Ma). According to Hubert and Renno (2006), the biogeographic signature is high levels of endemism in elevated areas, which likely contained the distribution of the ancestral taxa, whereas lowlands would have high species diversity but low levels of endemism (Fig. 6.4c). Recently, an opposite pattern to this hypothesis was described for Curimatidae species, where the lowlands were considered the museum areas and, consequently, the highlands represented the dispersal centers during the Eocene and Oligocene (Melo et al. 2021).

## 6.3 Evaluating Neotropical Biodiversity at Multiple Scales

Given the intricacy of the neotropical biota assembly, any approaches aimed at assessing the underlying diversification processes should embrace this complexity by considering multiple evolutionary scales (McGill et al. 2019). However, there has been a historical division in the research fields of micro- and macroevolution, with the former usually focusing on investigating within-species (population) processes, while the latter has been more related to the analysis of biodiversity patterns above the species level. While this separation led to an important knowledge gap in the interface between these two poles of the speciation continuum (Li et al. 2018; Singhal et al. 2022), phylogeography emerged as a potential bridge to connect these two scales (Avise 2000; Hickerson et al. 2010; Edwards et al. 2022).

### *6.3.1 Intraspecific Scale*

The knowledge of intraspecific genetic patterns (structure and diversity), and that of how they came to be shaped, is of prime importance in evolutionary biology (Li et al. 2018) and biodiversity conservation (Carvalho et al. 2017). In the former, within-species lineages are considered basic units of diversification and could be key to understanding how microevolutionary events may translate into the patterns detected at the macroevolutionary scale (Alencar and Quental 2021). On the other hand, detecting independently evolving sets of populations is also critical to propose effective conservation and management guidelines to preserve the intraspecific genetic diversity and, consequently, to maintain a species' ability to respond to future selection pressures (Moritz 1994). Thus, to reach these goals at this

evolutionary scale, researchers provide insights into gene flow, historical effective population size, colonization routes, population differentiation, the phylogenetic relationship among populations, and the timing of divergence or demographic changes.

An interesting example is that of the neotropical otter (*Lontra longicaudis*), a medium-sized semiaquatic carnivore widely distributed in the neotropical region, from northwestern Mexico to Uruguay and across the north of Argentina (Rheingantz et al. 2017). Due to anthropogenic impacts caused mainly by hunting, habitat fragmentation, mining, and water pollution, the species was classified as Near Threatened by the International Union for Conservation of Nature (IUCN) in 2021 (Rheingantz and Trinca 2021). A phylogeographic study that sampled a large portion of the species' range identified at least four geographically structured phylogroups (Colombia, Bolivia, Amazonia, and eastern South America) that recently diverged, and which are partially congruent with a subspecies classification previously proposed for this species (Trinca et al. 2012). Given the limited sampling, the authors classified only the Amazonia and eastern South America phylogroups as ESUs. Because ESUs represent the main source of historical and adaptive genetic diversity within species, they receive special consideration in conservation efforts (Moritz 1994). The presence of phylogeographic breaks in *L. longicaudis* suggests that these populations should be managed independently.

Another noteworthy example is that of neotropical giant anteaters (*Mymercophaga tridactyla*), the largest anteater species widely distributed in the neotropical region, from Honduras southwards to northern Argentina (Aguiar and da Fonseca 2008). The species was classified as Vulnerable by the IUCN in 2021 (Miranda et al. 2014), due to anthropogenic impacts caused mainly by hunting, habitat loss, roadkill, and wildfires. In face of this critical scenario, Coimbra et al. (2022) implemented an integrative framework between phylogeographic analyses and species distribution modeling (SDM) to identify priority areas for conservation and understand the current genetic distribution of populations from four biomes (Amazon, Cerrado, Pantanal and Atlantic Forest). The authors observed two main genetic clusters, one formed with individuals from the Amazon, and another composed of the remaining populations, with the latter presenting a high genetic diversity level. The estimated historical gene flow revealed a unidirectional migration from the Amazon to the non-Amazonian cluster. Paleodemographic reconstructions detected an expansion in populations, influenced by climate changes during the Pleistocene, with more stable climatic conditions in areas occupied by the non-Amazonian lineage. Based on these results, the authors concluded that the Amazon lineage is at risk due to the loss of genetic diversity and the small size of its population. On the other hand, although the non-Amazonian populations showed more promise to persist in the future, their current range areas have been massively devastated and might not support viable populations (Barragán-Ruiz et al. 2021, for instance), which led Coimbra et al. (2022) to suggest the Brazilian Cerrado as a priority biome for species conservation.

### 6.3.2 *Interspecific Scale*

Moving towards higher hierarchical levels in the speciation continuum, an important aspect of connecting intra and interspecific scales is the assessment of species limits (de Queiroz 2007). This can be a challenging task, especially in complex radiations (Barley et al. 2013) and in the early stages of the divergence process, as incongruences among different organismal properties (e.g., reciprocal monopoly, reproductive isolation) might be observed (Carstens et al. 2013). Researchers previously attributed such differences to incompatible concepts of species, which could generate different species limits depending on the chosen criteria (Mayden 1997). De Queiroz (2007) proposed the unified species concept workaround for this conundrum, by dissociating the conceptualization from the process of delimiting lineages. Instead, the properties upon which the modern species concepts were based should be considered as lines of evidence that accumulate throughout the speciation process, and that can be used to support the assessment of species boundaries (de Queiroz 2007). Indeed, such conceptualization led to remarkable advances in the field of species delimitation, with several methodological approaches being proposed (reviewed in Carstens et al. 2013; Rannala and Yang 2020). In the neotropical region, species delimitation methods have been applied in several taxonomic groups, such as fish (Ochoa et al. 2020), frogs (Pie et al. 2019), lizards (Domingos et al. 2017), mammals (Fegies et al. 2021), and plants (Perez et al. 2022). Such studies are very important, especially in a region with such a spectacular and still poorly understood biodiversity, as they help establish more robust species boundaries and uncover cryptic lineages.

In this context, recent advances have also been observed in the study of species complexes (i.e., closely related lineages with a prevalent taxonomic uncertainty) that are suitable models for the understanding of the initial steps of the speciation crossroad (for a review, see Pinheiro et al. 2018). For example, the cactus species complex *Pilosocereus aurisetus* has a naturally fragmented occurrence, associated with the *campos rupestres* rocky formations within the Cerrado biome in the dry diagonal. This plant species complex was analyzed in a series of studies, and the results obtained suggested a very recent diversification, most likely associated with long-term isolation in interglacial refugia (Bonatelli et al. 2014; Perez et al. 2016a, b). As another interesting example, the widespread freshwater wolf fish (*Erythrinus erythrinus*) species complex showed a combined effect of long-distance allopatry and neo-sex chromosomes as potential mechanisms generating an incipient speciation stage (Souza et al. 2022).

Therefore, phylogeographic studies considering closely related species and species complexes are very important to provide information about the speciation process itself. This is extremely important to advance the understanding of diversification processes in the super-diverse neotropical region, for which studies are still underrepresented (Turchetto-Zolet et al. 2013).

### *6.3.3 Community Scale*

Phylogeography has traditionally been focused on the microevolutionary scale, i.e., within and among closely related species or species complexes. Indeed, studies focusing on particular groups have exponentially increased through time (Garrick et al. 2015), while there is still a small number of phylogeographic studies that adopt clade-wide approaches to extract generalities across multiple groups. Because of that, generalization efforts to explain large-scale genetic variation and biodiversity patterns have mostly been restricted to disciplines such as macroevolution, biogeography and macroecology. Advances toward clade-wide approaches come from the related field of comparative phylogeography (Edwards et al. 2022; Hickerson and Meyer 2008), which combines information from multiple co-occurring species to evaluate shared demographic events. At this higher evolutionary scale, phylogeographic studies can provide better information for conservation plans, because they are capable of identifying areas that should be prioritized to preserve evolutionary processes at the community level (Carvalho et al. 2017). For example, when signatures of shared events are detected, they can be associated with major historical episodes, such as geological or climatic modifications, which have the potential to affect several species in the community simultaneously (Oaks 2019). Moreover, this is a powerful framework to connect multiple evolutionary scales, as it allows for the explicit incorporation of microevolutionary processes (e.g., species-specific effective population sizes and migration) while integrating information from several co-distributed lineages to provide insights about potential community-level diversification mechanisms. For example, Prates et al. (2016) investigated responses to climatic fluctuations in three co-distributed lizard species. They detected idiosyncratic responses both for past and future climatic conditions, which reflected the unique organismal traits in each species (Prates et al. 2016). The response of species to climatic fluctuations was also assessed by Bonatelli et al. (2022), focusing on taxa associated with the dry diagonal. The results suggested a demographic expansion of most species during the glacial periods, but some of the analyzed organisms had the opposite response and showed signals of retraction (Bonatelli et al. 2022).

## 6.4 Assessing Old Phylogeographical Questions with Modern Tools

Traditionally, phylogeographic studies have made historical inferences using haplotype networks and gene trees to estimate divergence times among lineages/species and perform associations with known geological and climate events to uncover potentially relevant diversification processes based on patterns of genetic distribution. However, this workflow, based on *ad hoc* hypotheses, can lead to over-interpretations of the analytical results (Knowles and Maddison 2002). For example, consider the diversification hypotheses for neotropical fishes of the *Salminus* genus

(Machado et al. 2018). Based on riverscape transformations, two main hypotheses were formulated to explain the allopatric diversification of the genus (paleogeographic and hydrogeological hypotheses, see Sect. 6.2 for more details on their genetic signatures). Although they reflect different processes, they can show a similar pattern of genetic variation when new barriers emerge following headwater capture, or in the face of local extinctions, making it impossible to distinguish between both hypotheses (Machado et al. 2018). To solve an issue like this, it is important to implement a rigorous statistical framework of model-based inferences, employing coalescent models for parameter estimation and hypothesis testing. This field is named "statistical phylogeography" (Knowles 2009), and it has been largely applied in phylogeographic studies (Hickerson et al. 2010). Below, we will discuss some of the methods commonly employed to improve the understanding of the historical scenarios that can potentially explain diversification processes and the current genetic patterns in neotropical taxa.

### *6.4.1 Species Distribution Modeling*

Species distribution modeling (SDM) refers to statistical and/or mechanistic approaches used to predict species occurrence across space and time based on their abiotic preferences and tolerances (Elith and Leathwick 2009). It has been successfully integrated in phylogeographic studies to improve the comprehension of processes that shaped the genetic patterns (Alvarado-Serrano and Knowles 2014), and to assess future climate change impacts (Abreu-Jardim et al. 2021). Among the insights that SDM can bring to phylogeography in the neotropical region, we highlight studies predicting species distribution with paleoclimate reconstruction (Coimbra et al. 2022), indicating stability areas over time (Carnaval et al. 2009; Carvalho et al. 2021), identifying possible isolating barriers (Machado et al. 2019), and developing spatial biogeographic hypotheses that can be tested using statistical phylogeographic methods (Prates et al. 2016).

A solid example of how this integrative approach can be powerful was presented by de Oliveira et al. (2021), who employed SDM and Approximate Bayesian Computation (ABC; see below) to evaluate the role of Pleistocene climatic changes on the demographic history and genetic patterns of two endemic anurans (*Bokermannohyla alvarengai* and *Bokermannohyla oxente*) from the Espinhaço range in Brazil. According to paleodistribution modeling, both species represent a sky-island system in which populations expanded their sizes and ranges during glacial periods – specifically, during the Last Glacial Maximum – but suffered retractions during interglacial periods. The observed genetic structure was explained as the result of Pleistocene climatic fluctuations that affected each taxon in distinct periods. Using the obtained results and data from the literature, the authors built possible diversification scenarios to be tested using ABC analysis. Although both

species showed signs of being affected by historical climate changes, statistical phylogeography showed different results regarding their diversification processes and the time of their divergence. The authors suggested that conservation actions should involve strategies to preserve each independent lineage, mainly because of their higher probability of extinction, as observed for small-ranged vertebrates in a montane habitat.

### *6.4.2 Inference of Demographic Scenarios*

Hypotheses generated from SDM and from other sources of evidence can be assessed with robust statistical frameworks by using the large datasets generated with HTS (Knowles 2009). Hence, the number of sampled individuals and sequenced loci in phylogeographic studies increased exponentially (Garrick et al. 2015). Major advances were also achieved by using the principles of the coalescent theory (Kingman 1982), which allows for the incorporation of the stochasticity of the genetic processes (Knowles and Maddison 2002; Wakeley 2003) into demographic inferences, using different statistical strategies based on the likelihood function (Beerli and Felsenstein 2001; Hey and Nielsen 2004).

Another approach that has been applied to accommodate more intricate demographic histories, a feature that is particularly paramount to understanding the high complexity of alternative possible scenarios in the Neotropics (see Sect. 6.2), involves likelihood-free analyses based on simulations. Among them, ABC has been widely used in phylogeography (Bertorelle et al. 2010). It involves the simulation of genetic data under alternative demographic scenarios, which are then compared to the empirical data based on a set of summary statistics (e.g., the expected heterozygosity level or the site frequency spectrum) that are used to retain only a small proportion of the most similar simulations (Csilléry et al. 2010).

In the Neotropics, ABC was applied to the comparison of specific hypotheses associated with Pleistocene climate oscillations in several taxa. For example, the *Pilosocereus* (Cactaceae) species complex that includes populations associated with the sky-islands of dry habitats was investigated to assess the effects of past climate conditions on long-distance dispersal and vicariance. The results supported the vicariance hypothesis as a result of expanded ranges during glacial periods with a recent retraction to microrefugia (Perez et al. 2016a). Similar examples of ABC applications to elucidate the effects of past climate changes involve the demographic history of three other species from the Cactaceae family with a distribution associated with xeric enclaves in the Atlantic Forest (Franco et al. 2017), a bird species associated with wetlands (Miño et al. 2017), a spider species distributed in subtropical regions (Peres et al. 2015), and a freshwater fish (Mondin et al. 2018). Machado et al. (2018) explored changes in population size over time as a result of riverscape transformations, in order to distinguish between vicariance and geodispersal events. According to the authors, the expected demographic patterns for geodispersal events are characterized by founder events in colonized areas, followed by expansion in

population sizes. Conversely, vicariance events failed to leave signatures of founder events, source-basin identity and expansion event. Other interesting ABC applications for neotropical taxa species include species delimitation (Camargo et al. 2012), and comparative phylogeography testing for synchronous expansions in taxa occurring in dry habitats (Gehara et al. 2017).

### *6.4.3 Machine Learning for Demographic Inference and Predictive Phylogeography*

More recently, machine learning has increasingly been used in evolutionary studies (Schrider and Kern 2018; Borowiec et al. 2022). The flexible and data-hungry nature of these approaches are attractive features to analyze large and complex genomic datasets, typical of phylogeography studies. For example, Convolutional Neural Network (CNN) is a machine learning technique that can extract information from simulated genetic data, a strategy similar to ABC. One advantage of using CNN over ABC, in this context, is that the inferences rely on information from all simulated data (Flagel et al. 2019; Sanchez et al. 2021), without the need to perform a rejection step that only retains a small number of the simulations which are more similar to the empirical data (Csilléry et al. 2010). Furthermore, CNN algorithms automatically extract meaningful features from an "image" of the genetic data (Torada et al. 2019), which eliminates the need for an arbitrary set of summary statistics which may fail to capture all information from the data (Robert et al. 2011). As with other machine learning techniques, CNN performance usually improves with larger datasets, and it is not negatively affected by the high dimensionality of genomic datasets, as is the case with ABC (Prangle et al. 2018). Indeed, in a comparison between CNN and ABC applied to species delimitation, the accuracy of CNNs was higher, despite the modest-sized datasets concerning a plant species complex associated with dry vegetation areas (Perez et al. 2022). Recent attempts to apply CNN in phylogeography focused on assessing the history of the colonization of river basins in the Neotropics (Souza et al. 2019; Oliveira et al. 2020, see Chap. 21), and the demographic history of lizards (Fonseca et al. 2021). It is important to note that advances have also been made in the use of CNN for analyzing shared demographic histories in multiple co-occurring species (Kirschner et al. 2022).

Another promising application of machine learning that has recently been introduced is predictive phylogeography. These approaches seek to combine information from genomic, environmental and organismal trait data to make predictions even for species that have not been sampled or whose datasets are incomplete (Pelletier et al. 2018; Sullivan et al. 2019). In the Neotropics, applications of predictive phylogeography were used to gain insights on traits enabling adaptation to forested and open habitats in lizards (Lanna et al. 2022), and species features related to demographic responses of taxa associated with the dry diagonal to climatic changes in the Pleistocene (Bonatelli et al. 2022).

### 6.4.4 *Phylogeography as a Bridge to Connect Multiple Evolutionary Scales*

By combining elements from both microevolutionary (such as population and landscape genetics) and macroevolutionary disciplines (such as phylogenetics and biogeography), phylogeography functions as a bridge between these fields (see Sect. 6.3). Focusing on the suite of analytical approaches frequently used in phylogeography, we propose a framework that considers and integrates these distinct evolutionary scales (Fig. 6.5).

Starting with microevolution (Fig. 6.5a), tools from landscape genetics can be used to assess the spatial distribution of genetic variation at the intraspecific level, revealing patterns of genetic population structure (Pritchard et al. 2000) and their correlation with environmental (Forester et al. 2018) or geographic distances (isolation by distance; Perez et al. 2018). Population genetics approaches can reveal the effect of microevolutionary forces and estimate demographic parameters (e.g., effective population sizes, migration rates, and divergence times).

At the micro-macro evolution interface, species delimitation methods can be used in closely related species and species complexes. In such cases, it is important to adopt an integrative approach, performing inferences including multiple sources of data (e.g., genetic, morphological, and environmental) and analyses based on different assumptions (Carstens et al. 2013). The different resulting hypotheses from each method can then be used to generate an integrative species delimitation based on their congruence (Satler et al. 2013), or explicitly compared with modeling approaches (Perez et al. 2022).

Finally, at a macroevolutionary scale, multiple co-occurring species can be analyzed in a comparative framework to provide insights on historical events that affected the demographic history of several species in the community (Edwards et al. 2022; Oaks 2019).

## 6.5 Final Considerations

Overall, the great biodiversity present in the neotropical region is a consequence of complex interactions between biotic and abiotic processes. This chapter provided a brief overview of species diversification patterns and reviewed the development of ideas based on phylogeographic studies to explain the origin and maintenance of the rich biodiversity from this region. Although it is possible to observe an increasing number of empirical contributions, the full comprehension of evolutionary processes is still far from complete. There is a clear need to increase the number of studies considering wide phylogeographical data, multiple hierarchical levels, and applying large HTS datasets to generate results with higher resolution. It is also necessary to look more closely at how functional traits and ecology influence phylogeographical patterns and predict how current genetic diversity might respond to

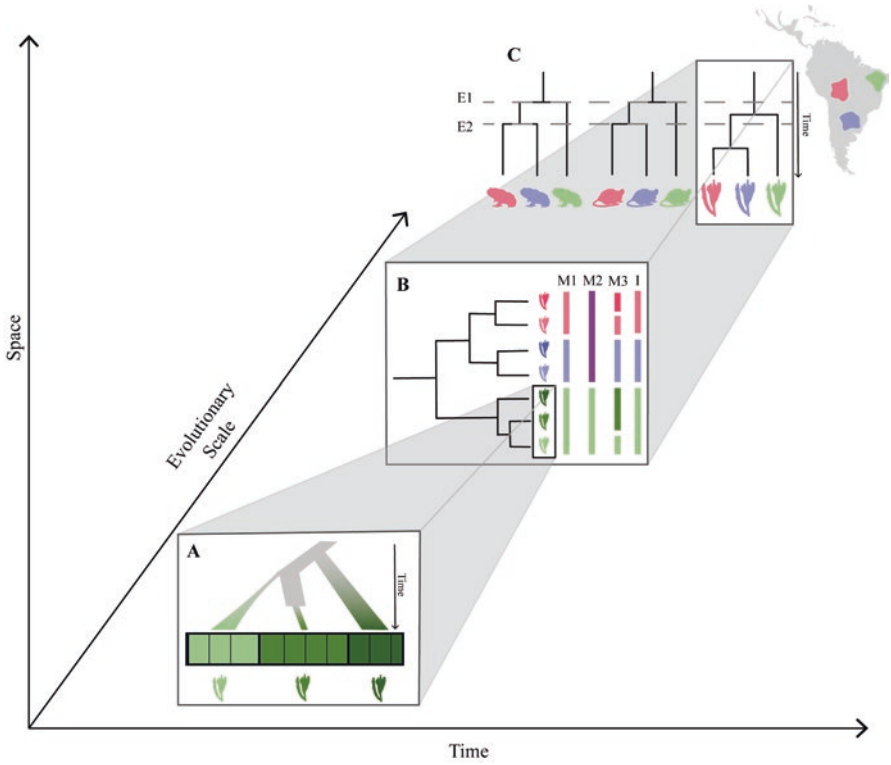


**Fig. 6.5** Schematic representation of a phylogeographical study spanning and connecting micro and macroevolutionary scales. (**a**) Multiple populations of the same species are analyzed to assess population structure (shown in different shades of green in the bar plot) and estimate demographic parameters according to the diversification scenario that can include microevolutionary events such as divergence times, fluctuation in effective population sizes, and migration. (**b**) On a higher evolutionary scale, taxonomic limits can be assessed by combining the species with closely related ones. Distinct sources of data and methods (M1-M3) can be analyzed and combined in an integrative species delimitation (I). (**c**) At an even higher scale, species with similar distributions can be analyzed comparatively to assess whether historical events (E1 and E2) caused shared demographic responses

anthropic impacts (Cavers and Dick 2013). The latter is particularly urgent because of the high risk of extinction that neotropical biodiversity faces as a result of habitat destruction and climate change. As aforementioned, genetic diversity is frequently regarded as the most fundamental dimension of biodiversity, providing the critical basis for evolutionary changes, such as adaptation to new environmental conditions. Thus, knowledge of the mechanisms that shape and sustain this diversity can help guide biodiversity management and conservation policies by providing, for example, information on biological diversity status, and identifying priority areas or taxa.

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# Chapter 7
# Landscape Genetics in the Neotropics


**Carolina da Silva Carvalho and Marina Corrêa Côrtes**

## 7.1 Introduction

Landscape genetics was first defined by Manel et al. (2003) as a research field that combines population genetics, landscape ecology, and spatial analysis to understand the genetic variation in space. Based on modifications proposed by Holderegger and Wagner (2006) and Storfer et al. (2007), Balkenhol et al. (2015) provided a more integrative definition that highlights the importance of quantitatively linking genetic variation with spatial heterogeneity. Thereafter, landscape genetic studies have employed multiple analytical tools to explicitly correlate genetic variation with environmental features, such as elevation and land use types. By doing so it is possible to quantify the effects of landscape composition, configuration, and matrix quality on microevolutionary processes, such as gene flow, drift, and selection, using neutral and adaptive genetic data. In this chapter we recapitulate fundamental concepts of landscape genetics and genomics and elucidate common analytical methods. We then provide a synthesis of landscape genetic studies and applications in the Neotropics, and finally, discuss some challenges and opportunities for future research.

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## 7.2 Integrating Genetic and Landscape Data to Elucidate Microevolutionary Processes

Landscape genetic studies require three general steps: (i) measuring spatial or temporal genetic variation using molecular markers, (ii) quantifying environmental heterogeneity to capture the composition, configuration, and/or matrix quality of the study landscape, and (iii) statistically linking landscape heterogeneity and genetic variation to explicitly test for landscape–genetic relationships. While different molecular markers have been used to measure genetic variation, until the early 2000s, microsatellites were the most used (Schlötterer 2004). With the advances of high-throughput sequencing technologies, however, genome-wide single nucleotide polymorphisms (SNPs) have become the marker of choice (Morin et al. 2004; Hall and Beissinger 2014; Zimmerman et al. 2020). While both SNPs and microsatellites can provide information about demographic processes, such as gene flow and genetic drift, SNPs occur across the genome (including coding or functional regions) and, therefore, can comprise adaptive loci for measuring direct and indirect effects on phenotypes, local adaptation, and selection (Hall and Beissinger 2014; Storfer et al. 2018).

With molecular marker data in hand, several parameters can be used to estimate genetic variation within and between entities (individuals or populations). The parameters used to measure genetic diversity may include heterozygosity, allelic richness and frequency, and inbreeding coefficient. Pairwise parameters should be used when measuring variation between entities and inferring genetic connectivity or relatedness, including genetic distance estimates (e.g. Nei), kinship or relatedness estimators (e.g., *r*), and genetic structure indexes (e.g. $F_{ST}$) (see Waits and Storfer 2015, Shirk et al. 2017, for more genetic parameter estimates used in landscape genetics).

A landscape can be a mosaic, containing patches of habitat, corridors, and matrices, or can be the representation of an environmental gradient (Metzger 2001). The spatial extent (area within the landscape boundaries) is chosen by the researchers based on the scale of action or perception of the study species (Metzger 2001). Therefore, it can vary enormously, from a plot to an entire biome, as long as the landscape has at least one measurable heterogeneity factor relevant to the study species (Metzger 2001). Once the heterogeneity factors are identified as explanatory variables, researchers should collect these environmental data either by doing fieldwork on the ground or by retrieving the information remotely from data sources or repositories. For example, to evaluate the effect of elevation on genetic diversity and gene flow between populations, one can use open-source rasters, like digital elevation models retrieved from the USGS Earth Explorer, available in high resolution worldwide (https://earthexplorer.usgs.gov/). Environmental data can be (i) treated in their raw form (elevation quota), (ii) averaged or converted to percentage within a delimited area (percentage of forest cover within a buffer), (iii) transformed in resistance values that are mapped in a surface (see below), among others. Importantly, spatial and temporal resolution need to be taken into consideration when choosing

the heterogeneity factors and how collected data is processed and mapped (Storfer et al. 2007; Cushman et al. 2015).

Deciding which statistical approach to use to link genetic variation to landscape heterogeneity depends on (i) whether the study is individual or population-based (Balkenhol and Fortin 2015; Waits and Storfer 2015), and (ii) the analysis level (node, link, neighborhood, or boundary) (Wagner and Fortin 2012). Classic population genetic studies analyze genetic differentiation among discrete groups, often considered (sub)populations. However, this approach can be challenging in species whose individuals are continuously distributed across the landscape and, hence, are not clearly partitioned into discrete populations. Landscape genetic studies are not restricted by experimental designs centered on discrete populations and, therefore, can measure neutral or adaptive genetic variation between individuals as well (Hall and Beissinger 2014; Waits and Storfer 2015). While population-based measures are applied to discrete groups of organisms or to groups determined analytically using clustering genetic methods (e.g. STRUCTURE), individual-based measures do not require that individuals are grouped a priori (see Hall and Beissinger 2014 for genetic measures applied for individual and population-based approach). Specifically, the individual-based approach has proven more powerful to detect the early influence of current landscape features on contemporary gene flow and connectivity compared to population-based approaches (Landguth et al. 2010).

Statistical analysis to link landscape heterogeneity and genetic variation can be performed according to four main approaches — node, link, neighborhood, and boundary levels — and each approach requires that data are processed differently (see Wagner and Fortin 2012 for more details). Briefly, node-level analysis assesses the influence of landscape features on genetic diversity or the presence of adaptive genes at a spatial location (i.e., node). For example, one can quantify the effect of forest amount on population allelic richness (Carvalho et al. 2015). Different statistical analyses can be applied, such as linear models and multivariate methods (e.g., Redundancy Analysis – RDA). It is also important to check and, if needed, account for spatial autocorrelation due to isolation by distance (Wagner and Fortin 2012, 2015).

Link-level analysis is a pairwise approach in which a matrix of genetic dis(similarities) between individuals or populations are modeled as a function of a single or multiple matrices with landscape distances. Landscape distances can represent differences in environmental conditions between sites, or the probability or cost to transpose the landscape between sites. The former is commonly used to test isolation-by-environment hypotheses (Sexton et al. 2014; Wang and Bradburd 2014), while the latter approach is widely used to test isolation-by-resistance (Fig. 7.1). A model of isolation-by-resistance predicts a negative relationship between gene flow and the resistance distance, which is a metric that takes into consideration quantitative information on habitat quality and its suitability for promoting organismal movement (McRae 2006). A model of isolation-by-environment predicts that the genetic distance between populations or individuals increases with differences in local environment between sites, independently of the resistance imposed by the landscape (Wang and Bradburd 2014).

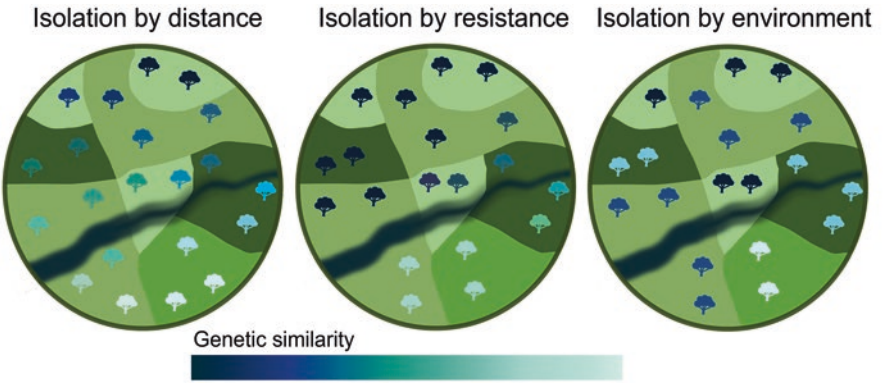


**Fig. 7.1** Schematic representing the spatial genetic variation in hypothetical trees under models of isolation-by-distance (IBD), isolation-by-resistance (IBR), and isolation-by-environment (IBE). Trees are color coded along a gradient of genetic similarity so that similarly colored trees are more genetically related. There are eight patches of four habitat types (green colored polygons) with different environmental conditions and one linear feature with thickness correlated with resistance

Landscape distances can be estimated using various methods depending on the study design and data availability, but the most popular relies on landscape resistance surfaces. Landscape resistance surfaces are spatially-explicit raster data layers that assign resistance values to landscape features, reflecting the degree to which that variable hinders or promotes movement (Zeller et al. 2012). To parametrize the resistance surface — that is, to assign cost values to each landscape feature — one can use expert opinion, estimates from environmental niche models, field-based data (e.g., capture-recapture, GPS telemetry), or infer based on genetic data assuming it is a proxy of functional connectivity (Zeller et al. 2012; Peterman et al. 2019). A powerful method that allows surface parametrization and optimization based on a genetic distance matrix uses a genetic algorithm and it is implemented in the R package ResistanceGA (Fig. 7.2) (Peterman 2018). After the surfaces are parametrized, landscape distances are estimated with least cost path, random walk commute time, or electrical circuit theory (Peterman et al. 2019). These distances are then used as explanatory variables to fit pairwise genetic distances, using linear mixed models that account for non-independency in pairwise data (Maximum-Likelihood Population Effects – MLPE, Van Strien et al. 2012). Although Mantel or partial Mantel tests have been widely used to correlate pairwise genetic and landscape distances, these approaches are no longer recommended as they have several drawbacks, such as high type-I error rates, the inability to model the effect of multiple covariates simultaneously, and the absence of a maximum-likelihood framework that allows for model selection (Monteiro et al. 2019).

Neighborhood-based methods relate genetic diversity at a spatial location with attributes of neighboring sites (Wagner and Fortin 2012). While the link-level approach addresses the question of how connectivity between patches affects gene flow, neighborhood-based approach analyzes how the average connectivity of each

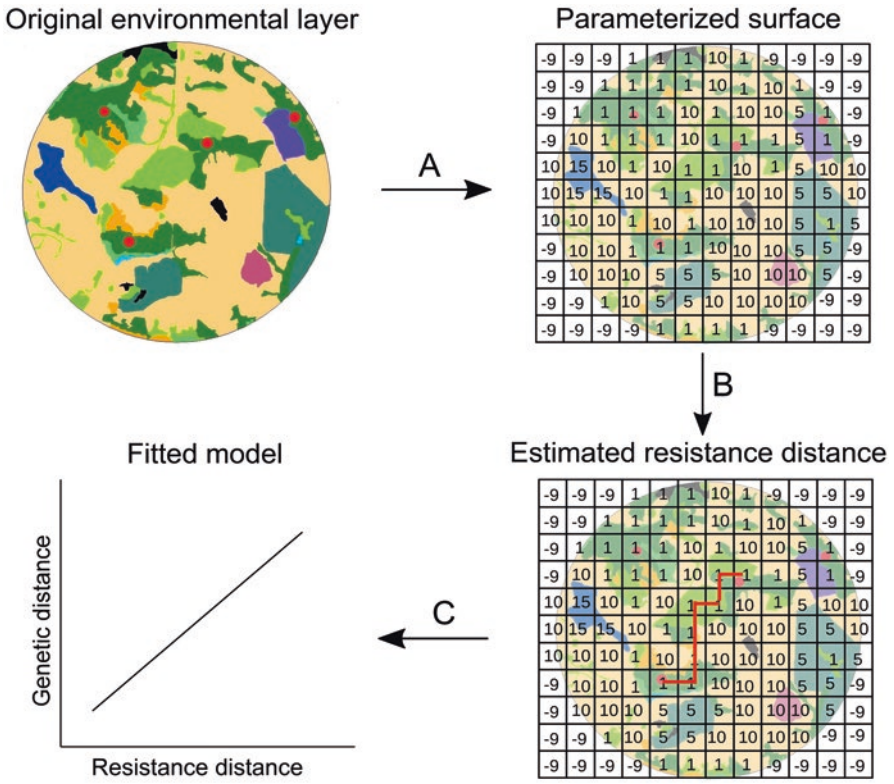


**Fig. 7.2** Methodological approach to model isolation-by-resistance using an optimization procedure such as ResistanceGA to construct resistance surfaces. (**a**) Parametrization: resistance values are assigned to each landscape class in a categorical surface (e.g., land use and cover types) or to scores along a continuous variable (e.g., elevation) following some type of transformation, (**b**) pairwise effective distances are calculated between sampled sites using least cost path, cumulative cost distance, or resistance distance, and (**c**) statistical models are fitted using genetic distance as the response and effective distance as the explanatory variable. The above steps are repeated several times and, in each iteration, a different resistance value is assigned to the landscape features (categorical or continuous) resulting in alternative parametrized surfaces. The model outputs of each iteration are compared using model selection criteria to determine the best-ranked model and respective optimized resistance surface depicting connectivity probability across the studied landscape

patch with all neighboring patches affects its genetic diversity. Gravity models are the most promising statistical approach, although they are still underutilized (Murphy et al. 2010, 2015). Finally, boundary-based methods are used to detect the presence and configuration of a barrier to gene flow. This is usually done using Monmonier's algorithm or Womble's bilinear algorithm, and once genetic boundaries are detected, they can be linked to specific landscape features (Manel et al. 2003).

## 7.3 Adaptive Genetic Diversity

In 2007, the term landscape genomics emerged to incorporate studies that use a large number of loci and those that integrate landscape genetics and population genomics to identify the environmental factors that shape adaptive genetic variation and the genes that drive local adaptation (Joost et al. 2007; Manel et al. 2010). SNPs have become the most used molecular marker in landscape genomic studies. Genome-wide SNP sequencing implies that loci at both neutral and functional regions are sampled. Therefore, it is important that researchers identify and separate the two subsets of loci to conduct either demographic-based or adaptive-based studies in landscape genetics. There are several statistical tools to detect putatively adaptive SNPs. Some tools are based on outlier detection methods, which identify loci with high genetic differentiation between populations, whereas others are based on associations between allele frequencies and environmental data (genotype-environment association – GEA) (Rellstab et al. 2015). Which method to use will depend on the study goal, experimental design, and availability of environmental data. Outlier detection does not depend on environmental data and candidate loci are solely identified by their values of population genetic differentiation (such as $F_{ST}$), which should be larger than a pre-defined confidence threshold (Luikart et al. 2003). When populations are suffering from disruptive selection, $F_{ST}$ values of adaptive loci are higher than neutral loci, whereas candidate loci present lower $F_{ST}$ values when suffering from stabilized selection (Luikart et al. 2003). It is important to highlight that the outlier detection method is always population-based. GEA, however, can be population or individual-based and relies on uni or multivariate analysis, such as LFMM (latent factor mixed model) and RDA, respectively (Rellstab et al. 2015). This approach is landscape genomics at its core as it aims to identify the environmental factors that shape the adaptive variability. Candidate loci are those that have allelic frequency correlating significantly with environmental variation (Forester et al. 2018). GEA are considered more powerful than outlier detection tests to detect adaptive genetic variation (Forester et al. 2018).

Regardless of the statistical method used to assess adaptive SNPs, it is extremely important to account for underlying neutral population structure to reduce false positive detections (i.e., considering a SNP as adaptive when it is not) (Rellstab et al. 2015). These false positives arise mainly when populations are strongly structured due to demographic processes (genetic drift and gene flow) but, coincidentally, the environment is also different between them. To reduce the rate of false positives, it is important not to use environmental variables other than the ones specified in the scientific hypothesis (Rellstab et al. 2015). Moreover, good replication along environmental gradients and within evolutionary lineages allow for more robust detection of candidate loci, increasing true positive rates (Rellstab et al. 2015). Running statistical tests over many loci also increases the rate of false positives and thus, it is necessary to correct for multiple comparisons (e.g., by using false discovery rates – FDR), regardless of the approach used (François et al. 2016). Finally, several uni and multivariate analyses take into account neutral population

structure (e.g., LFMM, BayeEnv, PCAadapt and partial RDA) and it is good practice to apply more than one method to detect candidate loci: the more methods converge to the same candidate locus, the greater the confidence that this locus is linked to a region of the genome that is under selection. Conversely, while this approach can increase the confidence of true positive detection, it can increase the rate of false negative detection by eliminating loci under weak selection (Forester et al. 2018). Then, the use of the intersections or sums of loci detections among statistical methods will depend on the objective of the study.

## 7.4 Landscape Genetics in the Neotropics

Landscape genetics has much to contribute to understanding the biodiversity in the Neotropics — home to outstanding diversity of habitats and species (Raven et al. 2020; Antonelli 2021). To assess the extent to which landscape genetics have been applied in neotropical systems, we synthesized the trends of landscape genetic publications produced in the American tropics. We searched for papers in the Web of Science database up to December 2021 using the keywords "*tropic*" followed by any of the following combinations "landscape", "seascape", "riverscape" and "genetic*" or "genomic*". Then we manually checked if the studies associated genetic parameters with landscape or environmental heterogeneity. A total of 123 papers were retrieved and 65 were selected based on our criteria. Most of the excluded articles were removed because they were carried out in other tropical regions. In other cases, the study did not perform a landscape genetic study, even though these words were listed in the title or abstract. For example, Castilla et al. (2017) used molecular markers to measure local kinship and pollen-dispersal distances, but genetic parameters were not treated as a response variable. Likewise, Torres-Vanegas et al. (2019) did not relate genetic pollen pool differentiation with any landscape variable. Conversely, there are other known landscape genetic studies that did not appear in our search because they did not include some of the keywords, usually related to tropical (e.g., Blair and Melnick 2012, Carvalho et al. 2021a, and additional Mexican studies compiled in Rico 2019).

The first retrieved paper was published in 2009 (Dixo et al. 2009) and since then we have observed an increase in publications, with papers from 2021 comprising 15% (n = 10) of the published articles (Fig. 7.3a). Among the most frequent terms used in the titles (excluding the search keywords) were structure, forest, gene flow, divergence, and population (Fig. 7.3b). These topics are consistent with the main types of studies being conducted in the Neotropics (see next section "Approaches in neotropical studies"), which are predominantly population-based and focused on demographic processes, such as gene flow and connectivity. In particular, very few studied microevolutionary processes at the individual-level (but see Moraes et al. 2018; Latorre-Cardenas et al. 2021) and just more recently, studies started addressing the landscape adaptive genetics of neotropical species (e.g., Lanes et al. 2018; Gallego-García et al. 2019). The scientific journal *Molecular Ecology* was the main

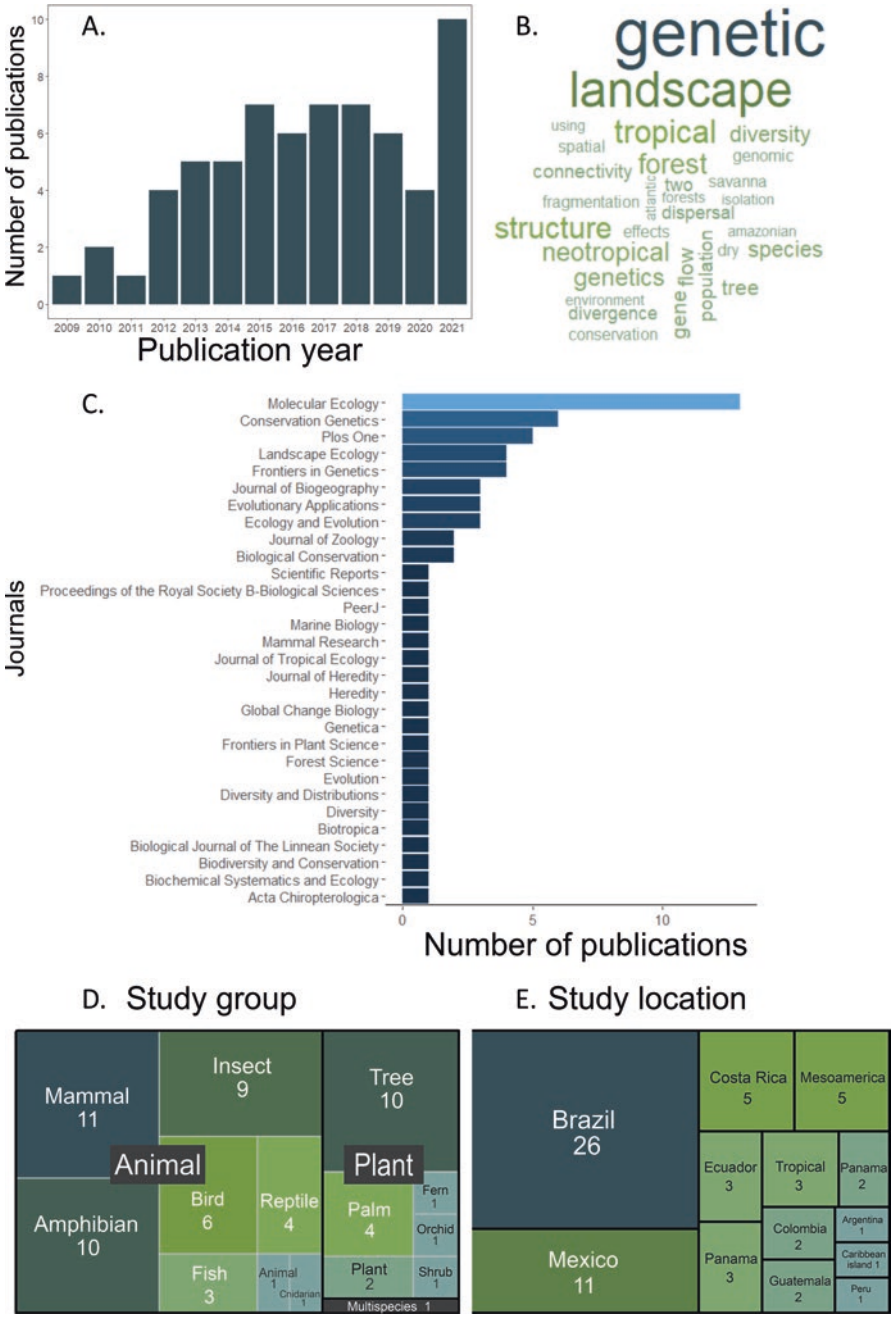


**Fig. 7.3** Trends of landscape genetic studies in the neotropics. (**a**) Number of publications from 2009 to 2021. (**b**) Word cloud based on titles of articles. (**c**) Number of articles published in different scientific journals. (**d**) Taxonomic focus of publications. (**e**) Countries or regions where studies were conducted

outlet for publication, followed by Conservation Genetics and PlosOne (Fig. 7.3c). Nearly 70% (n = 45) of the articles focused on animal species, 29% (n = 19) studied plants, and one paper conducted a meta-analysis of multispecies tropical gene flow (Monteiro et al. 2019) (Fig. 7.3d). Most neotropical countries lacked landscape genetic studies (Fig. 7.3e), while ten studies included multiple countries across a regional or global scale. Brazil was the most frequently studied country (40%), followed by Mexico (17%) (Fig. 7.3e).

## 7.5 Approaches in Neotropical Studies

Neotropical studies have used landscape genetic tools to elucidate ecological and evolutionary aspects of plant and animal species, and to guide conservation and management of fragmented populations. As identified in the synthesis above, many studies focus on understanding the landscape features that impact gene flow and genetic diversity. To do so, studies have compared models of isolation-by-distance (IBD), isolation-by-resistance (IBR), and isolation-by-environment (IBE) to evaluate whether gene flow is explained only by Euclidean distance, characterizing IBD, or if landscape features have an important role in promoting or hindering gene flow (Fig. 7.1). García-Rodríguez et al. (2021), for example, quantified the relative role of IBD, IBR due to topography and habitat suitability, and IBE due to climatic conditions, in shaping genetic differentiation in nine amphibian species in the Isthmian Central America (ICA). Rather than finding a consistent effect of factors influencing genetic differentiation in ICA, their results suggest that responses are idiosyncratic across species, likely related to differences in their life histories. Jaffé et al. (2016) also carried out a comparative landscape genetic study of 17 stingless bee species focusing on quantifying the relative role of IBD and IBR. In this case, they found that gene flow is limited by geographic distance, and that deforestation, elevation, precipitation, temperature, and rivers do not influence gene flow across species. Although comparative studies are important to understand general patterns, these are still scarce in neotropical landscape genetic studies.

Studies in the Neotropics have used different approaches to parametrize resistance surfaces to model IBR patterns, including expert opinion, habitat suitability models, and optimization procedures to assign cost value to each environmental feature. Khimoun et al. (2017) used the ResistanceGA (Fig. 7.2), a method of resistance surface parametrization and optimization developed by Peterman et al. (2014), to disentangle the effect of anthropogenic and natural landscape features (elevation, land cover and roads) on genetic differentiation in a bird species of Guadeloupe island in the Antilles. Along with evaluating which landscape features impose more resistance to genetic connectivity, Khimoun et al. (2017) also propose a framework to objectively define the spatial resolution (grain size of the raster layers that is ecologically relevant and computationally efficient), landscape thematic resolution (classification scheme of land and sea cover and use types to describe landscape variables), and spatial extent (area within the landscape boundaries). This is

important because the incorrect specification of spatial and thematic resolution, and spatial extent of the landscape may affect the strength of landscape-genetic relationships (Cushman and Landguth 2010).

Monteiro et al. (2019) carried out a review of 69 articles that explicitly tested the landscape effects on gene flow in tropical species, with the Americas harboring most species. Elevation, land cover, and forest cover were the most frequently assessed gene flow predictors. Climatic variables, such as temperature and precipitation, have also been tested, as well as environmental suitability models performed with these variables. Studies using riverscape and seascape genetic approaches, that is those that use landscape genetic tools to study the gene flow in aquatic or semi-aquatic species, have also been carried out in the Neotropics, although in much lower frequency compared with terrestrial studies. Latorre-Cardenas et al. (2021), for example, using a non-invasive genetic sampling of the neotropical otter (*Lontra longicaudis*) in Mexico, found that landscape-riverscape attributes, such as the hierarchical spatial organization of the stream networks, shape the species genetic structure. Because this species has a continuous distribution, a landscape genetics individual-based approach was used. Despite not requiring a priori delimitation of genetically discrete groups, the individual-based approach is still rarely used in neotropical studies.

In a much smaller proportion, landscape genetic tools have also been used to understand local adaptation and the distribution of adaptive variability (Santos and Gaiotto 2020). Medina et al. (2021), for example, examine genome-wide variation and ecologically important phenotype traits in a neotropical frog distributed across an environmental gradient. By using outlier detections and genotype-environment associations, they identified candidate SNPs associated with temperature and body size. Genomic differentiation coupled with phenotypic variation followed a clinal pattern, suggesting a strong selective processes for the species inhabiting this region. Neotropical studies focusing on understanding adaptive variability mostly use restriction site-associated DNA sequencing (RADseq) to detect loci involved in local adaptation (Santos and Gaiotto 2020). This approach has been used in landscape genomic studies because it is a cost-effective method for acquiring a large number of polymorphic markers in many individuals. However, RADseq usually samples only a small proportion of the genome, missing many loci underlying adaptation (Lowry et al. 2017). There are alternatives, such as whole genome sequencing, but this approach is still cost prohibitive for most landscape genomic studies (Catchen et al. 2017). Santos and Gaiotto (2020) reviewed the knowledge status and evaluated the importance of sampling design for studying landscape genomics of wild plants. They reviewed 35 papers that evaluated *in situ* local adaptations with SNPs and only 6% of the studied species were from Central and South America. Because they found that the number of SNPs and individuals sampled influence the detection of loci underlying local adaptation, authors recommend pool-seq approaches to increase sample size and improve the likelihood of detection of SNPs under selection. Pool-seq is a technique in which equimolar amounts of DNA from different individuals are pooled for sequencing (Santos and Gaiotto 2020). Although

this technique reduces DNA sequencing costs, it has some limitations such as not providing information for individuals separately.

Habitat loss and fragmentation are the main threats to biodiversity and are a matter of particular concern in the tropics where deforestation has alarmingly accelerated during the last decades. Rapid deforestation can impact all levels of biodiversity, including intra and interspecific diversity. Neotropical studies have explicitly assessed the impact of habitat loss and fragmentation on genetic outcomes in plant and animal species, including gene flow and genetic differentiation, and adaptive variability and selection. Studying the critically endangered Dahl's Toad-headed turtle, Gallego-García et al. (2019) found that urban areas and roads were the dominant features restricting gene flow among populations. Moreover, fragmented populations in the deforested grasslands presented lower levels of adaptive and neutral genetic diversity and were adaptively different from populations in more forested areas. Collevatti et al. (2020) and do Amaral et al. (2021) took a step forward and analyzed the effect of habitat loss and fragmentation on adaptive quantitative traits and evolutionary potential in savanna trees (*Tabebuia aurea* and *Caryocar brasiliense*, respectively) using controlled experiments. They found that habitat amount decreased some adaptive quantitative traits, which may limit evolvability and jeopardize species long-term persistence.

The landscape effect of habitat loss and fragmentation was also investigated at local scales. Soares et al. (2019) used a multiple scale approach to assess the impacts caused by local (logging) and landscape (forest loss and isolation) scale disturbances in the genetic diversity and structure of an endangered palm tree, *Euterpe edulis*. They found that tree logging modified the genetic diversity more rapidly than other hypothesized variables, whereas genetic structure was more influenced by landscape-scale modifications. *E. edulis* is one of the most studied palm species in the Neotropics and many landscape genetic studies have already been carried out in Brazil. Carvalho et al. (2015), for example, found that genetic differentiation between sites within landscapes is most likely due to contemporary changes caused by habitat loss and fragmentation, with matrix resistance being the strongest predictor of genetic differentiation. Conversely, several studies have found that genetic diversity and inbreeding in *E. edulis* are less impacted (or effects are harder to detect) by habitat loss and fragmentation (Santos et al. 2015; Carvalho et al. 2015, 2016). The landscape genetic toolbox can also be used to assess fine-scale ecological processes, such as the ones emerging from reproduction and parentage. Carvalho et al. (2021b) characterized the spatial distribution of maternal progenies in a frugivore-generated seed rain of the palm *E. edulis* across landscapes subjected to different levels of frugivore impoverishment and habitat loss. They found that medium-sized birds may play an important role in mixing maternal genotypes where large-sized frugivores have been extirpated, which may explain the lack of effects of habitat loss and fragmentation on genetic diversity.

Some neotropical studies have focused attention on highly modified and anthropogenically managed systems in agricultural or mining settings. Otero-Jiménez et al. (2020), for example, examined gene flow of the rodent *Heteromys desmarestianus goldmani* within a coffee agroecosystem with different management

intensities in Mexico and found that tree cover and slope were the features that best promoted connectivity. Carvalho et al. (2019) assessed the genetic consequences of extreme habitat loss driven by mining in two herbaceous plant species in Brazil (*Brasilianthus carajensis* and *Monogereion carajensis*), accounting for the confounding effects of habitat fragmentation. They conclude that these species are resilient because genetic diversity and gene flow were unaffected by habitat loss and, thus, highlight that we should be careful to not generalize the genetic consequences of anthropogenic-led transformations. Cleary et al. (2017), in turn, evaluated the impacts of expanding pineapple plantations on two frugivores bats with different mobility in a biological corridor in Costa Rica. While the recent expansion of pineapple has begun to disrupt movement and gene flow of the small, less mobile, frugivore (*Carollia castanea*), the biological corridor is effective and facilitates functional connectivity of the larger and more mobile bat (*Artibeus jamaicensis*).

Urban landscapes are among the most transformed and artificial environments to wildlife and, therefore, can impact different dimensions of biodiversity (Grimm et al. 2008). Despite covering 3% of the global land (Grimm et al. 2008), landscape genetic studies in these novel environments are still underrepresented in the literature (Storfer et al. 2010; Fusco et al. 2021). Richardson et al. (2017) evaluated genetic connectivity among Norway rats (*Rattus norvegicus*), a key pest species responsible for seasonal outbreaks of leptospirosis in the tropics, in the city of Salvador, Brazil. Although they had not explicitly correlated landscape and genetic data, they identified that high-traffic roads and topographic relief likely play a role in impeding rat movements with populations structured in home valleys. Informed by the results, the authors were able to identify units of eradication for future intervention campaigns. Other neotropical studies have also used landscape genetics to contribute to conservation and management strategies. Thomassen et al. (2010) map environmentally associated genetic and phenotypic variation in a bird species (*Glyphorynchus spirurus*) in the Ecuadorian Andes and demonstrate the utility of a spatially explicit framework for prioritizing areas for conservation. By using a framework for conservation prioritization based on preserving ecological and evolutionary processes, they found little overlap between currently protected areas and regions predicted to be important in maximizing adaptive variation. Carvalho et al. (2021a) propose a comprehensive landscape genomic approach to assist in the restoration of moderately disturbed and highly degraded sites in mining landscapes in Brazil. Specifically, they identified genotype-phenotype-environment associations, mapped adaptive genetic variation, and predicted the adaptive genotypes associated with the environmental conditions of restoration sites. They found that local provenances were optimal to restore moderately disturbed sites, whereas a mixture of genotypes was seen as the most promising strategy to recover highly degraded mining sites.

The microevolutionary consequences of anthropogenic transformations are central in landscape genetics. Novel features of the Anthropocene are thus considered key drivers of contemporary genetic diversity and gene flow (Manel et al. 2003). However, neotropical studies have also applied landscape genetic and genomic tools to understand the historical underpinnings of phylogeography and speciation. In

these studies, the spatial extent usually encompasses the entire species distribution (i.e. Mastretta-Yanes et al. 2018), while typical landscape genetic studies focus on smaller landscapes of hundreds of km (i.e. Lanes et al. 2018) or replicates of landscapes of 2–5 km each (i.e. Carvalho et al. 2015). Vasconcellos et al. (2019), for example, coupled landscape genomics with paleoclimatic distribution models to understand the consequences of past climate changes on the present-day tropical biota. Specifically, they evaluate whether climatic stability since the Pleistocene explained population differentiation in the treefrog *Hypsiboas lundii*, a species endemic of the South American Cerrado. To test the hypothesis of isolation-by-instability, they parameterized a stability resistance surface depicting the cost imposed by climatic instability due to local changes in environmental suitability over time. Mastretta-Yanes et al. (2018) integrated species distribution modeling for glacial/interglacial cycles, landscape genomic tools, and analyses of demographic history to test the hypothesis that tropical mountains have facilitated the differentiation and *in situ* persistence of alpine-grassland species (*Juniperus monticola* and *Berberis alpina*) from the Trans-Mexican Volcanic Belt during the climate fluctuations of the Pleistocene.

## 7.6 Challenges for Neotropical Landscape Genetics

Historically, scientists from the global south, which comprise many of the neotropical countries, are underrepresented in the scientific literature pertaining to ecology and conservation (Wojciechowski et al. 2017; Maas et al. 2021; Nuñez et al. 2021). In a literature search conducted between 1998 and 2008 approximately 5% of the landscape genetic studies were based in South America, with 39% of the studies being conducted in North America (Storfer et al. 2010). A more recent review on urban landscape genetics also found large geographic bias in the 32 compiled publications, with only one study in South America (Fusco et al. 2021). Even the studies conducted in tropical systems are frequently led or co-led by researchers from the global north. In the bibliometric analysis conducted on neotropical landscape genetics we found that less than half of the multiauthored publications (30 studies, 46%) had, simultaneously, first and last authors based on neotropical countries, with 39 studies first authored and only 31 studies with senior researchers from neotropical institutions. In a crude simplification, first authors contribute the most, whereas last authors are usually the intellectual driving force, providers of mentorship, and funding (Tscharntke et al. 2007). Strikingly, only 24 studies (37%) were conducted solely by neotropical-based researchers. On one hand, this can be the positive outcome of international scientific collaboration, on the other, the underrepresentation of local researchers can configure as "helicopter science" (when researchers from wealthier countries collect data from developing countries and publish results without involving local scientists), which is considered a facet of neocolonialism (Dahdouh-Guebas et al. 2003; Nuñez et al. 2021).

Despite the low relative contribution of neotropical studies and researchers to the scientific literature in ecology, conservation and landscape genetics, the American tropics concentrate most of the world's and tropical biodiversity (Raven et al. 2020; Antonelli 2021) while also suffering from one of the highest rates of land cover and use conversion (Hansen et al. 2013; Curtis et al. 2018). So why are there fewer publications conducted in neotropical systems? Nuñez et al. (2021) and other recent studies have been raising awareness of geographic biases in the ecological literature and providing recommendations to increase representation at the global scale. We will not discuss in detail all the barriers and possible solutions here, instead we will highlight the issues that are more prominent for ecological studies that rely on genetic and molecular analysis.

Apart from the language barriers (Valenzuela-Toro and Viglino 2021; Khelifa et al. 2022) and cultural legacy, considering that traditional scientific endeavors initiated in the global north, there are other factors that most likely influence this common geographic bias in publications in landscape genetics. The first, and possibly the most obvious, is the amount of funding and infrastructure for conducting science and conservation (Ciocca and Delgado 2017; Valenzuela-Toro and Viglino 2021). Indeed, wealthier countries with a higher gross national income and higher Research & Development budget allocation produce more ecological studies in general (Martin et al. 2012). This is particularly relevant for laboratory and equipment dependent research, such as landscape genetics, for it requires biotechnology and specialized reagents and supplies. Many public universities, where most of the scientific research is conducted in the Neotropics, do not possess the latest technology such as high-throughput sequencers given that acquisition costs are prohibitive even for well-funded projects and because of lack of specialized personnel to manage these platforms (Ciocca and Delgado 2017). In these situations, researchers either employ a less modern but cheaper technique, which may result in less impactful science, or choose to outsource the biomolecular and bioinformatics analysis to international service providers, being susceptible to unfavorable and often unstable currency conversion rates. Likewise, many supplies are not produced locally and need to be imported as well. On top of transaction costs and associated unpredictability, shipping biological material and importing supplies from abroad demand that administrative and bureaucratic procedures are also taken care of by the researcher. These hurdles combined make genetic-based studies more uncertain and riskier and certainly contribute to slower productivity and fewer publications over time by neotropical scientists.

A second challenge that factors in is field and molecular sampling. Landscape genetics focuses on intraspecific variation, usually at the individual or population level, and, for that, it requires robust experimental design to properly sample spatial, genetic, and environmental distributions. This means that a sufficiently large sample size is necessary to conduct a strong study. The Neotropics are mega biodiverse (Antonelli et al. 2018; Pillay et al. 2022) and, as such, communities often present a skewed species frequency distribution, with few hyperabundant species (e.g., plants, ter Steege et al. 2013). In this scenario, it becomes very challenging to set up a study design that is both ecologically and logistically feasible if the focal species is

naturally rare or threatened to extinction, habitat specialized with limited distribution, cryptic, or with erratic occurrence in space or time. Frequently, researchers that aim at a landscape genetic approach end up conducting a population genetic study due to insufficient sampling of populations, landscape, or spatial variation. Additionally, tropical systems are subjected to more rainfall, heat, humidity, higher ultraviolet levels, and microbial activity. All of these contribute to more contamination and faster degradation of nucleic acids in the environment, particularly when samples are collected in a non-invasive manner. This issue results in lower yields and quality of isolated DNA and higher failure rates during sequencing and amplification of molecular markers, further decreasing the sample size.

Finally, another reason that complicates landscape genetic studies is availability and access to environmental and landscape remote data. In the last few years there has been an improvement in data availability in Latin America (e.g., MapBiomas in Brazil, Souza et al. 2020) and an effort to provide data at the global scale (Hansen et al. 2019). However, compared to North America or Europe, we still lack access to spatial and temporal high resolution environmental rasters, especially for biomes that span multiple countries such as the Amazon.

## 7.7 Opportunities for Neotropical Landscape Genetics

The opportunities for neotropical landscape genetic studies are partially due to the fact that there are still very few studies relative to its enormous biodiversity. Landscape genetics offer a useful tool set to understand patterns and processes of biodiversity and, therefore, it is a powerful approach to better understand the ecology and evolution of many dominant or poorly known species in rapidly changing and dynamic landscapes. As previously mentioned, georeferenced environmental maps are becoming increasingly available in Latin America. Likewise, there are global and regional efforts to make the occurrence and spatial distribution of species from different taxa publicly available (e.g., ATLANTIC: Data Papers from a biodiversity hotspot). Having access to both types of data sets is paramount to delineating a powerful study design for sample collection, assuring that enough environmental, spatial and population variation are sampled and that confounding factors, such as habitat loss and fragmentation metrics (Fahrig 2003), are avoided.

In particular, local adaptation plays a major role in species capacity to persist in changing environments. By using landscape genomics, it is possible to detect significant variations in allele frequency at specific loci that could be directly or indirectly associated with phenotypes that influence individual fitness and, therefore, local adaptation through natural selection. Despite its usefulness, adaptive landscape genomics is still incipient in Latin America in general (Santos and Gaiotto 2020). This approach holds great promise for fully understanding the contribution of different processes such as gene flow and natural selection on diversity and structure of native populations. Moreover, it can provide the first step to pinpointing

important genomic regions that could explain phenotypic variation and fitness, with real implications for conservation measures.

Another aspect worth delving into in the neotropics is the genetics of biotic interactions. Biotic interactions are considered stronger and more important towards the equator and, thus, follow the latitudinal diversity gradient, with several hypotheses posing that those interactions are driving forces of species diversity in the tropics (Schemske et al. 2009 and references therein). There are multiple molecular ecology approaches to studying biotic interactions (Symondson and Harwood 2014; Roslin et al. 2019) and landscape genetics could contribute by dissecting the influence of environment and space on the spatial and temporal patterns of interacting species which holds consequences for local adaptation, fitness, trait correlations, and intrapopulation diversity. Likewise, landscape community genetics (Hand et al. 2015) is a forefront in the field that should be explored in the neotropics. Again, a combination of molecular approaches, such as metabarcoding associated with population genomics, could inform about the interplay between assembly of species-rich communities, interaction networks, and population-level diversity in a spatial context.

## 7.8 Final Remarks

Considering that landscape genetics is an interdisciplinary field it is important that neotropical scientists establish collaborations to combine expertise on different fronts, including population genetics, geographical information system and geotechnologies, spatial and statistical analysis, and ecology of the study species. International networks should be established and nurtured among institutions in the American tropics, especially when habitat or species geographical range studied spans multiple countries. Researchers should have a clear understanding of what landscape genetics entails so that the proper tools are employed, and appropriate reporting is done in publications.

The steady increase in publications of neotropical landscape genetics is already an indication that the field will soon be blooming in the region and, therefore, we expect to see a consolidated body of literature and expertise in the near future. The resulting knowledge will provide the grounds for comparison with other ecoregions, contribute to analytical and conservation methods that are more suitable for tropical areas and, most importantly, generate novel ecological and evolutionary hypotheses relevant to mega biodiverse, yet human-modified, landscapes.

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# Chapter 8
# Integrative Cytogenetics, A Conservation Approach in Atlantic Fish: Concepts, Estimates, and Uses

**Wagner Franco Molina, Clóvis Coutinho da Motta-Neto, and Gideão Wagner Werneck Félix da Costa**

## 8.1 Introduction

The genes modulating the organismal adaptability are located on the chromosomes. Thus, the detection of cytogenetic changes is an integral part of genomic analysis and decisively contributes to the understanding of the evolutionary process. Metaphasic chromosomes consist of two chromatids, each one representing a chromatin fiber associated with histone proteins and anchored in a proteinaceous scaffold. These structures are large, orthologous syntenic regions that retain the extensive changes occurring in the genome, due to evolutionary processes that design and model genetic variations. The chromosomes relate to population variability and biological diversification processes. Since chromosomes are more than DNA molecules, they play an important role in segregation dynamics as gene carriers and as key points in the equalization of genetic material between two daughter cells.

Casualty and adaptability are evolutionarily involved in the origin and inheritance of chromosomal rearrangements. Karyotypic alterations can change the fitness of individuals, affecting gene flow patterns and promoting speciation. Thus, karyotypic aspects of the species are also associated with the historical changes (Fig. 8.1) that shape their geo-dispersion, the environment, and the set of evolutionary factors (migration, mutation, genetic drift, natural selection) involved in the diversification of groups.

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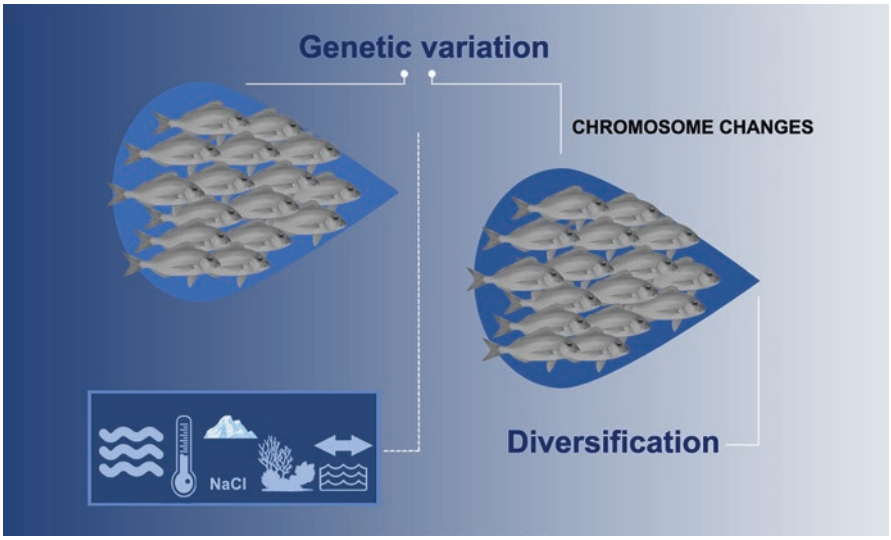


**Fig. 8.1** Variations in organization and structure of chromosomes of marine fish can be associated with environmental and biological factors, and can act in the maintenance of gene flow, adaptation, and speciation processes

## 8.2 Marine Environment and Fish Chromosome Diversification

The distribution of marine fish groups is historically modeled by a mixture of vicariant and ecological processes on a large scale (Floeter et al. 2008). They interfere with gene flow in populations and fix chromosomal variations between oceanic regions (Amorim et al. 2017), with parapatric karyomorphic distributions being the result of secondary contact (Accioly et al. 2012). Chromosomal variations result in the amplification of adaptive aspects of species (Liu et al. 2017; Berg et al. 2017; Yuan et al. 2018; Kess et al. 2020) and are linked to the diversification of fish groups in the marine environment (Affonso et al. 2014; Almeida et al. 2017).

The marine environment, by extending to over about 70% of the planet's surface, decisively influences the climate (Koutavas 2012). At first glance, it may seem that there is a uniform distribution of the oceans in relation to the Equator; however, while 81% of the Southern Hemisphere is covered by the oceans, they cover only 61% of the Northern Hemisphere.

In the marine environment, biogeographic barriers are determined by physical oceanographic factors (currents, barriers, temperature, salinity, etc.) and biological conditions (physiological, dispersal, and ethological adaptations) that modulate the distribution of species and influence gene flow between regions (Grant and Bowen 1998; Lessios et al. 1999; Lecchini and Galzin 2003). In this context, biogeographic regions are defined by taxonomy, species composition, and endemism rate (Bowen et al. 2016; Toonen et al. 2016). At least 53 biogeographic regions are recognized in

the oceans, distributed into 30 biogeographic domains (Toonen et al. 2016; Costello et al. 2017). Reef fish are models for delimiting oceanic biogeographic regions due to their well-defined geographical distribution and taxonomy (Floeter et al. 2008; Kulbicki et al. 2013).

The level of effectiveness and the evolutionary role of biogeographic barriers in marine environments have been established through genetic comparisons between populations and species divided by these barriers (Rocha 2003; Bowen et al. 2013; McClain and Hardy 2010). In the Atlantic, some species maintain panmictic populations over thousands of kilometers, while others exhibit genetic multipartition in small, more restricted geographic areas (Shaw et al. 2004; Vasconcellos et al. 2008; Silva et al. 2015), until the speciation process is fully complete (Rocha 2003).

A set of abiotic and biotic oceanographic factors delimits the biogeographic regions of the Brazilian coast, Caribbean, Mid-Atlantic Ridge, and the western Atlantic (Floeter et al. 2008). These biogeographic regions are limited by five well-defined biogeographic barriers: Mid-Atlantic Ridge (Briggs 1974), Red Sea Land Bridge (Steininger and Rögl 1984; Bellwood and Wainwright 2002), Isthmus of Panama (Coates and Obando 1996), Benguela Current (Shannon 1985, Marlow et al. 2000), and the plume formed by the Amazon and Orinoco rivers (Hoorn et al. 1995).

Especially relevant with the biogeographic regions, the Provinces of Brazil and Caribbean are influenced by the freshwater plume and sediments formed by the Amazon and Orinoco rivers, which have intermittent or semi-porous effects on the gene flow between these regions (Rocha 2003) (Fig. 8.2). This is corroborated by

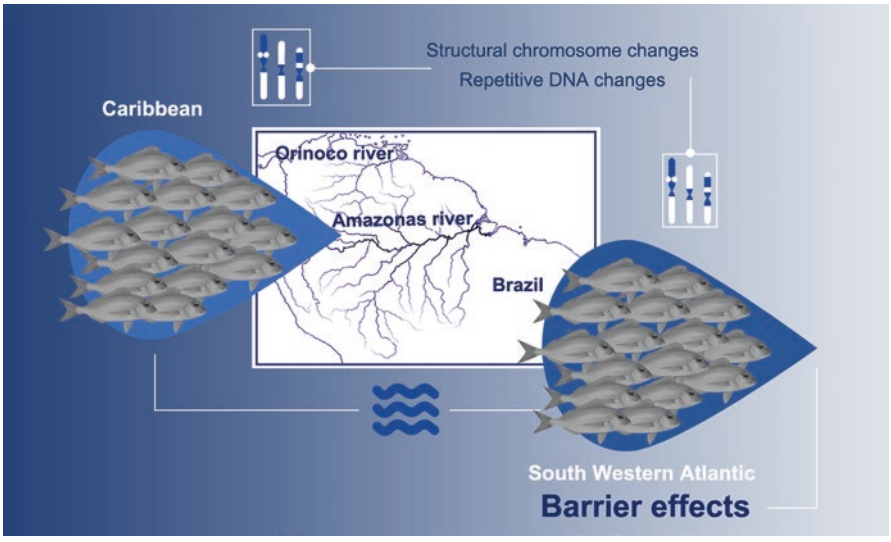


**Fig. 8.2** Biogeographic barriers, such as the Amazon River plume, show varied effects on the gene flow of marine fish groups in the Atlantic. In some groups, the lineage subdivision promoted the fixation of conspicuous karyo-evolutive divergences

the species distribution found on the Brazilian and Caribbean coasts, and by related species observed in both regions (Floeter and Gasparini 2000; Rocha 2003; Floeter et al. 2008).

One of the examples of phylogenetic split probably caused by the discharge of Amazon River/Orinoco waters is observed in the genus *Gramma* (Grammatidae), a small genus of the western Atlantic with only five known species (Froese and Pauly 2019; Fricke et al. 2020). Two of these species, *Gramma loreto*, which occurs in shallow waters and deep reefs of the Caribbean, and the Brazilian sister species *Gramma brasiliensis* (South western Atlantic) are very similar and allopatrically divided by the river plume, between 11.8 and 11.3 Mya, in the mid and late Miocene (Figueiredo et al. 2009). They present striking morphological similarities and a blue and yellow pattern, sharing homologous rDNA-bearing chromosomes. However, they display conspicuous structural chromosomal changes due to geographic isolation. In fact, *G. loreto* (2n = 48, and fundamental number, FN = 58), located in the center of family dispersion, presents a more conserved karyotype pattern based on the basal Percomorpha pattern, while *G. brasiliensis* (2n=48, FN=60), the only species in the South Atlantic, differs by the presence of a pericentric inversion and by a higher GC-rich heterochromatin content (Molina et al. 2012a). These phylogenetic and cytogenetic divergences are clearly associated with the barrier effect, however it may not manifest in many other Percomorpha groups (Molina 2007).

The marine environment has peculiarities in the biological diversification of its main vertebrate component, the fish. Specific physical and biological factors govern these regions by promoting numerous hotspots with an extraordinary number of species compared to less diverse regions. From a geographical point of view, the oceans have always been under a slow and constant change due to tectonic events (Rea et al. 1990), ocean floor remodeling, recurrent glaciations (Goldner et al. 2014), promoting waves of population expansion and contraction (Souza et al. 2015). These events affected population dynamics and influenced the chromosomal patterns.

In a scenario of environmental continuity, high dispersal potential, and high population density, several marine groups show a common avoidance response to chromosomal changes. This process, known as karyotype stasis, is demonstrated by the extensive sharing of a basal karyotype, through tens of millions of years (Molina et al. 2014a). Low chromosomal divergence causes quite homogeneous and symmetrical karyotypes (e.g., small size difference between the smallest and largest karyotype chromosomes), with repetitive DNA regions varying little among species, genera, families, and orders (Molina et al. 2014a; Motta-Neto et al. 2019).

In contrast, cases of karyotypic diversification include some fish groups with low vagility, less dispersal potential, and reduced effective population size that can present intense chromosomal reshuffling processes (chromoanagenesis). These conditions are well illustrated in Gobiiformes (Ene 2003; Lima-Filho et al. 2017), which have high chromosomal evolution rates (Molina et al. 2014a) associated with the species diversification process itself (Lima-Filho et al. 2016). Speciation in these groups occurs not only due to the accumulation of changes in DNA composition, but also due to physical rearrangements in divergent genomes (Nosil and Feder 2012).

Evolutionary changes in marine fish lineages reveal phylogenetically shared traits, with stochastic chromosomal rearrangements prevalent in some groups (Molina and Galetti Jr 2004; Getlekha et al. 2016, 2018) occurring solely or in concert (Molina and Galetti Jr 2004; Affonso et al. 2014) and derived from meiotic events, which cause specific rearrangements (Molina et al. 2014a, b; Sodeland et al. 2016).

Intra- and interpopulation chromosomal variability in marine fish promotes diversity in protein profiles and DNA sequences, thereby reaching new adaptive norms. Considering the different rates of changes among chromosomal regions in eukaryotic chromosomes (Ruiz et al. 1997; Renaut et al. 2012), the comparison of a significant spectrum of genomic sequences through fluorescent *in situ* hybridization protocols (FISH) increases the chances of detection of variations involving large structural and functional areas of the genome (Phillips and Reed 1996).

The most frequent gene regions used to study variations between populations and species are 18S and 5S rDNA sequences, histone genes (H1, H2B, H2A, H3 and H4), microsatellites, transposons, and retrotransposons. When combined with structural chromosome patterns, they reveal conspicuous intra and interspecific cytogenetic signatures.

Both the independent and concerted evolution of these repetitive chromosomal sequences (Merlo et al. 2012), is not yet fully understood in most fish species. However, cytogenomic architecture comparisons within and between marine fish lineages have proved to be crucial to understanding their implications for the process of evolutionary divergence.

## 8.3 Chromosomes Under the Aegis of Evolutionary Factors

Evolutionary factors involved in the spatial structuring of genetic diversity are one of the main pillars of conservation genetics. Gene flow, mutations, genetic drift, and natural selection act on the chromosomal patterns of fish (Matoso et al. 2002; Soares et al. 2014; Berg et al. 2017; Costa et al. 2019). In fact, part of the phenotypic expression of organisms is enclosed in their chromosomes, with characteristics associated with the processes of biological variation and diversification (Fig. 8.3) represented in different syntenic gene groups (i.e. groups of genes located on particular chromosome segments).

Population cytogenetic approaches, aimed at an estimation of the occurrence of chromosomal polymorphisms in marine fish, should consider larger and more geographically diverse samples. In this context, chromosomal markers are used in marine fish to indicate barriers to dispersion, quantify diversity, characterize invasive species, and estimate patterns of change in historical and contemporary scenarios. The occurrence of chromosomal polymorphisms, in general, constitutes a contemporary condition and identifies the initial steps in the process of karyotype divergence.

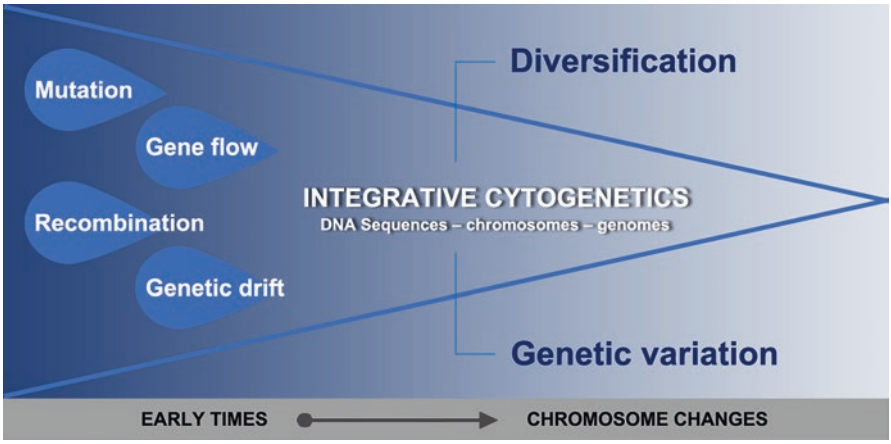


**Fig. 8.3** Integrative cytogenetics refers to the analysis of evolutionary factors on chromosomal patterns of marine fish species, and their genetic variations over time

Conspicuous cytogenetic variations within and between populations are often found in neotropical freshwater species, due to a markedly fragmented spatial distribution, which promotes various population sets (Moreira-Filho and Bertollo 1991). The accumulation of genetic divergences between small populations of continental fishes, by action of the multiple evolutionary factors (i.e. natural selection, genetic drift, mutation, etc.), can quickly drive it forward to speciation (Matoso et al. 2002; Costa et al. 2019). Similarly, marine species with populations showing restricted gene flow due to biological, ecological, or abiotic factors may show greater karyotype diversification. These groups represent models particularly appropriate for understanding the microevolutionary changes in a biogeographic scenario, including estimation of contemporary gene flow and other evolutionary processes during phylogenetic divergence.

In general, gene flow restrictions are related to spatial barriers enhanced by the physiographic complexity of the environment (marine currents, marine relief, thermoclines, etc.) or ocean spaces (Cunha et al. 2014), specific ecological scenarios (Oliveira et al. 2014), or low potential of dispersion of the species associated with short larval pelagic period or low migration capacity of adults (Neves et al. 2016).

The numerous marine environments, structurally multidimensional due to the intervention of marine currents, temperature, depth, physical and ecological barriers, and ecological interactions, act differently on gene flow and chromosomal diversification. Such diverse environmental features give rise to a more integrative than a punctual view of the cytogenetic analysis of marine fish diversity. These complex environments often give opportunity to apply population and phylogenetic approaches and studies of divergence rates, considering the geographical context of the species and their populations. The integrative cytogenetics applied to the understanding of microevolutionary processes, such as gene flow, allows inference on the role of barriers in population delimitation and biological patterns that govern

evolutionary change rates, both with implications to biological conservation in the marine environment (Fig. 8.4).

About 200 fish species, representing 6% of the karyotyped species, have differentiated sex chromosome systems (Arai 2011). Sex chromosome systems in marine and estuarine fish are apparently not correlated with spatial distribution patterns, occurring in fish groups from polar to tropical environments in both hemispheres (Ghigliotti et al. 2013; Lima-Filho et al. 2014). In these groups, sex chromosome systems may result from independent evolutionary events (Ueno and Takai 2008; Zhang et al. 2018) or as demonstrated for various groups as a shared character (Ozouf-Costaz et al. 1991; Soares et al. 2014). The origin of heteromorphic sex chromosomes has important evolutionary consequences, ranging from phenotypic divergence and reproductive isolation of sympatric species (Kitano and Peichel 2012), to adaptation (Chen et al. 2014), and biological conservation.

Sex determination can be polygenic due to cumulative effects of genes located in autosomes, a condition that can persist until a dominant factor of sex determination is established on the W or Y chromosomes (Moore and Roberts 2013; Bachtrog et al. 2014). The absence of heteromorphic sex chromosomes in most fish species indicates that the acquisition of a sex-determining allele on specific chromosomes is a rare event (Graves 2008; Moore and Roberts 2013). However, linkage groups can differentiate from a pair of autosomes, where one of the homologous acquires a sex-determinant locus, whose alleles confer some advantage to one sex and a disadvantage to the other (Adkins-Regan and Reeve 2014) favored by the restriction of this

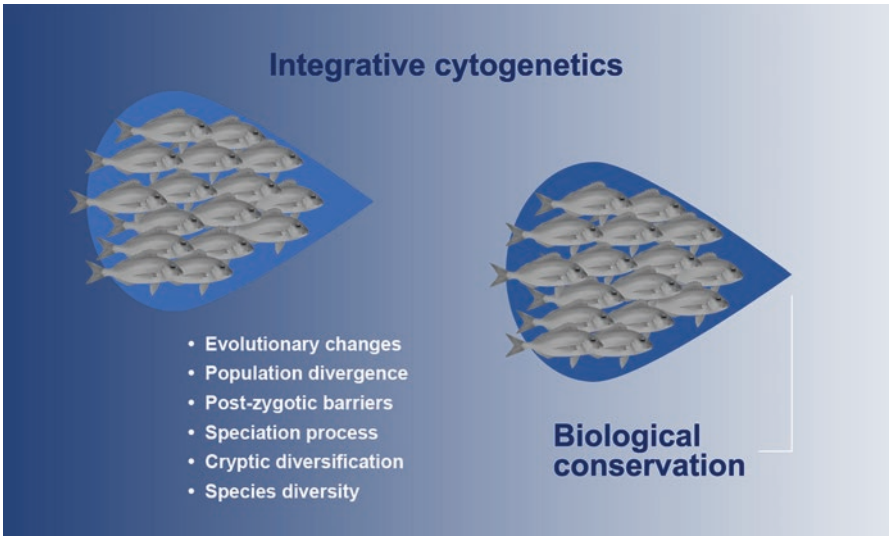


**Fig. 8.4** Integrative cytogenetic analysis through comparison of multiple chromosomal regions within populations and species under different environmental conditions allow access multiple evolutionary aspects of species

locus to one sex by recombination suppression (Devlin and Nagahama 2002; Bachtrog 2006; Graves 2008).

Inversions, centric fusions, and heterochromatinization processes participate in the differentiation of sexual systems in fish, including marine groups (Salvadori et al. 2009; Lima-Filho et al. 2014; Peichel et al. 2004; Natri et al. 2019). The absence of recombination leads to retrotransposable element invasions in sex chromosome regions, promoting marked amplification of repetitive sequences (Charlesworth et al. 1994; Volff et al. 2007), especially in the early stages of chromosome evolution (Volff 2006; Chalopin et al. 2015a, b).

Despite occurring in a low frequency, sex chromosome systems in fish are extremely diverse, showing both male and female heterogamety in single or multiple systems (Almeida-Toledo et al. 2000a, b). The lability of sex chromosome determination in fish is revealed by the presence of well-established or nascent sex chromosomes (Darolti et al. 2019), as well as by distinct systems between close related species or even between populations of the same species (Moreira-Filho et al. 1993; Devlin and Nagahama 2002; Bertollo et al. 2004) and by female and male heterogamety in the same phylogenetic group (Salvadori et al. 2009).

The fixation of sex chromosomes plays a decisive role in the reproductive isolation and permanent individualization of lineages (Barske and Capel 2008; Kitano et al. 2009; Kitano and Peichel 2012). The minor incidence of sex chromosome systems in fish reduces the effects of the Haldane rule — "when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous (heterogametic) sex" (Haldane 1922; Schilthuizen et al. 2011) — in this group, contributing to a long acquisition period of post-zygotic reproductive isolation (Russell 2003). Thus, anthropic actions promoting contact between allopatric species, phylogenetically close or with incipient diversification, can be harmful by the high risk of hybridization and genetic introgression.

In fish, gonochorism precedes hermaphroditism (Pla et al. 2020) as a reproductive strategy. Hermaphroditism arise independently in this animal group and this polyphyletic condition is present in about 2% of extant teleost species, involving more than 20 taxonomic families (Avise and Mank 2009). Several clades showing hermaphroditism as a preferred reproductive strategy generally show no morphologically differentiated sex chromosomes. In fact, large groups like Labridae, which exhibit gonochoristic and protogynic strategies, and bi-directional sex changers — Pomacentridae: gonochoristic and protogynic; Serranidae: gonochoristic, protogynic, and hermaphrodite synchronic; and Sparidae, which have gonochoristic species and both types of sequential hermaphroditism — present no or very few reports of chromosome sexual systems among their species (Arai 2011; Zhang et al. 2018). Given its evolutionary relevance, this correlation deserves more detailed analyses.

## 8.4 Karyotype Evolutionary Processes

Many key questions about the biodiversity of fish species, especially in marine environments, arise due to incessant human interference in the aquatic ecosystems. One of the basic pillars of conservation is the query, "how many species are actually present in these environments?" Even with interdisciplinary efforts, the answer remains largely incipient, considering the increasing number of new descriptions (Fricke et al. 2020).

Molecular markers have revolutionized the evolutionary approaches applied to biological conservation (Ferraris and Palumbi 1996). Complementarily, chromosomal analyses have offered varied approaches to understanding the evolutionary aspects of fish groups (Lima-Filho et al. 2016; Nirchio et al. 2017).

The evolutionary history of life forms can be described through three main evolution processes: anagenesis, cladogenesis, and reticulate diversification. Anagenesis, or phyletic evolution, consists of the gradual differentiation of one species into another through the establishment of new characteristics. Thus, the ancestral species is succeeded by a derived form without temporal coexistence, and reproductive isolation between species is not a concern, since the change is gradual and the isolation between them is strictly temporal. In the second process, cladogenesis, a species is divided into two lineages, and the ancestral species may initially coexist with the derivative, requiring the existence of pre- and post-zygotic isolation in case of sympatry to ensure that each species maintains its specific chromosomal and genetic characteristics (Stuessy and Hörandl 2014; Allmon 2017). In contrast, reticulate evolution promotes the formation of a new species resulting from the genetic introgression of two species (Fig. 8.5). In this latter case, some level of reproductive isolation is established between the hybrids and the parental species, followed by proper adaptive processes (Mallet et al. 2015; Gallardo 2017). Hybridization processes in the marine environment is frequent (Montanari et al. 2016; Tea et al. 2020), but less directly associated with speciation processes (Rocha et al. 2008; Litsios and Salamin 2014), and must have contributed to the interspecific transfer of genes in several groups.

The huge morphological diversity found in fish species is often associated with accumulation of cytogenetic modifications (Affonso et al. 2014), including a high divergence in the diploid numbers ranging from 2n = 12 to 2n = 446 (Arai 2011). In addition, fish show an extraordinary variation in genomic content, ranging from 0.7–266 pg (Gregory and Mable 2005), constituting the smallest and largest known genomes for vertebrates, respectively.

Large genomes tend to present specific ecological tolerances, and in some groups, such as plants, birds, amphibians and mammals, their size is negatively associated with the metabolic rate or speciation (Kozłowski et al. 2003; Knight et al. 2005; Lertzman-Lepofsky et al. 2019; Grzywacz and Skórka 2021). However, the evolutionary significance of this variation in fish is not yet well understood (Hardie and Hebert 2004).

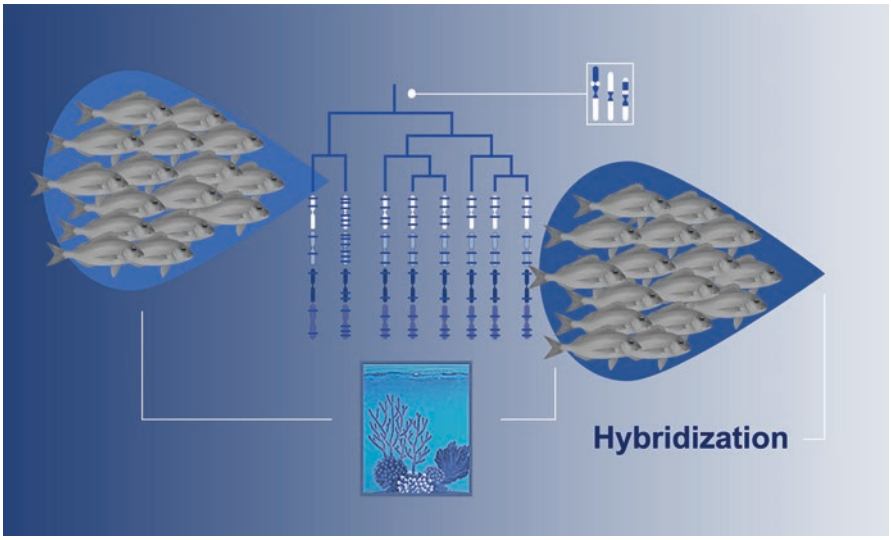


**Fig. 8.5** Numerical and structural chromosomal conservatism resulting from karyotypic stasis processes may provide lower post-zygotic block levels in some marine fish groups (e.g., Chaetodontidae, Pomacanthidae), facilitating remarkable processes of natural hybridization under favorable conditions (species sympatry or recontact, overlapping spawning areas, and sex ratio distortions between closely related species)

Of the mechanisms in the karyotypic and genomic diversification of fish, polyploidy plays an important role, acting on phenotypic complexity, diversity, and evolutionary novelties. Phylogenomic analyses indicate that, during the evolution of vertebrates, the whole genome went through two rounds (2R) of whole genome doubling (WGD) (Smith et al. 2015). Around 350 Mya, the fish experienced a third round, known as fish-specific genome doubling (FSGD or 3R), which occurred in the ray-finned fish (Actinopterygii) lineage after its separation from tetrapods (Jaillon et al. 2004; Meyer and van de Peer 2005; Pasquier et al. 2016).

Polyploidy may occur sporadically in one or few individuals of a population, in some species, and can evolve to post-zygotic reproductive isolation and lineage diversification. Interspecific hybridization, with the fusion of two or more genomes (allopolyploidy) (Alves et al. 2001), or genome duplication in a species or population (autopolyploidy) (Liu et al. 2017) are associated with the origin of polyploidization events in fish. In fact, changes in the number of whole chromosome set in hybrids, generating reproductive barriers with the parental species, can result in polyploid speciation (Gregory and Mable 2005).

Stochastic polyploid events are frequent in continental fish groups, but apparently absent in exclusively marine groups, in which small variations in abiotic factors, large population numbers, and reduced amount of gene flow barriers possibly provide fewer opportunities for polyploid deme fixation.

Among the marine fish groups, meta-analysis approaches have distinguished groups with slow, intermediate, or accelerated karyotypic evolution rates (Molina

et al. 2014a). Some groups of vertebrates, such as cats, seals, certain primates, some marsupials, distinct lineages of birds (King 1993; Sumner 2003), and several groups of fish (Molina 2007), reveal only inconspicuous karyotypic changes. Karyotypic stasis occurs through extensive sharing of the ploidy level, chromosomal number and structure, and genomic organization along the phylogenetic differentiation (Kahl 2015).

In Percomorphs, some groups show varying degrees of conservatism in relation to a karyotype considered basal, composed of 48 chromosomes formed mostly or entirely by acrocentric elements (Ozouf-Costaz et al. 1997; Brum and Galetti Jr 1997; Galetti Jr et al. 2000). Processes of slow chromosome divergence over long periods of time characterize a bradytelic evolution, whose resulted conservatism is characterized by only subtle variations in the organization of repetitive sequences, as in Lutjanidae (Costa et al. 2016) and Haemulidae (Motta-Neto et al. 2011a, b, 2019).

Bradytelic karyotype evolution patterns have been more precisely investigated in the Eupercaria series (Molina 2007; Molina et al. 2014a; Motta-Neto et al. 2019), one of the nine Percomorpha series. Eupercaria is the most diverse group, composed of >6,000 species and 163 families (Hughes et al. 2018), with long periods of divergence starting in the Late Cretaceous (Alfaro et al. 2018). Basal karyotypic characteristics are shared by 88% of Eupercaria clades, from the oldest to the most recent, including large orders, such as Perciformes, Gerreiformes, Labriformes, Centrarchiformes, Chaetodontiformes, and Spariformes (Calado et al. 2013; Molina et al. 2014b; Costa et al. 2016; Motta-Neto et al. 2019). The species of this group are distributed over extensive oceanic regions (Nelson et al. 2016), becoming an excellent model to analyze bradytelic karyotype evolution under space-time contexts.

In Eupercaria clade, most species share a stable karyotype composed of 2n = 48 acrocentric chromosomes, reduced heterochromatic and mainly centromeric content, besides the presence of one chromosome pair carrying the nucleolus organizing region, with small variations (Galetti Jr et al. 2000; Motta-Neto et al. 2011a, b, 2012; Calado et al. 2014; Costa et al. 2016). This evolutionary pattern indicates a high level of chromosomal synteny, sharing orthologous organizations from different classes of DNA, often inferred through replication bands and genomic analyses of linkage groups (Ellegren 2010; Zhang et al. 2019).

Slow evolutionary rates in the linkage groups are likely to arise in fish groups historically distributed in stable and geographically poorly stratified environments, formed by large ecological continuums, where large panmictic populations occur (Motta-Neto et al. 2019). Additionally, aspects of the chromosomal composition of certain fish groups (intrinsic factors, e.g., transposable elements, multigene families, microsatellites) may play some role in chromosomal evolutionary dynamics (Motta-Neto et al. 2019). Under diametrically opposite conditions, groups with continental distribution, formed by small populations, stratified by geographical barriers, with reduced or absent gene flow, and subject to diverse environmental conditions have propitious conditions for fast and intense karyotypic diversification (Moreira-Filho and Bertollo 1991; Costa et al. 2016).

One of the evolutionary consequences of karyotype stasis may be the slow acquisition of post-zygotic reproductive isolation (Molina et al. 2013), leading to the extensive occurrence of natural hybrids in many families, such as Chaetodontidae, Centrarchidae, Carangidae, Lutjanidae (Koppelman 1994; Murakami et al. 2007; Randall 2007; Batista et al. 2012), among others.

Some groups of marine fish share numerous cytogenetic characteristics with the known basal karyotype associated with evolutionary novelties, indicating an intermediate rate of chromosomal changes. This horotelic pattern, with intermediate rate of changes, is shown in speciose families such as Labridae, Serranidae, and Acanthuridae (Affonso et al. 2014; Amorim et al. 2017; Almeida et al. 2017; Fernandes et al. 2021). In fact, 60% of the Serranidae species have Percomorpha-like karyotypes, but a significant number of karyotypes demonstrates cytogenetic discrepancies involving the organization of repetitive sequences (18S rDNA, 5S rDNA, transposable elements (TEs) and microsatellites (SSRs) associated with variations resulting from pericentric inversions (Amorim 2020). This pattern indicates that extensive synteny still occurs, but with micro- and macro-structural karyotypic diversification processes established during expansion and speciation events in the group. In Labridae, the most abundant group of tropical reef fish, with more than 600 species (Fricke et al. 2020), a peculiar chromosome variation occurs, which reflect different evolutionary histories deeply associated with coral reefs (Floeter et al. 2017). Labridae exhibits a gradation of karyotype structure patterns varying in 2n and FN (number of chromosomal arms) values, revealing a moderate to high rate of chromosome changes based on pericentric inversions, fissions and fusions (Alvarez et al. 1986, Ueno and Takai 2000, Amorim et al. 2016, Almeida et al. 2017). Karyotype divergences in the distribution of 5S and 18S ribosomal DNA sequences within this family have been associated with these changes in the karyotype macrostructure (Amorim et al. 2016, 2017).

Contrastingly, other Percomorpha groups have high rates of chromosome changes, characterizing a tachytelic evolution. Some marine groups, such as Gobiiformes, exhibit extraordinary diversity (>2,200 species) and a remarkable rate of karyotype evolution (Amores et al. 1990; Molina et al. 2014a, b). Physical characteristics of the marine environment, use of specific niches, or intrinsic biological characteristics of the species, which promote ecological and geographical fragmentations, may be associated with these marked chromosome change processes (Galetti Jr et al. 2000; Lima-Filho et al. 2012; Auvinet et al. 2020). Such huge diversity includes the diploid number (2n = 30 to 56), karyotype macrostructure (Vasiliev and Grigoryan 1993), diversification in the composition and distribution of repetitive sequences, and sex chromosome occurrence (Lima-Filho et al. 2012, 2014, 2017).

Several mechanisms participate in the karyotypic diversification in marine fish groups, mostly pericentric inversions (Galetti Jr et al. 2006). In the family Pomacentridae, pericentric inversions modulate very distinct karyotype patterns among subfamilies and species (Molina and Galetti Jr 2004; Getlekha et al. 2018). Although the importance of paracentric inversions in fish is largely underestimated by cryptic detection conditions (Borges et al. 2019), pericentric inversions stand out

for involving large portions of the genome (Getlekha et al. 2018), and for its well-known association with adaptive processes (Wellenreuther and Bernatchez 2018).

Tetraodontiformes (Eupercaria) represents another group with marked species diversity, whose divergence processes were associated with a wide range of chromosome changes. This group includes species with the lowest DNA content per cell among vertebrates (Fischer et al. 2000; Jaillon et al. 2004). Its families have marked variations in the number of chromosomes, such as Triacanthidae (2n = 48, FN = 48-50), Balistidae (2n = 40 to 46, FN = 40 to 48), Monacanthidae (2n=33 to 40, FN=34 to 40), Ostraciidae (2n = 34 to 50, FN = 48-54), Tetraodontidae (2n = 28 to 46, FN = 36 to 72), and Diodontidae (2n = 46 to 52, FN = 58 to 68) (Arai 2011). A combination of specific chromosome rearrangements, such as centric fissions and fusions, pericentric inversions, and variation in the amount of heterochromatin (Brum et al. 1995; Noleto et al. 2007; Martinez et al. 2011), are associated with the distinct karyo-evolutive tendencies of the group (Sá-Gabriel and Molina 2005).

In summary, the karyotype patterns of marine fish are modeled by complex factors, resulting from the synergistic action of attributes inherent to the chromosomes, simultaneously with their biological characteristics and the geographical conditions in which the species occur (Molina 2007; Molina et al. 2014b). In addition, the increased knowledge on the organization of different repetitive DNA sequences, such as SSRs and mobile elements, among others, has been allowing us to understand the evolutionary dynamics of these sequences and its association with the chromosome differentiation (Artoni et al. 2015; Costa et al. 2015). Therefore, the enlargement of the phylogenetic spectrum and mapping distinct classes of repetitive DNA, enclosing large genome portions, have been providing a reliable scenario for the comprehension of chromosomal evolution of this group.

## 8.5 Population Cytogenetics

Chromosome changes in the population level can have important evolutionary consequences related to adaptability and speciation, and even though several marine groups show a high chromosome stability, numerical and structural karyotype variations among populations can occur. In this sense, the knowledge on the interpopulation chromosome variation can pose a way to reduce the considerable threat of the translocation practices involving specimens between geographically distant regions.

One of the most conspicuous intraspecific cytogenetic variations is the occurrence of varying numbers of chromosomes within and between marine fish populations. The numerical chromosome variations in the marine fish populations are largely dissociated of euploidy processes (involving whole sets of chromosomes), have a little participation of B chromosomes, but are highly represented by numerical and numerical-structural polymorphisms (involving one or few chromosomes).

### *8.5.1 Supernumerary Chromosomes*

Supernumerary or B chromosomes are facultative chromosomes whose occurrence may vary between individual cells, individuals in the same population, or populations. They occur in approximately 15% of eukaryotes (Camacho et al. 2000), of which 5% are fish species (Oliveira et al. 2009).

These chromosomes origin from A (autosome) chromosomes (Artoni et al. 2006; Jones 2018) and can have a common phylogenetic origin in related species (Melo et al. 2017). As a facultative component of the genome, they present remarkable evolutionary variation and have been used in interpopulation analysis in several neotropical groups (Oliveira et al. 2009). Genomic studies involving B chromosomes reported a variable composition, mainly associated with repetitive DNA (e.g., ribosomal DNA, satellite DNA, and mobile elements) unique to or shared with A chromosomes (Ahmad and Martins 2019).

Although reported in several freshwater fish groups in the neotropical region, B chromosomes are relatively rare in marine species (Galetti Jr et al. 2000), as in some families of Tetraodontiformes (Alves et al. 2008; Martinez et al. 2011; Noleto et al. 2012).

The chromatin evolutionary dynamics in Tetraodontiformes promoted remarkable genomic reductions, resulting in considerable chromosome size and heterochromatin amount differences among species (Noleto et al. 2009). The evolutionary reduction of the genomic content in Tetraodontidae (Brainerd et al. 2001), for instance, indicates an important role of the repetitive sequences both in the genomic compaction and in its expansion in karyotypes of different clades.

Reports of B chromosome occurrence in some species of Tetraodontiformes stimulate its investigation in more exhaustive population comparisons. Interindividual variations in B micro-chromosomes have been reported in the bandtail puffer *Sphoeroides spengleri* (Tetraodontidae) of distinct geographic regions in the Atlantic (Fig. 8.6). Although B chromosomes were not detected in the populations of Southeast Brazil (Brum et al. 1995), a progressively increased frequency was observed toward the south, suggesting a clinal distribution of 0 – 2B/cell in populations of the São Paulo state region (Alves et al. 2008), and 0 – 3B/cell in the coast of the Paraná state (Noleto et al. 2012), the most southern region in Brazil where the species was analyzed. This B chromosome polymorphism is rarely reported in marine fish and could indicate a clinal variation associated with the adaptive conditions of this species.

The B elements can be quite variable in fish (i.e. in number, size, chromosome type, presence/absence of active genes), harbor a complex portion of repetitive sequences (Ziegler et al. 2003; Utsunomia et al. 2016), and can have adaptive effects (Ahmad and Martins 2019). Thus, its unusual presence in marine fish raises intriguing questions related to its origin, phylogenetic amplitude and adaptive effects in the peculiar conditions presented by the oceanic environments.

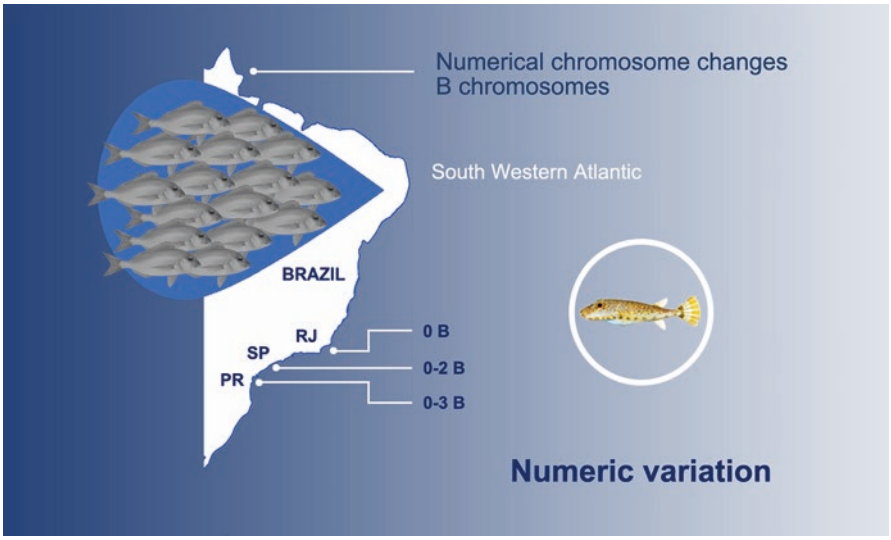


**Fig. 8.6** Numerical karyotypic variations related to B chromosome frequency in *Sphoeroides spengleri* (Tetraodontidae) populations in Western Atlantic regions

### *8.5.2 Chromosome Inversion Systems*

Peri- and paracentric inversions are chromosome rearrangements involving break and subsequent insertion of chromosomal segments in inverted position in the chromosomes. Pericentric inversions involve the centromeric region, representing the main karyotypic diversification mechanism in major marine fish groups (Galetti Jr et al. 2000; Ueno and Takai 2000).

On the other hand, paracentric inversions, which do not involve the centromere, are largely underreported in fish karyotypes for not changing chromosome morphology, and by infrequent involvement of detectable markers in the inverted region. Thus, while polytene chromosomes of insects (e.g. *Drosophila*) show a higher occurrence of paracentric inversions easily detected (Coyne et al. 1991), in fish chromosomes, on the other hand, they have a reduced detection rate (Borges et al. 2019).

Inversions are associated with major evolutionary processes, such as environmental adaptation (Barth et al. 2017), opening opportunities for niche enlargement, reproductive isolation, sex chromosome evolution, and speciation (Wellenreuther and Bernatchez 2018; Costa et al. 2019).

Genomic analyses have revealed inversion polymorphisms associated with local adaptation to novel environmental conditions (Berg et al. 2017; Cayuela et al. 2020). In marine fishes, the occurrence of these rearrangements can be highly divergent between clades of the same genus (Getlekha et al. 2018), associated from gradual chromosome changes to concerted events (Molina and Galetti Jr 2004). The growing use of FISH (Fluorescence In Situ Hibridization) methodologies in marine

species trends to improve the detection of inversions within linkage groups, and explore questions related to its tremendous evolutionary importance on the genome modeling of species.

### *8.5.3 Chromosome Translocation*

Robertsonian translocations, also known as centric fusions, involve breaks close to the centromeres of two acrocentric chromosomes and their subsequent fusion, resulting in a new chromosome. This structural rearrangement reduces the diploid number but maintains the number of chromosomal arms. This new gene set, although rearranged, is potentially maintained and functional. In humans, it is the most common structural change (Therman et al. 1989), often not causing health changes (Song et al. 2016). In some marine fish families, they constitute an evolutionary trend of changes and one of the main chromosomal variation and diversification mechanisms.

Contrastingly, *in tandem* fusion is an "end to end" fusion which involves two chromosomes attaching to each other at the telomeric regions, with loss or inactivation of one centromere (Popescu and Hayes 2000). In most *in tandem* translocations, however, no more than two or three original chromosomes participate in forming one product, resulting in a dicentric or tricentric element (Wang et al. 1987). Records about *in tandem* translocation cases are better documented in humans generating deleterious results as congenital abnormalities – syndromic relation (Down's syndrome), development delays (motor, speech and hypotonia) and malformations (craniofacial anomalies), but are more common in neoplasms (leukemias) (Sachdeva et al. 1971; Brito-Babapulle and Catovsky 1991; Meschede et al. 1998; Lee et al. 2001). In vertebrates, its effects are also described mainly regarding abnormalities related to fertility problems – sex-chromosomes aneuploidy or sex reversed specimens (e.g. *Peromyscus eremicus*, *Equus cabalus*) (Hsu et al. 1978; Long 1996); congenital genital abnormalities and heart dysfunction in special human-manipulated breeds (e.g. *Bos taurus* – Holstein cattle) (Iannuzzi et al. 2020). Despite being less reported in marine fishes, these chromosome rearrangements contributed significantly to karyotypic diversification in some groups, such as the Antarctic teleost fish of the family Nototheniidae (Notothenioidei) (Auvinet et al. 2020). In Atlantic tropical regions, *in tandem* fusion events have been associated with the speciation process in Atlantic surgeonfish species (Acanthuridae) (Affonso et al. 2014; Fernandes et al. 2015, 2021). In the genus *Trematomus* (Nototheniidae), *in tandem* fusions, in combination with centric fusions, accompanied speciation events and likely played an important adaptive role in their ecological, genetic, and morphological diversification (Pisano and Ozouf-Costaz 2000; Auvinet et al. 2020).

Population variations in the number of chromosomes in Atlantic fish majorly result from polymorphic (heteromorphic) Robertsonian translocations. This transitory condition is perceived by the presence of odd 2n and more than one diploid value in the same species, without changing the FN. Their transitional occurrence

promotes conspicuous population polymorphisms in diversified reef fish groups, and constitutes one of the main mechanisms of karyotype diversification in many groups (Azevedo et al. 2005; Vasconcelos and Molina 2009; García-Angulo et al. 2018). Additionally, centric fusions have great importance in the origin of multiple sex chromosome systems in fish (Almeida-Toledo et al. 2000a, b; Henning et al. 2011; Kitano et al. 2009), contributing to phyletic diversification (Fig. 8.7).

In Gobiidae, the intense evolutionary dynamics through centric fusions and deletions resulted in at least six karyomorphs in *Gobius paganellus* (2n = 48/FN = 48; 2n = 47/FN = 47; 2n = 46/FN = 46; 2n = 47/FN = 48; 2n = 46/FN = 48; 2n = 46/FN = 47) (Amores et al. 1990), with spatial distribution differences between the Mediterranean and Atlantic being interpreted as environmental adaptive patterns.

The damselfishes of the genera *Dascyllus* and *Chromis* (Pomacentridae) also exhibit striking examples of diploid number variation derived from centric fusions (Ojima and Kashiwagi 1981; Takai and Ojima 1995), evidencing an evolutionary trend of the Chrominae subfamily (Molina and Galetti Jr 2002; Getlekha et al. 2016).

From the population point of view, chromosomal variations in the genus *Chromis* can be very distinct between species showing differences related to number and morphology (acrocentric, a; metacentric, m; submetacentric, sm) of the chromosomes. In fact, cytogenetic comparisons between populations of *Chromis multilineata* (2n = 48 a) distributed in vast areas of the Brazilian coast and of the archipelagos of Fernando de Noronha and São Pedro e São Paulo (Meso-Atlantic region) present no discernible karyotype variation and rDNA sites distribution (Cunha et al. 2014). In contrast, the karyotypes of *Chromis jubauna* (2n = 46/47; 3-4m + 6sm + 36-38a;

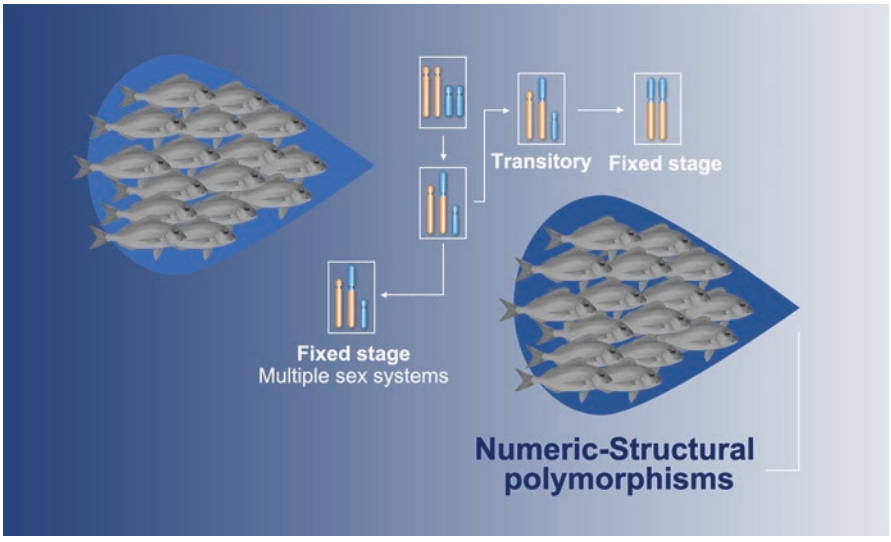


**Fig. 8.7** Numerical-structural polymorphisms resulting from centric fusion events are particularly common in some marine fish groups, representing the initial steps to establishment of new karyotypes and sex chromosomes systems

FN = 56) and *Chromis flavicauda* (2n = 39; 9m + 6sm + 24a; FN = 54) (Molina and Galetti Jr 2002), suggest a more diversified karyotypic scenario. In the genus *Dascyllus*, distributed in Indian and Pacific regions, numerical-structural polymorphisms occur between populations or within the same population of the same species (Ojima and Kashiwagi 1981; Kashiwagi et al. 2005). Extensive chromosome variations have been reported in several species, such as *Dascyllus aruanus* (2n = 27/28/29/30/31/32/33), *Dascyllus reticulatus* (2n = 34/35/36), and *Dascyllus trimaculatus (*2n = 47/48). The gene reorganization promoted in these transitional phases of the karyotype, can be adaptively fixed between populations and affect gene flow between them.

During the centric fusion process, the acrocentric chromosomes involved may lose telomeric sequences (Molina and Galetti Jr 2002; Caputo et al. 2003), or they may be maintained or lost in different chromosome pairs, indicating possible independent and asynchronous events (Getlekha et al. 2016). Peculiarities in the composition of the centromeric regions may hinder or favor the fusion processes in some fish groups. While AT-rich heterochromatic regions can compact DNA (Canapa et al. 2002), preventing centric fusions (Garrido-Ramos et al. 1995), GC-rich DNA regions associated with rDNA regions may favor them (Molina and Galetti Jr 2002; Caputo et al. 2003; Getlekha et al. 2016).

The labrid *Xyrichtys novacula* shows chromosome number and genetic divergence variations along its distribution in the Mediterranean (Italy, 2n=48, NF=56), Caribbean (Venezuela, 2n=45-48, NF=56), and western Atlantic (Brazil, 2n=48, NF=56) (Vitturi et al. 1989; Almeida et al. 2017; Nirchio et al. 2019). The numerical chromosome variations exclusive to the Venezuelan population apparently constitute a polymorphic karyotypic condition, showing the occurrence of genetic stratification of Atlantic lineages.

Widely dispersed along the eastern Atlantic coast and common in the Mediterranean and Black Sea, *Uranoscopus scaber* (Uranoscopidae) reveals intrapopulation variation caused by Robertsonian translocations (2n = 27/28/29/30) (Vitturi et al. 1991; Caputo et al. 2003), while in other locations it is monomorphic (2n = 26) (Sofradzija 1985). The variation within this species suggests adaptive processes to environmental multipartition throughout its distribution area.

Karyotypic changes undergo transient polymorphisms until their fixation in a population or species. In dioic fish, Robertsonian polymorphisms can fix karyotype changes and promote sex chromosome fixation if exhibit positive sex selection (Sember et al. 2021). Diverse groups of marine fish have reproductive strategies associated with asynchronous hermaphroditism (androgyny, they begin life as males and revert to females; and protogyny, the opposite process) (Godwin 2011). In fact, chromosome polymorphisms under this circumstance probably could have an accelerated dissemination, since the same heterozygous individual can act bidirectionally as male and female in the reproductive group. This condition highlights an attractive and currently unknown analysis on its role on fixation and dissemination of structural polymorphisms.

### *8.5.4 Microstructural Variations in Repetitive Sequences*

The karyotypes of several groups of marine fish are characterized by remarkable chromosomal stability. In these cases, the prospection of chromosome structural variations have been focusing on more dynamic microstructural chromosomal markers, such as repetitive DNA, whose lower adaptive stringency can result in conspicuous levels of intraspecific variation.

The FISH technique is routinely used in the analysis of specific DNA sequence dynamics within chromosomes and genomes. Its use in cytogenetics (mainly clinical cytogenetics and cytotaxonomy) also extends to developmental biology and genetic improvement (Liehr et al. 2002, 2006; Yang et al. 2009).

Several repetitive DNA sequences are more frequently investigated in comparative studies on marine fish chromosomes (18S rDNA, 5S rDNA, H1, H2, H3, H4, microsatellites, transposable elements, GATA, telomeric and centromeric sequences, among others). With rapid evolution rates, repetitive DNA regions offer many possibilities as population and cytotaxonomic markers.

Heterochromatic regions present huge variation and diversification among several groups of neotropical freshwater fish (Galetti Jr 1998; Artoni and Bertollo 1999; Hashimoto and Porto-Foresti 2010; Prestes et al. 2019). However, in general this DNA fraction has a very more discrete role as chromosomal markers in marine fish groups (Canapa et al. 2002; Costa et al. 2016), based on C-banding analysis. Likely, the reduced heterochromatic content, positively correlated with a more reduced genome size in marine fishes (Hardie and Hebert 2004; Yi and Streelman 2005; Yuan et al. 2018), contributes to this.

The mapping of repetitive sequences by FISH allows wider analyses in different regions along the chromosomes, revealing an unpredicted diversity of chromosomal variation in marine groups.

Among the classes of repetitive DNA, the multigene families offer multiple perspectives for evolutionary analysis of chromosomes. Multigene families are sets of similar genes that encode proteins with similar functions, formed from evolutionary events involving gene duplication. Due to their varied mechanisms of changes, organization and functional interactions these sequences have their own evolutionary rates and can disperse in the genome through chromosomal rearrangements. In fact, a significant number of pseudogenes (sequences like coding genes, but that lost their functionality due to mutations) are byproduct of the evolution of multigene families. The action of chromosomal rearrangements and evolutionary processes (duplication, inactivation or loss) on these DNA fractions promote unique karyotypic patterns in the individuals of a population or species.

In eukaryotes, conserved rDNA coding sequences are efficient phylogenetic markers (Gornung 2013), and the most employed as cytotaxonomic markers in marine fishes.

Ribosomal RNA genes are organized into two distinct multigene families composed by hundreds of thousands of *in tandem* arrangement copies, in which the non-transcribed spacers (NTS) show high interspecific variations in consequence of

insertions/deletions, mobile elements, minisatellites, and pseudogenes (Campo and Garcia-Vazquez 2012; Rebordinos et al. 2013; Costa et al. 2013, 2016). As they have several divergent domains, the multiple copies of 45S rDNA sequences can vary considerably between clades being used in the reconstruction of relatively recent divergences (Hillis and Dixon 1991). In most teleostean fish, about 70% of the previously karyotyped species have in the major rDNA regions a single locus of the small ribosomal subunit 18S rDNA (Gornung 2013), a condition assumed to be plesiomorphic in fish (Amemiya and Gold 1988), and used as basal to investigate diversification processes.

Comparative analysis of the Ag-NOR sites evidenced clear discrepancies in frequency and position of these sites between populations of *Trachinotus falcatus* and *Trachinotus goodei* (Carangidae) of the north-eastern and south-eastern Brazilian coasts (Accioly et al. 2012) (Fig. 8.8). Conspicuous interpopulation variation in the frequency of rDNA sites was also found between populations of *Halichoeres radiatus* (Labridae), of two insular Atlantic regions, the archipelagos of Fernando de Noronha, and São Pedro and São Paulo (Amorim et al. 2017) (Fig. 8.9). The Ag-NORs/18S rDNA population variations contribute to identifying restrictions to gene flow between different geographic contexts along the western Atlantic.

The 5S rDNA sequences, *tandem* arrays composed of a 5S rDNA coding region and a variable NTS, have also been extensively employed in populational and phylogenetic inferences (Long and David 1980; Hillis and Dixon 1991). Although highly conserved even between unrelated species, the 5S rDNA sequences varies

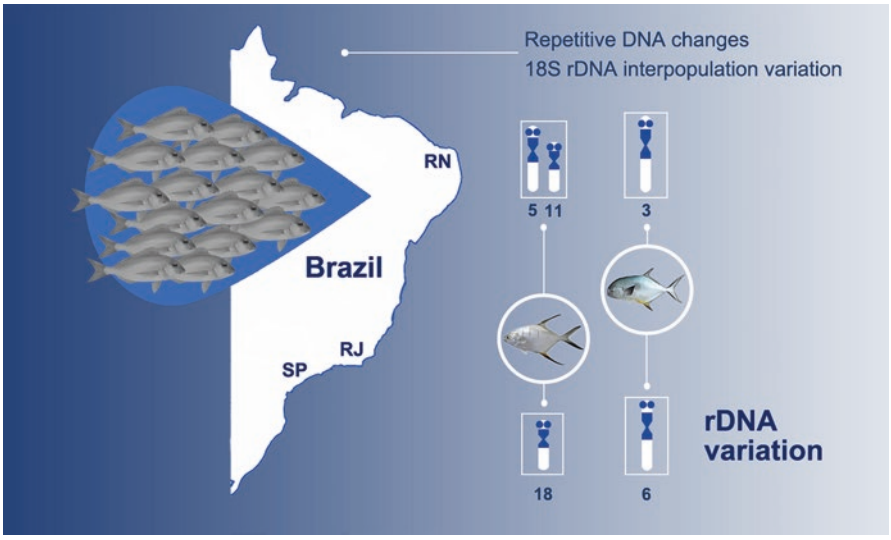


**Fig. 8.8** Numerical and structural polymorphisms of rDNA loci in Atlantic fish. Ag-NORs (boxes) site variations between populations of the species *Trachinotus goodei* (on the left) and *Trachinotus falcatus* (on the right) from the northeast and southeast coasts of Brazil (Adapted from Accioly et al. 2012)

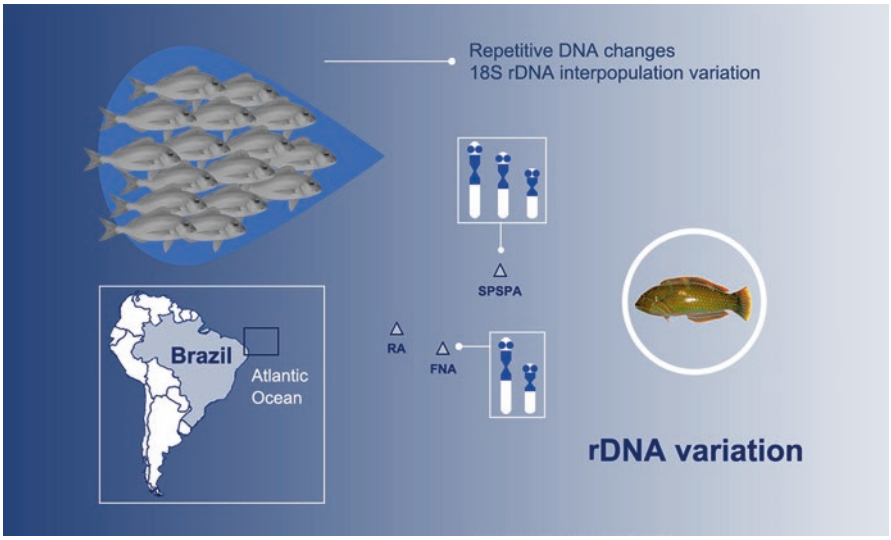


**Fig. 8.9** The combined analysis of multiple chromosomal regions with varying degrees of evolutionary dynamism allows mapping subtle populations in the marine environment. The highlighted example shows varying rDNA site frequencies between the *Halichoeres radiatus* (Labridae) populations in two Brazilian marine islands, the Archipelagos of São Pedro and São Paulo (SPSPA), and Fernando de Noronha (FNA)

with respect to the occurrence of insertions/deletions in the NTS and minirepeats, and occurrence of pseudogenes (Leah et al. 1990; Sajdak et al. 1998)

An important component of rDNA in eukaryotes is the transposable elements, which are capable of intragenomic mobility (Charlesworth et al. 1994; Jurka et al. 2005). Transposable elements include retrotransposons, whose flanking sequences occur in the long terminal repetitions (LTRs) moving through the genome via reverse transcriptase, and non-LTRs, which have no terminal repetitions (known as LINEs – long interspersed nuclear elements, and SINEs – short interspersed nuclear elements) (Bohne et al. 2008; Ferreira et al. 2011). These elements have a strong association with heterochromatic regions and act in gene regulation and repair, and in the chromosome rearrangements and sex chromosome differentiation due to their repetitive nature, influencing genomic and karyotypic evolution (Cioffi et al. 2010; Ferreira et al. 2011; Valente et al. 2011). Although they are important genomic elements in several fish species (Xiao et al. 2020), smaller genomes in marine fish seem to be poorer in transposable elements, which constitute about 3–5% of the genome (Aparicio et al. 2002; Chen et al. 2014).

At the forefront of cytogenetic studies, molecular techniques have significantly expanded the knowledge about large-scale genomic organization and epigenetic chromatin modeling (Fuchs et al. 2006). In this context, DNA methylation markers have been used to investigate several functions such as transcription inhibition and elongation, homologous recombination suppression, gene expression regulation, epigenetic control of genomic imprinting, cell differentiation, chromatin dynamics

in sex chromosomes and morphogenesis, among others (Martin and Zhang 2005; Kouzarides 2007; Dyachenko et al. 2010; Marques et al. 2011; Schmid et al. 2016).

Differential DNA methylation expression has revealed epigenetic aspects associated with chromatin dynamics of B chromosomes (Barbosa et al. 2015), as well as the functional characterization of specific autosomal regions of chromosomes of marine species. One of these regions, called the BOD region, represent a shared trait present in one pair of chromosomes of species of the genus *Bodianus* (Labridae) (Molina et al. 2012b). This exceptional region hypomethylated consist in a pseudo-NOR locus, exceptionally uncondensed, heterochromatic, argentophilic, GC-neutral, and, in contrast to classical secondary constrictions, shows no signals of hybridization with 18S rDNA probes. Hypomethylation of the BOD region is associated with the precise co-location of *Tol2* and *Alu* transposable elements, suggesting their active participation in the regulatory epigenetic process (Motta-Neto et al. 2018). Inferences on their role as evolutionary or phylogenetic markers are still unknown but may help understand their origin and evolutionary effects.

## 8.6 Final Considerations

A growing body of new evidence indicates that neutral or adaptive genomic modifications arising from rearrangements occurred during the karyotypic diversification of marine fish species. Chromosome polymorphisms, increasingly accessible through the analysis of multiple genomic regions, are the initial steps towards the establishment of distinctive patterns between groups, showing the effect of environmental or biological factors of a given clade on gene flow and lineages differentiation. Cytogenetic variations are associated with different evolutionary factors, such as genetic drift, including founding effect, gene flow, and natural selection. The presence or absence of chromosomal changes have interfaces with adaptive patterns, protecting the genetic cohesion of species, or individualizing divergent paths between groups. Exclusive aspects of the marine environment provide the maintenance of large populations, strict ecological requirements, and variable gene flow profiles resulting from the dispersal potential of the species, making marine fish groups informative models to investigate change rates and evolutionary interfaces associated with the karyotypic patterns.

Decisively, cytogenetic analysis has been proved an efficient tool for prospecting hidden diversity within neotropical fishes. Chromosome number, structure and its gene organization are basic features of the eukaryotic genome, with consequences for the aspects associated with adaptation and speciation, such as recombination and segregation. In fact, advances in the cytogenetic techniques have contributed to identifying divergent evolutionary units and new species, resolve taxonomy uncertainties and provide effective contributions to biological conservation of ichthyofauna in the Neotropics.

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# Chapter 9
# *In Situ, Ex Situ* and *On Farm* Conservation of Plant Genetic Resources in Neotropics


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## 9.1 Introduction

Nikolai Vavilov (1887–1943), geneticist, botanist, geographer, agronomist was responsible for determining the origin centers of domesticated plants and considered South America as a great center of diversity, outlining well-defined subcenters: (i) Peruvian, Ecuadorean, Bolivian; (ii) Chile and (iii) Brazilian-Paraguayan (Debrenne and Debrenne 2003; Levina et al. 2005). Vavilov included Brazil as one of these subcenters due to the large number of plant species that can be found in the country, highlighting some well-known ones such as cassava (*Manihot esculenta*), pineapple (*Ananas comosus*), and passion fruit (*Passiflora edulis*), among others (Vrugtman 1994). In that time, Vavilov and his colleagues were responsible for

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exploring countless regions of the world, understanding the cultivation processes and who were the people who maintained these species. Although Vavilov's dream of ending world hunger was unfulfilled, he was the forerunner in using genetics to generate 'superplants', to create the world's first seed bank in Leningrad (now known as St. Petersburg) and to implement the concept of *ex situ* conservation, that is, to conserve biological materials that are conserved outside their origin place (Birstein 2008; Dzyubenko 2018).

During 1932 and 1933, Vavilov collected seeds and parts of native and cultivated plants in Central/South America, including Brazil, recording in that countries a diversity of domesticated and semi-domesticated plants (Loskutov 1999). The history of cultivated plants in Brazil is extremely rich in detail and possibly begins with the first indigenous peoples on the South American continent (Alcantara-Rodriguez et al. 2019). From their migratory routes, these communities expanded these food crops to other unoccupied territories, thus diversifying food species that were previously restricted to some areas (Simmonds 1976; Bedoya et al. 2017; Clement et al. 2021). It is believed that this process was drastically interrupted by the invasions of Europeans, mostly Portuguese and Spanish, who were also responsible for bringing a large part of European agricultural crops, but also from several regions that were part of the spice route (Clement et al. 2010; Moya 2018). However, Clement (1999) describes that 4–five million indigenous peoples in the Amazon cultivated or managed at least 138 plant species, at some stage of domestication. The genetic erosion of these crops guided by the extinction of these populations reflects an unprecedented loss that persists nowadays.

As a country with the largest territorial extension, greatest agrobiodiversity, and a high volume of information we will focus on Brazil in this Chapter not undermining the immense genetic resources in neighboring countries.

In addition, over three centuries and half, with the process of slavery, it is believed that Brazil imported approximately 5 million Africans, but also their cultural habits and various food plants (Araujo 2016). In the recent past, from the migrations, several cultures (including Japanese, Italian and German), new types of agriculture were designed in Brazil, bringing these peoples a range of cultivated plant species that were incorporated in their new cultivation areas, and today, they are part of many markets and fairs in the country (Lesser 2013).

It is worth noting here the great role played by various indigenous cultures, many extinct in Brazil, and which played a crucial contribution in the domestication and maintenance of many plant species (Hanazaki et al. 2018). For centuries, these peoples developed agroforestry systems in a large part of the national territory, selecting species of wild plants, and from continuous selections of seeds, roots, and tubers, they diversified their food habits and incorporated these plants into their diets (Miller and Nair 2006; Levis et al. 2018).

The importance of heterogeneous traditional Brazilian cultures was, and still is, maintained by *quilombolas*, *caiçaras*, *ribeirinhos*, indigenous peoples and other profiles of family farmers, who made different agricultural systems possible, and which culminated in the immense agrobiodiversity in the country we know (Ávila et al. 2017; Santos and Barros 2017). These different agricultural systems,

particular to each people or traditional community, have allowed, through the in *situ/on farm* conservation process, over the centuries, many cultivated species still resist and persist (Alercia et al. 2014).

However, due to the rapid technological development of agriculture in Brazil (Pereira et al. 2012), in which varieties with higher performance gain prominence, many traditional varieties have disappeared, which increasingly requires conservation efforts, underling the importance of conservation *ex situ* and *in situ*/*on farm*. Kahane et al. (2013) conclude that not only a change in policy is needed to influence behaviors and practices but also strong leadership able to synergize the various initiatives and implement action plans. Against this backdrop, agrobiodiversity has received increasing attention in conservation programs around the world (Frison et al. 2011). As the impacts of climate change on food generation are discussed, large *ex situ* seed conservation banks have a global responsibility (Westengen et al. 2013), however, traditional communities play a crucial role in bank conservation of local seeds (Maharjan and Maharjan 2018).

In recent decades, Brazilian scientific institutions have developed research to measure this genetic erosion, simultaneously identifying the degree of diversity that many species have. Ultimately these actions have been proposing conservation measures that reduce the social, economic and cultural impact of this genetic erosion of cultivated plants brings to the country (Ming et al. 2010; Barbieri et al. 2014). Some of these studies demonstrate the high genetic diversity of seeds, roots and tubers, the importance of these small-scale agricultural systems, and the enormous importance of conserving these materials *ex situ* due to continued genetic erosion (Veasey et al. 2011; Siqueira et al. 2014).

## 9.2 The Genetic Erosion in Plant Resources

Genetic erosion corresponds to the loss of variability in crop plant populations and can occur at the species, landrace and allele level (van der Wouw et al. 2009). The replacement of landraces with a few genetically uniform varieties depletes genetic diversity and provides ideal conditions for diseases and insect pests that are called genetic vulnerability (Keneni et al. 2012). The genetic diversity of crop plants is extremely vulnerable to climate change (Altieri and Koohafkan 2008; Ramankutty et al. 2018; Sparovek et al. 2018; John and Babu 2021) and the neotropical zone is not an exception. A recent survey carried out a diagnosis involving three regions of Brazil (Minas Gerais, Mato Grosso do Sul and Rio Grande do Sul) and a region of Uruguay (Tacuarembó), with the objective of identifying the main challenges that family farmers face in the conservation of agrobiodiversity, especially in relation to the landraces component (Silva et al. 2020). The main difficulties for conservation, which can be considered indicators of genetic erosion, were mainly associated with: (i) lack of projects and public incentives for local farmers associations; (ii) the production model, such as the expansion of coffee, maize, soy, sugarcane and tobacco monocultures; (iii) the rural exodus; (iv) lack of succession in family farming

(disinterest or lack of opportunity for young people in the countryside); (v) environmental phenomena and climate change; (vi) the essential aspects for the maintenance of life, such as access to water; and (vii) the permanence in the territories (territorial insecurity, in the case of indigenous communities).

In the southern, southeastern, central-western, northern and northeastern regions of Brazil, family farmer producers of yam (*Dioscorea* ssp.) (Bressan et al. 2005; Castro et al. 2012; Nascimento et al. 2015; Silva et al. 2017), sweet potato (*Ipomoea potatoes*) (Bressan et al. 2005) and cassava (Amorozo 2008; Marchetti et al. 2013) reported that they used to plant more landraces earlier than they currently plant, suggesting the occurrence of genetic erosion events in these locations. Similar reasons reported by Silva et al. (2020) were mentioned by these farmers, who also considered other factors, such as changes in the physical and chemical conditions of the soil over time, the lack of technical assistance on the proper management of the crop, the increase in production costs and the influence of eating habits, leading to a preference for certain landraces due to their flavors and colors. A study involving bean (*Phaseolus vulgaris*) producers in the state of Rio de Janeiro observed that the number of landraces cultivated by farmers ranged from one to 11, and this variation was influenced by socioeconomic, ecogeographic and cultural factors (Cavalcanti et al. 2021). In three of the five mesoregions studied, family agriculture has been affected by climate change. In these three regions, there was a high frequency of farmers who stopped cultivating beans in recent years and farmers who cultivated a low crop diversity. To face the risk of genetic erosion, farmers, researchers and extension workers involved in this project are building strategies to support the conservation of this germplasm in the region (Cavalcanti et al. 2021).

The southwestern of the Amazon, due to events of European conquest and colonization and the decimation of indigenous populations in the region (Mann 2005), suffered consequences of loss and extinction of genetic resources (Clement 1999), which involved local races and landraces of maize (Brieger et al. 1958). This process was further intensified with the expansion of the rubber economy in this area (Lacerda 2006). For a long time, it was hypothesized that locally developed maize races became extinct as a result of these processes. A study showed that a richness of landraces, classified as the *Entrelaçado* (in English means Interlocked) race, continue to be conserved by traditional and indigenous farmers who inhabit the region, who attribute local and indigenous names to this race, and have typical culinary uses (Costa et al. 2021). In addition, the southwestern Amazon is considered an important center of maize diversification (Kistler et al. 2018), and this research draws attention to the importance of maize conservation in this region, where one of the oldest races of South America is still cultivated (Costa et al. 2021).

*In situ/on farm* conservation corresponds to the conservation of components of biological diversity in their natural environments. This conservation strategy involves the conservation of ecosystems and natural habitats, the maintenance and recovery of viable populations in their natural environments and, in the case of domesticated or cultivated populations, in the environments where they have developed their characteristic properties (CBD 1992). Likewise, it allows crop species to continue subject to evolutionary processes, promoting the adaptive development of

species in terms of climate changes and biotic factors over time (Brush 2000). The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) considers that genetic diversity must be maintained in local agricultural systems, within which the participation of farmers is essential (ITPGRFA 2009). Traditional Agricultural Systems (TAS) are formed by interdependent elements such as cultivated plants and animal husbandry, social networks, food systems, knowledge and other associated manifestations. These elements involve managed landscapes and agroecosystems, agricultural products and associated culture (Altieri and Koohafkan 2008; Koohafkan and Cruz 2011). TAS conserves important genetic variability, cultural traditions and a wide diversity of uses associated with agrobiodiversity (CBD 1992; Altieri and Koohafkan 2008; Koohafkan and Cruz 2011).

In 2002, the Food and Agriculture Organization of the United Nations (FAO) introduced an international partnership initiative called Globally Important Agricultural Heritage Systems (GIAHS), with the aim of laying the foundation for international recognition, dynamic conservation and management sustainability of TAS (Altieri and Koohafkan 2008; Koohafkan and Cruz 2011; FAO 2021). In 2018, the Serra do Espinhaço de Minas Gerais TAS, also known as "Sempre-viva (Always Alive) Flowers Gatherers", was the first brazilian TAS internationally recognized as world agricultural heritage by the GIAHS program (FAO 2021). Areas with high biodiversity, rich in endemic species, which are threatened with extinction are called *hotspots* (Myers 1988, 1990; Myers et al. 2000). The term hotspots was used for the first time with a focus on tropical forests (Myers 1988) and later studies expanded the indication of these areas involving different ecosystems (Myers 1990; Myers et al. 2000). In recent years, the term has also been used in the context of agrobiodiversity, that is, agroecosystems, which involve species, wild relatives, races and landraces that are important for food and agriculture (Pacicco et al. 2018; Maxted and Vincent 2021). The establishment of zones indicated as agrobiodiversity *hotspots* (Ruiz 2009; Pacicco et al. 2018; Costa 2020; Cavalcanti et al. 2021), can promote the conservation of crops and enhance the genetic and cultural heritage conserved in these areas. In Brazil, five agrobiodiversity hotspots related to maize were identified, involving the Amazon, Caatinga, Cerrado and Atlantic Forest biomes (Costa 2020), and one for beans in the Atlantic Forest (Cavalcanti 2018; Cavalcanti et al. 2021). In Pampa, three were identified related to the maize crop, in different regions of Uruguay (Costa 2020).

In Brazil, despite the challenges faced by genetic erosion in recent decades, a wide diversity of landraces has been conserved under cultivation in different regions, involving: (i) bean, in the state of Rio de Janeiro (Cavalcanti et al. 2021); (ii) cassava, in Alto Rio Negro, Amazonas (Emperaire et al. 2008) and in Mato Grosso (Marchetti et al. 2013); (iii) pumpkins (*Cucurbita maxima*), in Rio Grande do Sul (Barbieri et al. 2014); (iv) and yam, in the southern, southeastern, central-western, northern and northeastern of Brazil (Castro et al. 2012; Nascimento et al. 2015; Silva et al. 2017). Different micro-centers of maize diversity have been identified in different sociocultural and eco-geographic contexts in Brazil and Uruguay (involving the Amazon, Cerrado, Caatinga, Atlantic Forest and Pampa biomes), with the aim of indicating priority areas for the conservation of the species in these territories

**Fig. 9.1** Maize landraces diversity conserved in a diversity micro-center in the Atlantic forest/Pampa Ecoton, Brazil. Archive of the interdisciplinary group of agrobiodiversity study – InterABio. Maize races of South American lowlands project. (Photo: Flaviane Malaquias Costa)

(Fig. 9.1) (Costa et al. 2017a; Costa 2020). Micro-centers of diversity correspond to very restricted geographic areas, within which significant diversity is accumulated (Harlan 1971, 1992). The environmental and human components related to these areas have intrinsic peculiarities which shape the agrobiodiversity and sociocultural values present in each location, which makes each place particular within the scope of *in situ/on farm* conservation.

*Ex situ* conservation corresponds to the conservation of components of biological diversity outside their natural habitats (CBD 1992). This conservation strategy occurs through germplasm banks, inside which samples of seeds or other parts of plants are stored, mainly under controlled conditions of temperature and humidity, whose main objective is to conserve the greatest possible genetic diversity. The conservation of orthodox seeds (which will survive drying and/or freezing during *ex situ* conservation, as opposed to recalcitrant seeds, which will not) is carried out through storage in cold rooms, under medium temperatures for the short term, and low for the long term. Recalcitrant species or species that are vegetatively propagated are conserved under field conditions (*in vivo* conservation) or *in vitro* (Silva et al. 2007; Liu et al. 2020). The *ex situ* conservation of genetic resources is an important initiative to support food security and ensure the continued use of plant species that are important for human consumption in the future, as well as being important for the foundations of genetic improvement programs for crop plants (Silva et al. 2007).

In Brazil, the first institutions to constitute important germplasm collections were: the Agronomic Institute of Campinas (IAC), being the first agronomic research institute in Brazil; the 'Luiz de Queiroz' College of Agriculture of the University of São Paulo (ESALQ/USP), with funds from the National Academy of Sciences of the United States (Brieger et al. 1958; Paterniani and Goodman 1978); and the Federal University of Viçosa (UFV), which has a germplasm bank initially formed with resources from the Rockefeller Foundation. In addition to these, many other Brazilian institutions contributed to assembling collections with approximately 250,000 accessions that today make up the germplasm banks of the National Center for Genetic Resources and Biotechnology (Cenargen) of the Brazilian Agricultural Research Corporation (Embrapa) (Santonieri and Bustamante 2016). Embrapa's Germplasm Banks conserves at least 92 families, partially listed in Table 9.1, 425 genus and 1955 species. In 2004, aiming at the *ex situ* conservation of genetic diversity in the long term, the Svalbard Global Seed Vault was founded by FAO and Biodiversity International, in Norway. Currently, this bank conserves more than 1 million samples, originating from almost all countries, being considered the most diversified collection of food crop seeds in the world, and part of the brazilian germplasm collections (at least 15,046 accessions originating from Brazil) are also conserved in this place (Crop Trust 2021).

Since the beginning of the establishment of international systems for the conservation of genetic resources, the guidelines proposed by the FAO and the Consultative Group on International Agricultural Research (CGIAR) have placed a high emphasis on *ex situ* conservation, with a focus on the main crops that form the basis of

**Table 9.1** Number of accessions of the main botanical families conserved ex situ in the germplasm banks of the Brazilian agricultural research corporation (Embrapa)

| Family | Number of accessions | Family | Number of accessions | Family | Number of accessions |
|---|---|---|---|---|---|
| Poaceae | 99,003 | Convolvulaceae | 1401 | Celastraceae | 209 |
| Fabaceae | 85,851 | Pinaceae | 1213 | Clusiaceae | 209 |
| Cucurbitaceae | 9679 | Amaryllidaceae | 1130 | Lamiaceae | 195 |
| Solanaceae | 9065 | Brassicaceae | 1080 | Malpighiaceae | 184 |
| Malvaceae | 8849 | Rosaceae | 970 | Orchidaceae | 176 |
| Euphorbiaceae | 6701 | Bromeliaceae | 941 | Annonaceae | 160 |
| Arecaceae | 6591 | Meliaceae | 928 | Aquifoliaceae | 138 |
| Asteraceae | 6068 | Rutaceae | 788 | Rubiaceae | 121 |
| Piperaceae | 4130 | Lythraceae | 632 | Proteaceae | 119 |
| Amaranthaceae | 3141 | Passifloraceae | 628 | Polygonaceae | 118 |
| Pedaliaceae | 2376 | Araucariaceae | 410 | Apocynaceae | 116 |
| Vitaceae | 2038 | Musaceae | 384 | Caricaceae | 111 |
| Anacardiaceae | 1711 | Sapindaceae | 374 | Araceae | 101 |
| Myrtaceae | 1533 | Cactaceae | 368 | | |
| Total | | | | | 259,940 |

Source: platform Allele (http://alelobag.cenargen.embrapa.br/AleloConsultas/Passaporte/familia.do)

nutrition human, while traditional communities, indigenous peoples and family farmers around the world continued the evolutionary processes and agricultural diversification at a local level, conserving, under cultivation, several varieties of plants important for food and agriculture (Santonieri and Bustamante 2016). *In situ/ on farm* and *ex situ* conservation strategies have specific advantages and disadvantages. Therefore, it is essential that conservation strategies are complementary so that the containment of genetic erosion happens safely and efficiently.

## 9.3 Genetics as a Framework for *Ex Situ* and *In Situ* Plant Conservation

Conservation of plant genetic resources spread worldwide from the 1970s, becoming popular in 1992, after the Convention on Biological Diversity (CBD) in Rio de Janeiro. Thereafter, decisions about conservation genetics strategies started to demand multiple scientific aspects. As molecular and data processing tools developed, it was feasible to approach essential subjects in the area, increasing public policies and research (Hoban et al. 2020).

In addition to species number, the CBD guidelines comprises biological diversity as ecosystems, between species and within species variety. Even though diversity may hold many hierarchical levels – for plant genetic resources conservation, the genetic level is generally the most used. Its usefulness lies in the fact that genetic variability loss is one of the main threats to species resilience and evolutionary potential. Genetic uniformity triggers vulnerability, in both farming and natural populations, as it increases the likelihood of inbreeding depression and allele loss at each generation, due to genetic drift. Moreover, it decreases the adaptive value reducing natural or artificial selection prospects. Therefore, genetic variability is valuable. Given the climate changes predicted for the next decades, it becomes vital to safeguard it (Parmesan and Hanley 2015).

Genetic variability must be maintained and manipulated for conservation strategies. First, to estimate it biotechnology tools such as molecular markers can be used, revealing different variants of a gene (alleles). Such variants can occur between individuals or populations as a base change in DNA sequence, in a specific genome location. By evaluating these DNA changes, we detect the species genetic variability (see Chap. 20 for plants). Over time, many markers have been developed. The first popularized markers were the biochemical ones, such as isoenzymes and alloenzymes, making it possible to distinguish different proteins in a gel, identifying intragenic variation. Even though these isoenzymes are less polymorphic, before the PCR technique they were widely used to infer levels of genetic variation and their distribution, allowing investigation of populations' genetic structure (Brown 1978; Hartl and Clark 2007).

Other markers use restriction enzymes to recognize and cleave specific small DNA sequences, creating different sizes of cuts that can be compared in RFLP

(Restriction Fragment Length Polymorphism). Combined with PCR, these restriction sites result in markers such as AFLP (Amplified Fragment Length Polymorphism), in which DNA is digested by restriction enzymes and selectively amplified by primers. Among other markers, there is the amplification of DNA fragments with random primers, the RAPD (Random Amplified Polymorphic DNA). However, the most popular markers in conservation studies were repetitive, highly variable, and codominant sequences. These sequences are found in the literature as microsatellites, VNTRs (Variable Number of Tandem Repeats), STRs (Short Tandem Repeats) and SSR (Simple Sequence Repeats). Also popular is the ISSR (Inter Simple Sequence Repeat) technique, which uses microsatellite sequences as primers to reveal polymorphisms (Grover and Sharma 2016). In Brazil, easier access to isoenzymes, thermocyclers and analysis in automatic capillary electrophoresis tools provided most of the methodologies applied throughout the 1980s and 1990s, persisting until today (Turchetto-Zolet et al. 2017).

Lastly, automatic sequencing technology accelerated genome projects and some plant examples will be examined later (see Chap. 20). Early in the twenty-first century began a race among companies for new generation sequencing methodologies, revealing polymorphisms in the entire genome, usually SNPs (Simple Nucleotide Polymorphism) (van Dijk et al. 2014). Two fronts can be considered in the evolution of genetic markers: resolving power by increasing polymorphisms and increasing genome coverage (Grover and Sharma 2016). For plant genetic resources, the genomic methodologies coupled with geospatial methods, open new possibilities for conservation by considering the variability into time and space (Diniz-Filho et al. 2016b). However, the combination of new methodologies is not exclusive to the most recent markers. The applications exemplified in the next paragraphs demonstrate there is no single choice for marker and no impairment for geospatial methodologies association, although the resolution needed to explore the genetic variability differs according to the chosen marker (Diniz-Filho et al. 2020). In situations where research funding to access genetic diversity is not as much as necessary, which is commonplace in the country with the greatest biodiversity on the planet, tools considered outdated may be the only feasible ones.

More recent techniques, such as whole genome sequencing, often are used with a focus on genome characterization, lacking discussion and inferences towards species conservation. Basic research is required, but applied research is still shy and needs to be expanded.

As an example of molecular markers used to estimate genetic diversity, there are studies in pitanga (*Eugenia uniflora*), whose variability was accessed in different populations by AFLP (Margis et al. 2002; Salgueiro et al. 2004) and RAPD (Aguiar et al. 2013; Guerra et al. 2016). Natural populations of mangabeira (*Hancornia speciosa*) had their variability assessed by isoenzymes (Martins et al. 2012) and ISSR markers, showing high polymorphism in northeastern (Nunes et al. 2021) and in Cerrado regions by SSR (Costa et al. 2017b). Another application can be found in baru tree (*Dipteryx alata*), in which SSR markers detected allogamy, self-compatibility and gene flow with pollen donor dispersal distance for *in situ* and *ex situ* conditions (Guimarães et al. 2019).

Population structure and phylogenetic relationships in turnip (*Brassica rapa*) have been recently addressed (Sammour et al. 2021) with isoenzymes. Using SSR markers, it was possible to observe a significant low level of interpopulation genetic variation in palm heart (*Euterpe edulis*) and high levels of gene flow, reaching up to 22 Km. Furthermore, inbreeding was not significant, with implications for the species *in situ* and *ex situ* conservation (Gaiotto et al. 2003). For cassava, alleles from SSR markers enabled the genetic characterization of traditional varieties (Siqueira et al. 2009), in addition to spatial pattern of genetic diversity distribution, important for management and conservation of the species in the Amazon Forest (Alves-Pereira et al. 2018). Another native tuber with considerable use in Brazil and Latin America is the yam (*Dioscorea trifida*). In this species, ISSR and SSR markers were used to infer genetic diversity of accessions maintained by family farmers (Nascimento et al. 2013).

In two decades, studies in cagaiteira tree (*Eugenia dysenterica*) show how to increase knowledge using different markers in a no model species. Evidence of adaptive variability in cagaiteiras was observed with isoenzymatic markers (Telles et al. 2001a, b), also showing the species allogamous preference (Telles et al. 2003). In these studies, the genetic diversity among subpopulations is explained by genetic drift, however there is a correlation among phenotypic differences, spatial distribution, and edaphic patterns. The genetic structure of populations was investigated using RAPD markers, showing the neutrality model as a cause of phenotypic variations (Trindade and Chaves 2005), with restricted gene flow (Zucchi et al. 2005). High genetic diversity was also found using SSR markers (Zucchi et al. 2003). After extensive sampling, it shows a continuous northwest-southeast gradient in population genetic differentiation with niche ecological modeling (Diniz-Filho et al. 2016a), not explained by a simple stochastic differentiation process (Barbosa et al. 2015).

In the last paragraphs, several methodologies for genotyping DNA polymorphisms demonstrated new ways of approaching genetic variability, complementing each other. Most of the variability assessed by genetic markers is considered neutral, influenced only by genetic drift. However, sometimes the genetic difference, mainly the genetic variability accessed by phenotypes and quantitative characters within an experimental design, is influenced by both drift and natural selection. Such differences should be considered a priority for conservation, as they reflect local adaptations. Thus, common and localized alleles, supposedly resulting from selection, should be of greater importance than rare and sparse alleles, theoretically resulting from stochastic processes (van de Wouw et al. 2009). The relative effect of neutral and adaptive evolution forces must be considered for in situ conservation and sampling strategies for *ex situ* conservation (Sebben 2003).

Among botanical varieties of mangabeira, in addition to low levels of divergence, molecular markers associated with quantitative traits revealed divergent selection as a structuring factor. Genetic drift and local adaptive selection are likely causes of variation among subpopulations within varieties (Chaves et al. 2020). In cagaiteira progenies, populations with less genetic diversity are peripheral to the southeast region, and populations with greater diversity are in the northwest. However, when

the analysis was performed with parent trees' DNA, the pattern of diversity changed. This modification highlights that conservation strategies must be planned considering different sources of information, allowing inferences not only about the stand genetic diversity but also the evolutionary forces (migration, selection and drift) that are shaping variation across populations (Boaventura-Novaes et al. 2018). Genetic drift causes most phenotypic differences among subpopulations, and uniform selection is dominant for traits relevant to Cerrado climate adaptation. Understanding the evolutionary process that shapes the variability of populations *in situ* is important for management, especially in face of climate change (Boaventura-Novaes et al. 2018).

After addressing some general aspects of genetic diversity, it is worth to highlight the molecular tools from the perspective of monitoring and managing plant genetic resources *in situ* and *ex situ*. For *in situ* conservation, there is maintenance of as many alleles as possible, allowing evolutionary processes and response to environmental changes. This conservation strategy considers population dynamics, taking into account the full picture of genetic structure in space. A study on palm hearts (*E. edulis*) illustrates these perspectives. Three *in situ* conditions were analyzed: secondary forests, rural backyards, and protected areas. Surprisingly, on farm populations (rural backyards) showed greater variation in genetic diversity indicators, with the presence of alleles not found in protected areas (Milanesi et al. 2021). Another study with data from SSR markers in Brazilian nut (*Bertholletia excelsa*) emphasized the importance of connectivity areas in forests with different degradation degrees (Chiriboga-Arroyo et al. 2021).

*Ex situ* conservation may preserve intraspecific diversity alleles for centuries, especially for species with wide geographic distribution. Although this approach does not effectively capture the genetic variation of natural plant populations (Wei and Jiang 2020) nor its biological aspects (Kovacs et al. 2021), planned collections with genetic data and a well-performed sampling challenge these findings. This is the case of the *ex situ* baru tree germplasm collection at Federal University of Goiás (UFG), which presented more alleles than an *in situ* population (Guimarães et al. 2019). Conservation and selection strategies make it possible to maximize effective size and minimize genetic diversity loss, inbreeding, and inbreeding depression, important to a germplasm collection. This approach was used in cagaiteira progenies, culminating in recommendation of at least 30 individuals to conserve the *in situ* collection to meet an effective population size of 100 trees (Rodrigues et al. 2016). An *in vivo*, *ex situ* germplasm collection of cagaiteira was established with an effective size of 56 at the UFG (Boaventura-Novaes et al. 2021) (Fig. 9.2).

Within *ex situ* conservation, characterization of germplasm banks must be highlighted as essential for efficient use of genetic resources. SSR markers are the most employed markers to characterize genetic diversity and similarity among accessions, although next-generation sequencing has been increasingly employed (Nybom and Lãcis 2021). Among numerous results, some examples of characterization include accessions of garlic (*Allium sativum*) (Cunha et al. 2014), jenipapo (*Genipa americana*) (Silva et al. 2014), mangaba (Silva et al. 2019) and their progenies (Soares et al. 2018) and umbu (*Spondias tuberosa*) (Santos et al. 2021). In cultivated species, germplasm banks are better characterized, so there are countless


**Fig. 9.2** Fruits (**a**) and germplasm collection of cagaiteira (**b**) located in Federal University of Goiás (UFG), Brazil. (Photo: Carolina Ribeiro Diniz Boaventura Novaes)

studies with different markers. We find the use of SSR for nuclear collections of rice (*Oryza sativa*) (Borba et al. 2009), soybean (*Glycine max*) (Mulato et al. 2010), cassava (Costa et al. 2020), grape (*Vitis* spp.) (Oliveira et al. 2020), passion fruit (Cerqueira-Silva et al. 2015) and DArTseq for bean nuclear collection (Valdisser et al. 2017). In addition to characterization of genetic diversity, molecular markers can be used to group accessions with high genetic similarity, to construct nuclear collections without genetic redundancy, to estimate gene flow and effective size.

Often seemed as mutually exclusive, integration of *ex situ* and *in situ/on farm* conservation offers the opportunity to combine advantages. Priorities for genetic conservation of cagaiteira was established based on geographic information, response to climate change and habitat loss (Diniz-Filho et al. 2020). The authors presented a model of populations that should be conserved *in situ* and populations that should be sampled to complement the *ex situ* collection, which already exists for the species. Authors developed a simple R script to implement this approach in studies with abundant genetic data. It identifies the most efficient way to conserve as many alleles as possible with a minimum number of populations (Diniz-Filho et al. 2016b).

Understanding intra and interspecific levels of genetic diversity from genotypic and phenotypic data is essential to know, conserve, manage and make sustainable use of biodiversity. Therefore, molecular biology tools are essential for long-term maintenance of populations, considering their demographic and ecological sustainability. Unfortunately, most species lack basic information such as heterozygosity, reproductive system, and genetic structure. The modest science and technology structure applied for *ex situ* and *in situ/on farm* conservation and the large number of species, especially in tropical ecosystems, are the main causes of this knowledge gap. The public policies and research that have been implemented need to be carried out so that humanity can enjoy the benefits resulting from the conservation of plant genetic resources.

## 9.4 Global Strategy and Local Actions to Safeguard the Valuable Genetic Inheritance of Food Plants

Since 1984, Brazil has been a member of the Commission on Genetic Resources for Food and Agriculture, the permanent intergovernmental body, headquartered in Rome, that brings together 170 countries. The Commission aims to reach international consensus on policies for the sustainable use and conservation of genetic resources for food and agriculture.

In November 2001, the Commission adopted the International Treaty on Plant Genetic Resources for Food and Agriculture. Brazil is one of the 148 parties of this Treaty which aims to guarantee food security through the conservation, exchange, and sustainable use of the world's plant genetic resources for food and agriculture (PGRFA), as well as the fair and equitable benefit-sharing arising from its use. It also recognizes Farmers' Rights, subject to national laws to: *i*) the protection of traditional knowledge relevant to PGRFA; *ii*) the right to equitably participate in sharing benefits arising from the utilization of plant genetic resources for food and agriculture; and *iii*) the right to participate in making decisions, at the national level, on matters related to the conservation and sustainable use of PGRFA. The Treaty establishes the Multilateral System of Access and Benefit-sharing to facilitate plant germplasm exchanges and benefit sharing through Standard Material Transfer Agreement (SMTA).

It is also the responsibility of the Commission to conduct global assessments on plant genetic resources, which are carried out every ten years. Those are based on the systematization of the information sent by each member country about the conservation of genetic resources along with data on uses, factors that contribute to genetic erosion and the challenges and opportunities involved in conserving it, as well as how to use them sustainably to contribute to food security.

The most recent global assessment available covered the period between 2012 and 2019. In Brazil, the Cenargen was responsible for build, in a participatory way, the document that aggregated Brazilian data on the *in situ* conservation, *on farm* management and *ex situ* conservation of Plant Genetic Resources, as well data on the sustainable use of genetic resources and the construction of institutional and human capacities.

The Second Brazil Report on the state of the art of conservation of Plant Genetic Resources for Food and Agriculture (2012–2019) pointed to the continental dimension and the Brazilian cultural and biological diversity as factors that make carrying out surveys and inventories onerous and difficult to execute. Despite the increase in the number of projects carried out by educational and/or research institutions with the participation of farmers, a comprehensive inventory has not yet been carried out, at the national level, that portrays the reality of genetic resources conserved *in situ* and *on farm* in Brazil.

The reason identified for such a small number of inventories of local agrobiodiversity is the lack of policies to value the conservation of genetic resources performed by farmers (*in situ*/*on farm* management) which makes the farmers

vulnerable. This fact, associated with the lack of trained professionals and funding to carry out these surveys, especially in the context of participatory mobilization supporting communities so that they make these inventories themselves, has been reflected in the low number of inventories in the National territory.

The data collected on the questionnaires applied virtually allowed identify the protagonism of farmers in taking decisions, for instance, on the selection criteria for the best planting materials. The report accounted for the management of 3268 varieties local/creole/traditional of more than 20 species on the *in situ* and *on farm* conservation. A significant advance was the increase in participatory genetic improvement projects distributed in all Brazilian geographic regions, which accounted for 29.45% of the 143 projects reported. Simultaneously, activities were carried out to access traditional knowledge (57.3%), characterization and evaluation of local varieties (51.7%), structure analysis and population dynamics (22.4%), distribution of multiplied seeds (54.5%) and evaluation of the use and management of local varieties (50.3%), as well as evaluations of the utilization, management, socioeconomic and environmental of local and improved varieties.

Among the highlighted initiatives for the development and strengthening of systems to monitor and safeguard genetic diversity and minimize genetic erosion is the recognition of traditional agricultural systems as global heritage under the GIAHS Program. This program points out five criteria for recognizing a System Agricultural as a global heritage: food security, conservation of agrobiodiversity, traditional knowledge, social organization and cultural landscape. The Brazilian strategy to identify traditional agricultural systems with potential for recognition as GIAHS is the implementation of an award, sponsored by the National Bank for Economic and Social Development – BNDES to recognize good relative practices in TAS.

In the same context, another strategy that can contribute to the conservation of genetic resources is the registration of traditional and biodiverse agricultural systems as Intangible Heritage. The TAS of Rio Negro, in the Amazon, was the first to be registered as Intangible Heritage by the National Historical and Artistic Heritage Institute (IPHAN 2019). In 2012, with the construction of its Safeguard Plan, a process for monitoring the conservation of genetic resources was initiated, consisting of actions to safeguard heritage assets in a multi-ethnic and multilingual context in which indigenous groups share forms of transmission and circulation of knowledge, practices, environmental services and products.

The application file deposited at IPHAN states that the TAS of Rio Negro is understood as a structured set, formed by interdependent elements: cultivated plants, spaces, social networks, material culture, food systems, traditional knowledge, duties and rights. This cultural asset is anchored in the cultivation of cassava. The wealth of knowledge in this system, as well as the diversity of plants, the circulation networks, the autonomy of families and the sustainable way of producing, are some specificities that guarantee the conservation of the forest and present, as a social base, the more than 22 indigenous peoples who inhabit the region (IPHAN 2019).

In 2018, the IPHAN Advisory Board recognized the 2nd TAS as Brazil's intangible cultural heritage. The TAS of *quilombola* communities in Vale do Ribeira, in the state of São Paulo, consists of knowledge and ways of doing things related to

agricultural practices that these communities maintain in their territories (IPHAN 2020). Some of these practices date back to centuries of existence and are not only related to the act of planting and harvesting food. They are also related to the itinerant *coivara* gardens, the diversity of managed plant species, the methods and material culture related to food preparation, local productive arrangements, exchange and marketing networks, the contexts of knowledge transmission and food consumption that involve expressions of music and dance. Therefore, the TAS of Vale do Ribeira is a cultural expression that has multiple dimensions (IPHAN 2020).

The practice of *coivara* is defined by the cutting of vegetation and subsequent burning for agricultural purposes combined with forest management. *Coivara* is fundamental to the system and represents the accumulation of knowledge over generations, having been adapted and improved by communities in the Vale do Ribeira (The main agricultural foods managed in the TAS and cataloged are the 12 varieties of maize, 22 of cassava, 23 of rice and 21 of beans, in addition to a variety of important crops, but without a systematic survey, for example, sugarcane (*Saccharum officinarum*), yam and sweet potato (Andrade et al. 2019).

A greater number of scientific papers have adopted the name "seed guardians" to farmers and traditional peoples who manage and conserve agricultural biodiversity. The seed guardians, in addition to conserving agrobiodiversity, promote the selection of genetic material and agro-cultural processes such as ways of preparing the land and food, accomplishing something very important for humanity, the co-evolution of plant species (Pinheiro et al. 2020).

The Cenargen, in its most recent regulation for the Germplasm Curatorship System, which is the main administrative instrument used to manage the actors responsible for maintaining the network of germplasm banks, recognizes the role of guardians of seeds, and assigned a seat to a representative of the guardians on their Scientific Technical Committee.

The monitoring of the genetic diversity cultivated by traditional farmers has been carried out through projects that involve NGOs, educational and/or research institutions, and agrobiodiversity guardian farmers themselves. In 2015, the Brazilian Semiarid Articulation (ASA), a network formed by more than three thousand civil society organizations of different natures, which defends, disseminates, and puts into practice, including through public policies, the project of coexistence with the semiarid, started the Semiarid Seeds project, which aims to stimulate the dynamics of self-management of seeds in rural communities, supporting the strengthening of community seed houses, as well as their networking. The project includes training farmers for the production and multiplication of seeds stored in community seed banks and community management of the genetic diversity of crops, aiming to diagnose the most adapted seeds that are cultivated and stored by families, as well as the genetics erosion rate. The project supports and monitors 859 community seed banks in the Brazilian semiarid region (Fig. 9.3).

In the Semiarid Seeds Program, for example, it was possible to identify that backyards are environments of high diversity. This is because the backyards are an environment for children's education, testing and conservation of new varieties. This space is historically managed by women and is generally located around the

house. Urban backyards also function as repositories that represent a part of the genetic diversity of species present in the region.

Widely spread in Brazil (and in Neotropics in general), seed fairs also represent an important strategy for the conservation of genetic resources, as they expand the spaces for exchange of seeds and exchange of associated traditional knowledge, helping to strengthen community management of agrobiodiversity and access to missing species and varieties. Fairs take place in all regions of the country and are responsible for promoting the introduction of new crops in local agricultural systems (Fig. 9.4).

**Fig. 9.3** Facade and interior of a local seed bank located in the semiarid region of Brazil. (Photos: Patricia Goulart Bustamante)


**Fig. 9.4** Seed fairs in different regions of neotropics: (**a**) La Paloma/Rocha/Uruguay (**b**) Arara/Paraíba/Brazil. (Photos: Archive of the interdisciplinary group of agrobiodiversity study – InterABio. Maize races of South American lowlands project)

Considering the strategic importance of genetic resources for food and agriculture and the need for greater coordination between *ex situ, in situ*, and *on farm* genetic resources conservation strategies, it is necessary to adopt innovations in governance with mechanisms that promote shared management collections of genetic resources conserved *ex situ* to intensify collaborative actions and advance the democratic governance of these resources by raising awareness among researchers and public managers on the role of seed guardians and the vitality of traditional agricultural systems for conservation genetic resources.

## 9.5 Final Considerations

Throughout this chapter, some examples and recommendations were presented that suggest some key principles that should guide strategic actions to safeguard the valuable genetic heritage of food plants. They are as follows:

- Intrinsic value of agrobiodiversity: The conservation and use of agrobiodiversity assure the continuity of generation/evolution of genetic variability required to meet present and future environmental, economic and socio-cultural demands. Therefore, agrobiodiversity has an intrinsic value, worthy of care in itself, regardless of its economic value or potential for immediate use.
- Complementarity between the different forms of conservation of genetic resources: The *ex situ* conservation, prioritized by public institutions so far, is strategic in the context of natural disasters, climate change, GMO (genetically modified organism) contamination and conflicts. In addition, the reintroduction of materials stored *ex situ* can contribute to restore the diversity of farming systems affected by genetic erosion. Although neglected, *on farm* conservation, carried out by men and women farmers, is also very important for the conservation of genetic resources. Therefore, recognizing the complementarity between *ex situ* and *on farm* conservation is a condition for the reversion of genetic erosion and to ensure food and nutrition security in the context of climate change.
- Participatory approach: In addition to environmental and economic factors, the diversity of culture, knowledge and people's needs are intrinsically linked to the biological diversity present in food production systems. Thus, the participatory approach should be a crosscutting element in actions aimed at the conservation of genetic resources and agrobiodiversity. The efficacy of measures to stop genetic erosion, either through technology development or through public policies implementation, depends on the integration of knowledge and perspective of farmers, researchers and consumers.
- Respecting and valuing traditional knowledge: Traditional knowledge is part of agrobiodiversity. Actions seeking the conservation of genetic diversity must recognize, respect and value traditional knowledge and associated practices. Therefore, the conservation of genetic resources requires an approach that

includes both the biological dimension and the associated knowledge and cultural values.

- Farmers' rights: Indigenous peoples, traditional communities, men and women farmers have played in the past, play in the present, and will continue to play a major role in the conservation of genetic resources and agrobiodiversity. Consequently, they have full right to their territories. The rights include accessing, using, managing and exchanging genetic resources, as well as free will to decide on access to resources that are under their custody and benefit sharing arising from their utilization. The recognition and enforcement of such rights are essential for them to continue contributing to the conservation and development of genetic variability of staple food species, and thus ensure food and nutrition security.
- Gender equity: Women play a key role in *on farm* conservation of genetic resources and the management of agrobiodiversity. Therefore, conservation strategies should ensure the participation of women, not only for what they have to offer with their knowledge and skills, but based on a reciprocal relationship that promotes equal rights, equal opportunities and poverty reduction.
- Present and future contribution of youth: The knowledge associated with genetic resources and agrobiodiversity result from intergenerational transmission processes. Accordingly, strategies and methodologies that seek the conservation of these resources should recover and reinforce intergenerational knowledge networks, fostering the full participation of youth, their economic and social development, and strengthening their cultural identity.
- Social control: The participation of society in public management is vital for democratic governance and for better effectiveness of government policies and programs. Given the strategic role of conservation of genetic resources for the resilience of food production systems and adaptation to climate change, it is essential that the management of public seed banks incorporates the principles of good governance, adopting a co-management system with broad participation of different stakeholders, particularly those responsible for *on farm* conservation.
- Partnerships and networking: The efforts to curb genetic erosion that threatens the present and the future of food and nutrition security cannot be considered in isolation from other cross cutting issues. The complexity of this challenge demands coordinated action, involving different sectors of government, the scientific community, and custodian farmers of agrobiodiversity, civil society, private sector and consumers. Therefore, the effectiveness of measures for the conservation of genetic resources and agrobiodiversity in a climate change context requires a networking strategy that promotes intersectoral partnership, both at the national and international levels.
- Precaution: In order to safeguard the integrity of the genetic diversity conserved *ex situ* and *on farm*, and the food production systems, it is necessary to enforce the precautionary principle in decision-making processes involving commercial release of GMOs into the environment.
- Sustainability of agrifood systems: Along with soil and water, genetic resources are the basis of food production systems. The pursuit of sustainable agrifood

systems, from the stages of upstream production to distribution and consumption, is imperative for the reduction of pressure over natural resources. In this sense, the promotion of high-nature value traditional farming systems are key steps to achieve the sustainability of agrifood systems, creating the conditions for the conservation of genetic resources and agrobiodiversity.

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# Chapter 10
# Genetic Management Applied to Conservation of Reduced and Fragmented Wild Populations

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## 10.1 Introduction

Many wild populations, especially of endangered species, are found in human-modified landscapes characterized by habitat loss and fragmentation. In these landscapes, populations tend to become small and isolated. Their genetic structure is strongly influenced by genetic drift and inbreeding. Inbreeding promotes changes in the genotype frequencies, increasing homozygosity, which may favor the expression of deleterious alleles (Edmands 2007; Frankham 2010); furthermore, it can push the loss of rare alleles or allele fixation leading to potential population genetic differentiation. Losses in genetic diversity can reduce fitness and the potential for ecological adaptation over time, consequently affecting the average time to species extinction (Frankham et al. 2002; Frankham 2010).

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Studies in neotropical landscapes indicate that habitat loss and fragmentation contribute to the loss of genetic diversity and population genetic differentiation – e.g., lion tamarins (Grativol et al. 2001; Martins et al. 2012; Ayala-Burbano et al. 2017; Moraes et al. 2018a, b), and woody Cerrado species (Antiqueira and Kageyama 2014). For species with greater ecological restrictions (e.g., related to dispersal ability and social behavior), these effects may occur faster than have been expected by researchers – e.g., golden lion tamarin (Moraes et al. 2017). Against this background, genetic management can help prevent and rescue alleles lost in the wild in these endangered species (Moraes et al. 2017; Ralls et al. 2020).

The International Union for Conservation of Nature (IUCN) and the Convention on Biological Diversity (CBD) have highlighted the relevance of genetic diversity for Biological Conservation (Hoban et al. 2020). Commonly, genetic variation has been inferred by estimates of heterozygosity and inbreeding coefficient using pedigree and/or molecular data; the latter mainly based on neutral loci (e.g., Kirk and Freeland 2011). It is noteworthy, however, that there are concerns about the true correlation between neutral genetic diversity and loss of fitness; once homozygosity can be beneficial by eliminating deleterious alleles from populations, and thus, inbred populations with low levels of heterozygosity may have adaptive advantages (e.g., García-Dorado and Caballero 2021; Teixeira and Huber 2021). Despite this potential benefit, conversely, studies have demonstrated that low levels of neutral genetic diversity have a negative effect on population fitness (e.g., Ralls et al. 2020; Reed and Frankham 2003). Therefore, the importance of measuring levels of heterozygosity and inbreeding, even through the use of non-adaptive markers, as a parameter of population viability is still defended by the scientific community (e.g., Hoban et al. 2020; Ralls et al. 2020).

It is important that conservation biologists discuss the implementation of management strategies for the conservation of wild populations within the reduced and fragmented neotropical landscapes (e.g., Niebuhr et al. 2015). In order to contribute to this process, we discuss in this chapter the following topics: (i) When genetic management is a viable conservation strategy and limitations of in situ genetic management in neotropical landscapes; (ii) Planning genetic conservation management actions; (iii) The importance of genetic management to cope with climate change in the Neotropics; (iv) Case studies: What did we learn?; (v) Recommendations for the management of neotropical populations.

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## 10.2 When Genetic Management Is a Viable Conservation Strategy and Limitations for In Situ Genetic Management in Neotropical Landscapes

In situ management may be a necessary conservation strategy when the species is no longer able to sustain itself in its original habitat. Overall the main objectives of in situ management are to promote viability and sustainability in small (or inbred) and fragmented populations, avoiding genetic deterioration. Some questions that can help conservation biologists to decide for an intervention: (1) Has species the effective population size small? Has it experienced significant bottlenecks? (2) Has it lost genetic diversity? (3) Is it suffering the effects of depression and/or genetic drift? (4) Is it fragmented? After identifying the problem, it is necessary to indicate the best conservation intervention. Some of these strategies could be (1) increasing the available habitat area, (2) increasing gene flow through reintroductions or translocations, and (3) re-establishing the functional connectivity of the landscape. Also, it is necessary to investigate whether the environmental and ecological conditions of the landscape that will be managed can sustain the species over time and the genetic and evolutionary aspects of the species conservation (Kleiman et al. 1986; Kleiman 1989; Kierulff et al. 2002; Frankham et al. 2002).

The Neotropics have large areas of natural remnants comprising many highly threatened tropical ecosystems. Consequently, many of their species are threatened with extinction (Mares 1986; Loyola et al. 2009). In addition, the lack of information about the real conservation status of the habitats and the basic biology of the species in the Neotropics are an additional problem to plan conservation efforts. Issues related to historical, political, socio-ecological, economic, and scientific factors hinder also the advancement of research and conservation (Mares 1986). Thus, the solution to this matter involves a conservation policy and financial investments that should include governments and experts from all over the world (Mares 1986; Loyola et al. 2009).

## 10.3 Planning for Genetic Conservation Management

### *10.3.1 Pedigree Versus Molecular Analysis: What Genetics Can and Cannot Do*

The most common way to improve a species' long-term viability (Ramirez et al. 2006) and promote its conservation is to reduce risks of extinction of wild populations by in situ conservation efforts. Alternatively, ex situ captive breeding programs have also been recognized as a powerful strategy for rescuing neotropical endangered species and aiding biological conservation (Rudnick and Lacy 2008; Frankham 2010). Further, for species that were extinct in the wild, such as the Spix's macaw (BirdLife International 2019), ex situ programs are mandatory.

The main goal of an ex situ conservation program is to establish self-sustaining population capable of minimizing inbreeding and loss of genetic diversity over generations, thus maintaining its adaptive potential (Frankham et al. 2002). This is based on the premise that by maintaining a gene pool in captivity that is representative of the species, individuals that will be selected for reintroduction into their natural habitat will carry proper genetic diversity and possibly have a higher probability of survival (Robert 2009). However, the maintenance of animals in captivity can compromise the ability of these individuals to reproduce and survive in nature, since inbreeding depression and adaptation to captivity can promote a rapid modification of the genetic structure in captive groups, which, in general, have a reduced population size (Frankham et al. 2002).

Traditionally, genetic management of captive breeding programs has relied solely on studbooks (Fienieg and Galbusera 2013), which keep detailed pedigree information about all the individuals that are kept in captivity. Although pedigree analyses based on studbooks can provide relevant information for the management of species in captivity, the effectiveness of these approaches depends on their completeness and depth of data (Ayala-Burbano et al. 2020). However, studbooks are often incomplete and may contain errors or data gaps (Ito et al. 2017) and this may result in erroneous genealogical estimates. This is because pedigree analyses assume, among other things, that the founder individuals are unrelated (Lacy et al. 1995), and therefore, the true level of genetic diversity in subsequent generations is likely to be lower than in the theoretical model used (Rudnick and Lacy 2008). Besides, estimations of the individual coefficient of inbreeding are highly susceptible to the quality of pedigree information available (Goyache et al. 2003) and are influenced by the fact that endangered captive populations, in general, remain closed (without regular influx of new individuals) for long periods. As a result, inbreeding can be underestimated. Despite this, for decades pedigree analyses have been the most used method to provide historical summaries, descriptions of current status, guidance on setting population goals and the selection of couples, and projections of future trends for both demographic and genetic aspects of captive, domesticated, and laboratory populations (Lacy et al. 1995; Fernández and Toro 1999; Frankham 2010).

Although the primary use of studbooks data is to improve captive management, pedigree data can provide baseline information on species' life history (e.g., longevity or reproductive lifespan) that is essential in Populations and Habitat Viability Assessments (PHVA). Currently, computer programs that analyze pedigrees are accessible and are widely used in the zoo community (e.g., ARKS, SPARKS, PopLink, PML, PMx Endog). In addition, database platforms developed by the International Species Information System (ISIS) made keeping records easier. Species managers and studbook keepers primarily use these programs to assess the status of a population and to evaluate the effects of management measures adopted (Princée 2016).

The genetic management based on studbook data can use various estimates to monitor the amount of genetic diversity in a population (e.g., Mickelberg 2011). Genetic variation is an important requirement for both short–term and long–term survival of endangered species as it provides a key resource to cope with constant

environmental pressures. However, given the limitations of studbook-based analyses (such as missing data or assumptions equal levels of (un) relatedness among founders), captive breeding programs have included both molecular and pedigree analyses (e.g., Gautschi et al. 2003; Ivy et al. 2009; Mcgreevy et al. 2011; Henkel et al. 2012; Ferrie et al. 2013; Ito et al. 2017).

Molecular data allows to identify unknown parentage and reconstruct pedigrees, and then estimate more accurate genetic diversity parameters for founders and generations born in captivity. In this context, some authors have recently suggested using a genetic data-based index as a complement to the Mate Suitability Index (MSI), that considers differences in genetic diversity, kinship, inbreeding coefficient, and unknown ancestry, all calculated only by pedigree data (Ballou et al. 2001; Ralls and Ballou 2004; Lacy et al. 2012). Thus, the inclusion of molecular data as complementary parameters to evaluate mating pairs would improve management decision-making of conservation proposes – e.g., black lion tamarin (Ayala-Burbano et al. 2020).

In summary, an integrative approach could result in more accurate genetic diversity estimates of captive populations. Molecular markers-based data – such as heterozygosity estimates, allele richness, private allele number, population structure, inbreeding, and kinship – could be monitored over generations helping to identify individuals for reproduction in captive aiming the reintroduction within the neotropical forests (Moraes et al. 2017; López-Cortegano et al. 2019). However, in the Neotropics, these integrative approaches in management programs are still scarce. Despite this, a recent study in the endangered black lion tamarin (BLT), *Leontopithecus chrysopygus*, combined pedigree and molecular data to infer remaining genetic diversity and heterozygosity to the founder generation (F0) of the captive population of the species. Furthermore, a molecular analysis performed on animals identified by PMx analyzes as potentially less harmful mate-pairs, showed that the selected BLTs had a low Internal relatedness (IR) index, indicanting high individual heterozygosity (Ayala-Burbano et al. 2020). Recommendations for the captive breeding program of the species included the translocation of animals from two Brazilian institutions (Primatology Center of Rio de Janeiro and Zoological Park of São Paulo) to the Jersey Zoo (UK), the only place outside of Brazil that keep the species under human care (Wormell and Marques 2020). This ex situ management allowed the influx of new animals that reproduced and resulted in births after about 10 years without offsprings at that institution (https://www.durrell.org/news/durrell-celebrates-the-birth-of-endangered-monkeys/).

### *10.3.2 Reintroduction Versus Translocation Strategies*

Reintroductions and translocations are important management strategies for the conservation of threatened species (Griffith et al. 1989; Fischer and Lindenmayer 2000). Kleiman (1989) defined reintroductions as the release of captive-born animals into the original distribution area of the species where it is currently extirpated, and translocations as the transfer of free-living animals to another location within

the historical distribution area. The principal objective of these conservation strategies is to establish viable populations over time (Griffith et al. 1989; Kleiman 1989; Sigg et al. 2005). So, they must contribute to the increase of population size and the re-colonization and establishment of new populations (Kleiman 1989; Rout et al. 2007), acting directly on both the genetic composition and the demography of population (Kleiman 1989).

The biology of reintroduction emerged as a conservation action around the 1970s and 1980s when charismatic vertebrates were reintroduced within their natural habitat – e.g., golden lion tamarin in Brazil (for review see Seddon et al. 2007). Since then, the number of reintroduced and translocated animal species has increased (Fischer and Lindenmayer 2000), as has the number of successful programs around the world (e.g., Parker 2008; Kierulff et al. 2012; Wright et al. 2014; Michaelides et al. 2015). However, the genetic consequences of these interventions remain often unknown, particularly regarding the genetic diversity of neotropical species (e.g., Moraes et al. 2017).

The maintenance of genetic diversity is essential to species conservation (Brekke et al. 2011). One of the main advantages of translocation and reintroduction is the increase of genetic diversity and the reversal of the effects of inbreeding on small populations. However, despite exerting less pressure on the population than inbreeding, exogamic depression may also be a risk. Exogamic depression occurs when individuals from well-differentiated populations are mixed and the fitness of the population decreases, thus reducing the capacity of a reintroduced or translocated population to adapt to the habitat (Moritz 1999). In such cases, outbreeding should be avoided.

Chacón-Vargas et al. (2020), for instance, evaluated the genetic makeup of ex-situ collections of living palms (*Ceroxylon quindiuense*) and wild populations from Colombian Central Cordillera to select the ex-situ juveniles for reintroduction. However, their results showed that each evaluated population should be considered and protected as a different evolutionary unit (i.e., they were genetically differentiated populations between themselves) and outbreeding was not recommended.

New populations established through translocation have a greater rate of success than those reintroduced (Griffith et al. 1989; Fischer and Lindenmayer 2000). As an example, survival rates (Kierulff et al. 2002) and estimates of effective population size and genetic diversity were higher in populations established from translocated than reintroduced individuals of golden lion tamarins in the Atlantic Forest. On the other hand, there was a greater loss of rare alleles in the translocated than reintroduced set of golden lion tamarins over time (Moraes et al. 2017). These authors showed that loss of alleles may be more pronounced in the first years after the translocation of small ($\leq$11 individuals) and structured groups of golden lion tamarins. It is thus important to monitor and avoid a possible reduction of genetic variability over time, particularly of populations with small founder sizes, because this may compromise the local viability of the species if it is exposed to stochastic events.

In general, larger release groups of individuals are more successful to establish in the wild than smaller ones (Griffith et al. 1989). Programs that could release 100

or more founders had a higher percentage of success than those with lower numbers (for review see Fischer and Lindenmayer 2000). The minimum viable size of founders recommended to avoid inbreeding in newly established populations is around 50 to 5000 individuals depending on the target species (Franklin and Frankham 1998). However, less than 50 individuals are generally used in reintroduction and translocation programs (for review see Fischer and Lindenmayer 2000). The problem is worse if such populations remain small and isolated for a long time, consequently losing genetic diversity and eventually becoming differentiated due to founder effects and genetic drift (e.g., Moraes et al. 2017).

Reintroduction and translocation programs only have positive effects on demographics and genetic composition if the external factors that limit the population expansion are also controlled. Therefore, after a new population has been established, it is imperative to monitor it. So, translocation and reintroduction may be useful conservation strategies as long as they are done in combination with other strategies such as habitat restoration, to guarantee a minimum population size and gene flow among the populations (Kleiman 1989; Moraes et al. 2017).

### *10.3.3 Neotropical Landscape Management Applied to Genetic Conservation*

The expansion of anthropogenic activities has a destabilizing effect on natural populations (Bender et al. 1998). Landscape configuration affects key ecological processes such as dispersal, mortality, and movement of individuals (Baguette and Van Dyck 2007; Baguette et al. 2013; Cote et al. 2017; Jacob et al. 2020). Consequently, habitat loss and fragmentation can reduce genetic diversity and disrupt evolutionary processes, leading to higher vulnerability to extinction (Aguilar et al. 2008). Landscape fragmentation has consequences on habitat quality, occurrence probability, distribution, and abundance of species (Arroyo-Rodríguez and Mandujano 2006). In this context, the size of forest fragments may be related to genetic diversity. For example, Dixo et al. (2009) evaluated populations of the toad *Rhinella ornata*, an endemic species of the Atlantic Forest in Brazil, and compared the genetic diversity between forest fragments of different sizes. They found that population genetic diversity was positively related to fragment size and haplotype diversity was lowest in smallest fragments, likely due to decreases in population sizes. However, a positive correlation of fragment area and genetic diversity is not always observed. A study carried out in the Atlantic Forest of southern Bahia by Ganzhorn et al. (2015) analyzed the population genetics of *Manilkara maxima*, an important endemic and endangered tree, to better understand the effects of fragmentation on density and genetic diversity. They observed that the size of the forest fragments was positively correlated with the variation in adult and young plant density, but not with genetic diversity. Instead, density accounted for 80% of allelic diversity and 70% of allelic richness in both life stages. Finally, the authors concluded that small

forest fragments also had a considerable density, but the larger fragments are of unique conservation value because they hold the greatest number of reproductively mature individuals, the ones necessary for the recruitment of new individuals.

Another important parameter is the location of the habitat remnant in the landscape, if it is isolated from other suitable areas and the matrix is impermeable for the species this will limit gene flow (e.g., Moraes et al. 2018b). The exchange of genes across the landscape is a key process facilitated by landscape connectivity (Benz et al. 2016). Landscape connectivity – the degree to which the landscape facilitates or impedes the movement of individuals among resource patches (Taylor et al. 1993) – has a structural component (e.g. spatial arrangement of landscape elements) and a functional component that considers the behavioral responses of the individuals to landscape elements (e.g. fragments of habitat and edge) (Goodwin 2003). Movement restriction is the main response of animal species to the process of landscape modification (Lindenmayer and Fischer 2006). If movement is limited or interrupted due to isolation of habitat patches, the population can lose genetic diversity by drift and become structured over time (Habel et al. 2015).

Resistance movement estimation models have been used in corridor planning to restore the gene flow between wild populations (Rabinowitz and Zeller 2010; Sawyer et al. 2011). In these models, the first step consists to develop a resistance surface, in which each pixel receives a value that corresponds to the degree of facilitation or impediment to the movements of individuals in each unit of a heterogeneous landscape (Taylor et al. 1993; Adriaensen et al. 2003). In the resistance surface, the identification value of the pixels of each class (e.g. land cover/land use) is transformed into the relative movement cost of the individuals for each class. Castilho et al. (2011) used the patterns of gene flow of the lion *Puma concolor* to parameterize habitat permeability and identify migration areas in southern Brazil.

In a review, Covarrubias et al. (2021) evaluated the effects of natural and anthropogenic elements of landscape on gene flow across temperate, tropical, and subtropical landscapes for anurans. In studies at tropical landscapes, agricultural activities (e.g. Nowakowski et al. 2015), urban settlements (e.g., Zancolli et al. 2014; Eterovick et al. 2016) and habitat fragmentation (e.g., Arroyo-Lambaer et al. 2018) had a negative effect on the genetic patterns of anuran populations. However, there are several examples of studies carried out with different taxa that found low genetic differentiation between populations in landscapes with different degrees of isolation, particularly within the neotropical environments. For example, Costa et al. (2021) investigated the impact of landscape features on the genetic structure of bee populations (*Eulaema nigrita*) among fragments of the Brazilian Atlantic Forest. They found that there was significant but low genetic differentiation between populations, which was attributed to the high-dispersal capacity, together with insufficient time in isolation.

In another study, Campos Telles et al. (2007) found that an anuran species, *Physalaemus cuvieri*, exhibited high levels of gene flow in fragmented landscapes in Brazil, which was attributed to moderate dispersal due to its ecological and life-history characteristics. Jiménez et al. (2020) showed patterns of population

structure and weak isolation-by-distance of the rodent *Heteromys desmarestianus goldmani* in forest fragments permeated by crop fields in Guatemala. There are many sources of variation influencing the detection of landscape effects on gene flow in tropical landscapes, such as species-specific differences in dispersal ability and reproductive systems, historical processes underpinning genetic differentiation, different sample sizes, the resolution of the spatial data (pixel size), the extent of the study area, sampling design, and time-lags in the responses to landscape changes (Monteiro et al. 2019).

Some information generated from landscape genetics studies are essential to plan conservation and management actions, such as identification of dispersal capacity of the species in the landscape (e.g. Moraes et al. 2018b), the capacity of usage of forest corridors by species (e.g., Garrido-Garduño et al. 2016), identification of landscape elements that impact gene flow (Khimoun et al. 2017), identification of suitable areas where reforestation efforts could be extended to reestablish the population connectivity – e.g., via ecological corridors (Alexandre 2018) and bridges over roads (Ascensão et al. 2019) –, and forecast the impact of future climate and land cover changes on gene flow (Thomassen et al. 2010; Velo-Antõn et al. 2013).

Conservation and management strategies can be fundamental for the persistence of populations. For example, Moraes et al. (2018b) found that the most plausible model explaining the variation in the kinship of the golden lion tamarin, included the influence of management (translocations and reintroductions) and landscape resistance. Thus, human-mediated dispersal events characteristic of conservation management strongly affected the genetic structure of this species. However, resistance landscape was also an important variable explaining the gene flow of this species, confirming that the anthropic elements of the landscape can limit its dispersal. These results indicated that human modifications in landscape connectivity may interfere in the genetic conservation of species with high ecological constraints, such as lion tamarins, in a short time – e.g., ≤30 years (Moraes et al. 2017, 2018b). So, the authors emphasized that management strategies, such as translocations and reintroductions, are emergency conservation actions, but they are not able to guarantee by themselves long-term population viability if they remain isolated. Therefore, forest management that increases the matrix's permeability, such as forest corridors and stepping stones, is fundamental to avoid the isolation of populations, especially arboreal animals. Indeed, maintaining or improving landscape connectivity is essential to allow species to follow long-term environmental changes (McGuire et al. 2016). Landscape genetics studies from neotropical areas are still underrepresented (Manel and Holderegger 2013; Monteiro et al. 2019; Covarrubias et al. 2021), and investment in multi-species research applied to management is essential to support the conservation of biodiversity in the Neotropics.

## 10.4 The Genetic Management Importance to Cope with Climate Change in Neotropical Regions

Changes in the climate are widespread, rapid, and intensifying. Human activities are resulting in global temperature warming, rising sea levels, glacial retreats, increasing the frequency of climatic extreme events, like droughts, hurricanes, and heatwaves, and their impacts are projected to worsen (IPCC 2021). The magnitude and rate of the current climate changes are already resulting in a global-scale biological response, affecting marine, freshwater, and terrestrial organisms, and all levels of biodiversity, from organisms to communities (Welbergen et al. 2008; Urban 2015). Nonetheless, the effect of climate change depends on the species' characteristics, such as dispersal capacity and life cycle. For instance, long-life and sessile organisms like trees may not have enough time to adapt to climate changes, and populations located in no longer suitable sites can become extinct. So, climate-related local extinctions have already been observed in hundreds of species (Wiens 2016).

To avoid extinction, species can respond to climate changes in different ways: adaptation through phenotypic plasticity, by moving to a new area with suitable habitat they are adapted to, or by genetic adaptation to cope with new climatic conditions (Dawson et al. 2011). The ability of a species to adapt to the new climatic conditions is associated with the levels of genetic diversity within and among populations. Genetic diversity is the most basal level of biodiversity, represents the evolutionary potential, and is crucial for the maintenance of species over time. However, studies have shown that if the current pattern of greenhouse gas emissions are maintained, most genetic lineages of different groups of organisms will be lost by the end of the century (Ravenscroft et al. 2015; Brown et al. 2016; Lima et al. 2017).

According to the Coupled Model Intercomparison Project 6 (CMIP6) concentration-driven global temperature projections, in the intermediate scenario - Shared Socio-economic Pathway (SSP) 4.5 – which suggests a greenhouse gas emissions peak around 2040 and then they decline by the end of this century, the mean surface air temperature will be 1.6 °C ± 0.4 higher than the mean observed in 1986–2005 (Collins et al. 2013). Under the worst scenario, SSP8.5, in which greenhouse gas emissions continue to rise through the century, the mean temperature in the tropics would be 3.3 °C ± 0.6 higher by 2100. In the tropics, climate change projections are critical. The greatest surface ocean warming is projected to occur in the subtropics and tropics, if the temperature rises by more than 4 °C, about half of the tropical marine species may become locally extinct. Climate change has a significant impact on tropical forests, manifesting in the form of extreme events like droughts, heatwaves, and frequent fires. These events cause tree mortality, hamper tree growth, and limit the forest's overall ability to thrive (Brando et al. 2014). Also, warming is causing changes in the tropical tree communities and their distributions, leading to a shift from moist to drier forest in places like the Amazon and the relocation of species from lower to higher elevations (Fadrique et al. 2018; Aguirre-Gutiérrez et al. 2020).

Neotropical ecosystems contain exceptional biodiversity levels, including high numbers of endemic species (Myers et al. 2000). Moreover, numerous studies have quantified the increasing fragmentation of neotropical forests over time, which complicates the species adaptation to environmental changes (Malhi et al. 2008; Brodie et al. 2012; Corlett 2012). For instance, large-scale agricultural production, mainly involving soybeans, maize and livestock, is a major factor in the decline of both flora and fauna species richness in neotropical forests. This conversion of forests into agricultural land affects the ability of local communities to adapt or disperse as a response to climate change (Keenan 2015). Therefore, it is crucial to develop actions in forest management to preserve or even enhance the resilience of tropical species against climate related challenges and to prevent extinctions.

There are many reasons why climate change increases the need for species management: (i) environmental changes to which species are not adapted; (ii) decrease in reproductive and survival capacities; (iii) increase in demographic catastrophes; and (iv) acceleration of the extinction vortex. Thus, for species unable to rapidly adapt to climate change in their current location or move to another suitable location, some type of management may be necessary (Dawson et al. 2011; Bellard et al. 2012). For this, it is required to identify where new genetic diversity can be obtained, and then use this information to implement management programs to improve the species ability to adapt following climate change. In addition, there is the possibility to translocate populations to more suitable sites. However, other aspects such as interspecific interactions must be considered. Among others, climate change can result in phenological shifts in flowering plants and insect pollinators, culminating in mismatches between them, changing the structure of plant-pollinator networks (Rafferty and Ives 2010).

The conservation consequences of climate change are a current concern. Climate change is accelerating genetic loss and many species will be unable to adapt to new environmental conditions. For this reason, genetic management in those populations is urgent. Assessing genetic diversity combining predictive models is crucial to propose adequate conservation strategies, mainly in regions under massive environmental fragmentation, such as the neotropical region.

## 10.5 Case Studies: What Have We Learned?

### *10.5.1 Golden Lion Tamarin*

The golden lion tamarin (GLT), *Leontopithecus rosalia*, is an arboreal primate endemic to the Atlantic Forest of the state of Rio Janeiro (RJ), Brazil. Originally, it was distributed throughout the RJ coastal lowlands – below 550 m altitude (Kierulff and Rylands 2003). Due to the loss and isolation of its habitat, its main current distribution area is within the São João River basin (Fig. 10.1), located in the central-northern region of the RJ state. In 1964, the GLT was included in the IUCN list of

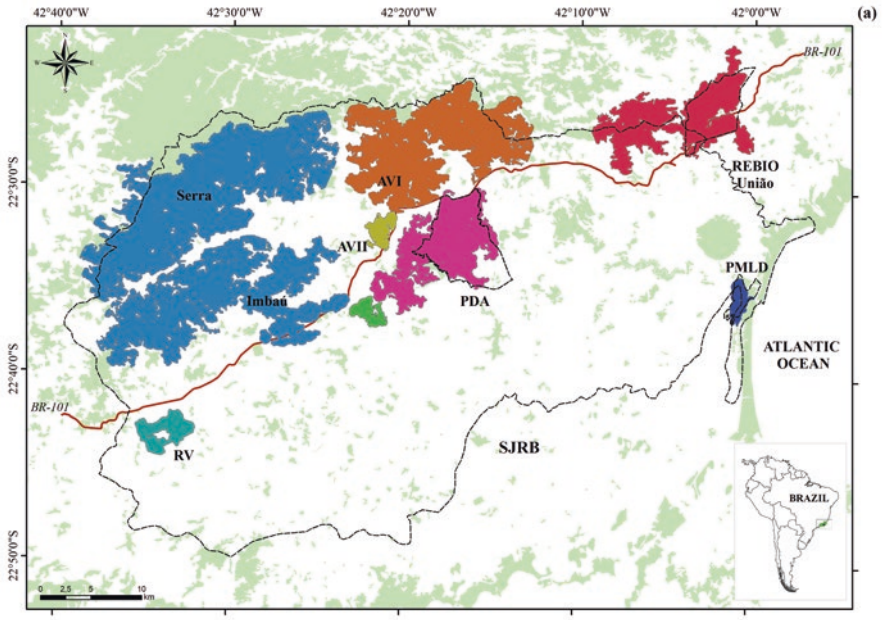


**Fig. 10.1** Simulated areas indicated by different polygon colors where the golden lion tamarin could potentially disperse freely considering that it can travel over distances of up to 8 km. The largest black dashed polygon delimits the São João River Basin, and the smallest black polygon outlines delimit the biological reserves of Poço das Antas (PDA) and União (REBIO União) both within the Atlantic Forest, RJ, Brazil

endangered species. In 1975, only 10% of dense forest and 30% of degraded forest remained in its distribution area. Conservation efforts began in 1974 with the establishment of a captive breeding program led by the Smithsonian's National Zoological Park and the designation of the Poço das Antas Biological Reserve (PDA) for the conservation of the GLT. The PDA was implemented in 1984 with 3000 ha and later expanded to 5000 ha (Brooks et al. 2002; Kierulff et al. 2012). In situ conservation began in 1984 with the golden lion tamarin conservation project (GLTCP), led since 1993 until today by the Associação Mico Leão Dourado (AMLD). The GLTCP/AMLD conservation efforts have included ecological and behavioral studies, environmental education, community participation, reforestation, habitat management for connectivity (reforestation and corridors), and population management (reintroduction and translocation) (Kierulff et al. 2012; Ruiz-Miranda et al. 2019). The establishment of a strong long-term conservation program recovered the species from a "critically endangered" population of 200 animals in the 1980s to an "endangered" population of 3700 animals in 2015, then was reduced in 2016–17 by 32% by a yellow fever epidemic (Kierulff et al. 2008, 2012; Ruiz-Miranda et al. 2019). The project is an example of a multi-stakeholder conservation program aimed at

establishing a multi-use conservation landscape to secure a long-term viable population of the species.

This population management aimed to increase the population numbers in protected or semi-protected areas and to conserve the genetic diversity of wild populations (Kierulff et al. 2002). The reintroduction project consisted of the release of 146 captive-born F1 generation individuals (from 33 founders of 30 zoos) between 1983 and 2000 into forest fragments in privately-owned land. In this way, landowners were motivated to protect their forests and to engage in other conservation activities of the project. The translocation actions were carried out in 1994 to rescue 42 individuals from six social groups that were isolated in fragments of 0.2–2 $km^2$ on the coast of RJ and release them into a 2400 ha forest at Fazenda União (currently the União Biological Reserve – REBIO União), municipality of Rio das Ostras, RJ. Pedigree's analysis showed that the reintroduced population retained 96% of its genetic diversity of the original population (Mickelberg 2011). Analyses of molecular genetic data showed that the reintroduction of captive animals rescued the genetic diversity that was lost in the wild, or that was in the process of being lost (Grativol et al. 2001; Freitas 2012). Over time the reintroduced and translocated populations showed different changes in genetic structure. The translocated population increased its effective size and lowered its inbreeding coefficient over time when compared to the reintroduced population. On the other hand, the reintroduced population had higher retention of alleles over time, when compared to the translocated and native population (i.e., unmanaged) (Moraes et al. 2017).

Landscape structure is one of the factors that still affect the genetic structure of the populations; increasing connectivity is one of the major goals of the conservation program. A study of landscape genetics (Moraes et al. 2018a, b) showed that the dispersal potential of GLT decreases as the complexity of the landscape decreases (i.e., deforestation is greater). Furthermore, some individuals can travel long distances of up to 8 km more frequently if the landscape becomes sufficiently permeable to their movement. These results can be used to direct landscape management plans to increase the functional connectivity of the landscape (for concepts see Tischendorf and Fahrig 2000) and reestablish gene flow between isolated populations.

A map of areas where GLT movements can be reestablished and populations could be functionally connected was generated (Fig. 10.1) using the cost distance tool in the ARCGIS 10.3.1 software (ESRI), the resistance map for the movement of GLT (Moraes et al. 2018a, b), and the occurrence locations of the 2014 survey (Ruiz-Miranda et al. 2019). Four large areas (Serra, AVI, REBIO União, and PDA) were identified and should be the focus of habitat restoration assuming that the GLT is capable of occasional dispersal over 8 km if the connectivity of the landscape is reestablished. In addition to the daily movements that keep individuals within a genetically connected population, many species need less frequent movements over long distances to persist over time (Lindenmayer and Fischer 2006).

### *10.5.2 Spix's Macaws*

The Spix's macaw (SM), *Cyanopsitta spixii*, is one of the most threatened bird species in the world. According to the IUCN (BirdLife International 2019), the SM was initially considered threatened in 1988, up listed as critically endangered in 1994, and was once again up listed as extinct in the wild in 2019 (Barros et al. 2012).

The first known specimen was collected in 1819 and few sightings were reported since (Collar et al. 1992). Thus, it seems that the species has always been rare (Juniper 2002; Collar et al. 1992). In 1986 and 1987 three and two individuals, respectively, were sighted by Paul Roth at Curaçá, in the Caatinga of the state of Bahia, northeast of Brazil (Barros et al. 2012). However, in 1990 Tony Juniper and Carlos Yamashita reported only one individual in this locality (Juniper 2002). This bird was monitored by field biologists from 1991 until it disappeared in 2000 (Barros et al. 2012).

As in most cases of threatened species, the destruction of the habitat of the SM is one of the main threats for the species. More specifically, logging along wooded creeks since the beginning of the nineteenth century had an important impact on the habitat (Juniper 2002). Additionally, capture for the trade was also an important factor, as since its discovery, the SM has been reported in the national and international trade (Juniper and Yamashita 1990, 1991; Collar et al. 1992; Bampi and Da-Ré 1994), despite being so rare.

Since 1990 the Brazilian government coordinates official groups to plan the recovery of the species. These groups were constituted by representatives of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), institutions that keep captive SM, scientific institutions, and conservation organizations. In 2012 the National Action Plan (PAN, acronym in Portuguese) for the Conservation of the SM was published (Barros et al. 2012). The main objective of this PAN is to "Carry out [the] reintroduction of … Spix's macaws in their original area of occurrence until 2024, seeking …[a] continuous population increase and conserving habitats with [the] involvement [of the] community…" (ICMBio 2021). The specific objectives include studies focused on reintroduction methods, the reduction of local capture and hunting of wild fauna, the recovery of the habitat, and the management of the ex situ population. Among the actions listed in the PAN, one is directly involved in the genetic management of the captive population: "Confirm [the] identification of birds, determine the degree of genetic similarity and review the pedigree…".

The official captive reproduction program coordinated by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) and latter by ICMBio started with 17 individuals from the wild (hereafter called as founders), but unfortunately only seven of them reproduced (Barros et al. 2012). Additionally, there was a large imbalance of reproductive success among these founders. For example, the known pedigree in 2008 showed 116 individuals, 15 of them were founders, nine of these founders did not leave any descendant and one pair (male 11 and female 12) produced 21 offsprings and 30 other individuals were direct

descendants of this pair (Barros et al. 2012). At that time, as the population of SM was small, the group coordinated by ICMBio decided that it was more important to produce more individuals than to try to separate inbred but productive pairs.

The genetic analyses (based on minisatellites, Caparroz et al. 2001; and based on microsatellites, Presti 2006, Monteiro 2015) of six founders revealed that they presented higher pairwise genetic similarity than expected between unrelated individuals, which is expected given the original small population. Besides the general low genetic variability observed in the species, it was possible to rank the best pairs based on their genetic similarity: the lower pairwise genetic similarity the better potential reproductive pair (Barros et al. 2012). However, establishing such pairs is complex. First, the birds are kept in institutions in different countries (as an example, currently there are SM in Brazil, Germany, Belgium, and Singapore; C. Lugarini, pers. comm.), and transporting them is expensive and involves issuing many permits. Second, some birds may not be healthy or are too old to reproduce. Also, SM, like other psitacids, choose their partner and this compatibility is very important to achieve reproductive success. Thus, pairwise genetic similarity is only one of the parameters that have been used to recommend the best pairings.

This scenario changed in the middle of 2010s when Al Wabra Wildlife Preservation (AWWP, Qatar) started to use artificial insemination techniques with SM. At that time AWWP held the majority of individuals in the world and, based on microsatellite data (Presti 2006; Monteiro 2015) and artificial insemination results, the AWWP team identified two genetic lineages. When sperm from a different lineage was used to fertilize a female, the reproductive success was higher than when sperm from the same lineage as the females was used in the artificial insemination (C. Purchase, pers. comm.). Thus, this procedure confirmed the importance of using genetic similarity data to achieve higher rates of reproductive success.

Currently, the birds that were once in Qatar are in Germany, where artificial insemination is not used anymore, but chicks are still being born there and in Brazil. The number of SM reached 202 individuals at the end of 2021 (C. Lugarini, pers. comm.) and the first SM were reintroduced in 2022 in Curaçá, BA. The people of Curaçá are thrilled to see their Caatinga sky with SM flying free again.

### *10.5.3 Eugenia Dysenterica: A Tree Species from Brazilian Cerrado*

The Cerrado, a savanna in Central Brazil is the second largest Brazilian biome and one of the world's biodiversity hotspots with a high level of plant endemism (Myers et al. 2000). However, over the last 50 years, more than 50% of its vegetation cover has been cleared or transformed for human uses (e.g., pasture, cash-crop agriculture) (Klink and Machado 2005), putting at risk long-term conservation of various species. Studies combining predictions of geographic range dynamics from Species Distribution Models (SDMs) with genetic analyses have proved to be a promising

approach to better understand the effects of human-induced climate change and habitat loss on the spatial structure of genetic diversity of species in complex scenarios such as in the Cerrado biome.

To predict the effects of climate changes in habitat loss more accurately, Diniz-Filho et al. (2020) applied a framework incorporating information on geographic range shifts in response to future climate and land use changes to establish priorities for genetic management of populations of *E. dysenterica*, a widely-distributed tree species in the Cerrado. In short, they used a matrix of allele frequencies for 23 localities (transformed into a binary 0/1 matrix of allele presence, PA). Around each locality, using a buffer with 10 km radius, the proportion of natural remnants was estimated. SDMs based on species' occurrences are used to estimate environmental suitability in current and future scenarios, whose comparison allows defining the environmental stability for each population. The PA matrix was then used to obtain all possible combinations of localities in which all alleles are represented at least once, and the sets with the smallest number of localities are selected. The frequency of localities in these sets is the irreplaceability of localities. The best solutions are a combination of the sets with the proportion of natural remnants and environmental stability. Finally, these best solutions are discussed in the context of in situ and ex situ genetic management (Fig. 10.2).

The best solution constrained to maximize the mean climatic stability and mean proportion of natural remnants combined includes 14 localities with higher irreplaceability. In this case, to an efficient genetic management planning it is necessary to be creative, once the authors found that some regions will become more unstable under climate change and the regions where it will be climatically suitable in the future represent strongly modified and human-dominated habitats. To conserve all

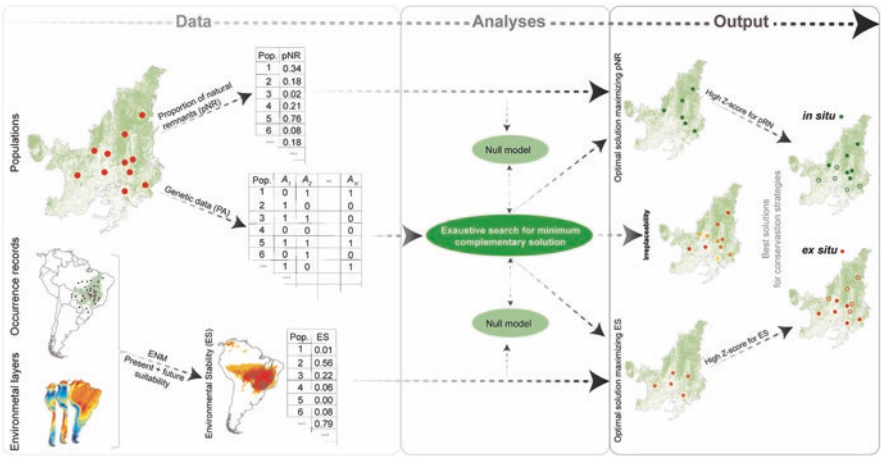


**Fig. 10.2** Schematic representation of the methodological steps to propose a genetic management framework. The framework is based on a complementarity analysis using genetic data, the proportion of natural remnants and Ecological Niche Models (ENMs). (Figure from Diniz-Filho et al. 2020)

genetic diversity of *E. dysenterica* the solution is to adopt in situ conservation strategies in the northwestern part of the species, regions with higher natural remnants. Despite the expected reduction in climatic stability in this region, a suitable microclimate may be enough to maintain these populations. In opposition, the southeastern region, despite the climatic stability, is extremely fragmented with few natural remnants. Thus, here the best strategy may be to adopt *ex-situ* conservation strategies to maintain the genetic diversity from this region.

## 10.6 Recommendations for the Management of Neotropical Populations

- Local factors such as ecological (e.g., carrying capacity, habitat quality), level and kind of threat (e.g., areas where there is illegal poaching) need to be considered when planning management programs and this needs constant evaluation and eventual changes – for reintroduction strategy view more details in Kleiman (1989);
- An integrative approach, including molecular markers-based and pedigree analyses, should be prioritized in captive breeding programs that aim to produce individuals for reintroduction. This will allow well-informed decisions to be made saving money, resources, and, most importantly, species (Fienieg and Galbusera 2013);
- Due to drift, rare alleles are lost more quickly than heterozygosity when populations experience a bottleneck effect (Maruyama and Fuerst 1985). Therefore, whenever possible, before reintroduction or translocation, an increase is recommended for the population size and the frequency of alleles of the founder populations (Grativol et al. 2001), without failing to control the parentage degree between the founders and the possible effects of inbreeding on the new established population;
- Genetic monitoring post-management is important for the delimitation of management strategies that will guarantee the conservation of wild species. We recommend caution when using short-term census data to evaluate the long-term success of translocated or reintroduced populations as a source for the establishment of new populations, which can further aggravate the genetic bottleneck.
- Functional connectivity management must be performed to maintain long-term population viability by restoring interpopulation gene flow and avoiding the negative effects of small population size. For this, multispecies research on the dispersal behavior and landscape genetics should be intensified within the neotropical region to support forest management programs;
- To propose efficient conservation strategies to minimize the vulnerability to climate change, it is necessary to combine population genetics, spatial ecology, and predictive models (through species distribution models) to incorporate genetic data into climate change forecasts and to evaluate the climate-adaptive potential of populations.

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# Chapter 11
# Chromosome Variability of Manatees (*Trichechus* spp.) from Brazil: The State of the Art, Challenges and Perspectives for Management and Conservation

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## 11.1 Introduction

The only extant members of the Order Sirenia are the dugong and three species of manatees – West African, Amazonian, and Antillean (Marsh et al. 2011). Like many other marine and freshwater mammals worldwide, sirenians are in danger of extinction (de Oliveira et al. 2012; Dill 2012; Brum et al. 2021). In fact, a fifth sirenian species, Steller's Sea cow, was exterminated by humans during the eighteenth century (Marsh et al. 2011). There is no reliable data concerning the number of

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individuals for any of the populations of sirenian species, and except for dugongs in Australia, which such estimates are in the tens of thousands, in other countries, especially in developing countries, the estimates are in the low hundreds (Marsh et al. 2011).

Two species of sirenians occur in Brazil: The Antillean manatee (*Trichechus manatus*) and the Amazonian manatee (*Trichechus inunguis*). Both are listed as vulnerable by the IUCN and are critically endangered in several countries despite legal protection (de Oliveira et al. 2012). While *T. manatus* is found on the coast from Amapá to Alagoas state, the Amazonian manatee is endemic to the Amazon River, distributed within numerous rivers and lakes from their source to the mouth in Peru, Colombia, Ecuador and Brazil (Bonvicino et al. 2019). However, manatees can also be found close to the coast at Marajó archipelago, within Pará state, Brazil, which is a complex estuary with several islands, lakes, and rivers, where the mixing influence of fresh and saltwater is distinctive. Remarkably, saltwater intrusion in Marajó Bay and surroundings varies according to the season, with peaks in September to November (dry season) and negligible in the rainy season (January to May). This particular region is considered a sympatric area for the two species of manatees (Domning 1981; Luna 2001; Luna and Passavante 2010; Bonvicino et al. 2019).

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### *11.1.1 Genomic Tools for Conservation in the Manatees – Comparative Cytogenetics from the Hybrid Zone Between South American Manatees*

A better knowledge of the genetic diversity of populations living in geographic regions susceptible to environmental changes is essential for assertive actions for their conservation. This may be the case of the area where the two species of South American manatees are found in sympatry: The Amazon delta, which is influenced by significant human and environmental processes that may progressively damage the status of a non-vulnerable area as it is presently considered (Bonvicino et al. 2019; Anthony et al. 2021). The use of genomic approaches to solve problems related to genetic variation, population viability, and management of local populations is the core of conservation genetics, termed more recently as conservation genomics (Woodruff 2001).

Conservation genomics can inform managers of the optimal actions needed to conserve species and more rigorous and detailed genetic studies may assist managers in making policy decisions that could have long-term consequences for a species (Bonde et al. 2012). In this regard, the conjunction of data generated by different techniques, from more classical approaches to newly developed one, is an essential tool in the process of describing and identifying appropriate taxonomic and population units, being essential for conservation and management programs (Allendorf et al. 2010; Romiguier et al. 2014).

Among these approaches, karyotypical studies have proved to be an important tool in the identification of distinct chromosomal characteristics between species, especially cryptic ones, or even between populations of the same species. This fact shows that cytogenetic studies can have a great impact on management programs for threatened species. Therefore, in breeding programs, when species are difficult to discriminate phenotypically, there is the possibility of the occurrence of crossing between individuals chromosomally different, resulting in hybrids with reduced fertility and loss of the taxonomically pure species (Lukhtanov et al. 2011; Cioffi et al. 2012; Potter and Deakin 2018).

*T. manatus* and *T. inunguis* are found in sympatry in the zone near the Marajó archipelago. Indeed, some studies pointed to the occurrence of hybridization in this area (Garcia-Rodriguez et al. 1998; Vianna et al. 2006; Santos et al. 2016; Lima et al. 2019). Considering that these species have very distinct karyotypes, with 2n = 48 in *T. manatus* and 2n = 56 in *T. inunguis*, hybrids between these species could be readily identified by cytogenetic analyses (Valeri et al. 2021; de Oliveira et al. 2022). In fact, despite the controversial results obtained by different molecular approaches, the unequivocal occurrence of hybrids between *T. manatus* and *T. inunguis* has recently been demonstrated by cytogenetic analyses in individuals found in nature (de Oliveira et al. 2022). These findings show that karyotypical analysis is still essential as part of conservation and reproduction programs of threatened species of mammals. The absence of these data can lead to the formation of hybrids which can present reduced fertility in many cases. Hence, since cytogenetic data

have shown that none of the identified hybrids correspond to F1 products, it is important to analyze this issue more carefully, in order to understand the possible consequences of this process in the conservation of South American manatees.

### *11.1.2 The State of Art: Genetic Variability and Possible Hybridization in* Trichechus *Species*

Most population genetics data belong to *T. manatus*, especially the populations found in the southeastern USA. So far, the genetic homogeneity of manatees inhabiting Florida has been supported by alloenzyme studies (McClenaghan and O'Shea 1988), as well as by mtDNA D-loop sequencing (Garcia-Rodriguez et al. 1998), indicating a recent population bottleneck or a recent colonization from the West Indies. This information formed the basis for implementation of management and conservation measures in the USA (Garcia-Rodriguez et al. 1998). In the same way, the genetic variability observed in *T. manatus* from Mexico was also very low, probably because of the depletion of population stocks due to extensive exploitation up to the 1960–1970 (Nourisson et al. 2011). Interestingly, in comparison to *T. manatus*, mtDNA D-loop sequencing in 68 individuals of the Amazonian manatee, *T. inunguis*, showed a relatively high genetic diversity and effective population size, although this species had also a history of extensive hunting (Cantanhede et al. 2005).

The complex Amazon estuary in Pará and Amapá states has been described as a sympatric area for the two manatee species (Garcia-Rodriguez et al. 1998; Luna 2001; Luna et al. 2008; Bonvicino et al. 2019). Some studies based on mitochondrial DNA have indicated the occurrence of interspecific hybrids between these two species in the estuarine sympatric area, close to the Amazon River mouth (Garcia-Rodriguez et al. 1998; Vianna et al. 2006; Luna 2013; Santos et al. 2016; Lima et al. 2019). In this framework, despite the limited sample size, and absence of inclusion of nuclear markers, it was suggested that the hybridization was a common occurrence in this area (Lima et al. 2019). However, a recent study assessing not only mitochondrial DNA but also 13 nuclear microsatellite markers has found no indication of nuclear hybridization, except for a single captive individual of unknown origin, previously identified as a hybrid by both mtDNA and karyotyping (Luna et al. 2021). This specimen of *T. manatus*, named as "Poque", was the only indication of hybridization analyzed so far, being supported with chromosome and molecular evidences (Vianna et al. 2006; Luna 2013; Lima et al. 2019; Luna et al. 2021). In addition, another recent study based on cytogenetic studies have confirmed that two out of three individuals found stranded in the Marajó island and Amapá coast corresponded to hybrids, due to the presence of heterozygous chromosome pairs (de Oliveira et al. 2022). Therefore, the occurrence and frequency of natural hybrids remain unclear and deserve special attention by researchers and managers.

Several studies have shown that hybridization could threaten endangered species and small populations due to genetic swamping, assimilation, or outbreeding

depression (Rhymer and Simberloff 1996; Rieseberg and Carney 1998; Bohling and Waits 2015). Therefore, hybrids and hybridization zones are usually neglected and considered a threat to conservation goals (Draper et al. 2021). However, studies detailing the negative consequences of introgression and genetic assimilation are still scarce (Chan et al. 2006). Regarding the possible occurrence of hybridization in manatees, it is mandatory to proceed with supplementary studies with the aim of clarifying possible controversial results. In case of confirmation, Draper et al. (2021) suggest that studies should target the biology and stability of the hybrid population, and to determine how this process could threaten the conservation of the parental species. In this sense, chromosomal analyses of individuals collected in nature and verification of possible fertility barriers should be highly considered.

### *11.1.3 Chromosomal Variability in* Trichechus *Species*

There are few reports on cytogenetics of *Trichechus* species, and except for three studies (Kellogg et al. 2007; Pardini et al. 2007; Valeri et al. 2021), the analyses were based only on conventional staining and banding techniques (Loughman et al. 1970; White et al. 1976; Assis et al. 1988; Gray et al. 2002; Hunter et al. 2012; Barros et al. 2016). Although a limited number of individuals were studied cytogenetically, it is well-established that there is a great difference in chromosome number, with the Florida manatee presenting 48 chromosomes (White et al. 1976; Gray et al. 2002; Kellogg et al. 2007; Hunter et al. 2012; Barros et al. 2016; Valeri et al. 2021), while the Amazonian manatee has 2n = 56 (Loughman et al. 1970; Assis et al. 1988; Luna 2013; Valeri et al. 2021). Preliminary results from a study in progress of comparative chromosome painting using whole chromosome probes from *T. manatus* on *T. inunguis* indicate the occurrence of at least one fission involving pair 6, which hybridized on pairs 15 and 27 of *T. inunguis* (Tavares et al. 2021). No further information was provided by the authors.

The first reference to an individual of *T. manatus* with diploid numbers divergent of 2n = 48 was presented by Vianna et al. (2006), who analyzed the karyotype of three captive manatees in Brazil, classified as *T. manatus* based on morphological traits. The results showed a typical 2n = 48 in two of them, however, one of them, named Poque, was a supposed hybrid between *T. manatus* and *T. inunguis* as previously mentioned, with 50 chromosomes. It was suggested that this chromosome number could be the result of an F2 backcross, due to breeding between a F1 hybrid female (2n = 52) and a male *T. manatus* (2n = 48), because this individual presented a *T. inunguis* mtDNA. More recently, two males rescued from natural environments within the area of sympatry were reported with 2n = 49, with chromosome features indicating they were hybrid individuals (de Oliveira et al. 2022).

Apart from numerical differences, it is worth mentioning that karyotypes can also diverge due to morphological differences, mostly identified by difference in arm ratios, and consequently, centromeric position. These differences can be found between close related species, and between different populations of the same

species. Given this, although cytogenetic studies in *Trichechus* species have been performed in a relatively small number of individuals, there is evidence for intraspecific chromosome differences in *T. manatus*. On this regard, Barros et al. (2016) described differences in the morphology of two chromosome pairs in individuals of *T. manatus* from the northeast Brazilian coast when compared to the standard karyotype of this species, based on populations from Florida (Gray et al. 2002). This morphologic variation in chromosomes also contributes to genetic variation within a species, and the real significance of its presence deserves further studies. Indeed, some studies focusing on the effect of chromosome inversions have shown that these processes have possibly an important role in the chromosomal evolution of a wide range of animals (Stefansson et al. 2005; Dobigny et al. 2015). For instance, the findings of Barros et al. (2016) corroborate geometric and morphometric results observed in the Brazilian population. They are also in accordance with the conclusions obtained by mtDNA (Garcia-Rodriguez et al. 1998; Vianna et al. 2006).

### *11.1.4 Poque, the First Described Hybrid*

The hybrid individual was named "Poque" referring to Oiapoque, a city near French Guiana's border where it was found by the CMA/ICMBio staff during an expedition along the Amapá coast. He was illegally held in captivity in an outdoor enclosure with another manatee in Oiapoque city. While the ICMBio team was planning the transfer of the manatees to an appropriate facility, the other manatee had died and Poque survived. Luna (2013) proceeded with an interview with the owner of the facility where Poque was kept, Mr. Wilton de Oliveira Caluf. He explained that Poque's mother was hunted and a calf measuring 1.20 m in length was sent to him. Therefore, this evidence supports Poque's wild origin. In addition, due to some morphological characteristics, the ICMBio/CMA assumed that "Poque" could be a hybrid. The comparison with other *T. manatus* specimens found at the ICMBio/CMA facility showed that Poque was smaller and had only three nails on each pectoral flipper (all other specimens at ICMBio/CMA had four nails). However, according to Reynolds and Odell (1991), *T. manatus* may have three to four nails on each flipper.

Usually, pink or white patches in the skin of *T. inunguis* and their absence in *T. manatus* were also cited as a morphological distinction between these two species (Jefferson et al. 1993, 2008). However, some individuals of *T. manatus* rescued on the northeast coast of Brazil had a white or light pink patch on their bellies. These calves were originally from CE, RN and PB states, which are more than 1000 km distant to the sympatric area. Surprisingly, fourteen individuals of *T. manatus* rescued in Mexico also presented pink marks in the skin, like the patches observed in the Amazonian manatee (Nourisson et al. 2011). Chest marks have also been observed in *T. manatus* from Belize, Puerto Rico and Florida (Luna 2013). Concerning Poque, although the first analysis has suggested the presence of two small white spots in the belly, a more careful examination has proved they corresponded in fact to scars from previous injuries (Luna pers. observation).

### *11.1.5 Conservation Genetics Foster Manatee (*Trichechus *spp.) Management in Brazil*

Even though population genetics is essential in the development of management plans for threatened species (Frankham et al. 2002), studies focusing on the genetic variability of sirenian are still scarce. Despite the increasing number of reports dealing with population genomics, most of the studies have limitations, such as small sample size, or the use of restricted genetic markers. In view of the controversial interpretations concerning the occurrence of hybridization between *T. manatus* and *T. inunguis* in the region where these species are sympatric and considering that karyotype can be used as a primary tool to identify hybrids between species with distinct karyotypes, we performed molecular and chromosomal analyses of individuals of both species of *Trichechus* from different geographical areas. The results were compared to previous studies, to support or not the occurrence of hybridization and to help understand the consequences of this process to the parental species.

## 11.2 Material and Methods

### *11.2.1 Sample Collection*

Samples submitted to cytogenetic analysis for karyotypic characterization were obtained from rescued manatees in Northern Brazil (Omar, Victor, Arari, Ana and Vitória) and captivity ones (Poque, Folião and Bela) at CMA facilities at Ilha de Itamaracá, PE, northern Brazil. The locations of the specimens analyzed are shown in Fig. 11.1; the ellipse punctuates the potential sympatry zone between *Trichechus* spp.

### *11.2.2 Samples*

Blood samples were collected from eight specimens of *Trichechus* spp. (Table 11.1) using disposable syringes and transferred to tubes containing heparin. All procedures for the collection and transport of biological material were licensed by ICMBio through the SISBIO (Sistema de Autorização e Informação em Biodiversidade): No 30327-1 and No 54305-1 to Grupo de Estudos de Mamíferos Aquáticos da Amazônia (GEMAM) of Museu Paraense Emílio Goeldi (MPEG) and SISBIO ICMBio No 55433-7, No 70710-1 and 25473-1 to CMA (Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos). Samples analyzed in the United States were executed under Cites export license 09BR003661/DF (Brazilian) and US import license 08US808447/9.

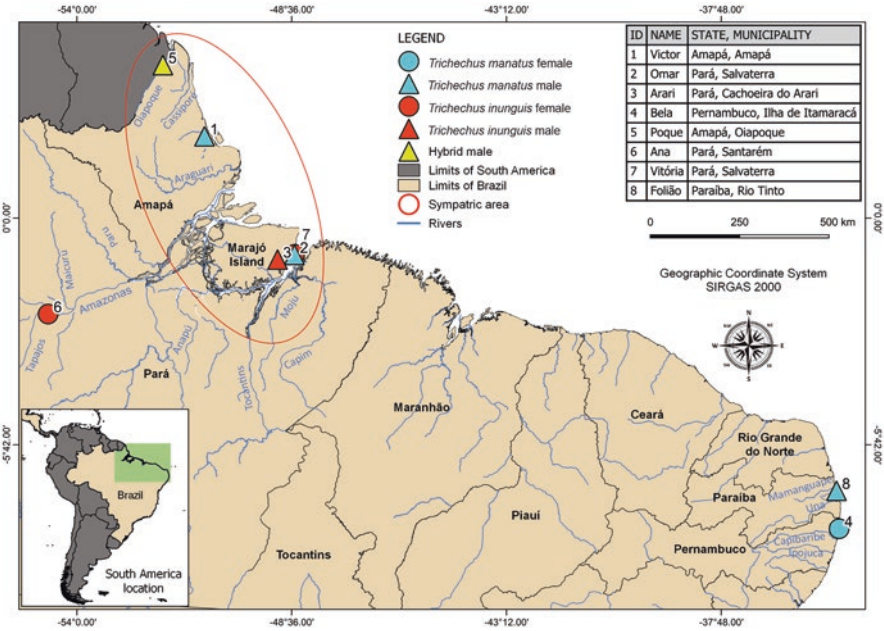


**Fig. 11.1** Locations of the *Trichechus* spp. specimens along the Brazilian coast submitted to cytogenetic analysis for karyotypic characterization. The circle-shaped symbols represent females and triangles represent males. The blue color indicates *Trichechus manatus*, the orange color *Trichechus inunguis* and the yellow color, a hybrid. The sympatric area of *T. inunguis* and *T. manatus* encompassing part of the Amapá state and Marajó island are represented by the red ellipse. This area includes the rivers and bays that receive both marine and the Amazon River influence, favoring the coexistence of the two species

### *11.2.3 Chromosome Obtention*

Lymphocyte culture followed Moorhead et al. (1960) with modifications. Ten drops of whole blood were added to tubes containing 5 ml RPMI medium enriched with 10% bovine calf serum and 100 µl phytohemagglutinin and incubated at 37 °C for 92 hours. After 91 hours of incubation, 150 µl of colcemid (10 µg/ml) were added, and the tubes returned to the incubator for 1–2 hours. Then, the tubes were centrifuged for 10 minutes at 1400 rpm. The supernatant was discarded, and 5 ml of hypotonic solution (KCl 0,75M) at 37 °C were added. After incubation for 20 minutes at 37 °C, ten drops of fresh and cold fixative (3 methanol: 1 acetic acid) were added and the material was gently resuspended. Next, the material was centrifuged, and the supernatant was discarded. This step was repeated for three times, or until the suspension was clean enough to be used.

**Table 11.1** Manatees analyzed in this study, including diploid number (2n) for the animals with previous chromosomal studies

| Specimen | Species | Sex | Geographical origin | 2n |
|---|---|---|---|---|
| Victor | *T. manatus* | Male | Rescued at Macapá municipality, Amapá state | 2n = 49 (de Oliveira et al. 2022) |
| Omar | *T. manatus* | Male | Found stranded at Salvaterra municipality, Marajó island, Pará state | 2n = 49 (de Oliveira et al. 2022) |
| Arari | *T. inunguis* | Male | Found stranded at Cachoeira do Arari municipality, Marajó island, Pará state | 2n = 52 (de Oliveira et al. 2022) |
| Bela | Possible hybrid, considered Poque's calf | Female | Captive born, ICMBio/CMA, Itamaracá, Penambuco state | 2n = 48 (Luna 2013) |
| Poque | Hybrid according to karyotype and molecular DNA analyses. | Male | Oiapoque, Amapá state (captive born or wild population?) | 2n = 50 (Vianna et al. 2006; Luna 2013) |
| Ana | *T. inunguis* | Female | Found stranded at Santarém, Pará state | 2n = 56 (Luna 2013) |
| Vitória | *T. inunguis* | Female | Found stranded at Salvaterra, Marajó island, Pará state | 2n = 56 (Luna 2013) |
| Folião | *T. manatus* | Male | Found stranded at Rio Tinto municipality, Paraíba state, Brazilian northeast coast | 2n = 48 (Luna 2013) |

The classification of *Trichechus manatus* and *Trichechus inunguis* was based on morphological traits and geographical origin

### *11.2.4 Cytogenetic Analyses*

Diploid number and chromosome morphology were determined after subjecting the metaphase plates to conventional staining: one drop of the chromosome suspension was added to a clean glass slide, dried at room temperature, and stained with Giemsa solution (5% in buffer pH 6.8) for 5 minutes. Afterwards, the slides were washed in tap water and dried at room temperature.

Chromosome comparative analyses were based on G-banding patterns. To obtain G-banded chromosomes, the material was submitted to trypsin treatment, according to Seabright (1971), with modification – the slides were stained with Wright solution instead of Giemsa. Slides were analyzed in a light microscope under a 100× lens. The best metaphases were captured, and chromosomes were ordered using the GenAsis software (Applied Spectral Imaging). Chromosomes were numbered according to Gray et al. (2002) for *T. manatus* and Assis et al. (1988) for *T. inunguis*.

## 11.3 Results

### *11.3.1 Diploid Number and Karyotype Description*

We found diploid numbers similar to previous reports in the specimens clearly classified as *T. manatus* (2n = 48) (Figs. 11.2 and 11.3) and *T. inunguis* (2n = 56) (Figs. 11.4, 11.5, and 11.6). However, two animals tentatively classified as *T. manatus* showed 2n = 49 (Figs. 11.7 and 11.8), with an extra small element in their karyotypes. For the hybrid individual Poque, we confirmed a 2n = 50. The results are summarized in Table 11.2.

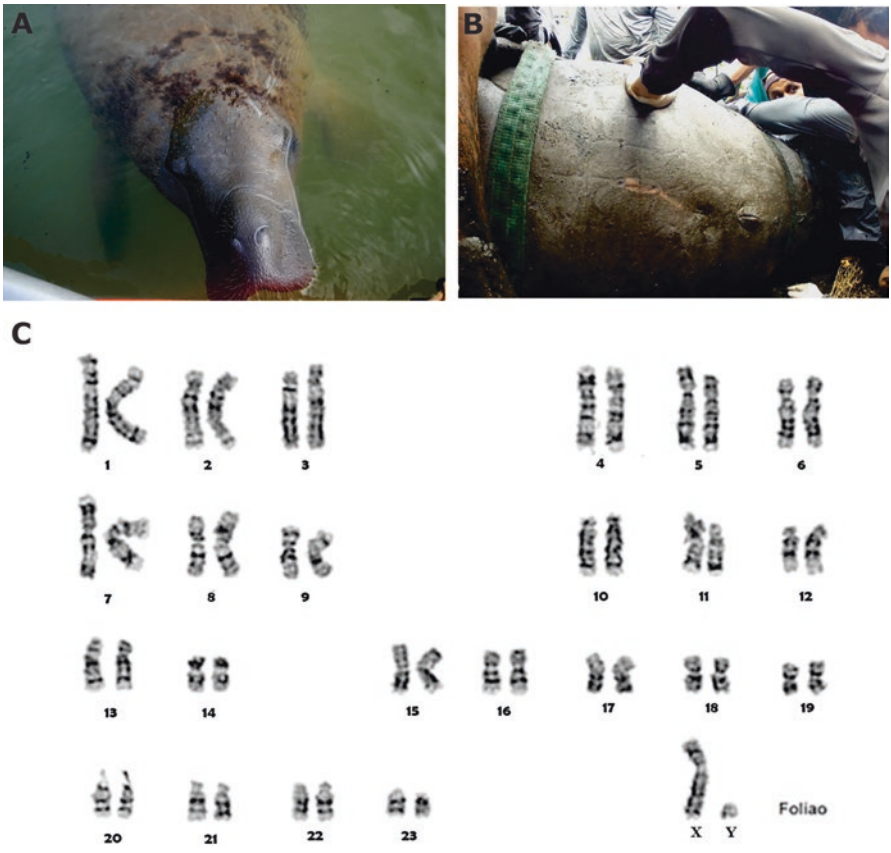


**Fig. 11.2** Folião is a released Antillean manatee with gray-brownish coloring because of the algae attached to its body (**a**). It has nails and a small ventral belly patch (**b**). (Photos by Alexandra Costa and ICMBio collection). Folião presented a chromosome complement typical of a male, *Trichechus manatus*, (**c**)

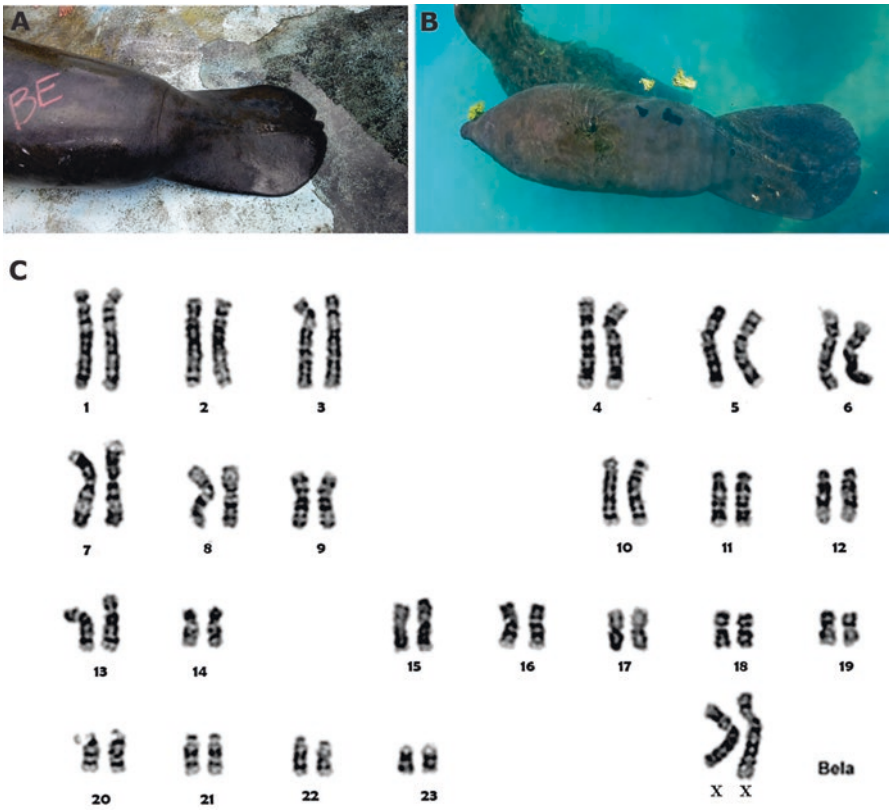


**Fig. 11.3** Bela, a captive born manatee with characters of *Trichechus manatus*. Dark gray coloring with nails, its paddle is more elongated than rounded (**a**, **b**), It is considered an alleged Poque's calf. (Photos ICMBio collection). However, we found a chromosome complement typical of a female *T. manatus* (**c**)

### *11.3.2 G-Banding Comparison*

G-banded chromosomes allowed us to suggest the occurrence of four events of fusion/fission rearrangement involving pairs 1, 6, 8 and 9 (*T. manatus* numbers as reference), which could explain the different diploid numbers 2n = 48 and 2n = 56 for *T. manatus* and *T. inunguis*, respectively. Hence, considering the chromosome alternative form observed in each species, we termed each type of rearrangement as "AA", "BB", "CC" and "DD" for the bi-armed element observed in *T. manatus* and their possible homologous pairs in *T. inunguis* as "aa", "bb", "cc", "dd", as observed in the Fig. 11.10.

We also observed heteromorphism in pair 1 of two possible hybrids – Omar and Poque, as indicated in Figs. 11.8 and 11.9, not related to their hybrid origin. This chromosome morphology may be present in the population of *T. manatus* as

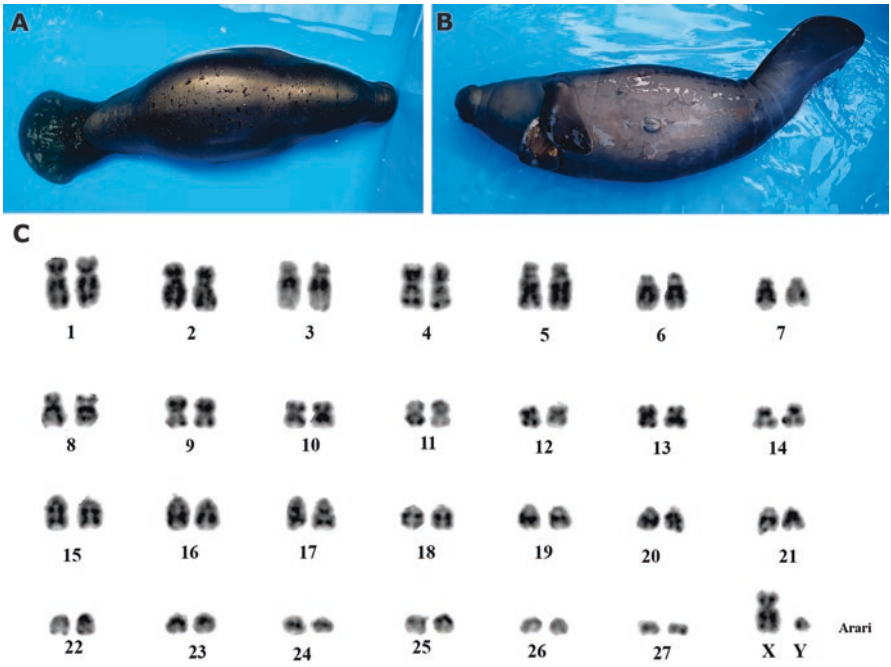


**Fig. 11.4** Arari is a *Trichechus* specimen rescued in Cachoeira do Arari, Pará, has an Amazonian phenotype, rounded paddle tail, but without spots or nails (**a**, **b**). Arari presented a chromosome complement typical of a male, *Trichechus inunguis* (**c**)

polymorphic or heteromorphic state resulted from a pericentric inversion or other mechanism that alter the centromere positions, such as centromere repositioning drift or neocentromere.

## 11.4 Discussion

### *11.4.1 Chromosomal Variation*

Within genetic studies, the revolution in molecular techniques over the last decades had undervalued other traditional fields, including cytogenetics. However, there are cases of intraspecific chromosome polymorphism, usually following geographic patterns, and contributing to the genetic variation of a species (Lukhtanov et al. 2011). In addition, karyotype analyses can be used as a primary tool to identify hybrids between species with distinct chromosome complements, especially in closely related species presenting sympatric distribution (Bulatova et al. 2007). It is also important to consider that chromosomal differences play an important role in partitioning genetic variance, and hence contributing to or directly cause

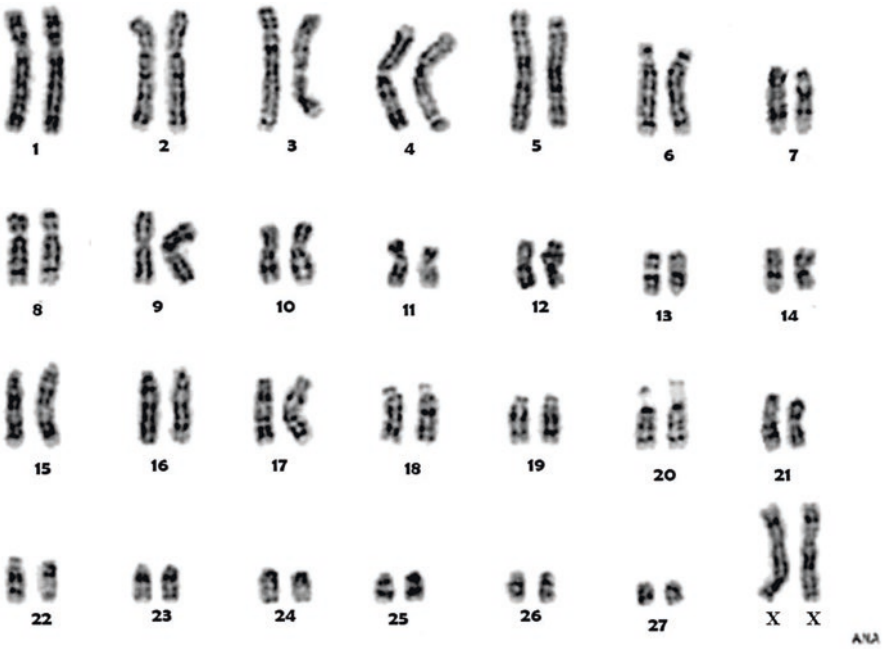


**Fig. 11.5** Chromosome complement of a female, *Trichechus inunguis*, Ana

reproductive isolation (Ellegren and Galtier 2016; Korunes and Noor 2019). Therefore, conservation projects that do not contemplate chromosomal variation risk failure and consequent species extinction (Rowell et al. 2011).

Obviously, the lack of application of cytogenetics in biodiversity research and conservation programs results in poor or only limited knowledge about the actual number and distribution of hybrids (Ráb et al. 2007), although chromosome rearrangements may act as an important mechanism of evolution and maintenance of isolation population in sympatry. As an example, little attention has been given to this subject despite the possibility of hybridization in the zone where *T. manatus* and *T. inunguis* occur.

G-banding is a rapid and cheap technique that allows to characterize the genome organization, arrange karyotype and determine the rearrangements occurred during the karyotypical differentiation within a specific group, especially in mammals (Ferguson-Smith and Trifonov 2007). This technique is not efficient in comparing very divergent karyotypes, in cases where the species are phylogenetically distant or that have extremely rapid chromosome evolution (Graphodatsky et al. 2011). However, G-banding is very useful when the large chromosome segments are conserved between species (Graphodatsky et al. 2011). To facilitate our cytogenetic interpretation of the possible hybrid between *T. inunguis* and *T. manatus*, we compared these species to identify the possible chromosome rearrangements occurred in

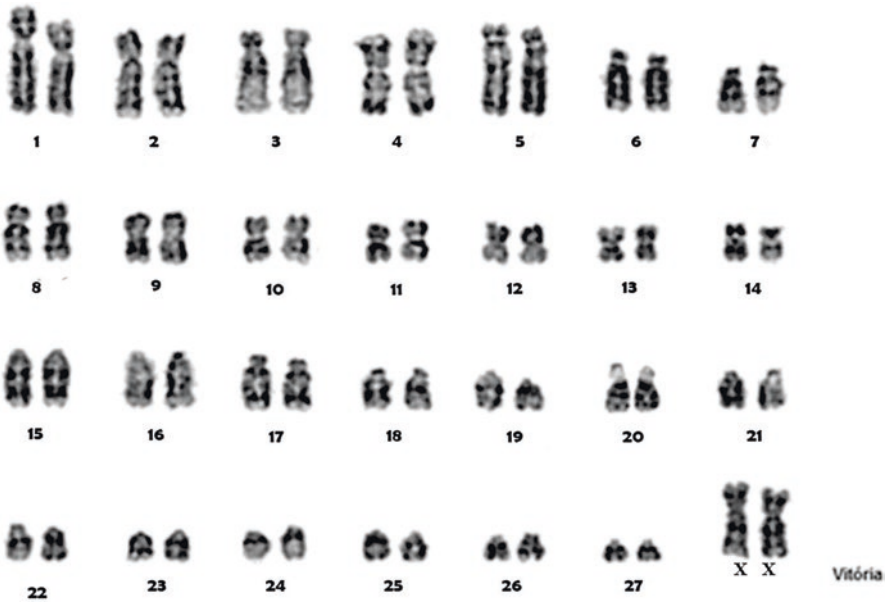


**Fig. 11.6** Chromosome complement of a female, *Trichechus inunguis*, Vitória

their chromosomal differentiation and used these chromosomes as marks to identify possible hybrids (Fig. 11.10).

As previously suggested by Vianna et al. (2006), the specimen named "Poque" would be a F2 backcross resulting from a cross between a F1 Hybrid female with male of *T. manatus*. The F2 backcross would be any variations among 2n = 52–48, due to different possibilities of segregation of the F1 female (genotype "AaBbCcDd"), while the male (genotype AABBCCDD) would produce gametes with n = 24 (ABCD). Following this system, Poque would correspond to the condition "AABBccDD" and 2n = 50.

As already demonstrated by de Oliveira et al. (2022), two of the males originally from the Marajó island and Amapá coast, an area corresponding to the sympatric zone for the two species, showed 2n = 49 (Figs. 11.7 and 11.8). In addition, these two specimens had a mixture of morphological traits. Although, the similar karyotypes and the presence of a small unpaired element led us to propose firstly a possible presence of a B-chromosome, the results of the G-banding analysis indicated these karyotypes were originated by hybridization. Interestingly, Omar shows a heteromorphism in pair 1, also observed in Poque's correspondent pair. However, this heteromorphism is not related to different parental origin, since this syntenic group corresponds to two acrocentric pairs in *T. inunguis*. Hence, the heteromorphic elements would have the same parental species, *T. manatus*, and may indicate the existence of morphological variation in pair 1. In fact, Barros et al. (2016) had already detected polymorphism in pairs 4 and 10 of *T. manatus*. However,

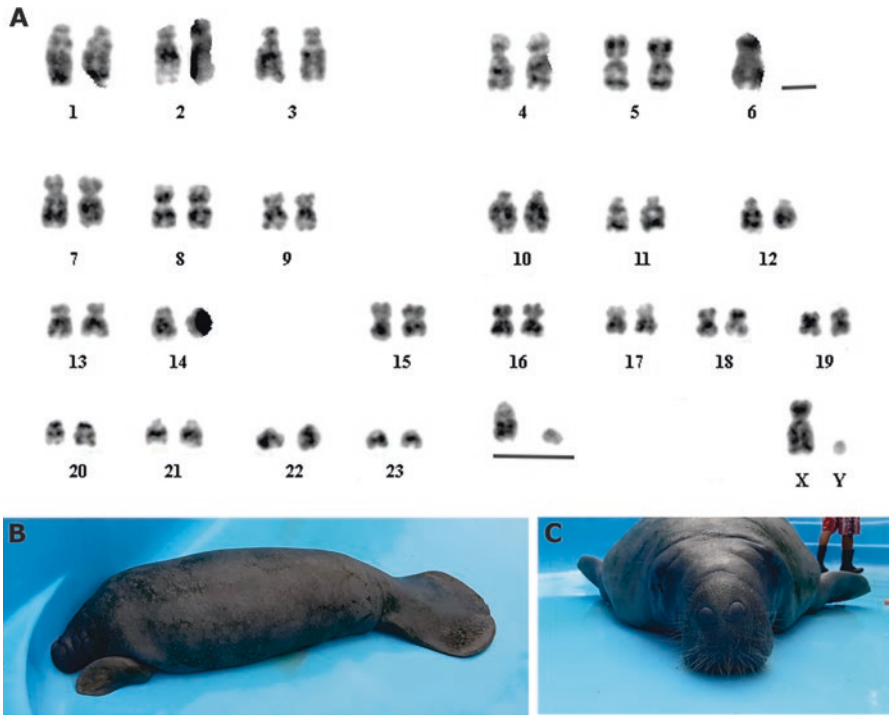


**Fig. 11.7** (**a**) Chromosome complement of a male, *Trichechus manatus*, Victor. Bar indicates the supposed absence of one of the homologous of pair 6 and two extra acrocentric chromosomes, following Assis et al. (1988). This specimen has an Antillean manatee phenotype, with nails, rounded paddle, coloration and thick leather typical of *T. manatus*. (**b**, **c**). (Photos by Fernanda Attademo during clinical management, Amapá state, Brazil)

it is intriguing that both individuals exhibiting this polymorphism show hybrid karyotypes. Again, the small number of individuals studied cytogenetically, representing only restricted areas of their geographical distribution, make further studies necessary to confirm any particularity of this finding.

Following the nomenclature proposed in Fig. 11.10, "Victor" has a 2n = 49, and the analysis of G-band reveals a rearrangement in pair 6, corresponding to the genotype AABbCCDD, while the other male, "Omar", also with 2n = 49, would possibly have the genotype AABBCcDD, and a rearrangement involving pair 8. These findings would reinforce the occurrence of natural hybrids in the sympatric zone, and additionally, considering that Poque, Victor and Omar do not correspond to F1 individuals, but probably were originated by backcrosses, it would imply in unavoidable fertility of hybrids between *T. manatus* and *T. inunguis*.

The other specimens analyzed in this study showed typical *T. manatus* karyotypes (2n = 48, AABBCCDD), as observed in Bela (female) and Folião (male), or typical *T. inunguis* (2n = 56, aabbccdd), observed in females Ana and Vitoria, and in the male Arari. Interestingly, there is a doubt concerning the possibility of Bela

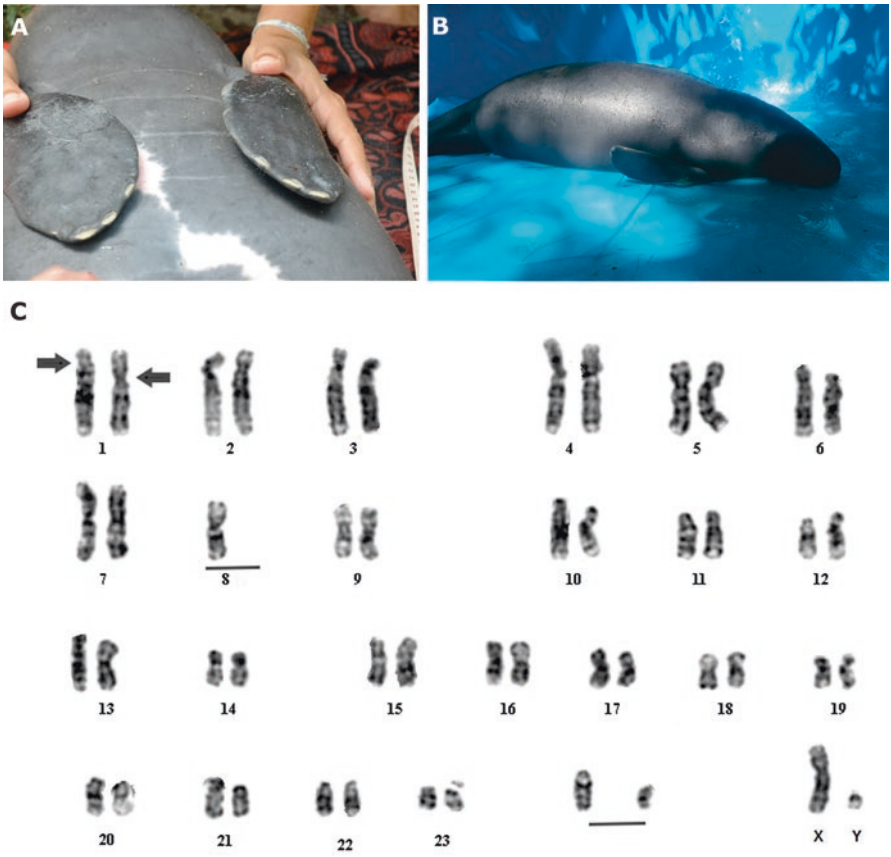


**Fig. 11.8** (**a**) Omar, a specimen held in captivity in Salvaterra, Marajó island, Pará, has an Amazonian phenotype with two spots, one in the belly and one on the ventral part of the rounded paddle. It has nails, and its coloring and skin texture is typical of the Amazonian species (**b**). (Photos IBD collection and Alexandra Costa). However, it presented a chromosome complement typical of a male, *Trichechus manatus*. Arrow indicates heteromorphism in the centromere position of pair 1. Bar indicates the supposed absence of one of the homologous of pair 8 and two extra acrocentric chromosomes, following Gray et al. (2002)

being daughter of the hybrid Poque. Bela has 2n = 48 with typical G-banding pattern of *T. manatus*. If Poque has an AABBccDD genotype, we must discard this possibility. However, it is important to consider that, due the chromosome condensation and the limited G-band patterns observed in the pairs 8 and 9 obtained in the Poque's karyotype, we cannot discard the possibility of an AABBCcDd genotype. If it is so, its meiotic segregation could produce a *T. manatus* karyotype as observed in Bela. Therefore, more analysis would be necessary to clarify the paternity of Bela, as well as to confirm the genotypes observed in Victor, Omar and Poque.

**Table 11.2** Results of the chromosomal analyses in *Trichechus* specimens, including diploid number (2n) and chromosome morphology

| Specimen | Species | 2n | M, SM chromosomes | A, T chromosomes | X | Y |
|---|---|---|---|---|---|---|
| Victor | *T. manatus* (Hybrid?) | 49 | 37 | 10 | SM | A |
| Omar | *T. manatus* (Hybrid?) | 49 | 37 | 10 | SM | A |
| Arari | *T. inunguis* | 56 | 30 | 24 | SM | A |
| Bela | Hybrid? | 48 | 38 | 8 | SM | — |
| Poque | *T. manatus* (Hybrid) | 50 | 36 | 12 | SM | |
| Ana | *T. inunguis* | 56 | 30 | 24 | SM | — |
| Vitória | *T. inunguis* | 56 | 30 | 24 | SM | — |
| Folião | *T. manatus* | 48 | 38 | 8 | SM | A |

Autosomes were classified in Biarmed (metacentric, M – submetacentric, SM) and One-armed (acrocentric, A – telocentric, T) chromosomes. Sex chromosome morphology is presented separately

### *11.4.2 Chromosomes and Hybridization*

Horn et al. (2011) proposed that hybrid zones were characterized according to the meiotic configurations expected in the hybrids. This proposal is based on the fact that it has been assumed that karyotypical differences act as one of the main post-zygotic isolation mechanisms due to disruption of meiosis in hybrids, and isolation barriers are expected to increase as a function of genetic divergence (King 1993; Rieseberg 2001), although hybrids between species with very similar karyotypes may also be sterile, due to genic mechanisms (Moore et al. 1999). Hence, the diploid number differences observed between *T. manatus* and *T. inunguis* would fit well with the assumption of a chromosome barrier in their interspecific hybrids. However, chromosomal studies in hybrids are restricted to the animals included in the present study, none of which corresponded to an expected F1 karyotype. In addition, no meiotic analyses have been performed so far.

It would imply that chromosomal impairments in hybrids are often associated with decreased levels of fertility. Nonetheless, instead of corroborating the karyotypical differences between *T. manatus* and *T. inunguis* as a barrier, our data suggest that their interspecific hybrids are fertile, as already explained based on chromosomal evidence. In addition, although karyotypical divergence and hybridization are usually associated to a role in suppressing recombination leading sometimes to speciation, there is still a lack of solid evidence (Faria and Navarro 2010).

Another aspect to consider is the fitness of a species, which is the result of the influence of natural selection in its habitat. Hence, *T. manatus* and *T. inunguis* hybrids would be expected to be less fit than their parental species. It would happen because the recombinant genotypes found in the hybrids have not been molded by natural selection and should be less well adapted in parental environments (Barton 2001). However, there is the assumption that in recombinant environments, hybrids could show a higher fitness than their parents (Anderson 1948). This could be the case of the region of Marajó archipelago and Amapá state, within the vast Amazon

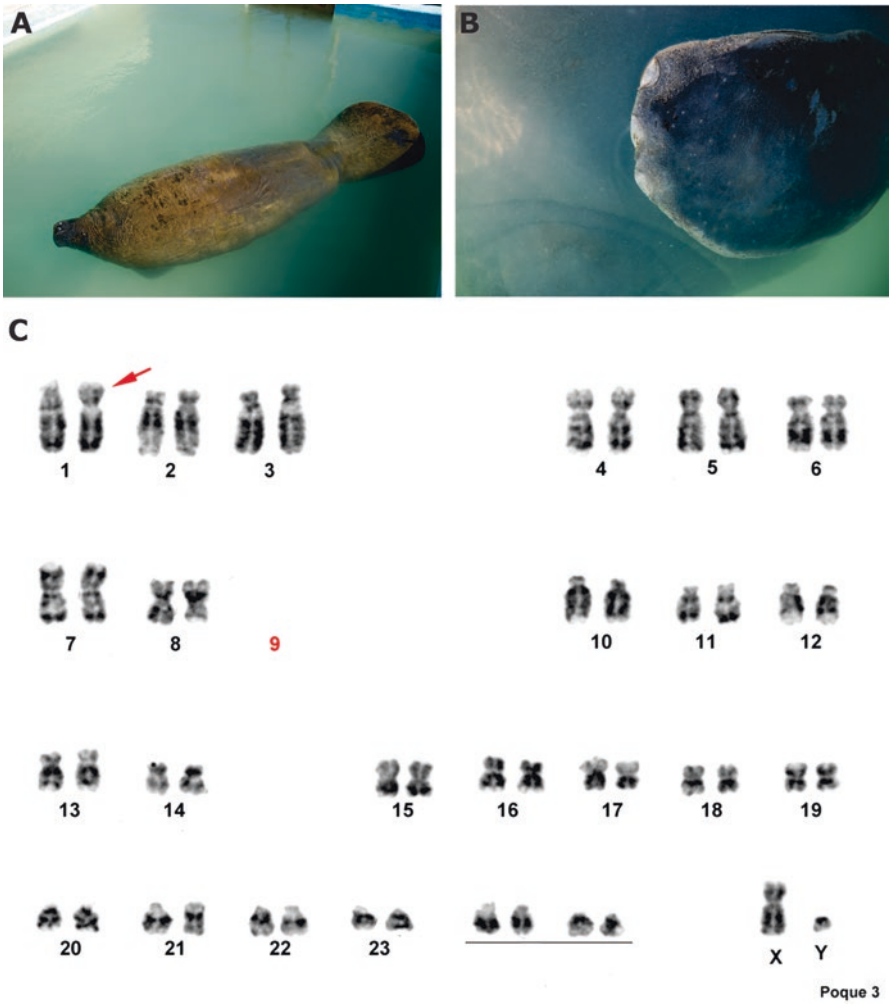


**Fig. 11.9** (**a**) Poque, held in captivity at CMA facilities at Ilha de Itamaracá, PE, shows typical *Trichechus manatus* characters despite being a hybrid. It presents dark gray coloring, is smaller in size than marine specimens of the same age, has nails and a rounded paddle. (**b**) Detail of the three nails, in this pectoral fluke. All pictures by ICMBio collection. (**c**) Chromosome complement found in Poque. Arrow shows heteromorphism in pair 1, not related to the hybrid condition. Note the absence of pair 9 and two extra acrocentric chromosomes (pairs 24 and 25), following Gray et al. (2002)

River Delta, where the freshwater outflow into the ocean causes changes in salinity, especially close to surface (about 4 m in depth) (Lambs et al. 2007) and seems to be commonly occupied by both Amazonian and Antillean manatees. Due to the influence of the Amazon River discharge, in the peak of freshwater influx (January to May), *T. inunguis* occupies the coastline and adjacent lakes.

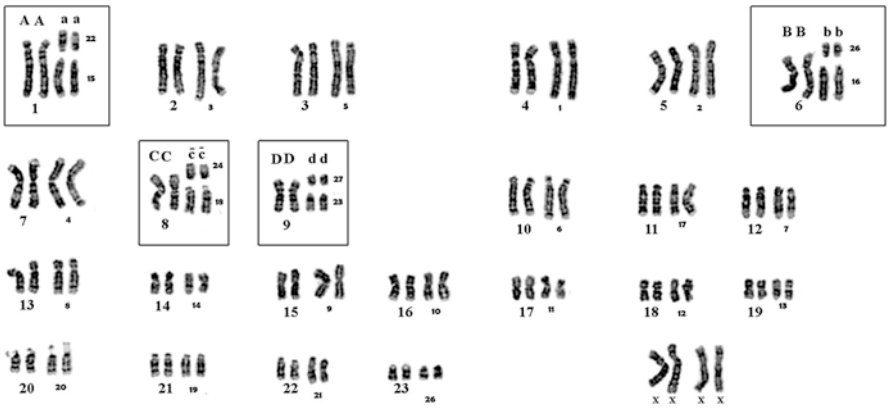


**Fig. 11.10** Comparative analysis of G-banded chromosomes from *Trichechus manatus* (left) and *Trichechus inunguis* (right). Four events of fusion/fission rearrangement would be involved in the differentiation of the two species of manatee (see box). Karyotype was arranged according to Gray et al. (2002). Letters in box represent the homozygous conditions for each rearrangement

The dispersal of Amazonian manatees northwards the mouth of the Amazon River, reaching as far as the French Guiana coast is plausible and could clarify the occurrence of hybridization in this zone. This is reinforced by genetic results indicating none of the possible hybrids – Poque, Victor nor Omar – are first generation hybrids, implying that F1 individuals are fertile, although they could be less fertile than parental individuals. Yet assuming that the coastline from eastern Pará state, the mouth of the Amazon and Amapá coastline is a hybrid zone is premature, as only a few specimens assumed to be hybrids were found so far (Lima et al. 2019).

### *11.4.3 Conservation Issues*

Abundance estimates of the Amazonian manatee are limited to a few areas within the vastitude of its distribution in the Amazon realm. It has been suggested that between 1935 and 1954, over 140,000 Amazonian manatees have been killed (https://marinemammalscience.org/facts/trichechus-inunguis/). For the Antillean manatee population occurring along the northeast coast of Brazil estimates are around 500–1000 individuals (Lima 1997; Luna 2001; Luna and Passavante 2010; Alves et al. 2015; Luna et al. 2018). These numbers suggest how imprecise the population estimates are for both species. However, it thus proposes that, overall, the Amazonian manatee population is much larger than the Antillean manatee. This scenario can draw possible arrangements for the co-occurring zone of Amazonian and Antillean manatees along the mouth of the Amazon River and surrounding Marajó island. *T. inunguis* disperses seasonally into Marajó Bay, as well the Amapá coastline, during the rainy season (Bonvicino et al. 2019). Recent observations of

Amazonian manatees copulating near Belém, and the occurrence of several stranded specimens including newborns (Emin-Lima et al. 2021) strongly argue for the regular use of brackish waters by this species. This study also confirms "pure" Amazonian manatees occupying lakes in Marajó island, such is the case of Arari.

Shurtliff (2011) reviewed the Mammalian hybrid zones and advocates using Mayr (1963) statement that a hybrid swarm is "a population in which parental forms have been fully replaced by individuals with admixed genomes". He continues citing Runck et al. (2009) that "a hybrid swarm is a self-sustaining population, not requiring continued gene flow from parental sources to maintain novel genetic combinations". Taking this in mind, it is fully questionable the assumption of Lima et al. (2019) that nominates the Guianas coastline and northern Brazil as hybrid zone of manatees, with so few specimens genetically analyzed.

We thus recommend that wild manatee's calves stranded and rehabilitated along the northern Brazilian coast could only be released in the same zone that they were rescued even if they are hybrids and if natural hybridization has naturally occurred. It is extremely important that specimens from different species or hybrids kept in captivity do not reproduce while they are held in facilities in order to not force an artificial hybridization.

The Antillean manatee habitat along the Brazilian coast continues to be intensely used by humans and depredation can force the species to seek other areas. This condition can increase the use of a sympatric area for the same periods of the year, also increasing the opportunity of mating between both species. It is necessary to protect the habitat of both species of manatees to avoid potential hybridization, as it can negatively impact both species that are at risk of extinction.

It is highly recommended the development of further investigation along the entire northern Brazilian coast in order to adequately define if there is a hybridization zone and how it affects the conservation of both species. Wild animals captured for health assessments and radio tagging are examples of approches that could be carried out on Marajó island and Amapá coast to define actions necessary to manage and conserve the manatees. Considering our findings and due to G-banding limitations, the use of other cytogenetic techniques (*e.g.*, Zoo-FISH, CGH) would be appropriate to corroborate data and clarify issues such as intraspecific polymorphism.

An extensive study in the area of the Amazon Delta and its drainage basin would be of great importance to characterize the type of hybrid zone existing between manatee species. The negative or positive impact of hybridization must be analyzed with caution, considering the importance of the hybridization zone in the process of speciation and its possible impacts in the parental populations. Thereby, this habitat must be protected and the Action Plans for those species needs to be fully implemented.

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# Chapter 12
# Supplemental Technologies for Freshwater Fish Conservation


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## 12.1 Introduction

The reduction of freshwater fish stocks is strongly associated with habitat destruction. The causes of unsuccessful reproduction and recruitment of certain trophic guilds are discussed in detail, emphasizing the neotropical region. By identifying the factors that prevent reproductive success and the recruitment of a given population, specific remedial actions can be implemented on regional and local scales, considering both conservation of biodiversity and economic development. For example, water level variation is a driving force for the life cycle of several neotropical species. However, simple water level management can locally favor the reproduction and recruitment of certain fish breeding guilds, thus reducing fish stocks in a way that indirectly affects the food chain according to the ecological role of each species. This chapter describes the impact of resultant changes in the trophic chain and actions that can mitigate them. Fish stocking has been successfully used as a management tool. This strategy is analyzed, along with suggested techniques to guide the most genetically favorable breeding for stocking. New technologies to improve the reproductive success of threatened species in fish production are also

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discussed, especially those technologies that minimize the impact of aquaculture on native fish species.

## 12.2 Habitat Manipulation to Favor the Reproduction and Recruitment of Specific Populations

Damming of rivers is a main threat to conserving the biodiversity of neotropical freshwater fish (Vörösmarty et al. 2010). By transforming river basins, dams alter river flows and create artificial lakes (Fig. 12.1). This modifies the connectivity of environments, prevents longitudinal and lateral migrations, fragments river networks, distorts natural pulses, and alters water temperature, thus reducing nutrient supply to downstream waters (Latrubesse et al. 2017; Winemiller et al. 2016). In addition to disrupting gene flow, damming isolates and fragments populations once genetically connected (Machado et al. 2022).

The consequences of river fragmentation on the fish community are enormous and often result in drastic reductions and even local extinctions of species. In addition, dams are almost insurmountable physical barriers, modifying large reaches of the rivers, both upstream and downstream, altering the temporospatial distribution of biodiversity and affecting the functions and services provided by aquatic ecosystems (Bem et al. 2021; Nilsson et al. 2005; Winemiller et al. 2016).

**Fig. 12.1** Hydroelectric power plant on the Canoas River, upper Uruguay River Basin, southern Brazil

### 12.2.1 Spawning and Recruitment

Reservoir formation reduces lotic environments and causes frequent artificial variations in water levels, both upstream and downstream of dams. These changes compromise the reproduction of several neotropical fish species, and for those that manage to spawn, larvae and juveniles may fail to develop, compromising recruitment. These riverine species present reproductive behaviors adapted to certain reproductive tactics that require specific habitats and various environmental cues. These cues include, for example, water level variation associated with turbidity, flood peaks, and temperature elevation. These conditions allow fish to successfully complete the different stages of reproduction, including gonad development, migration or mating behavior, spawning, egg development, and growth of larvae and juveniles (Agostinho et al. 2008; Gogola et al. 2010).

Reproductive and recruitment failures are most evident in highly migratory fish guilds because they require a mosaic of habits frequently found only in the free reaches of rivers. Consequently, these species perform seasonal migrations to spawning habitats generally located upstream of rivers and tributaries to complete the final process of gonadal maturation (Agostinho et al. 2004, 2015; Pelicice et al. 2015; Zaniboni-Filho and Nuñer 2004).

After successful spawning, recruitment will depend on the presence and accessibility of favorable areas for larval development. Despite reproductive plasticity, some species like *Schizodon nasutus* and *Rhamdia quelen* have a diverse array of reproductive tactics that require specific habitats for spawning and early development (Silva et al. 2012). Seasonal spawners are dependent on access to specific habitats, such as marginal lagoons, floodplain, the mouth of tributaries, and pool habitats, for eggs and newly hatched larvae to complete their different life cycle stages (Gogola et al. 2010; Lopes and Zaniboni-Filho 2019; Zaniboni-Filho and Schulz 2003).

Failure in spawning and recruitment can compromise the survival of large Siluriformes and Characiformes, including the dourado *Salminus brasiliensis*, pintado *Pseudoplatystoma corruscans*, pacu *Piaractus mesopotamicus*, jaú *Zugaro jahu*, piracanjuba *Brycon orbignyanus*, and suruvi *Steindachneridion scriptum*. Populations of these species tend not to persist in reservoir areas (Hoeinghaus et al. 2009).

### 12.2.2 Migratory Routes

Blocking migratory routes drastically affects all highly migratory fish in South American rivers. Species in the genera *Salminus*, *Prochilodus*, and *Pseudoplatystoma*, for example, have evolutionary characteristics that require them to undertake long upstream migrations to complete their life cycle. Species of these genera can migrate long distances if they are not presented with such

insurmountable natural barriers as waterfalls. However, recent studies have shown different genetic structures in migratory fishes, revealing different reproductive behaviors and life-history strategies based on different watersheds (Pereira et al. 2009; Ribolli et al. 2017; Rueda et al. 2013).

Because of the life-history strategies of migratory species, the installation of dams makes reproductive migration impossible along many stretches. Thus, migratory fish species must find ways to manage the requirements of spawning and recruitment and their confinement in reduced river stretches caused by damming. This, in turn, causes low population size and lack of genetic flow, thus compromising long-term viability (Ribolli et al. 2021; Machado et al. 2022). Consequently, migratory fish that remain in many fragmented stretches do not find natural conditions for spawning and development (Reynalte-Tataje et al. 2012), culminating in events that cause the collapse of stocks.

Interruption of lateral migration routes causes the disappearance of these remaining spawning and breeding sites of migratory fish. This compromises the development of species that depend on seasonal access to floodplain and marginal lagoon habitats, which are fundamental in basins with inundation areas, such as the Amazon basin and the upper Paraná Basin. Lateral migrations are associated with different life stages in species with different reproductive strategies and are essential for spawning, recruitment, and feeding (Castello 2008; Fernandes 1997; Gogola et al. 2022; Zaniboni-Filho and Schulz 2003).

### *12.2.3 Water Level Regulation: Upstream and Downstream Impacts*

Seasonal variations drive water level in river systems (Junk et al. 1989) and, as such, play a key role in the biology of several freshwater fish species (Baumgartner et al. 2020; Lowe-McConnell 1987). Dams are anthropogenic stressors that are seasonally altered by water level oscillations, both downstream and upstream of dams, but also altered by fluctuations in water level regulated by the demand for electricity (Agostinho et al. 2004; Graf 2006; Vannote et al. 1980). The neotropical region has distinct rainfall patterns, and fish species have adapted to them with specific strategies and tactics that differ among basins. However, artificial water level fluctuations interfere with these rainfall patterns and, consequently, affect the seasonal reproduction of fish (Lowe-McConnell 1987).

Species that spawn on river shores downstream of dams, on reservoir shorelines upstream of dams and those that build nests or use substrates to lay their eggs are drastically affected by variations in water levels (Agostinho et al. 2007). The successful recruitment of different fish breeding guilds is strongly affected by the annual water level fluctuation regime of reservoirs (Lima et al. 2017). Also, oscillations caused by power generation peaks lead to water level fluctuations along the

reservoir shore. This exposes nests built on the reservoir shoreline to the air, resulting in egg mortality (Chaves et al. 2009; Lima et al. 2017). Increased flow and water level below the dam also result from these changes, creating variable flow regimes and causing erosive processes owing to water velocity (Souza Filho et al. 2004). These events all lead to an increased mortality of eggs placed in nests in marginal regions.

At the same time, bottomless reservoirs immediately upstream of dams are entirely different from the original environments, and the vast majority of neotropical riparian species are not adapted to these new conditions (Agostinho et al. 2007). Furthermore, the sections upstream of dams tend to retain sediment and nutrients, limiting their access to downstream sections and, thus, reducing downstream biological productivity (Agostinho et al. 2008).

### *12.2.4 Riparian Deforestation*

Removing much of the natural riparian vegetation is necessary to fill reservoirs during dam construction. Furthermore, riparian trees are adapted to natural periodic changes from floods and droughts, and they do not persist in soils with permanent moisture or drought conditions (Agostinho et al. 2008) (Fig. 12.2). This riparian deforestation impacts aquatic ecosystems and threatens the seasonal migratory movements of frugivorous fish (Correa et al. 2007). Species of the genus *Brycon* are systematically impacted in that they perform both reproductive and trophic migration (Lima 2003; Smith et al. 2003). As a result of multiple anthropogenic impacts, *B. orbignyanus* has become just a remnant in small reaches of the Paraná and Uruguay basins, locally extinct from sites fragmented by dams (Oliveira et al. 2017). Before constructing the first dams on the upper Uruguay River, large schools of *B. orbignyanus* could be found, but after damming, these schools all but disappeared from the upper and middle Uruguay River. Currently, this fish is limited to short reaches in the Turvo Forest Reserve on the border between Brazil and Argentina.

## 12.3 Unsustainability of Populations Caused by Alteration in the Trophic Chain of the Fish Assembly

Even after meeting the requirements that ensure reproduction and recruitment of different fish species, the abundance of each species depends directly on food availability. Thus, changes in fish population dynamics are closely related to variations in pressures associated with the food chain, such as predation, competition, food availability, and both top-down and bottom-up effects. As a result, even small

**Fig. 12.2** Dry trunks of dead trees in the forest flooded by reservoirs

changes in the trophic chain can have an enormous impact on the entire fish community. These changes occur because freshwater ecosystems are exposed to a variety of anthropogenic disturbances (Lake et al. 2000).

Anthropic impacts can be direct, such as habitat loss and modification, river fragmentation, riparian forest removal, pollution, predatory fishing, exotic species introduction, and mining (Agostinho et al. 2008; Barletta et al. 2010; Bastos et al. 2015; Lobón-Cerviá et al. 2016). They can also be indirect, such as climate change (Araújo et al. 2022; Barbarossa et al. 2021).

Changes in biotic and abiotic variables result in the modification of biodiversity at different trophic levels (Jorgensen et al. 2013). A drastic reduction in the illiophagous *Prochilodus mariae* population had a direct effect on the reduction of organic carbon flux in the Orinoco River basin (Taylor et al. 2006). In the neotropical region, the main changes reported for freshwater fish communities are observed in hydropower reservoir environments wherein habitat alteration promotes the proliferation of opportunistic species and the decline of species with greater ecological demands (Agostinho et al. 1999), with migratory species being the most affected (Agostinho et al. 2003; Bem et al. 2021). Among the most recurrent and reported impacts on the trophic chain and consequent alteration in freshwater fish populations are dam construction and the introduction of non-native species.

### 12.3.1 Effect of Damming on Riverine Food Webs

After damming, a peak abundance of migratory and large species usually occurs. This is reduced and later replaced by the dominance and increased abundance of sedentary, medium, and small-sized species (Agostinho et al. 1999; Schork and Zaniboni-Filho 2017; Zaniboni-Filho et al. 2008). Pronounced population reductions also drastically affect trophic structure from the bottom up, such as detritivore-illiophagous (Agostinho et al. 1999), and top-down control, which is related to changes in the abundance of predators (Bem et al. 2021; Pelicice and Agostinho 2009). Among the main changes of trophic structure in these reservoir environments are frequency and abundance of small species (Schork and Zaniboni-Filho 2017), differentiation of abundance and nutritional condition of different trophic guilds (Pereira et al. 2017), and a reduction in the number of top-of-the-chain predators (Agostinho et al. 1999; Bem et al. 2021).

The adverse effects of river damming on neotropical long-distance migratory fish can be observed in drastic population reduction and even local extinction (Hoeinghaus et al. 2009; Petrere Jr et al. 2002; Schork et al. 2013). In the upper Paraná River, the composition of artisanal fisheries changed dramatically after the construction of the Itaipu dam. Before dam construction, landings were composed of eight migratory fish species, five of which were at the top and three at the base of the food chain. However, decades after damming, migratory species disappeared from the catches and were replaced by smaller species with less commercial value (Hoeinghaus et al. 2009). The structure and function of the food chain is directly impacted by the reduction in abundance or complete disappearance of less resilient species and indirectly influenced by top-down and bottom-up effects, respectively.

### 12.3.2 Non-native Fish Species

The introduction of non-native fish species has occurred deliberately in Brazil since the 1970s, as an incentive for sport fishing (Godinho et al. 1994) and as environmental compensation required by law. However, accidental escape from aquaculture activities, both cage units and conventional fish farming, can also be responsible for introducing non-native species (Souza et al. 2021; Zaniboni-Filho et al. 2018). In the Magdalena River basin, for example, the Colombian government introduced at least 29 non-native species in a program to revitalize fishing in the 1970s. This included fish species originating from other neotropical basins, such as *Cichla ocellaris* and *Colossoma macropomum*, and even species originating from other continents, such as *Oncorhynchus* sp., *Tilapia* spp. and *Oreochromis* spp. (Barletta et al. 2015).

The effects of such introductions on the local fish community are unpredictable. That is, once introduced, non-native species can establish, thrive, and alter ecological interactions, e.g., by predation on eggs, larvae, and adults or by competition for

resources and habitats (Kaufman 1992; Pelicice et al. 2017; Pelicice and Agostinho 2009). Some species become established in the environments where they are introduced owing to their voracity as predators, such as the piscivorous *Cichla kelberi* and *Pygocentrus nattereri* (Souza et al. 2021). However, other species have considerable adaptability, such as *Plagioscion squamosissimus*, which was widely introduced in rivers and reservoirs of São Paulo State/Brazil in 1966 and spread to reservoirs in neighboring states like Paraná and Minas Gerais. Although not a voracious predator, this species is a piscivore with feeding plasticity and competes for food resources with other native predator species (Bozza and Hahn 2010; Ferreira Filho et al. 2014). Apart from its adaptability, *P. squamosissimus* uses floating egg-laying, which favors recruitment in lentic reservoir environments.

There is a long history of introducing non-native fishes worldwide (Cucherousset and Olden 2011), and they are a recognized threat of extinction to native freshwater fishes (Dudgeon et al. 2006). The introduction of non-native piscivorous species was related to the local disappearance of *Moenkhausia vittata* and *Oligosarcus solitarius* in the Doce River (Souza et al. 2021). The introductions of *Oncorhynchus mykiss* and *Odonthestes bonariensis* may have extinguished *Orestias cuviehri* and *Trichomycterus rivulatus* in Lake Titicaca (Peru/Bolivia) (Ortega et al. 2007; Vila et al. 2007).

### *12.3.3 Removing Dams*

Although this chapter discusses the impacts of hydropower plants on the fish community, various types of dams, e.g., hydroelectricity, irrigation, water supply, navigation, flood, and pollution control (WCD, 2000), could adversely affect safety, ecology, or biodiversity. For example, the collapse of the Fundão tailings dam severely affected the Rio Doce system in southeast Brazil in 2015, dumping more than 40 million cubic meters of mine tailings, covering about 600 km (Gomes et al. 2017).

Brazil has the greatest number of dams installed in South America, followed by Argentina and Peru (FAO 2015). Currently, hydropower construction is booming globally (Zarfl et al. 2015), focusing mainly on large tropical watersheds (Winemiller et al. 2016). In the Amazon basin, more than a hundred dams are in operation, and more than three hundred projects are underway for new dams (Almeida et al. 2019).

The removal and decommissioning of hydroelectric dams remain unexplored issues in the neotropical region with no records in Latin America. However, a recent trend, particularly in the United States, involves the removal of hydroelectric dams that no longer serve a useful purpose, are too expensive to maintain safely, or have unacceptable levels of environmental impact (WCD 2000). According to the World Commission on Dams (WCD 2000), the number of decommissioned large dams has outpaced the number of dams built with more than 1000 dams removed in recent years in the U.S. (O'Connor et al. 2015). The main objectives of dam removal are to reduce environmental and social impacts, such as removing barriers to fish

migration, restoration, and river connectivity (WCD 2000). However, despite restoring migratory flow, rivers are unlikely to return to pre-dam environmental conditions because the effects of dams are amplified over time (Quiñones et al. 2015). Dam removal is itself a disturbance, and the release of sediment trapped by dams can be damaging in downstream stretches (Poulos and Chernoff 2017), affecting aquatic species and fisheries.

## 12.4 Replenish the Environment With Fish Stocking and/or Potential Breeders in Specific Habitats

In the search for management alternatives for fish populations with reduced supplies, fish stocking should be considered an alternative whenever environmental manipulation proves ineffective. The purpose of fish stocking is generally to recover stocks degraded by environmental changes or over-exploitation (Cowx 1999).

Among fish conservation strategies, stocking fish is one of the most conflicting. In Brazil, restocking programs began decades ago as compensation measures for the construction of dams. A 1927 law requires the construction of fish farms as compensation for damming. Even with repealing the law and rescinding the obligations it stipulates, many hydroelectric projects maintained periodic stocking without the adoption of additional monitoring measures or specific control actions. Since then, stocking has been carried out without clear objectives, scientific support, or evaluation of results (Agostinho et al. 2007, 2010). This precipitated a history of unsatisfactory results, as demonstrated by the numerous records proving the inefficiency of these actions (Agostinho et al. 2007, 2010), not to mention potential genetic damage to target fish populations (Toledo-Filho et al. 1992). Nevertheless, when appropriately done, fish stocking can mitigate and compensate for the reduction and disappearance of some species, reflecting positively on the fish community and riverine fishermen. Furthermore, stocking for mitigation and enhancement can re-establish a depleted fish population that previously played a fundamental ecological role in the sustainability of the fish community, such as top predator fish species. Thus, even in environments where fish cannot reproduce and rear young fish, the presence of these artificially stocked species can help balance the trophic chain (Bem et al. 2021) in parallel with supporting artisanal fishing and food security (Arantes et al. 2019; Hoeinghaus et al. 2009).

Given the history of problems associated with fish stocking in rivers and reservoirs, including the lack of clear objectives, genetic monitoring, and validation of recruitment (Agostinho et al. 2007, 2010), some suggestions for reversing this course are presented below, except stocking with non-native and exotic species (Pelicice et al. 2014, 2017). To recover the stock of native species, structure of the receiving environment and community and characteristics of donor and recipient stock, such as genetic composition, stocking density, and target species, are considered (Agostinho et al. 2010; Cowx 1999).

Stocking objectives can be subdivided into several categories (Cowx 1999; FAO 2015), among which we highlight the following: (i) Stocking for mitigation or compensation is carried out to compensate for disturbances, such as reservoir construction or similar perturbations, in cases where the natural population cannot be self-sustaining; (ii) Stocking for enhancement is used to maintain or improve natural stocks in situations where fishermen are dissatisfied with the amount of fishing or wish to increase stocks in specific stretches of the river; (iii) Stocking for restoration is conducted to recover a stock after an environmental disturbance, and in this case, commitment to environmental improvements, such as dam removal, is a must; (iv) Restocking is used in situations where rebuilding depleted populations is necessary; (v) Stocking for supplementation is applied to populations at risk of extinction or reduced genetic diversity; and (vi) Re-introduction is used to re-establish a locally extinct population.

These stocking actions are generally done with juvenile fish since the same investment allows the release of a larger number of fish. Additionally, the development of these specimens in the stocked environment allows for their interaction in the trophic chain of the managed environment. This affords the stocked population with the opportunity to fulfill an ecological role in both nutrient cycling and predator-prey interactions. While some actions aim to increase the stock for sport fishing, for example, others are performed to increase the reproductive potential of the managed population. In this case, adult specimens are stocked in places giving evidence of reproduction and recruitment of the target species.

The high fecundity of several species of interest for stocking allows the production of millions of larvae from a small number of fish. This condition substantially reduces genetic variability of the batch to be stocked and can damage the genetic quality of the recipient population. For example, deterioration of the genetic quality of the *P. mesopotamicus* stock was recorded in the Barra Bonita reservoir in southeastern Brazil after some decades of periodic stocking of this species (Toledo-Filho et al. 1992). Therefore, genetic monitoring to validate the effectiveness of stocking in rivers and reservoirs where fish were released is crucial (Agostinho et al. 2004, 2007, 2010). This evaluation has been done discreetly in some experimental restocking programs in reservoirs of the upper Uruguay River. In this case, releases are carried out with a small number of marked juveniles per species, and fishing is monitored through collaboration with local fishermen. Results revealed an increase in the catch of migratory fish species, such as *P. lineatus* and *S. brasiliensis*, that had been severely reduced by anthropic actions (Schork et al. 2012), or deemed locally extinct, such as *B. orbignyanus* (Zaniboni-Filho and Schulz 2003). All these stocking tests were carried out with fish individually marked with physical tags, T-bar anchor or hydrostatic tags (Harvey and Carolsfeld 1993), or dyes like oxytetracycline or alizarin red (Fig. 12.3) (Abreu et al. 2014; Hermes-Silva et al. 2017). However, it is recommended that the catch of repopulated individuals be monitored through genetic parentage analysis, as a validation step (Roques et al. 2018).

Although stocking is based on increasing fish production and fishermen's income, environmental restoration and mitigation may be a more viable strategy than stocking to sustain the fish community in rivers and reservoirs (FAO 2015).

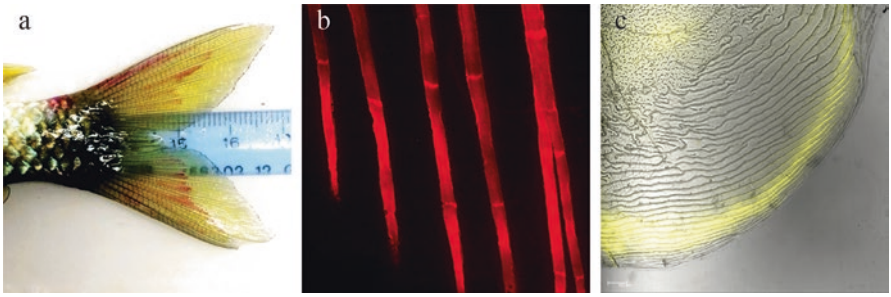


**Fig. 12.3** Fin of *Megaleporinus obtusidens* marked with alizarin red (**a**). Details of alizarin red on the caudal fin rays under a fluorescence microscope (**b**). Scales of *M. obtusidens* marked with the fluorochrome dye Oxytetracycline (OTC) (**c**)

## 12.5 Special Breeding Techniques to Promote the Most Genetically Favorable Breeding for Stocking

Historically, mitigating actions mainly comprise fishing control, construction of transposition ladders in hydroelectric plants, and fish farming for restocking practices (Agostinho et al. 2004). Fishing control aims to regulate the capture of fish during the spawning period, as well as controlling the capture of juvenile or immature fish. Little data are available to guide the effectiveness of transposition ladders since they are selective, and most species are not actually benefited by such engineering (Pelicice and Agostinho 2008). Stocking aims to restore the impacted population by introducing captive-bred fish to increase fish stock and promote catch. However, when performed without planning and/or technical-professional monitoring, stocking of any type can have unwanted consequences, such as the introduction of exotic species, the introduction of animals with different genetic compositions, potentially contaminating the natural population, introduction of fish from different locations with different behavior, either ecological or reproductive, introduction of pathogens, and risk of high inbreeding or ecosystem imbalance, along with the resultant extinction of local species/populations.

Artificial reproduction of captive specimens is a commonly applied management procedure that intends to restore, conserve and/or enhance wild populations. However, although this practice presents potential benefits for species recovery when well executed, studies have reported that artificial breeding has some critical limitations (reviewed in Snyder et al. 1996). Particularly, reproduction of captive breeders frequently leads to genetic, morphological, and behavioral conflicts between captive-bred and wild populations, which can result in failures to preserve wild populations (Price 1999; Snyder et al. 1996). Therefore, several studies have reported artificial breeding strategies that aim to preserve the genetic and phenotypic integrity of the target population (Duchesne and Bernatchez 2002; Theodorou and Couvet 2004). One such strategy is called “supportive breeding” (Wang and Ryman 2001) which implies maintaining a pool of locally adapted wild genitors,

i.e., genitors originating from the target population, in captivity, the offspring of which are released at an early developmental stage. Supportive breeding can offer several advantages. First, the genetic composition of the population is preserved from the use of randomly caught wild breeders at each breeding season (Ribolli et al. 2017), and an adequate breeding design is established (Fiumera et al. 2000, 2004). Second, this strategy avoids the introduction of a different gene pool into the target population for stocking. This kind of genetic contamination of the natural stock frequently occurs in Brazil because it is a common practice to release fish of unknown origin or a different origin from the target population. Third, the differences between captive-bred and wild animals as a result of domestication are limited by releasing first-generation captive animals at an early stage of life, thus limiting exposure to the selective pressures imposed by farming conditions (Kraaijeveld-Smit et al. 2006; Salonen and Peuhkuri 2006).

Assessing the accomplishment of captive-bred fish in nature and their effects on wild populations requires measuring the genetic risks which include inbreeding depression, change in allelic frequencies and/or the introduction of deleterious alleles in wild populations (Ford 2002; Ryman et al. 1995), as well as the morphological, physiological, and behavioral capabilities of captive-bred fish to survive in the wild. For example, parameters linked to genetic diversity are crucial for populations as they encounter environmental changes (Frankham 2008), and traits, such as body size and competitive ability, define an individual's ability to adapt to and survive in natural habitats (Håkansson and Jensen 2005; Hill et al. 2006; Kraaijeveld-Smit et al. 2006). Many studies have only considered one or two components linked to genetic diversity (Hill et al. 2006; Kraaijeveld-Smit et al. 2006; McPhee et al. 2004). However, an assessment of all traits is necessary in order to precisely forecast the success of captive-bred animals in the wild and the effects they may have on wild populations (Kraaijeveld-Smit et al. 2006).

Moreover, considering the high biodiversity of fish in neotropical rivers in Brazil, basic bio-ecological aspects, such as size and amount of the target species, as well as location and time of release, have been frequently neglected (Agostinho et al. 2004, 2007). Some stocking efforts reported in Brazil are conducted by people without any technical knowledge. This has sometimes resulted from court order owing to compensatory measures for anthropogenic activities or from penalties imposed owing to environmental offenses. Under such circumstances, an expert is generally not present to recommend the best stocking practices (Agostinho et al. 2010).

### *12.5.1 Genetic Variability Implications*

One harmful effect of poorly formulated stocking programs is the high degree of inbreeding caused by a high level of parentage between breeders, limited number of parents (sires and dams), or wrong mating strategies. This practice can collapse a stock by the development of a phenomenon called inbreeding depression. This condition results from the fact that biologically related individuals are more likely to

have deleterious recessive genes, causing a decrease in growth, survival, and viability, coupled with an increase in abnormalities (Kincaid 1983; Tave 1999). Therefore, inbreeding levels must be kept below critical limits in terms of biology and genetics since the probability of biological anomalies increases when the parents are related. A relatively high number of breeders should theoretically be used in captivity to avoid unfavorable genetic effects that may negatively affect the fitness of wild populations (Favé et al. 2008; Tenhumberg et al. 2004; Theodorou and Couvet 2004), but this number depends on the genetic background of the target population.

Therefore, for adequate stocking practices, it is essential to consider such factors as effective population number (Ne), genetic diversity, and kinship level between breeders. Effective population number is the number of individuals contributing genetically to the next generation (Nunney and Campbell 1993). In actual practice, however, a simple sampling of the number of individuals in a population often does not correspond to the true population size, which is often higher than the number of individuals estimated in the sample. Imprecise Ne occurs from counting immature and senile individuals in estimating the effective population size; differences in sex ratio; fluctuations in the number of individuals by generations and inequality in reproductive success (Frankham 1995).

The lack of genetic monitoring of breeding stocks and resultant reductions in genetic variability among juveniles released into the wild are common occurrences (Araki and Schmid 2010; Wasko et al. 2004). They can lead to bottleneck effects in natural populations and a reduction in effective population size (Lopera-Barrero et al. 2010). Considering the high fecundity of most neotropical fishes used in stocking programs, when a female in a single spawn can produce millions of eggs, it is common to use a single mating (one female *vs*. one male). This practice should be sufficient to meet the objectives of the storage program, and it is a common practice in Brazilian stocking programs, resulting in a stock composed of millions of full-sib animals released in rivers. At the same time, however, actions like this can lead to the loss of important genes linked to adaptation or resistance to diseases (Povh et al. 2008; Sønstebø et al. 2007) which, in the long term, may cause the extinction of the target species stocked (Innes and Elliott 2006). However, in most cases, stocking is carried out without using previously acquired genetic information about the natural population. Consequently, genetic information is also available for use in the creation of broodstocks (Cowx 2002), which is typically based only on the physical traits of animals (Povh et al. 2008).

Genetic tools are considered important resources for conserving neotropical fish species since the biology and population dynamics of many species still need to be discovered owing to insufficient research. Despite high diversity, some species have wide geographic distribution and differentiated population structure. Thus, populations with genetic variability can be found along neotropical watersheds. These include panmictic populations of long-distance migratory fish species, characterized by high gene flow (Ferreira et al. 2017), as well as restricted populations of local organisms with well-defined population structures (Piorski et al. 2008). Genetic structure is associated with reproductive behaviors (Braga-Silva and Galetti 2016; Pereira et al. 2009; Ribolli et al. 2017). This calls for an understanding of the

genetic diversity of neotropical fish and how it is maintained, as well as how populations are structured, in order to determine appropriate conservation steps or appropriate steps in stocking a target population. Many freshwater fish species have genetic variations with adaptive characteristics that increase survival and reproduction in particular environments and increase the ability of organisms to adapt to environmental changes and human activities (Templeton 2001). The production and stocking of a given species in a basin from breeders obtained in another basin was not appropriated, considering the above, but a common procedure in Brazil (Agostinho et al. 2010) without any scientific support.

Genetic variability in populations can be measured by heterozygosity, allelic richness, and frequency of alleles (Juanes et al. 2007; Mastrochirico-Filho et al. 2019). Intrapopulation variability is influenced by mutation, genetic drift, and natural selection factors. Anthropogenic activities, such as habitat fragmentation and pollution, increase the risks of genetic drift and reduce gene flow, decreasing the genetic variability of populations and interrupting the flow of adaptive genes that can lead to the extinction of some species (Templeton 2001). Thus, molecular genetic markers have emerged as a powerful tool to identify genetic variability in populations (Avise 1994). Genetic marking has opened up new possibilities for the genetic selection of stocks and monitoring of fish aimed at the practice of stocking (Lopera-Barrero et al. 2019; Povh et al. 2011). Among the genetic markers most used, microsatellites, or Single Sequence Repeats (SSRs), which represent a unique type of tandem repeated non-coding DNA genomic sequences, are abundantly distributed throughout the eukaryotic genome with high levels of allelic polymorphism (Oliveira et al. 2006). These molecular markers are codominant where the repeated sequences are of relatively small size (short sequences of 1–4 bp) that can be easily amplified by PCR (Polymerase Chain Reaction). Microsatellites generally span from twenty to a few hundred bases (Chistiakov et al. 2006). This marker is frequently used to assess the genetic variability of farmed populations, and it is aimed at stocking practices in Brazil, such as found in *P. mesopotamicus* (Povh et al. 2011) and *B. orbignyanus* (Lopera-Barrero et al. 2019). Recently, SNPs (single-nucleotide polymorphisms), which are point mutations that produce different alleles for a given nucleotide belonging to a specific locus, have been used as unique nucleotide substitutions of a sequence at a single site. Well characterized since the beginning of DNA sequencing, this marker is adaptable to the automation of genotyping (Mastrochirico-Filho et al. 2021), revealing hidden polymorphisms that other markers and methods do not detect. The SNP of a particular locus can contain up to four alleles (A, T, C, and G). However, most SNPs are generally limited to two alleles (often two C/T pyrimidines or two A/G purines) with codominant inheritance (Mastrochirico-Filho et al. 2021). The amount of polymorphism is not as high as in microsatellite markers (multi-alleles), but compensation for this disadvantage is made by its large quantity in the genome. Some studies were developed to characterize these markers in some neotropical fish (Mastrochirico-Filho et al. 2021), but the use of SNPs to evaluate broodstocks for stocking activities has not been abundantly reported.

### *12.5.2 Performance of Captive-Bred Fish to Survive in the Wild*

The capacity of captive-bred fish to adapt to wild environments is critical to forecast the benefits and genetic and ecological outcomes of supportive breeding programs. Supplementation practices may alter the fitness of the target species, such as those observed in salmonid populations via introgressive hybridization (Aprahamian et al. 2003; Araki and Schmid 2010; Levin et al. 2001). In addition, captive breeding may indirectly select disadvantageous phenotypes in the wild (Blanchet et al. 2008; Fraser 2008; Williams and Hoffman 2009). As salmonid populations are often locally adapted (Fraser et al. 2011; Garcia De Leaniz et al. 2007), the genetic admixture between wild and hatchery may result in a loss of local adaptation and reduced fitness in wild populations (Ford and Myers 2008; McGinnity et al. 2003; Milot et al. 2013).

In the upper Paraná River, promising results were reported considering the correlation between the number of stocked individuals and the fishing yield. This was shown when the weight of released fingerlings increased from 8 to 25 g for *P. mesopotamicus* and from 6 to 18 g for *P. lineatus* (Belmont et al. 2004). In terms of survival, stocking larger/older fish could result in a higher survival rate after release. However, the prolonged commitment required for farming increases the probability of domestication selection and the development of behaviors that are not adequate for the natural environment, such as schooling, increases in naivety or loss of competitiveness (Molony et al. 2003). Several studies have revealed the negative effects of hatchery rearing on the fitness of hatchery fish and reproductive success (Araki and Schmid 2010). In a study conducted with the Atlantic salmon *Salmo salar*, Milot et al. (2013) reported that modifications in survival and growth rates of hatchery-reared individuals alter life-history strategies at the cost of reproductive success. The authors encourage the adoption of more natural rearing conditions for captive juveniles and their release at a younger stage, such as unfed fry. However, studies to better understand the potential fitness decrease of hatchery-reared individuals of neotropical species are still lacking in the literature.

## 12.6 New Findings on Induced Spawning and Larviculture

The main objective of reproduction is the production of offspring that can reach adulthood and breed (Lowe-McConnell 1987). Therefore, fish must spawn when environmental conditions ensure maximum fertilization and larval survival (Harvey and Carolsfeld 1993). It is expected that successful reproduction depends on a timing adjustment to ensure that spawning occurs in the best place and at the exact moment when environmental conditions are most favorable for the survival of offspring. Thus, synchrony between the physiological processes of gonadal maturation and the occurrence of apt environmental conditions happens when a series of

adjustment mechanisms participate in gonadal maturation and spawning, which occur through precise hormonal controls (Zaniboni-Filho and Nuñer 2004).

The possibility of stimulating fish reproduction through environmental induction is viable since this mechanism triggers the entire process under natural conditions. The reproduction of captive fish can be controlled by manipulating water temperature, photoperiod, presence of shelters and nests for mating and spawning (Taranger et al. 2010), decrease of conductivity, water level, and rainfall simulation (Kirschbaum 1984, Chehade et al. 2015). However, the control mechanisms of gonadal development and reproductive behavior of some species, such as migratory species, make this simulation exceedingly difficult under captive conditions (Zaniboni-Filho and Nuñer 2004). Therefore, in most neotropical rheophilic species, reproduction in captivity depends on hormonal induction techniques (Almeida 2013).

One of the main challenges for fish reproduction in captivity is recognizing when the breeding stock is physiologically ready to receive hormonal induction (Carolsfeld 1989; Zaniboni-Filho and Nuñer 2004), and readiness varies according to fish species (Huergo et al. 2021). A subjective description for the selection of fit breeders exists, but the success of this selection depends on personal experience of the technician involved (Huergo et al. 2021). Generally, for most teleost, hormonal induction should occur when females exhibit an enlarged abdomen and rosy genital papilla, while males should release sperm when the abdomen is lightly pressed (Carolsfeld 1989; Woynarovich and Horváth 1983; Zaniboni-Filho and Nuñer 2004).

Several types of substances are used to induce spawning and spermiation, and each substance acts differently on the physiology of the fish. The substances used to promote reproduction in fish have been classified into seven categories (Huergo et al. 2021): (i) Antiestrogens are synthetic substances that compete with estrogens for their receptors, thus preventing their action; (ii) Gonadotropin-releasing hormone (GnRH) is a hormone that stimulates the release of gonadotropins by the adenohypophysis, with consequent increase in the levels of gonadal hormones. More than 3000 analogous forms of GnRH are produced by the industry, known as GnRH-a, and these analogues are 50–100 times more potent than GnRH (Harvey and Carolsfeld 1993). Of these 3000, three analogues are marketed for specific use in fish: (iii) The application of dopamine inhibitors stimulates the release of gonadotropins. They are generally used in combination with GnRH-a to potentiate their effects; (iv) Gonadotropins are substances rich in luteinizing hormones, such as dry pituitary glands (animal pituitary glands) or human chorionic gonadotropin, typically used to induce reproduction in fish; (v) Sexual steroids and corticosteroids are derived from cholesterol: (vi) The use of peptides to induce vitellogenesis is recent, and only a few results are available; (vii) Prostaglandins are fatty acid-derived substances used in species with complex reproductive behavior and multiple spawning.

A synthesis of the results obtained with different substances and protocols for the induction of fish spawning is presented by Huergo et al. (2021), who have provided information on several neotropical fish species. Many studies describe successful hormone induction protocols for the main neotropical migratory fish species and distinct categories of hormones (Almeida 2013; Carolsfeld 1989; Harvey and

Carolsfeld 1993; Pereira et al. 2018; Woynarovich and Horváth 1983; Zaniboni-Filho and Barbosa 1996; Zaniboni-Filho and Nuñer 2004). Studies have also been developed to support the replacement of dry pituitary glands by synthetic products for the induced reproduction of South American rheophilic species. However, results have been mixed, and neither consensus nor routine use of synthetic products in these species has been established (Pereira et al. 2018).

After hormonal treatment and the final maturation of gametes, the fertilization process can proceed in two ways (Woynarovich and Horváth 1983; Zaniboni-Filho and Nuñer 2004). One is keeping the parents in the same tank for courtship and natural release of gametes (semi-natural spawning). The other is stripping, when gametes are removed from males and females by a gentle abdominal massage of fish kept out of water. Gametes are mixed before adding water (dry fertilization), and the fertilized eggs are conditioned in appropriate incubators (Fig. 12.4). In semi-natural spawning, it is necessary to transfer the eggs from spawning tanks to incubators. However, this management practice causes a reduction in egg survival and loss in the quality of larvae (Bermudez et al. 1979). Nevertheless, studies comparing these two fertilization methods revealed that semi-natural spawning produced more remarkable survival of the parents from species sensitive to management (Lopera-Barrero et al. 2019; Reynalte-Tataje et al. 2013).

An alternative to optimize space in dry fertilization is the pooling of semen. Although successful, this alternative does not guarantee that the progeny will descend proportionally to the amount of semen used by each male (Ribolli and Zaniboni-Filho 2009). In addition, milt pools favor the gametes of some males over others, thus reducing the progeny's genetic variability.

The viability of obtaining fish produced in captivity, whether for restocking or forming *in vivo* banks, depends on larviculture, which is one of the most critical

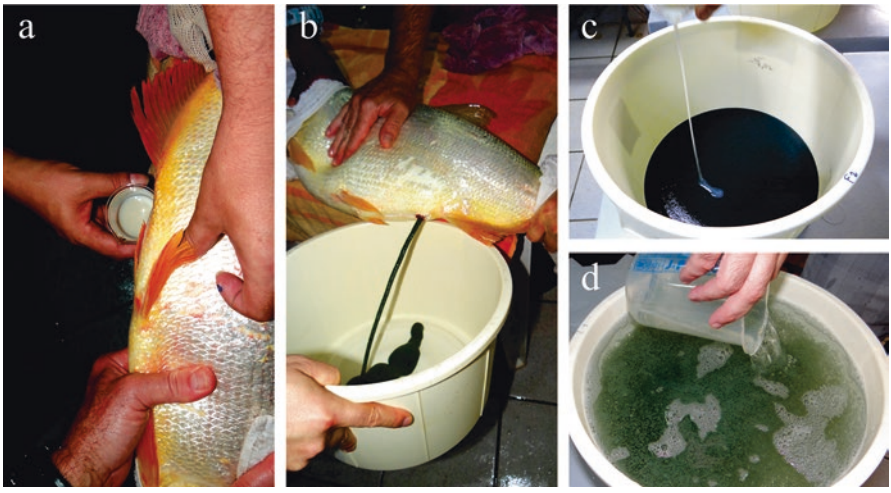


**Fig. 12.4** Dry stripping in male (**a**) and female (**b**) of *Salminus brasiliensis*. Dry fertilization with the mix of gametes (**c**), and addition of water to gametes to complete the fertilization (**d**)

stages of this process. The success of larval production depends on the establishment of variable larviculture protocols. This can be complex, since it depends on many factors, such as species-specific characteristics, environmental factors, feeding characteristics, and rearing conditions (Gisbert et al. 2022; Mukai and Lim 2011).

After spawning and fertilizing the oocytes, the eggs are transferred to incubators until the larvae hatch (Fig. 12.5). In this system, fertilized eggs remain under water flow, guaranteeing aeration of the eggs and the removal of metabolites. For some species, it is also necessary to move the eggs, thus imitating the current in the natural environment of the breeding areas. This technique is used for practically all neotropical fish species reproduced in the laboratory, albeit with some variations and a few exceptions, such as the larviculture of *Arapaima gigas*. Reproduction of this species happens naturally in fishponds, and the larvae are collected and transferred to the laboratory (Gonçalves et al. 2019).

In general, eggs have a high supply of food (yolk reserve; Fig. 12.6), which is used by larvae in the first hours of life and continues until the beginning of exogenous feeding (Oliveira et al. 2004). The beginning of external feeding is the most critical phase in larviculture. The larvae may present morphological limitations to capturing food and physiological limitations for its digestion and assimilation. The most important species in South American aquaculture present altricial characteristics and depend on live food supply during the initial feeding phase (Portella and Dabrowski 2008).

Multiple strategies have been used to reduce losses and produce more robust larvae. One involves keeping the larvae in the indoor culture during the whole period, allowing better water and food quality control. It is also possible to keep larvae in the indoor system for a few hours or days until they pass the critical stage of larviculture, thus reducing initial mortality, and then transferring them to outdoor ponds. The other strategy consists of transferring the larvae from incubators directly to outdoor ponds prepared for the reception of larvae.

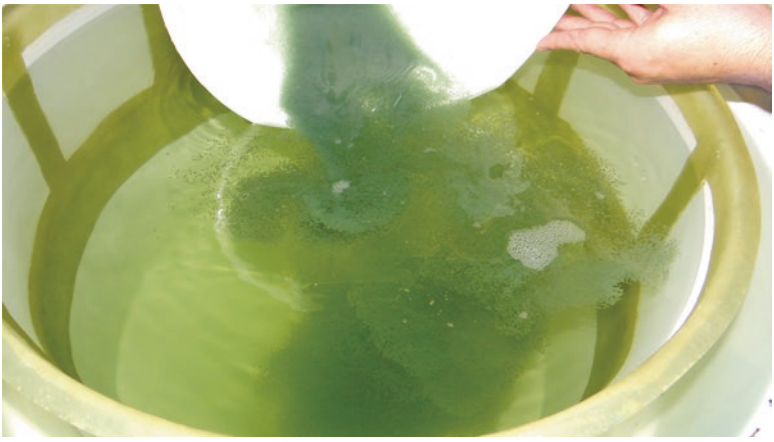

**Fig. 12.5** Fertilized eggs of *Salminus brasiliensis* in incubators under water flow

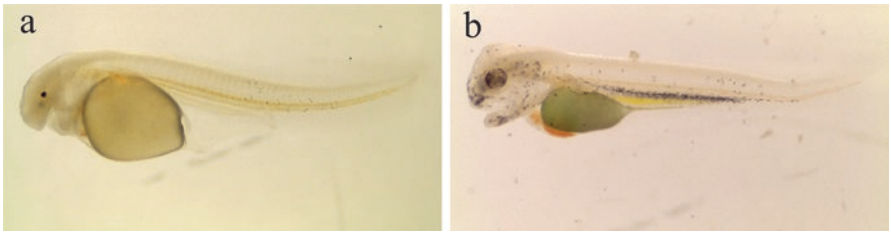


**Fig. 12.6** Newly hatched larvae with a yolk reserve. *Steindachneridion scriputm* (**a**) and *Salminus brasiliensis* (**b**)

In indoor conditions, food with adequate size, density, and composition can be more easily provided for feeding larvae, such as brine shrimp Artemia spp. nauplii, which can be enriched with fatty acids (Léger et al. 1987). Even before outdoor larviculture, a transitional period from endogenous to exogenous feeding is critical for survival before yolk-sac depletion, mainly for the *Pseudoplatystoma* genus (Andrade et al. 2016; Gisbert et al. 2014). At this stage, live food is offered together with dry food for a transition, with slow reduction of the live food up to 100% of dry pellet. This practice is indicated for species with cannibalistic behavior at first feedings, such as *Brycon* (Atencio-García et al. 2003), *Salminus* (Weingartner et al. 2020) (Fig. 12.7), and *Steindachneridion* genera (Fig. 12.8). Adding of probiotics and prebiotics in the feed has been implemented to improve larval health and zootechnical performance (Oliveira et al. 2022).

Larviculture performed entirely in outdoor ponds is widely used by farmers based on the greater operational ease, as well as lower costs. Nevertheless, it presents greater production instability and lower economic yield (Jomori et al. 2003). Some studies have shown advantages in starting indoor larviculture, enabling the larvae to develop physiologically and assimilate inert (dry) food. In the case of *S. brasiliensis*, the digestive ability for inert food appears on the fifth day after the start of exogenous feeding (Vega-Orellana 2006), the period when the best results are obtained for stocking larvae (Mai and Zaniboni-Filho 2005).

## 12.7 Cloning Gametes to Improve Reproductive Success for Threatened Species

The advancement of molecular biology and genomics, together with advances in new tools and approaches, allows increased knowledge of biology and a significant advancement for the conservation of threatened species. Biotechnology provides genetic management to improve reproductive success, such as genome engineering techniques and cryobanks relevant to the survival of threatened species. However, knowledge of basic biological aspects is fundamental, allowing the informed management of captive reproduction of rare or endangered species or species with

**Fig. 12.7** Cannibalistic behavior of *Salminus brasiliensis*

unknown reproductive biology, large size, difficult breeding captivity or very long-life cycle. However, many fish species do not have consistent protocols for inducing oocyte recruitment, development, and maturation (Holt et al. 2004).

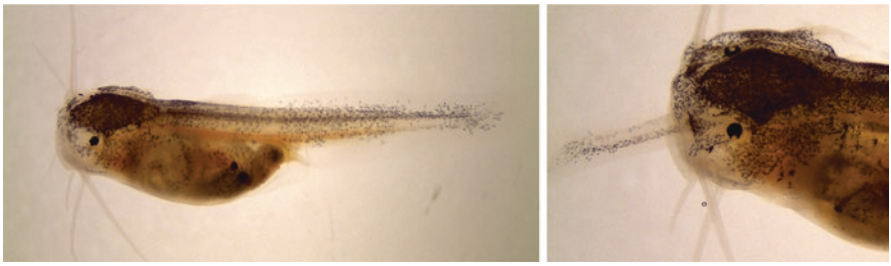

**Fig. 12.8** Cannibalistic behavior of *Steindachneridion scriputm*

### *12.7.1 Cryopreservation*

Cryopreservation of fish sperm for freezing at liquid nitrogen temperature for indefinite storage began through experimental trials with carp half a century ago (Sin 1974). As the practice became more widespread, it has provided the opportunity to establish cryobanks and the distribution of gametes (Carolsfeld et al. 2003). Currently, cryopreservation is a widely established practice and is the primary tool for the long-term conservation of gametes by helping to reduce inbreeding depression and the amount of space needed for broodstock growth (Chandra and Fopp-Bayat 2021).

Like other biotechnological methodologies, such as cloning or germ cell transplantation, cryopreservation of fish sperm needs to be adapted or validated for each species. The first studies with neotropical fish species were initiated in the 1980s, using migratory fish species of the *Leporinus* (Coser et al. 1987), *Prochilodus*, and *Salminus* genera (Coser et al. 1984). Currently, protocols are well established and adapted (Caldas et al. 2021; Motta et al. 2022; Palhares et al. 2021). However, cryopreservation only preserves semen of the parental lineage. Cryopreservation techniques for mammalian embryos became more widespread, thus providing an opportunity to establish cryobanks and the distribution of gametes. However, this methodology for fish embryos has not been successful (Tsai and Lin 2012).

The importance of gene banks for endangered species, when added to aquaculture interests, has encouraged more research on fish embryo cryopreservation, which has now been performed with some species, such as zebrafish, carp, and rainbow trout (Ahammad et al. 2002; Cabrita et al. 2003; Khosla et al. 2020). Research with neotropical fish species started in the 2000s (*P. mesopotamicus*, Streit Jr. et al. 2007; *P. lineatus*, Ninhaus-Silveira et al. 2009). However, the biological characteristics of teleost fish embryos, such as complex membrane structure, large yolk, distinct osmotic properties for each embryo compartment, low permeability of the chorion, and high sensitivity to cooling of fish embryos, particularly in the early stages, are the main barriers to successful cryopreservation of fish embryos (Rall 1993; Valdez et al. 2005; Zhang et al. 2003). In addition, studies have reported morphological lesions in the chorion of embryos after freezing, such as that observed in *P. mesopotamicus* embryos subjected to freezing with intra- and extracellular

cryoprotectants (Fornari et al. 2010; Neves et al. 2012). Despite advances, cryopreservation of neotropical fish embryos is still in its early stages.

On the other hand, oocyte cryopreservation currently presents as the best way to preserve the maternal lineage. Recent studies have reported success with the ultrafast freezing technique, called vitrification, in sea lion oocytes (Hamaratoğlu et al. 2004). However, as observed in fish embryos, high sensitivity to cooling (Tsai et al. 2009) and low membrane permeability remain the main obstacles to the cryopreservation of fish oocytes (Guan et al. 2008). Despite the obstacles, research to achieve satisfactory results in freezing and thawing oocytes, including neotropical fish species, is ongoing.

### *12.7.2 Cloning*

Parallel to the progress in gamete cryopreservation, cloning is one of the indispensable advances in genetic engineering and gamete manipulation (Fatira et al. 2019). Cloning is an asexual reproduction method that reflects the propagation of replicated biological material in various forms (Wakamatsu and Ozato 2002). In fish species, fertilization basically occurs with the union of oocyte and sperm, and cloning manipulations are based on this process. The primary cloning methods in fish are nuclear transplantation, androgenesis and gynogenesis.

The technology of nuclear transfer subverts fertilization by replacing the female genetic material of an unfertilized egg, which is genetically enucleated using irradiation with gamma rays, X-rays, UV rays, or cold-shock (Hou et al. 2015), with the nucleus from a different cell for the generation of a new individual genetically identical to the somatic cell donor (Gurdon and Colman 1999). The specific germline cells (eggs and sperm) have a complete set of genes and offer an avenue of possibilities for cloning.

Androgenesis is the development of embryos without genetic contribution from oocytes. As a first step to inducing androgenetic doubled haploids, i.e., organisms derived from a single progenitor, the eggs are genetically enucleated and subsequently fertilized with viable sperm (Das 2014). The combination of cryopreserved sperm and androgenesis in fish is exceptionally advantageous as it allows for the transport and conservation of male gametes for an indefinite period. In specific situations, androgenetic cloning of fish can be a conservation strategy, such as that performed in salmonid eggs fertilized with heterospecific sperm (Nagoya et al. 2010) or used for the induction of interspecific androgenetic cloning (Pandian and Kirankumar 2003).

In the gynogenesis process, progeny is exclusively composed of the maternal genotype with no contribution from the male genetic material (Komen and Thorgaard 2007). Gynogenesis has important potential for the genetic conservation of female species with a heterogametic sexual system. The population of the sturgeon *Acipenser nudiventris* is critically endangered. This species has a heterogametic system of female sex determination (ZW), allowing its gametes to produce both

females and males. Researchers successfully induced *A. nudiventris* gynogenesis using sperm DNA from the Siberian sturgeon *Acipenser baerii*, which was degraded using UV irradiation and used to inseminate the *A. nudiventris* oocyte (Saber and Hallajian 2014).

In neotropical fish species, androgenesis and gynogenesis still emerging. Recent studies have proposed a protocol for induced gynogenesis in *Astyanax altiparanae* (Nascimento et al. 2020b). Although very promising, cloning technology is still not very efficient owing to a combination of biological and technical challenges, such as the lack, or even absence, of information about species physiology (Keefer 2008). Furthermore, protocols for inducing oocyte recruitment, development, and maturation simply do not exist for many species (Holt et al. 2004). However, despite important advances in mammals (Lanza et al. 2000), cloning in fish has more discrete progress, with a focus on species on the brink of extinction, such as sturgeon (Fatira et al. 2019) and model species, such as zebrafish (Lee et al. 2002; Siripattarapravat et al. 2009).

A key advantage of fish cloning is the high fecundity of females of most species compared to mammals. In each reproductive cycle, multiple eggs are produced at each ovulation, making it possible to test the best techniques and repeatability. Some neotropical species produce more than 200,000 eggs/kg in each reproductive cycle, such as *C. macropomum, P. lineatus, B. orbignyanus,* and *S. brasiliensis*. The high external fecundity in most fish species avoids the necessity of introducing the fertilized embryo *in vitro* in the recipient for its incubation. In the future, individuals will be generated from viable cells and cryopreserved owing to the advancement of cloning technology, allowing us to move away from the necessity of maintaining reproductive cells (Watson and Holt 2001). This represents a powerful tool for the long-term preservation of genetic resources of endangered fish.

## 12.8 Germ Cell Transplantation Technology

Germ cell transplantation (GCT) is a powerful technology developed first in mammalian models (Brinster and Zimmermann 1994) with the primary goal of studying stem cell biology to assist biomedical sciences and support the development of biotechnologies. Today, the technique is applied in a broad range of livestock animals and endangered species, including teleost, for studies involving germ cell culture, germ cell development, transgenic approaches, biotechnology for reproduction, and genetic preservation of valuable specimens or endangered species. However, despite being a revolutionary technology for biodiversity conservation, its application is still new and limited in neotropical fish.

The technique is based on the digestion of gonadal tissue from a donor to obtain a suspension of stem germ cells (Ryu et al. 2022a), which is then purified and injected into a receptor. Ideally, the germ cells find an optimal environment in a germ cell-free host gonad (recipient) where they can establish and develop a new germline, culminating with the production of fertile gametes. Gametes carry the

genetic background of the donor, even if the donor species is different from that of the recipient (Takeuchi et al. 2003). To account for the appeal of this technique, small, short-lifespan, domesticated and/or non-endangered species can rapidly and repetitively produce gametes of a large, later-maturing, low fecundity and/or threatened species.

Also named as surrogate broodstock technology, GCT in fish was primarily developed using model species (medaka *Oryzias latipes* and zebrafish *Danio rerio*; Tsai and Lin 2012), and over the past two decades, it has been studied and applied in a wide range of fish species. The technique is revolutionary in fish breeding technology, especially for maintaining the genetic resources of both endangered and aquaculture species (Yoshizaki and Lee 2018; Yutaka et al. 2020). Furthermore, different approaches can be used for GCT in fish (Ryu et al. 2022a) based on the type of donor cell types and recipients.

### *12.8.1 Blastula Cell Transplantation*

This method is performed by aspirating the blastula cells of a donor embryo and injecting them under the blastoderm of the recipient blastula embryo. At this stage, the blastomere cells are still pluripotent and retain a high capability to adapt to different environments (Saito et al. 2010). After injection, these cells differentiate into primordial germ cells (PGCs), migrate, and colonize the gonadal primordium, establishing the new germline. However, some recipients display low survival rates as a result of several manipulations, especially chorion manipulation (Ryu et al. 2022a). Another disadvantage is the low number of PGCs that efficiently realize migration and colonize the gonadal primordium of the host embryo (Saito et al. 2010). For endangered species, this approach is not very suitable. For most threatened species, artificial reproduction for the early collection of embryos is not always viable, and the high number of embryos necessary for the success of the technique may not justify the effort in such cases. However, some trials have been made with the model species *D. rerio* (Ciruna et al. 2002), *O. latipes* (Shinomiya et al. 2000), *Carassius auratus* (Yamaha et al. 2001), the commercial rainbow trout *O. mykiss* (Takeuchi et al. 2001) and the critically endangered sturgeon *Acipenser sinensis* (Ye et al. 2017).

### *12.8.2 Primordial Germ Cell Transplantation*

Primordial germ cells (PGCs) are precursors of the germline stem cells, oogonia, and spermatogonia. PGCs are the ideal material for transplantation owing to their capacity to transmit the female and male genomes to the next generations, while being very plastic in adapting to a new environment, due to their pluripotency. In PGC transplantation, microinjection labels the embryo donor (one cell stage) with

fluorescence. The labeled PGCs are then harvested from the embryo at the somite stage or from the hatched larvae and purified. The selected fluorescent PCGs are then transplanted into the receptor by microinjection in the blastoderm of denuded blastula embryos (Saito et al. 2010) or in the peritoneal cavity of anesthetized larvae (Takeuchi et al. 2003). Labeling PGCs allows the identity of the transplanted cells to be visualized, warranting better purity of the transplanted cells. Generally, the efficiency of this chimeric method is higher than that for transplanting blastula cells. PGC transplantation was used in *C. auratus*, pearl danio *Danio albolineatus* (Saito et al. 2010) and the rainbow trout *O. mykiss* (Takeuchi et al. 2003).

### *12.8.3 Gonadal Germ Cell Transplantation Into the Peritoneal Cavity of Larvae or Into the Genital Pore of Adult Recipients*

Testis or ovary from donors can be used to obtain germ cell suspension for transplantation into different recipients, such as the peritoneal cavity of larvae or directly in the genital pore of adult specimens. After injection, the germ cells migrate to and colonize the depleted gonad, self-renew, and then differentiate into oogonia or spermatogonia, depending on the environment found in the hosting gonad, and finally develop into competent gametes (Okutsu et al. 2006).

The injection of germ cells into the peritoneal cavity of larvae was accomplished in the rainbow trout (Takeuchi et al. 2003), common carp *C. carpio* (Franěk et al. 2021), tilapia *O. niloticus* (Farlora et al. 2014), and in some threatened species like *A. sinensis* (Ye et al. 2022), the brown trout *Salmo trutta* and grayling *Thymallus thymallus* (Lujić et al. 2018). Gonadal GCT into the genital pore of adult fish was done in some neotropical species, such as pejerrey *O. bonariensis* (Majhi et al. 2009, 2014) and jundiá *R. quelen* (Silva et al. 2016).

The efficiency of GCT highly depends on the capacity to achieve a donor depleted of germ cells, but with an optimal environment to receive, host, and support the development of the transplanted cells. This means that the donor must have a functional testis or ovary, but free of its own germ cells, allowing the establishment and final maturation of donor gametes, exclusively. Hence, if the recipient gonad is totally germ cell-free, the mature host individuals will produce only donor-derived sperm and eggs in a number equivalent to that of a typical fish. Otherwise, the presence of even a few stem cells in the host gonad is sufficient to establish a germline. If endogenous gametogenesis occurs in the recipient gonad, a mix of donor and host gametes is produced, compromising the efficiency of the technique. Several approaches can be used to achieve complete sterilization (Wong and Zohar 2015), such as triploidization (Franěk et al. 2019; Okutsu et al. 2007), gene editing (Fujihara et al. 2022; Wargelius et al. 2016), hyperthermia (Pandit et al. 2015), and drugs (Siqueira-Silva et al. 2015, 2021).

Two other important factors that influence the efficiency of fish GCT are the amount and purity of transplanted cells (). In medaka (Seki et al. 2017) and zebrafish (Hayashi et al. 2019; Kise et al. 2012), high colonization indices were obtained with more transplanted cells. Among all cells present in the gonadal parenchyma, only the early germ cells, i.e., spermatogonia and oogonia, can colonize, multiply, and develop into functional gametes in a recipient gonad (Yano et al. 2008; Yoshizaki et al. 2010). Different methods have been developed to enrich germ cell number in cell suspensions, such as Percoll density gradient centrifugation, differential plating, centrifugal elutriation, and fluorescent and magnetic-activated cell sorting (Ryu et al. 2022b).

For neotropical fish species, *A. altiparanae* is considered a potential model for surrogate technology because it is a small species, simple to breed, with early sexual maturation and high growth rates. Recently, researchers have standardized protocols for gamete sampling, short-term storage, and *in vitro* fertilization in this species (Yasui et al. 2015) and evaluated the immune compatibility between donor and recipient to investigate the potential for surrogate technology (Levy-Pereira et al. 2020). Most recently, *A. altiparanae* was successfully used for germ cell xenotransplantation of *B. orbignyanus*, a threatened and valuable species (Siqueira-Silva et al. 2021).

## 12.9 Technologies to Minimize the Impacts of Aquaculture on Native Fish Species

In general, although the aquaculture industry is fundamental to food security, some biological risks are generally associated with these farming practices, such as the introduction of exotic pests and pathogens; destruction/modification of ecosystems and agriculture; introduction of invasive alien species, and genetic impacts on native stocks (Hallerman 2008). This section discusses the two latter issues and how biotechnology could be applied to solve the problems.

Two important effects result from fish escapes from aquaculture facilities. First, invasive alien species can compete directly with native species, resulting in global or regional biodiversity loss (Kitchell et al. 1997; Townsend 1996), as well as species substitution and decrease of native biomass. Moreover, non-indigenous species can interbreed with a reproductively compatible species in the natural environment, resulting in exogenous hybridization and, hence, threatening the genetic integrity of the native species (Rhymer and Simberloff 1996). Second, escapes are responsible for the negative effects of genetic introgression between the same species in which domesticated populations can interbreed with non-indigenous fish, contaminating the natural genetic pool. This can result in less genetic variability and a lower ability to adapt in the face of changing selective pressure, thereby increasing the likelihood of subsequent inbreeding and extinction (Hallerman 2008). To solve or reduce the impact of escapees on native fish species, several genetic approaches are now

available for inducing sterility and optimizing sterility application in the global fishery industry, such as triploids, interspecific hybridization, and CRISPR/Cas9.

### *12.9.1 Triploidy*

Among the biotechnologies that have reached the aquaculture industry, polyploidy deserves greater attention since triploids have numerous advantages, and studies have been carried out with several fish species (Dunham 2004; Piferrer et al. 2009). In addition, triploidy inductions in fish are of interest because of sterility, as they can yield improved products and serve as a potential method of reproductive confinement (Piferrer et al. 2009).

Polyploids can be defined as organisms with one or more sets of chromosomes in addition to the number found in nature. The phenomenon of polyploidy is lethal in mammals and birds. However, polyploidy is compatible with fish, and the occurrence of viable triploids has been frequently reported (Silva et al. 2007). Triploids can be found spontaneously in natural fish populations (Centofante et al. 2001; Silva et al. 2011) or artificially induced in farming systems by physical and chemical methods (Huergo and Zaniboni-Filho 2006; Tiwary et al. 2004).

Retention of the second polar body during the meiotic division of oocytes results in triploid individuals. Two sets of chromosomes are inherited from the mother and one from the father. Triploidy is induced by regular fertilization, followed by a process to force retention of the second polar body, ranging from temperature and hydrostatic pressure shocks to chemicals (colchicine and cytochalasin), which are applied shortly after fertilization (Piferrer et al. 2009). The success of treatments to induce polyploidy depends on the time of shock initiation, magnitude, and duration (Dunham 2004).

Another way of producing triploids is through indirect methods whereby regular eggs are fertilized with diploid sperm from a tetraploid male (Francescon et al. 2004). However, tetraploidy is an infrequent event, and the survival rate is low. Indeed, few species have viable and fertile adults (Yoshikawa et al. 2008). Furthermore, the risk of tetraploid escape into natural environments is high as they could reproduce freely with diploid individuals, resulting in sterile triploids and affecting the entire population.

Triploid fish are generally sterile by the lack of gonadal development caused by the incompatibility of chromosome sets. Sterility in triploid males has been established in some species, such as the European sea bass *Dicentrarchus labrax* (Peruzzi et al. 2004), turbot (Cal et al. 2006), gilthead sea bream *Sparus aurata* (Haffray et al. 2005), and arctic charr *Salvelinus alpinus* (Gillet et al. 2001). However, other aquaculture species, including *S. salar* (Benfey and Sutterlin 1984), coho salmon *Oncorhynchus kisutch* (Piferrer et al. 1994) and the tench *Tinca tinca* (Linhart et al. 2006), can produce aneuploid spermatozoa, although these are unable to generate viable offspring. The characteristic of sterility makes triploids an excellent model for use on a commercial scale. During early sexual maturity, reduced or inhibited

gonadal development may allow energy used in the reproductive process to be directed toward somatic tissue growth. Therefore, several studies have demonstrated the potential for increased growth rates, increased carcass yield, and meat quality (Piferrer et al. 2009).

Chromosomal manipulations for fish production began to affect the industry on a worldwide scale during the 1980s and 1990s. The main triploid species produced on a large scale are trout, salmon, and carp (Hulata 2001). However, in Brazil, these genetic biotechnologies have not yet reached the aquaculture industry for native species, as only experimental tests have been carried out. Some initial studies on triploids in Brazil were developed for the silver catfish *R. quelen* (Silva et al. 2007). In these experiments, the induction protocols allowed obtaining 100% triploids (Huergo and Zaniboni-Filho 2006). Recently, other protocols, mainly the use of heat-induced treatment, have been described for several species of neotropical aquaculture, such as *Brycon amazonicus* (Nascimento et al. 2021), *A. altiparanae* (Adamov et al. 2017), *Pimelodus maculatus* (Bertolini et al. 2020), *P. lineatus* (Yasui et al. 2020), and *C. macropomum* (Sato et al. 2020). Successful tetraploids were first described in a neotropical species for *R. quelen* (Garcia et al. 2017) and *A. altiparanae* (Nascimento et al. 2020a). In *A. altiparanae*, triploid males were not totally sterile and had no significant advantage (growth) over diploid males, but triploid females were sterile and presented increased carcass yield. Thus, these animals could be used commercially on a large scale and for conservation programs.

### *12.9.2 Interspecific Hybridization*

Interspecific hybridization is the mating (crossing) between different species, which has been used to produce aquatic organisms with specific desirable traits that perform better than those of either parental species (hybrid vigor or positive heterosis). Interspecific hybrids have been used for aquaculture and conservation to increase growth rate, combine desirable traits of two species, produce sterile fish, and increase overall robustness in farmed conditions (Bartley et al. 2001). With expansion of the aquaculture sector and the increased number of species being bred and farmed, interspecific hybrids now account for a substantial proportion of aquaculture production (FAO 2010). However, the information currently available needs to provide a clearer picture of the production level of all hybrids in aquaculture worldwide.

Approximately 20 types of fish hybrids have been produced in Brazil (Porto-Foresti et al. 2010). However, few hybrids have been commercially successful (Hashimoto et al. 2012). Nevertheless, the results of these hybridizations have allowed essential development in the aquaculture of native species since hybrids have been well accepted in the consumer market. The main interspecific crosses involve the Serrasalmidae species *C. macropomum, P. mesopotamicus,* and *Piaractus brachypomus*. Among Pimelodidae species, those most exploited commercially in Brazilian aquaculture by interspecific crossings are *P. corruscans,*

*P. reticulatum, Phractocephalus hemioliopterus,* and *Leiarius marmoratus* (Hashimoto et al. 2013).

In theory, crossings between different species would result in a sterile hybrid. However, many freshwater and marine species hybrids may not be sterile (Verdegem et al. 1997). In Brazil, serrasalmid and pimelodid hybrids were reported as fertile and could backcross with their parental species (Hashimoto et al. 2014; Prado et al. 2012); therefore, they constitute serious genetic risks to wild and cultivated stocks of pure species (Prado et al. 2017).

### *12.9.3 Genome Editing by CRISPR/Cas9*

Genome editing can quickly introduce favorable variations to the genome, such as fixing alleles at existing trait loci, creating *de novo* alleles, or introducing alleles from other strains or species (Gratacap et al. 2019). The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated) approach has been a powerful tool for targeted genome editing (Gasiunas et al. 2012; Jinek et al. 2012).

The CRISPR/Cas9 system was discovered in bacteria and has been applied as an efficient, targeted strategy to edit any gene. The method produces a double-strand break (DSB) at a target locus, allowing imperfect or targeted repair to generate changes to the sequence of genomic DNA (Gratacap et al. 2019). Genome editing using CRISPR/Cas9 was successfully used "*in vivo*" and/or in cell lines of several major aquaculture species of Salmonidae, Cyprinidae, Siluridae, as well as the Pacific oyster *Crassostrea gigas, O. niloticus*, and gilthead sea bream *Sparus aurata* (Gratacap et al. 2019).

Primordial germ cells (PGCs) are the precursor cells of female and male gametes. PGCs are initially produced outside the gonad area and afterward migrate to the genital ridges (Saito et al. 2008; Sawatari et al. 2007; Xu et al. 2010). Genes associated with the development and migration of PCGs are crucial for maintaining viable germ cells. Silencing these genes has led to the failure of PGCs to properly migrate during embryonic development, which results in sterility (Slanchev et al. 2005; Weidinger et al. 2002). Dead-end protein (dnd) is one of the fundamental genes for migration of PGCs; therefore, knockout or knockdown of *dnd* can be done to mismigrate PGCs, resulting in sterility.

Recently, some studies have focused on applying CRISPR/Cas9 to obtain sterile fish by knockout of *dnd* gene, such as in the sturgeons *Acipenser ruthenus* (Baloch et al. 2019) and *S. salar* (Wargelius et al. 2016). Other proteins of interest are the luteinizing hormone (LH) and Piwil1, which were successfully edited to sterilize the channel catfish *Ictalurus punctatus* (Qin et al. 2016) and the Atlantic salmon (Almeida et al. 2022), respectively. So far in Brazil, no study has been conducted to test this method in native species.

## 12.10 Some Recommendations

### *12.10.1 Mitigating Harmful Habitat Changes*

Regional planning of dam installation involving the entire watershed can reduce impacts and balance conflicting energy and biodiversity interests (Winemiller et al. 2016). Essential measures for the reproduction and recruitment of natural populations should take priority in the creation of protected areas, including riparian vegetation, natural water flow, access to tributaries, and flood areas, allowing the reproduction of specialized and vulnerable species (Schork and Zaniboni-Filho 2017).

Lotic habitats with natural flow regimes and habitats with fast water flow may benefit the reproduction and recruitment of species highly impacted by lentic reservoirs, such as trophic specialists, nest builders, and species with high migratory reproductive behavior. Therefore, the identification of reproductive fish reaches and nursing grounds (Ávila-Simas et al. 2014; Silva et al. 2012; Ziober et al. 2015), may be crucial to identify hot spots that can preserve target species. The maintenance of rivers sections with the presence and access of free tributaries has already been shown to be efficient for spawning migratory fish (Reynalte-Tataje et al. 2012; Silva et al. 2012, 2015).

Since variation in the water level affects the recruitment of different fish breeding guilds in different ways (Lima et al. 2017), it is possible to take actions to manage the water level of reservoirs and the flow of downstream stretches to favor the recruitment of specific populations of fish, actions which can help to mitigate imbalances in the fish community. Certainly, this same strategy can be used to reduce the recruitment of species that are locally overpopulated.

Considering only those species that present problems in reproduction and/or recruitment in a certain part of the basin, it is expected that when this condition persists, the species will disappear locally within a few generations. Thus, the definition of strategies to reverse limitations in reproduction and recruitment can be crucial to avoid local extinction. Accordingly, the precise identification of key environments used for the reproduction and recruitment of these species, as well as environmental factors that regulate these events, may allow the use of management strategies that favor periodic recruitment as a way to guarantee the maintenance of local populations. A major advantage of this management approach compared to others, such as stocking fish, is the possibility of using the genetic potential of the local population and its natural processes of selection and evolution.

### 12.10.2 Mitigating Impacts of Non-native Species Introduction

Information on behavior, feeding ecology and reproduction can significantly help environmental and governmental managers take more proactive measures to prevent the introduction of non-native fish species and assist in decision-making that involves mitigating impacts (Souza et al. 2021).

The neotropical region needs international cooperative measures for the development and implementation of certified strains (defined based on zootechnical aspects and biological impact on the natural population), promoting the safe production of fish in ways that do not compromise the genetic composition of native fish species in case of escapes. The determination of which lines/species can be cultivated should be discussed within the context of each hydrographic basin. Because of overlapping boundaries, this often involves management authorities in more than one country, as in the case of the Amazon and Paraná Rivers. A review by Zaniboni-Filho et al. (2018) discusses environmental conflicts related to fish farming in cages in Brazilian reservoirs and presents options for mitigating the problems, in addition to suggesting preventive measures and better management practices.

Broad dissemination among environmental agencies and information provided to enforcement teams about the consequences of inappropriate introductions (i.e., releases for environmental compensation, as required by law, and either legal or illegal releases for fishing) are immediate measures for conservation. In addition to genetic impacts, many species resulting from releases become established, and others dominate the environment where they were introduced, substantially and devastatingly affecting the trophic structure of original fish communities.

### 12.10.3 Fish Stocking Guidelines

Stocking actions are complex, and both the increase of fish stock and maintenance of genetic variability of the receiving stock are key considerations. Therefore, to guarantee the success of this activity, guidelines for the management of stocking are given below, along with recommendations for reducing genetic and other risks associated with captive breeding programs.

(i) Identify the leading causes and threats to local fish populations, and when possible, conduct mitigation actions prior to stocking programs.
(ii) Define the main objectives for stocking programs and the target populations and species. This topic is crucial for planning and monitoring the steps that follow (Agostinho et al. 2004, 2010). More than one objective is likely to involve restocking, although one may be more critical and should be emphasized. Furthermore, defining stocking objectives will allow determining the optimal size of individuals to be released (larvae, juveniles, young adults), densities, the best period for release (seasons of the year), and community

participation to promote a responsible approach in stocking programs (FAO 2015).

(iii) Perform genetic characterization of the receptor population. In the case of cryptic species, use species delimitation approaches (i.e., DNA barcoding; for details see Chap. 2). Characterize the genetic population structure of the recipient stock, considering the life history and reproductive behavior of each species/genus (e.g., isolation by distance, isolation by time, and migratory species). Estimate the genetic diversity of the recipient population. Species with low genetic diversity are more prone to inbreeding and genetic drift (Coleman et al. 2018).

(iv) Collect representative broodstock. Genetic characterization of the recipient population is essential and constitutes one of the principal hurdles to genetically safe stocking programs (Ryman and Ståhl 1980). It is crucial that the broodstock genetically represent target populations (Allendorf et al. 1987), or the genetic goal of recipient populations.

(v) Maintain the broodstock number as high as possible. Captive breeding programs with large effective population sizes (Ne) are more successful in maintaining high genetic diversity (Frankham 2009; Fraser 2008; O'Reilly and Doyle 2007). As a general guideline, hatchery programs should be at least Ne > 500 individuals per generation to overcome the potential effects of genetic drift and inbreeding depression (Coleman et al. 2018). However, to maintain evolutionary potential, Frankham et al. (2014) suggest at least Ne = 1000 to maintain initial evolutionary potential in perpetuity. The deleterious effects of a small breeding stock may include increased genetic load owing to mutation accumulation and domestication selection (Goodman 2005; Lynch and O'Hely 2001) and decreased effective population size (Wang and Ryman 2001). Ignoring the expected effects of small Ne will increase the likelihood of promoting a severe bottleneck and consequent inbreeding depression in the recipient population (Allendorf et al. 1987; Allendorf and Phelps 1980; Leary et al. 1985).

(vi) Identify and characterize the broodstock genetically. One of the main genetic threats of stocking is inbreeding depression (Gilk et al. 2004; Lynch and O'Hely 2001). All broodstocks should be tagged and genetically characterized to avoid crosses with related breeders, resulting in inbreeding depression.

(vii) Minimize sperm competition and equalize sex ratios at breeding. Spawning for stocking of natural populations should minimize artificial selection in captive populations (Campton 2004). During spawning, oocytes from several females and milt from several males (pool) should not be combined in a single container to avoid sperm competition and highly unequal genetic contributions from male breeders (Ribolli and Zaniboni-Filho 2009). Sperm competition substantially reduces the effective genetic number of breeders (Nb), and results in artificial selection related to sperm potency and fertilization success. Considering the high fecundity of most neotropical fishes used in stocking programs and the difficulty of managing many individuals in a single spawning event, the Factorial (Matrix) Spawning Protocol (Campton

2004) is suggested since it fits well with the objectives of maximizing genotypic diversity of the progeny, allows sex ratio maintenance and excludes pooled milt or oocytes from different individuals (for other protocols see Campton (2004)). Initially, gametes from males and females should be removed and held in individual containers and subsequently divided into aliquots of similar volume and weight. Subsequently, the oocytes of each female aliquot should be fertilized according to a mating matrix and then each aliquot incubated in an individual incubator.

(viii) Genetic characterization of progeny. The systematic genetic monitoring of progenies for restocking purposes prevents genotypes more adapted to the breeding and hatching process from increasing in frequency (Allendorf et al. 1987). Tringali and Bert (1998) suggest that contributions per generation be modest in restocking programs; thus, several distinct spawning events can compose a stocking schedule.

(ix) Sperm cryopreservation. Cryopreservation techniques reduce losses of genetic diversity and fitness, minimize inbreeding, and reduce domestication selection (O'Reilly and Doyle 2007). Cryopreserved sperm could be used to fertilize female eggs in subsequent generations (Sonesson et al. 2002), which would allow maximizing the number of reproducers, as well as conserving genetic variability for an indeterminate period (Fraser 2008). There is a protocol for freezing the semen of the main species of migratory fish in South America (Carolsfeld et al. 2003).

(x) Conduct long-term genetic monitoring of release stocking. Genetic identification through parentage analysis of fish caught by fishermen and in stocking monitoring programs will allow managers to assess whether the fish are developing in the environment in which they are stocked.

(xi) Periodic introduction of new broodstock from the wild. The introduction of new wild breeders is a recommendation for traditional hatcheries that use multiple generations in the hatchery (Myers et al. 2004; Whiteley et al. 2015). In addition, fish removed from the broodstock for cause, such as sickness or death, must be replenished to avoid reducing effective size.

## 12.11 Limitations and Future Perspectives of Fish Biotechnology

The number of fish at risk of extinction has gradually increased over the years owing to overfishing and the destruction of natural habitats. Different biotechnologies have been used to conserve and breed endangered species, such as cryopreservation of gametes, tissue, and embryos and *in vitro* fertilization. In this scenario, xenogeneic germ cell transplantation is a good option as it offers the possibility of *in vivo* gamete production in a surrogate recipient. Although the technology is still not used on a large scale, the possibility of producing millions of fertile gametes of a valuable

species in a shorter cycle and using domesticated species ensures the use of this powerful tool to preserve endangered species. As a result of this potential, research efforts in germ cell transplantation of threatened fish species have been intensifying over past years. However, studies must be species-specific as each species has its own particular characteristics during germline establishment and development. In addition, the surrogate production of germ cells always requires previous knowledge regarding germ cell development of recipient and donor species (considering xenotransplantation), sterilization methods, and techniques for germ cell preservation and transplantation. Therefore, time-consuming and labor-intensive work are required before establishing a given protocol.

Neotropical species are numerous and present a vast diversity of reproductive strategies. This means that developing germ cell transplantation between different species still requires more investigation and elucidation of the reproductive biology, especially better understanding of fish gametes, fertilization, and developmental biology (Pereira dos Santos et al. 2016). The potential value of *A. altiparanae* is noted as an efficient recipient for germ cell transplantation from different relevant species, emerging as a promising valuable host species.

Sterile interspecific hybrids are important resources for neotropical aquaculture by boosting the production of native species, especially because they could be produced with the purpose of genetic confinement. However, further studies are still required to guarantee that hybrids can be used safely for total sterility; otherwise, if fertile, they represent a large-scale problem from the perspectives of both environment and conservation, particularly considering genetic introgression, as previously reported (Prado et al. 2017). Finally, advances in gamete cryopreservation and cloning are indispensable in genetic engineering and gamete manipulation and represent powerful tools for the long-term preservation of genetic resources for endangered fish.

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# Part IV
# Wildlife Forensic Genetics, Ecotoxicology and Conservation

# Chapter 13
# Giving Names to the Characters: Identifying, Tracing and Estimating the Multiple Use of Aquatic Wildlife in Brazil

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## 13.1 Introduction

The most prominent personage of Brazilian folklore is the "boto" or the "legend of the boto". In the Amazon realm, dolphins are regarded as special creatures and shapeshifters that can take human form and seduce women at night (Cravalho 1999; Slater 2001). The magical powers of dolphins are openly recognized for their intrinsic attributes as "human lovers" and thus associated with "love charms" (Slater 2001). The so called "dolphin love enchantment" has been deeply studied in anthropology as a cultural phenomenon and made public in a variety of Brazilian

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books for all ages (*e.g*., Larêdo 1981; Santos 1987, 2004; Simões and Golder 1995a, b, c; Monteiro 2002; Val 2007; Siqueira 2012; Campos et al. 2013; Xavier de Holanda 2013). This cultural attribute of dolphins in the Amazon has probably surfaced long ago, as earliest Americans have relied on aquatic fauna for protein and other uses for centuries or millennia. Not only dolphins, but freshwater turtles, capybaras, tapirs, ariranhas, otters, caimans, and many other animals were harvested for food, religious and medicinal uses. During colonial times, in Brazil, medicine was largely practiced by curators, Jesuits and shamans. Religious apothecaries formulated secret recipes, usually combinations of Indian and European components (Camargo 1976). Wild animal's parts were used in these recipes, as they are still in practice in current times, so frequently observed in low-income communities. Several examples were reported (Araújo 1977): deer antlers are burned for preparing antimicrobials and anthelmintics, caiman and lizard leather for incensing, preventing snake attacks, love charms, and sea turtle lard for healing joints. Cascudo (1981) said that African blacks, native Indians and Europeans merged in the "Brazilian" conception of magic, enchantments, prayers, Iberian witchcrafts, that comes and were transmitted orally. In the last decades, these figures have not changed so much as aquatic wildlife is still the focus of a prosperous commerce in a variety of ways, although aquatic vertebrates are legally protected in Brazil. These uses of aquatic fauna can be seen in many ways in the present time. Notably, dolphins' body parts are a commonplace in religious rituals, as a complementary practice that demands amulets, fragrances and balm ointment. Indeed, dolphin's eyes, genitals, teeth and bones are in the center of this cultural melting pot, used in many different ways. Forensic genetics has confirmed that Guiana dolphins (*Sotalia guianensis*) are the principal target of this trading, whilst incidentally

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captured specimens in the North and Northeastern Brazilian regions fuel a prosperous market of amulets and trinkets (Siciliano et al. 2018). Interestingly, tucuxi (*Sotalia fluviatilis*) and Guiana dolphins teeth appeared in the "neo-hippie" culture and their handicrafts, present in many Brazilian cities (Siciliano et al. 2018).

Based on the general conviction that dolphins, whales, manatees, and other aquatic vertebrates are mystical and powerful creatures, fostering a thriving market of all kinds of amulets, this chapter aims to bring the attention to the most widely coverage of the multiple uses of aquatic wildlife in Brazil, with emphasis on dolphins' love charms and handicrafts. Other popular and trending uses of aquatic vertebrates are observed in decoration pieces in houses and hotels, as health treatments available in body ointments, oils, creams and pills for health holistic treatments. As additional support to these investigations, age determination of dolphins' teeth used in bracelets and necklaces could be a valuable tool, as it makes it possible to obtain specific information about demographic groups that have been more exposed to this trade. Although other uses of aquatic mammals are currently a matter of intense debate, they were extensively treated in international forums (Report SC/IWC 2018) and will not be covered here. This includes the "boto-piracatinga issue" in the Brazilian-Colombian Amazon border (Mintzer et al. 2020), the once common use of dolphin blubber as bait in the shark fishery (Lodi and Capistrano 1990) and the punctual killing of dolphins (Barbosa-Filho et al. 2016) and manatees (Luna et al. 2008) for food or bait in scattered remote communities. Our focus is the current commerce of by-products derived from aquatic wildmeat, either real or fake ones, that can be purchased in markets and fairs, traced to their origin by using genetic and biochemical analyses, and show a current trend of use in a multi-scale magnitude of social and cultural layers of the Brazilian society.

## 13.2 Data Collection and Analysis

### *13.2.1 Field Work*

Field research was conducted from 1999 to 2021, in search of any use of aquatic wildlife for multiple purposes, including food, religious, handicraft or any other. Open markets in all major cities in North, Northeastern, Southeastern and South Brazil were visited for checking *in loco* the products available, despite their origin or composition. In order to enlarge our search, we checked numerous websites announcing products labeled as made of aquatic wildlife. It was found a great variety of sites and products on sale, mostly essences and Bach flower remedies made from aquatic animals, such as dolphins, whales, manatees, capybaras, caimans, sucurijús (or anacondas) and poraquês (electric eels). A list of cities visited and articles purchased (Fig. 13.1), in addition to products offered on the website, are provided in Supplementary Material (Table 13.1). Some of these products, especially the one called "love charms", were purchased on site for further lab analysis

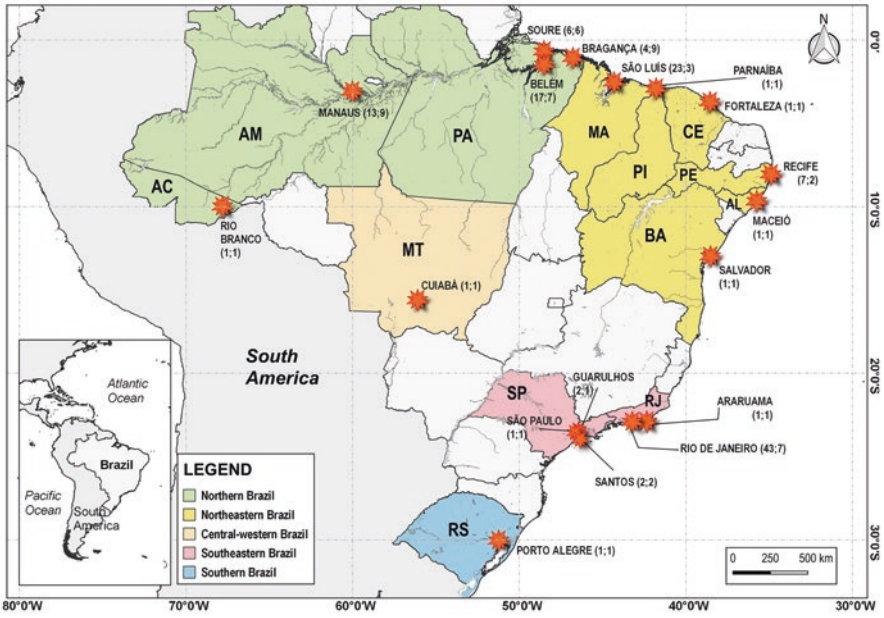


**Fig. 13.1** Locations surveyed for investigating the aquatic wildmeat products and uses, including dolphin love charms, perfumes, handicrafts, food, decoration and any other purpose throughout Brazil. Collecting points are distributed in Northern: Rio Branco-Acre (AC), Cuiabá-MT (Mato Grosso), Manaus-AM (Amazonas), Belém, Bragança and Soure-PA (Pará) and São Luís-MA (Maranhão); Northeastern: Parnaíba-PI (Piauí), Fortaleza-CE (Ceará), Recife-PE (Pernambuco), Maceió-AL (Alagoas) and Salvador-BA (Bahia); Southeastern: Araruama, Rio de Janeiro-RJ (Rio de Janeiro), Guarulhos, Santos and São Paulo-SP (São Paulo), and South regions: Porto Alegre-RS (Rio Grande do Sul), Brazil

of their content, which includes dried and wet tissues, teeth, bones, oil, balm ointment and pills. The "perfume" category, defined as fragrances with a piece of "dolphin" tissue inside the vial, has been observed in all places visited, among other items such as dehydrated eyes (Fig. 13.2), penis (or dried penis portions) and vaginas (Fig. 13.3). Dehydrated penises are usually sold as a single piece, although one shop in Rio de Janeiro offered them in thin slices. Less common items found in Pará, Bahia and Rio de Janeiro included vials containing "dolphin" and "whale" oils and small, sun-dried, pieces of blubber and muscle labeled as "botos". A very uncommon item was a newborn Guiana dolphin (*S. guianensis*) dried skull obtained in the Ver-o-Peso market in Belém, Pará (Fig. 13.3). During field monitoring carried out along the Brazilian coast, bones of small and large cetaceans used on display in homes and commercial spots were observed (Fig. 13.4).

**Table 13.1** List of locations visited along the North, Northeastern, Southeastern and South Brazil where aquatic wildlife products were obtained

| Locality | Product | Tissue | Storage | Local name |
|---|---|---|---|---|
| Rio Branco, AC | Perfume | Muscle | Fragrance with piece of muscle | Perfume do Boto/Perfume da Bota |
| Manaus, AM | Perfume | Muscle | Fragrance | Super Preparado Bota: Atrativo |
| Manaus, AM | Perfume | Muscle | Fragrance | Atração do Boto (Bôto)/Atração da Bota (Bôta) |
| Manaus, AM | Eye | Dried eye | Dried tissue | Patuá (do boto)/Olho do Boto |
| Manaus, AM | Oil | None | Oil | Óleo do Boto |
| Manaus, AM | Small dried pieces | Muscle | Dried small piece of muscle | Boto |
| Manaus, AM | Lotion | None | Glass bottle with perfumed water | Banho Atrativo Bôto Tucuxi Perfumaria Preto Velho Original de Umbanda |
| Manaus, AM | Lotion | None | Glass bottle with perfumed water | Banho Atrativo Atração do Bôto/da Bôta Perfumaria Preto Velho Original de Umbanda |
| Manaus, AM | Perfumed water | None | Vial with perfume | Super Preparado Boto/Boto Tucuxi: Atrativo |
| Manaus, AM | Dried tissue | Penis | Dried | Pênis do boto |
| Manaus, AM | Snake grease | ? | Cream | Banha de Cobra Sucuriju: Produto Original 100% Natural |
| Manaus, AM | Sucurijú pill | ? | Pill | Pílulas de Sucuriju |
| Manaus, AM | Freshwater turtle | ? | Cream | Banha de Tartaruga |
| Manaus, AM | Electric fish | ? | Cream | Banha de Poraquê 100% Natural |
| Soure, PA | Teeth | Teeth | Dried teeth | Dentes do boto |
| Soure, PA | Muscle in pieces | Muscle | In alcohol | Pedaço do boto |
| Soure, PA | Preparation | None | Glass bottle with perfumed water | Boto Tucuxi: Poderoso Banho de Atração |
| Soure, PA | Cream | ? | Plastic jar | Banha de Baleia |
| Soure, PA | Oil | Oil | Vial with perfume | Óleo da Bôta |
| Soure, PA | Perfume | None | Vial with perfume | Perfume da Bota |
| Belém, PA | Teeth | Teeth | Dried teeth | Cordão com dentes de boto e de gato-maracajá |
| Belém, PA | Teeth | Teeth | Dried teeth | Cordão com dentes de boto e de jacaré |
| Belém, PA | Teeth | Teeth | Dried teeth | Pulseira com dentes de boto |
| Belém, PA | Teeth | Teeth | Dried teeth | Dentes do boto |
| Belém, PA | Skull | Skull | Dried and cleaned skull | Caveira do boto |

(continued)

**Table 13.1** (continued)

| Locality | Product | Tissue | Storage | Local name |
|---|---|---|---|---|
| Belém, PA | Perfume | Muscle | Vial with perfume and essences | Atrativo do Sexo da Bota: Use para agarrar, conquistar e segurar a pessoa amada |
| Belém, PA | Perfume | Muscle | Vial with perfume | Perfume Atrativo do Bôto |
| Belém, PA | Perfume | Muscle | Vial with perfume | Perfume Atrativo da Bôta |
| Belém, PA | Perfume | None | Vial with essence | Preparado Atraente: Atração da Bôta. Original de Umbanda |
| Belém, PA | Perfume | None | Vial with essence | Preparado Atraente: Boto Tucuxi, Belém, Pará |
| Belém, PA | Oil | Oil | Vial with oil | Banha de Bota |
| Belém, PA | Water cologne | None | Glass bottle | Sexo do Boto para o Asseio do Sexo Masculino |
| Belém, PA | Water cologne | None | Vial with essence | Atrativo da Perseguida |
| Belém, PA | Perfume | None | Vial with perfume and leaves | Atrativo da Pesseguida [sic] |
| Belém, PA | Dried tissue | Eye | Dried | Patuá da Sorte Boto |
| Belém, PA | Balm ointment | None | Balm ointment for local massage | Banha Peixe-boi |
| Bragança, PA | Perfume | None | Vial with perfume | Perfume Boto Tucuxi, S. Bahia |
| Bragança, PA | Dried tissue | Muscle | Vial with perfume | Atrativo da Bota |
| Bragança, PA | Oil | Oil | Vial with oil | Boto Óleo |
| Bragança, PA | Oil | Oil | Vial with oil | Encantaria Bôta |
| Bragança, PA | Water cologne | None | Vial with essence | Água da Bota |
| Bragança, PA | Water cologne | None | Vial with perfume | Atrativo da Bota para Asseio do Sexo: Perfume da Perseguida |
| Bragança, PA | Water cologne | None | Vial with perfume | Perfume Atraente Boto Tucuxi S. Bahia |
| Bragança, PA | Water cologne | None | Vial with essence | Bota para Asseio do Sexo: Perfume da Perseguida |
| Bragança, PA | Perfume | None | Vial with essence | Atração da Bota: Agarradinho, Chega-te a Mim e Laços do Amor |
| São Luís, MA | Dried tissue | Eye | Dried in a purse | Legítimo Olho de Boto Tucuxi, Preparado Chama Atrativo, Belém, PA |
| São Luís, MA | Perfume | None | Vial with perfume | Perfume Atrativo Xoxotinha Perigosa, Preparado de Mironga |

(continued)

**Table 13.1** (continued)

| Locality | Product | Tissue | Storage | Local name |
|---|---|---|---|---|
| São Luís, MA | Perfume | None | Vial with perfume | Perfume Atrativo Atração do Boto, Preparado de Mironga |
| São Luís, MA | Perfume | None | Vial with perfume | Asseio da Bôta |
| São Luís, MA | Perfume | None | Vial with perfume | Perfume Atrativo Boto Tucuxi Oriental |
| São Luís, MA | Perfume | Muscle | Vial with perfume | Atração da Bota Oriental |
| São Luís, MA | Perfume | Muscle | Vial with perfume | Atração do Boto Oriental |
| São Luís, MA | Dried tissue | Eye | Dried in a purse | Legítimo Olho de Boto Tucuxi, Preparado Chama Atrativo, Belém, PA |
| São Luís, MA | Oil | Oil | Vial with oil | Óleo do Boto, Óleo da Bota |
| São Luís, MA | Muscle in pieces | Muscle | Perfume | Sexo da Bota |
| São Luís, MA | Muscle in pieces | Muscle | Perfume | Sexo do Boto |
| São Luís, MA | Dried tissue | Eye | Dried in a purse | Legítimo Olho de Boto Tucuxi |
| São Luís, MA | Oil | Oil | Oil in a vial | Óleo da Bota |
| São Luís, MA | Oil | Oil | Oil in a vial | Óleo do Boto |
| São Luís, MA | Perfume | None | Water cologne | Água de Boto |
| São Luís, MA | Perfume | Muscle | Muscle in a vial | Perfume Atrativo União da Bota. Oriental |
| São Luís, MA | Perfume | Muscle | Muscle in a vial | Asseio da Bôta |
| São Luís, MA | Perfume | Muscle | Muscle in a vial | Atração da Bôta |
| São Luís, MA | Perfume | Muscle | Muscle in a vial | Atração do Boto |
| São Luís, MA | Perfume | Muscle | Muscle in a vial | Perfume do Boto |
| São Luís, MA | Perfume | Muscle | Muscle in a vial | Perfume da Bôta |
| Parnaíba, PI | Teeth | Teeth | Dried teeth | Dentes do boto |
| Fortaleza, CE | Dried teeth | Teeth | Bracelet | Pulseira com dentes de boto |
| Recife, PE | Muscle in piece | Muscle | Vial with parfum | Perfume Sexo da Bota/do Boto |
| Recife, PE | Balm ointment | None | Balm ointment for local massage | Gel de Massagem Peixe Elétrico com Óleo de Copaíba |

(continued)

**Table 13.1** (continued)

| Locality | Product | Tissue | Storage | Local name |
|---|---|---|---|---|
| Recife, PE | Balm ointment | None | Balm ointment for local massage | Cartilagem de Tubarão Gel Massageador com Óleos e Extratos Vegetais |
| Recife, PE | Balm ointment | None | Balm ointment for local massage | Pomada Jacaré da Amazônia |
| Recife, PE | Balm ointment | None | Balm ointment for local massage | Gel Massageador Banha de Capivara Ação Refrescante |
| Recife, PE | Balm ointment | None | Balm ointment for local massage | Gel Suavizante Óleo do Peixe Elétrico com Óleo de Copaíba |
| Recife, PE | Balm ointment | None | Balm ointment for local massage | Banha de Sucuri Gel Massageador |
| Maceió, AL | Perfume | None | Perfume | Poção do Boto (Azul) e Poção da Bota (Rosa) |
| Salvador, BA | Muscle in pieces | Muscle | Perfume | Pedaço do Boto |
| Cuiabá, MT | Muscle in piece | Muscle | Vial with parfum | Perfume do Boto |
| Rio de Janeiro, RJ | Dried | Dried tissue | Dried penis in pieces | Pênis do boto em pedaços |
| Rio de Janeiro, RJ | Perfume | Muscle | Muscle in a vial | Perfume da Bôta |
| Rio de Janeiro, RJ | Perfume | Muscle | Muscle in a vial | Magia Aromática Perfume da Bota |
| Rio de Janeiro, RJ | Perfume | Muscle | Muscle in a vial | Magia Aromática Perfume do Boto (or Perfume do Boto) |
| Rio de Janeiro, RJ | Dried teeth | Teeth | Teeth in bracelet | Cordão com dentes de boto e de carnívoro |
| Rio de Janeiro, RJ | Perfume | Muscle | Muscle in a vial | Perfume da Bota/do Boto |
| Rio de Janeiro, RJ | Oil | Oil | Oil in a vial | Óleo Condicionador Espermacete de Baleia |
| Rio de Janeiro, RJ | Oil | Oil | Whale oil | Magia do Oriente: Óleo de Baleia [whale oil] |
| Rio de Janeiro, RJ | Lotion | Lotion | Whale spermaceti | Máscara Capilar Espermacete de Baleia [whale spermaceti] |
| Rio de Janeiro, RJ | Lotion | Oil | Whale oil | Espermacete de Baleia [whale spermaceti] |
| Rio de Janeiro, RJ | Dried | Dried vagina | Dried dolphin vagina | Vagina da bota |
| Rio de Janeiro, RJ | Dried | Dried penis | Dried muscle in a vial | Pênis do boto |
| Rio de Janeiro, RJ | Oil | Oil | Oil in a vial | Guiã Essências, Óleo do Boto |
| Rio de Janeiro, RJ | Oil | Oil | Oil in a vial | Guiã Essências, Óleo de Baleia |

(continued)

**Table 13.1** (continued)

| Locality | Product | Tissue | Storage | Local name |
|---|---|---|---|---|
| Rio de Janeiro, RJ | Perfume | None | Perfume | Magia Cigana, Perfume da Bota |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Magia Aromática, Perfume da Bota |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Magia Aromática, Perfume do Boto |
| Rio de Janeiro, RJ | Oil | Oil | Oil in a vial | Óleo do Boto |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Pura Magia Perfume da Bota Encanto Natural |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Bota |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Boto |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Oh! Mury Bota |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Oh! Mury Boto |
| Rio de Janeiro, RJ | Perfume | None | Perfume | Perfume do Boto |
| Rio de Janeiro, RJ | Oil | None | Oil in a vial | Mury. Boto |
| Rio de Janeiro, RJ | Oil | None | Oil in a vial | Sansara Óleo de Capivara |
| Rio de Janeiro, RJ | Oil | None | Oil in a vial | Sansara Óleo de Jacaré |
| Rio de Janeiro, RJ | Essence | None | Massage bath and essence | Essência de peixe-boi/Essência de baleia-azul |
| Rio de Janeiro, RJ | Balm ointment | None | Balm ointment for local massage | Banha de Capivara Gel para Massagem |
| Araruama, RJ | Dried muscle | Muscle | Perfume | Perfume da Bota/do Boto |
| Guarulhos, SP | Perfume | None | Perfume | Perfume da Bôta |
| Guarulhos, SP | Perfume | None | Perfume | Atração do Boto |
| São Paulo, SP | Perfume | None | Perfume | Perfume da Bota/do Boto |
| Santos, SP | Perfume | None | Perfume | Perfume da Bota/do Boto |
| Santos, SP | Muscle in piece | Muscle | Dried muscle in a vial | Perfume da Bota/do Boto |
| Porto Alegre, RS | Perfume | None | Fragrance with piece of muscle | Perfume da Bota/do Boto |

Brazilian states (*AM* Amazonas, *PA* Pará, *MA* Maranhão, *PI* Piauí, *CE* Ceará, *PE* Pernambuco, *AL* Alagoas, *BA* Bahia, *MT* Mato Grosso, *RJ* Rio de Janeiro, *SP* São Paulo, *RS* Rio Grande do Sul)

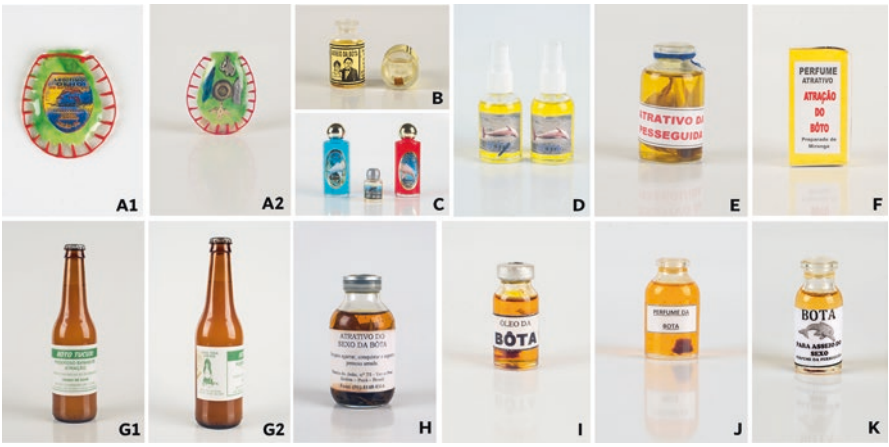


**Fig. 13.2** Examples of the most common multipurpose uses of aquatic wildmeat derived from dolphins in Brazil. (**a1** and **a2**) dolphin eye amulet, (**b**) dolphin love charm with dolphin piece inside; (**c**) dolphin perfume; (**d**) dolphin spray perfume; (**e**) dolphin love charm with dolphin piece inside and herbs; (**f**) dolphin love attraction perfume; (**g1** and **g2**) Dolphin potion for bathing purpose; (**h**) dolphin charm with multipurpose function (attraction, love, satisfaction, good luck, prosperity) with dolphin piece inside and herbs; (**i**) dolphin oil for love attraction; (**j**) dolphin perfume with large piece of dolphin inside and (**k**) dolphin attraction for sex cleanliness

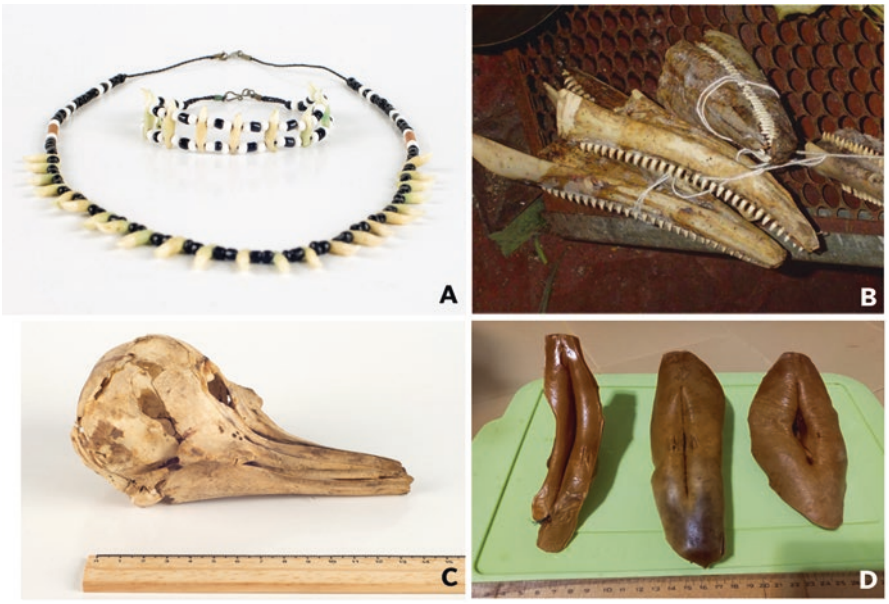


**Fig. 13.3** Other examples of the use of dolphins in Brazil. (**a**) Necklaces and bracelets adorned with teeth from Guiana dolphins (*Sotalia guianensis*), (**b**) dried mandibles of Guiana dolphins for sale as handicraft or religious uses. (**c**) dried skull of a newborn Guiana dolphin on sale at Ver-O-Peso Market, Belém, and (**d**) Guiana dolphins sun dried vaginas on sale for sexual attraction


**Fig. 13.4** Cetaceans as decoration and ornament uses in Brazil. (**a**) Guiana dolphin skeletons hanged in a house in Algodoal, eastern coast of Pará state, (**b**) Skull of a Bryde's whale (*Balaenoptera brydei*) used for house decoration in Balneário Barra do Sul, northern Santa Catarina state; (**c**) Humpback whale (*Megaptera novaeangliae*) skull used in decoration in a restaurant by the beach in Tamoios, Cabo Frio, Rio de Janeiro state and (**d**) Ribs and vertebrae of a blue whale (*Balaenoptera musculus*) used in decoration in a farm in Marajó island, Pará state, Brazil. (All pictures, except (**b**), are from the authors. Picture (**b**) is available from: g1.globo.com/sc/santa-catarina/noticia/2012/09/moradores-pegam-ossos-de-baleia-morta-para-decoracao-no-norte-de-sc.html)

### *13.2.2 Previous Forensic Identification*

Previous studies (Sholl et al. 2008; Siciliano et al. 2018) have sampled and conducted molecular identification of cetacean products (body parts or structures, and craft products) from popular and traditional Brazilian markets. Sholl et al. (2008) and Siciliano et al. (2018), in order to identify samples based on molecular data, investigated the mitochondrial gene cytochrome *b* (MT-CYB), based on available data from online databases. Cytochrome *b* following abbreviation is under HGNC rules (HUGO Gene Nomenclature Committee by National Human Genome Research Institute; Eyre et al. 2006). DNA was isolated according to Sambrook et al. (1989) phenol-chloroform protocol, and Polymerase chain reaction (PCR) were carried out to amplify the complete MT-CYB (ca. 1140 bp), with forward and reverse primer CB-out 1 and CB-out 2 (Cassens et al. 2000), as described by Siciliano et al. (2018). Sequencing reactions were performed with PCR primers, plus internal primers CB-in 1 and CB-in 2 (Cassens et al. 2000), labeled with XL and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and

loaded to an ABI Prism™ 3130 platform. Sequences obtained were analyzed and edited with Chromas (MacCarthy 1998), and assembled using Bioedit (Hall 1999). Assembled MT-CYB sequences were manually aligned with MEGA6 (Tamura et al. 2011; Kumar et al. 2016), converted to FASTA format and subjected to Basic Local Alignment Search Tool (BLAST–NCBI https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm taxonomic identification. Surprisingly, love charms samples encompassed not only dolphin tissue, but swine. Based on results obtained, the authors constructed two alignment datasets for MT-CYB comparisons; DATASET1, encompassing Siciliano et al. (2018) *Sotalia guianensis* sequences (GenBank accession numbers KY236017-KY236019), *S. guianensis* (EF488216 to EF488223, EF457552), *S. fluviatilis* (EF457551), *Inia geoffrensis* (EU554562), and *Tursiops truncatus* (AF084095); and DATASET2, encompassing Siciliano et al. (2018) *Sus scrofa* sequences (KY236020-KY236029) and (AB015065, AM492546, AM492548, AM492551, AM492569, AM492595, AM492597, AM92621, AM492653, KJ476220, KJ476229). For both DATASETs, genetic distance, haplotype estimates, nucleotide diversity and median joining (MJ) reconstructions network were carried out (Bandelt et al. 1999; Librado and Rozas 2009). Phylogenetic approaches were carried out for DATASET1 and parameters are described in Siciliano et al. (2018).

Moreover, two samples of "dolphin love attraction perfume" collected in a religious shop placed in the Public Market of Porto Alegre, South Brazil, in 2016 had their putative "dolphin" DNA extracted following the methods suggested by Sholl et al. (2008) and Gravena et al. (2008) and cytochrome oxidase *c* subunit I (coxI) was amplified using the primers COX1F (5′-TGCCTACTCGACCATTTTAC-3′) and COX1R (5′-TGAAACCTAGGAAACCAATA-3′) according to conditions described by Amaral et al. (2007).

### *13.2.3 Tooth Morphology and Aging*

Dolphins' teeth collected from handmade necklace, bracelets and earring sold at traditional markets and squares markets in three main cities from Northern and Northeastern Brazil (Belém in Pará state, São Luís in Maranhão state and Fortaleza in Ceará state), were used to assess the more frequent ages observed in the trade of artisanal object in these regions. The external characteristics of the teeth, which decorated the handicrafts, were utilized to ascertain the genus/species to which they corresponded. Moreover, as two distinct sizes of teeth from adult specimens were identified within the sample, four external measurements were employed to examine possible variations in tooth morphology that could signify the use of different species within the *Sotalia* genus for handicrafts (Ramos et al. 2000). These measurements included tooth total length (TL), measured from the apex of the crown to the end of the root; root diameter (RD), measured as the maximum width of the root; crown length (CL), measured from the apex of the crown to the root cingulum; and cingulum diameter (CID), measured as the diameter of the tooth at the intersection

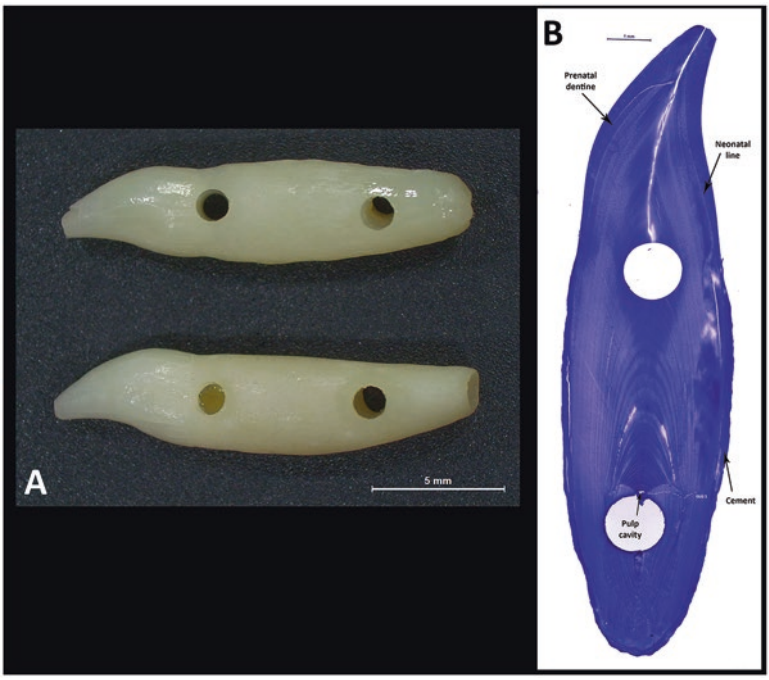


**Fig. 13.5** Teeth of *Sotalia* spp. dolphins obtained from various handicrafts commercialized in the North-Northeastern of Brazil. (**a**) teeth from a bracelet, (**b**) Thin section from a tooth used for the age readings. (Original figures by the authors)

area of the crown with the root. The age frequency was assessed by counting the Growth Layer Groups (GLGs) deposited in the teeth' dentine (Rosas et al. 2003; Rosas and Monteiro-Filho, 2002). Each tooth was subsequently wearing, decalcified with RDO®, cut with a manual freezing microtome in thin sections of 20–30 μm, stained with Mayer' hematoxylin, blued in Ammonia 2% and finally, mounted in glycerin 100% (Rosas et al. 2003; Conversani et al. 2020). Readings were made at three different times by researchers to determine the chronological age of each tooth. The GLGs were counted having in consideration a correspondence of 1 GLG and 1 year (Fig. 13.5) of life described previously for the genus *Sotalia* (da Silva 1994; Rosas et al. 2003; di Beneditto and Ramos 2004). The best age estimate was defined as the coincidental readings of at least two from the three times.

## 13.3 Results

Markets and religious shops visited in Rio Branco, Manaus, Belém, Recife, Salvador, Cuiabá, Rio de Janeiro and Porto Alegre had plenty of "dolphin love charms" available for purchase. A wide variety of amulets, sold in vials containing

perfumes or fragrances, were found in all markets and shops visited (Table 13.1, Fig. 13.2). Data indicated a wide spectrum of vials and textures, demonstrating that the commerce of cetacean products is more common in the North and Northeastern regions compared to the Southeast and South Brazil. Dried dolphin eyes, penis and vagina, all belonging to *Sotalia guianensis* were found in Belém and Soure (Pará), and São Luís (Maranhão), and also at the popular Mercadão de Madureira, in Rio de Janeiro.

It was noted that a large number of available items are offered to be used in holistic treatment, massage and soul healing, representing a diversity of aquatic creatures including manatees, whales, dolphins, capybaras, caimans, turtles, sucurijús and poraquês. No products available online were purchased, and consequently they were not analyzed. However, online catalogues announced several products of questionable authenticity, including essences called "BF Força das Baleias", which means "The power of whales", composed of "seven different types of whales" and other labeled as "manatee essence for treatment" (Fig. 13.6). According to some catalogues, the essences were originated from tucuxi dolphin (*S. fluviatilis*) and possible collecting locations were the rivers of the Amazon and Florianópolis, Santa Catarina, Southern Brazil (Fig. 13.7).


**Fig. 13.6** Alleged manatee (*Trichechus* spp.) balm ointment and lard on sale on the internet. Products were tested and later confirmed as vaseline


**Fig. 13.7** Commercial products sold as "Dolphins Bach Floral" that promise all kinds of healing, from helping childbirth, cancer treatment, sexual disorders, depression, hormonal dysfunctions, among others. According to site "http://floraisgolfinhos.com.br/", and in their Facebook homepage (Florais Golfinhos), both from the same company, the seller says that inside the vials that are essences from various animals, including dolphins, whales and manatees. The company also offers intensive courses to teach the beneficial uses of their products and techniques

### *13.3.1 Molecular Analysis*

Siciliano et al. (2018) identified dolphin love charms samples by molecular phylogeny. While several samples contained only liquids and scents, adorned with leaves or either very decomposed contents, could not be amplified. These authors have demonstrated that these samples belonged to two species: Guiana dolphins (*S. guianensis*) and swine *S. scrofa*. Successfully sequenced samples were compared to data from the online database. Three "love charms" samples were identified as *S. guianensis* (from Belém and Soure, Pará state, Brazil; and Rio de Janeiro, Rio de Janeiro state, Brazil). Siciliano et al. (2018) observed genetic distance from 0.001 to 0.008 among *S. guianensis* specimens, in which phylogenetic approaches demonstrated the monophyly of the genus (90 bootstrap, 0.97 pp) divided in *S. guianensis* and *S. fluviatilis* clades. Also, among *S. guianensis* specimens, median joining (MJ) reconstruction indicated a star-like topology with central haplotype including one sample sequenced from Soure (Pará) (Fig. 13.8). The DNA from two samples collected in the South region from "dolphin love attraction perfume" did not amplify, suggesting that the samples were too degraded or there were no DNA samples in the vials. The hypothesis of the absence of biological material is highly plausible, since the extractions resulted in an undetected product in 1% agarose gel stained with ethidium bromide and visualized under UV transillumination.

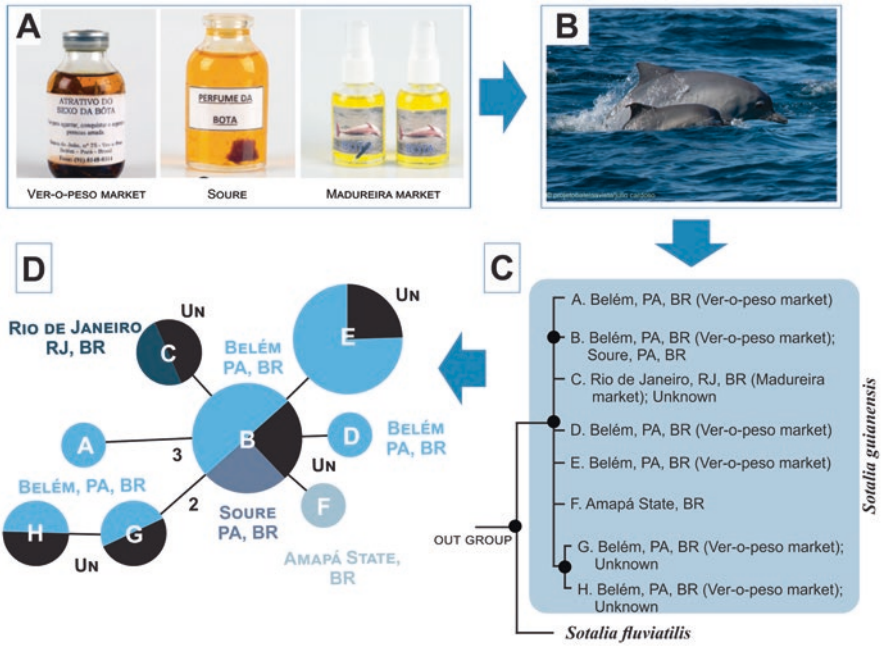


**Fig. 13.8** (**a**) Boto perfume bottles from Brazilian markets. (**b**) *Sotalia guianensis* photo by Júlio Cardoso. (**c**) Phylogenetic reconstruction based on MT-CYB sequences of *Sotalia guianensis*, carried out with TrN + I model, where black circles are bootstrap values greater than 80%. (**d**) Median joining network reconstructions based on MT-CYB sequences of *Sotalia guianensis,* where circle sizes correspond to the number of shared sequences of each haplotype, and circle and pie charts colors are relative to the purchase locality of each sample from Brazil; branch lengths are relative to haplotypes distance, and numbers above branches show mutational steps; branches without values showed only one mutation. *PA* Pará state, *RJ* Rio de Janeiro state, *UN* unknown locality of samples from GenBank. Information on haplotype composition, see Siciliano et al. (2018)

Ten *S. scrofa* samples (from Andaraí, Madureira and Tijuca, Rio de Janeiro state, Brazil) were identified with 100% and 95% cover and identity, and compared with other samples from DATASET2. Among *S. scrofa* specimens, Siciliano et al. (2018) observed genetic distance from 0.001 to 0.016, haplotype diversity of 0.8238 and 12 haplotypes, in which ten of them were singletons and two shared. No geographical structure was observed and curiously, one haplotype was shared by one sample from Andaraí, Rio de Janeiro, and one sample from Sri Lanka (Fig. 13.9).

### *13.3.2 Age*

Aging teeth included 64 teeth belonging to *S. guianensis* and 42 probably from *S. fluviatilis* (Ruenes et al. 2022). Observed ages ranged between 2 and 30 years being consistent with the age interval described for both species (Fig. 13.10).

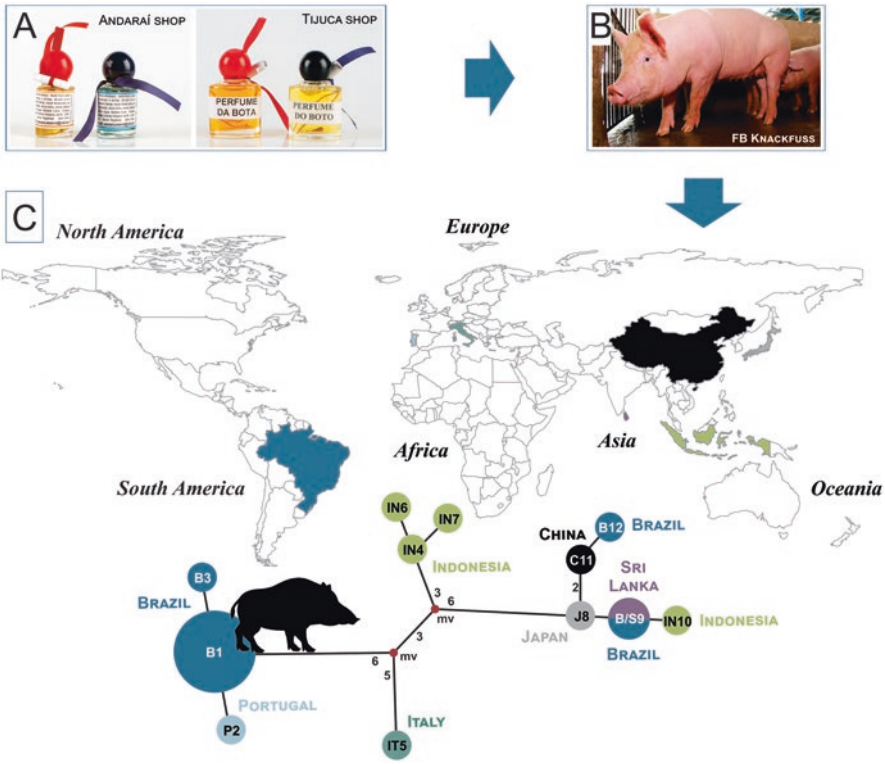


**Fig. 13.9** (**a**) Boto perfume bottles from Brazilian religious shops. (**b**) Domestic *Sus scrofa*, photo by Fabiana B. Knackfuss. (**c**) Median joining network reconstructions based on MT-CYB sequences of *Sus scrofa*, where circle sizes are relative to the counts of each haplotype and circle and pie charts colors are relative to the country of origin of each sample, as indicated in the map; branch lengths are relative to haplotype distance, and numbers above branches show mutational steps; branches without values showed only one mutation. *mv* median vector. Information on haplotype composition, see Siciliano et al. (2018)

Although artisanal object included different ages, some ages were more frequent in all handicrafts. The more frequent ages for both types ranged between 2 and 6 years, being classified as juveniles, and between 8 and 16 years, being classified as young adult animals.

## 13.4 Discussion

Remarkable differences were noted in the recent use of aquatic mammals in the vast Brazilian territory. These differences will be treated in topics, including the trade of dolphins' products, genetic identification of products, age of specimens used in the trade, and natural products derived from aquatic wildlife.

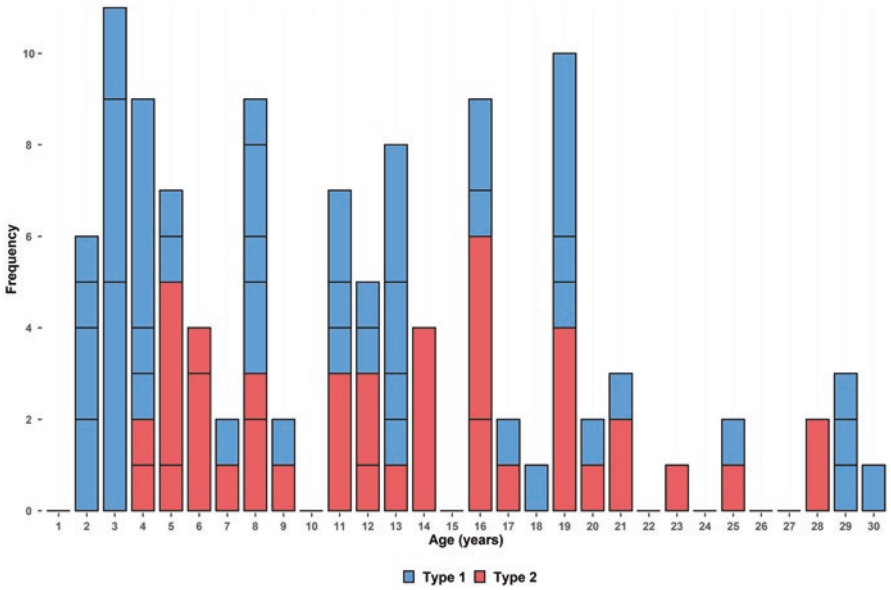


**Fig. 13.10** Ages classes for the two morphotypes of *Sotalia* dolphins (Type 1: *Sotalia guianensis*; Type 2: *Sotalia fluviatilis*) teeth detected in the trade of artisanal objects in the North and Northeast regions of Brazil. Branches indicated the frequency of each age class in the handicraft objects

### *13.4.1 Trade of Dolphins' Products in Brazil*

The highlight of the visits conducted at the most traditional public markets in Northern, Northeastern, Southeastern and Southern Brazil are the religious and magical items on sale, including amulets and perfumes, available at low cost to tourists. For example, these beaten path places to visit in Brazilian Amazonia, known as Ver-o-Peso Market in Belém, the Adolpho Lisboa Municipal Market of Manaus, the Mercado de São José, in Recife, the Mercadão de Madureira, in Rio de Janeiro and the Mercado Público de Porto Alegre are the sites where myth and folklore get together and are ready to be sold as souvenirs of the forest. But even with no sample positively identified in South Brazilian markets, it is still outstanding that this kind of product reached the most important public market in south Brazil, which is 3188 km far away from the Amazon markets (such as Ver-o-Peso), where dolphins are the epicenter of such fetishes, magical creatures that can heal your soul and give lovers a great passion, according to product's instructions or even to shop staff. Although there is no legend of the boto in the culture of South Brazil, the arrival of these love charms in the markets of this region should be observed carefully, suggesting that it is a standard item of widespread consumption of these religious stores.

Identification of the species used for a variety of purposes and the dating of such commerce are not an easy task. During the 1980s, authors have targeted the Amazon River dolphin (*Inia geoffrensis*) as the most frequently traded species in public

markets mentioned above (Best and da Silva 1989). Siciliano (1994) has pointed out that dolphin's eyes and genitals, attributed to Guiana dolphins (*S. guianensis*), originated from the fishing town of Vigia, located on the Eastern Pará coast. Subsequently, there have been reports on the use of *Sotalia* dolphin fat for therapeutic purposes in Northeastern Brazil, in the states of Piauí, Paraíba, and Maranhão (Alves and Rosa 2006, 2007). Another study also indicated the use of *Sotalia* dolphins for medicinal and magic/religious purposes in Northern Brazil based on short surveys conducted at Belém and Soure in 2005 (Alves and Rosa 2008; Alves et al. 2013). Authors referred to the specimens as tucuxi dolphins (*sensu S. fluviatilis*), without any further analysis. Sholl et al. (2008) conducted genetic analyses using cytochrome *b* sequences in samples from the Ver-o-Peso Market and proved that they were all love charms from Guiana dolphins (*S. guianensis*). According to these authors, *S. guianensis* specimens incidentally captured in gillnets set off the Pará and Amapá coasts were the main source of the religious and magical products found in regional markets. Footage of dozens of Guiana dolphins incidentally captured in gillnets, then handled and stored in the hull of the boat by fishers off the coast of Amapá, Northern Brazil, in 2007 has shocked the national and international community (Fig. 13.11). But certainly, this was the result of uncontrolled fishery activity, and lack of law enforcement. Further, Siciliano et al. (2018) confirmed this scenario of use of dolphin samples to produce love charms in Belém, Soure and Bragança, Pará state, all of them belonging to Guiana dolphins. Testing samples from Pará, Amazonas and Rondônia, other forensic studies have reached the same results confirming that

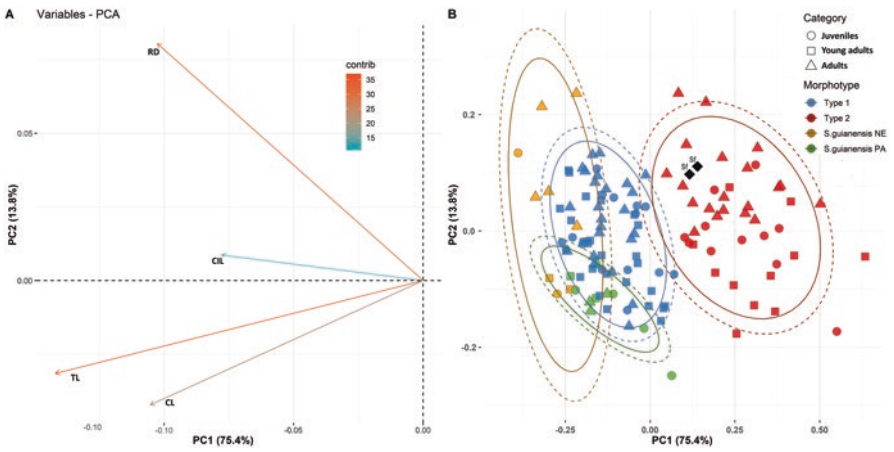


**Fig. 13.11** Principal component analysis for teeth measurements obtained from samples of *Sotalia* dolphins from North and Northeastern Brazil. (**a**): Contribution of the variables total length (TL), root diameter (RD), crown length (CL), and cingulum diameter (CID) to the principal component dimensions (PCs). (**b**): Scores distribution in the two dimensions of the PCs. The category indicates juveniles, young adults, and adults in the sample, and colors indicate the teeth morphotypes or species. The localities of Pará coast (PA) and Northeast Brazil (NE) included only stranded specimens of *Sotalia guianensis* preserved at MPEG Museum, PA. The black diamonds represent teeth from an adult specimen of *Sotalia fluviatilis* preserved at the Museu Nacional, Rio de Janeiro


**Fig. 13.12** By-caught Guiana dolphins *Sotalia guianensis* off the coast of Amapá, North Brazil, 2007. These specimens feed the trade of dolphin love charms and other products in Brazil. (Photo courtesy of Prof. Rosália Cutrim/UFRA)

Guiana dolphins are the supplier for this religious and magical trade all over the places visited (Gravena et al. 2008; dos Santos et al. 2018). High levels of incidental captures of Guiana dolphins throughout the Northern coast can turn this species into the principal supplier of the intense trade of love charms, and, thus, requires urgent measures from Brazilian environmental agencies (Fig. 13.12).

### *13.4.2 Tooth Morphology and Age*

Handicrafts have shown a very interesting result. The more frequent age classes for both teeth types, *S. guianensis* and *S. fluviatilis*, were juveniles and young adults (Ruenes et al. 2022), which is similar to age-structures of *Sotalia* dolphin populations that were long-term affected by fishery interactions (Rosas et al. 2003; Di Beneditto and Ramos 2004; Moura et al. 2009; Meirelles et al. 2010; Lima et al. 2017). The previous genetic forensic research using tissue samples from the dolphins-derived artisanal object sold at the same traditional markets indicated *S. guianensis* as the only aquatic mammal supplying this trade throughout Brazil. Thus, age-related results are consistent with the spatial range of *Sotalia*-derived products traded in Northern and Northeast Brazil, and it reveals that specific age classes of the populations are under pressure, being the most frequently used in the trade. On the other hand, tucuxi *S. fluviatilis* teeth are probably purchased in Amazonian fishing villages and distributed by traveling salesmen or neo-hippies, who are common figures in cities of the North and Northeast regions (Siciliano et al. 2018). Upon comparing the morphological characteristics of teeth present in the handicrafts with those of known specimens of *S. fluviatilis* and *S. guianensis*, housed in the Museu Nacional (Rio de Janeiro, Brazil) and the Museu Paraense Emílio Goeldi – MPEG (Pará, Brazil), two distinct groups, corresponding to each of the aforementioned species, were able to be discerned within the objects.

Dolphin carcasses, stranded and/or accidentally caught in fishing gillnets, has been previously suggested as a source for the manufactured products found in traditional markets on Northern and Northeastern Brazil (Siciliano 1994; da Silva and Best 1996; Sholl et al. 2008; Siciliano et al. 2018), as well as their use for human consumption and as bait in shark fishing (Siciliano 1994; Tosi et al. 2009; Zappes et al. 2009; dos Santos-Filgueira et al. 2021; Briceño et al. 2021). Based on these results, it is possible to suggest that artisanal objects commercialized as part of dolphin-derived products trade in Northern and Northeastern Brazil and *Sotalia* dolphins' mortality by accidental captures in gillnets seems to affect the same population segments, and are possibly associated in these regions.

### *13.4.3 Forensic Analyses*

Genetic analyses of love charms from Rio de Janeiro markets revealed a double cheating. First, the perfume of dolphin charm is proved to be ineffective and second, they contain pieces of domestic pig (*S. scrofa*) inside. Amazingly, the pieces of pig inserted in the vials are of multiple breeds as previously indicated. Several *Sus* races raised in Brazil belong to different breeds and origins, including Asian (Meishan, Jiaxing and Jinhua), Portuguese (Alentejana and Bísara), Spanish (Galega and Perijordina), and Italian (Napolitana) breeds, among others (Castro et al. 2002). This figure clearly indicates a widespread consumption and high magnitude production of such trinkets, sold without sanitary control.

### *13.4.4 Natural Products Derived from Aquatic Wildlife*

Among the species traded, it was noted a recent trend of alleged "natural" products, labeled as balm ointment, pills, and creams, derived from manatees, dolphins, capybaras, caimans, sharks, rays, poraquês, sucurijús, and freshwater turtles. It is rather common to find in green or organic markets, and online shopping, throughout Brazil, a variety of products labeled after these "exotic" wildlife. It is unlikely that this sort of item is of truly wildlife origin, although some were not tested, but are rather misleading products. Indeed, tested manatee balm ointment and cream ("Pomada de Peixe-boi" and "Gordura de peixe-boi") consisted of Vaseline with eucalyptus scent, containing no animal origin (Luna et al. 2004). Historically, manatees have been used in Brazil by native people. Interviews conducted with local people on the Amazon basin along 20,000 km from 2000 to 2008 confirmed the most diverse uses of manatees (Luna et al. 2001; Sartor et al. 2004; Luna 2007). Some regions use bones as a spoon to cook or even as a food tray. The blubber was widely used as medication, to conserve food (*e.g.*, manatee meat, used for consumption) which is important to the region as there is no refrigerator or even light on those communities and also as an oil to fry any kind of food. More recently, Siciliano

et al. (2021) have described similar uses of manatees in rural communities of the Amazon coast and inland, from religious to medicinal function.

## 13.5 Conclusions/Final Remarks

The Guiana dolphin (*S. guianensis*) plays the lead role in the national trade of aquatic wildmeat. Either bycaught or stranded specimens feeds an active commerce of amulets, handicrafts and trinkets, mostly in the North and Northeastern regions. This activity fall into the status of unaccounted income in a national scale, very hard to track back and quantify. The next step for research should be an evaluation of such trade by region and try to figure out its trends. It seems that prices can rise upon demand and scarcity, as dolphins' eyes, for example, can be sold as of November 2021 by R$ 200,00 (Brazilian reais = 35 US dollars) in the Rio de Janeiro religious shops. The market has its tricks, faking products using domestic animals, or simply not using the traditional piece of dolphin inside the vials. In the wake of natural health treatments, charlatanism thrives, offering a wide variety of potions, essences, balms, labeled after aquatic animals. Interestingly, the products are assigned "Registration free product" and advertised as "Product of the Brazilian Pharmacopeia". This makes clear a scenario of numerous health items for natural treatments widely available but uncontrolled by Brazilian sanitary institutions nor have been tested in official laboratories.

Summarizing, the trade of aquatic animals in Brazil remains unregulated, uncontrollable, unsustainable and untrammeled. We shall have to wait and see how the decision makers will cope with this scenario in the near future.

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# Chapter 14
# Wildlife Forensic Genetics: A Tool for Resolving Wildlife Crimes and Support Species Conservation

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## 14.1 Introduction

Wildlife conservation has been persistently challenged by a wide range of human activities (Ribeiro and Silva 2007; Webb 2001), threatening biodiversity worldwide, and increasing the extinction risk of a huge sort of organisms on the planet (Haddad et al. 2015; United Nations Economic Commission for Europe 2020; Wilkinson et al. 2018), which characterize a global biodiversity crisis (Dirzo et al. 2014; Otto 2018; Waters et al. 2016). Crimes against wildlife are among the most important threats to biodiversity, representing a serious threat to the different taxonomic groups and also affecting the security, political stability, economy, natural resources, and cultural heritage of many countries (International Consortium on Combating Wildlife Crime 2020). A few tens of thousands of international shipment seizures have been annually reported in the world wildlife seizures database by international traffic crime (World WISE) (UNODC 2020), however, these values, as well as for other crime types, as poaching and hunting, are still underestimated. Wildlife crime

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detections are usually hampered by remote locations, absence of eyewitnesses, and low amount of biological and physical evidence for accurate identification of the organism (Raymond et al. 2010; UNODC 2009). Added to this, the punishments against wildlife crimes are so weak that even when the police investigation successfully collects testimonial and physical evidence to submit to the court, the criminals prefer to pay the punishment rather than stop the criminal activity.

Early developed to the resolution of legal conflicts between humans (Butler 2010; Goodwin et al. 2011; Houck 2010), since the late 1990s forensic DNA analysis has been used to provide insights for the resolution of illegal activities against fauna and flora (Blevins and Edwards 2012), mainly when other techniques are not resolutive (Goodwin et al. 2011). Although wildlife and human forensic DNA analyses differ on the species involved, both approaches focus on similar questions (Moore and Frazier 2019). Thus, wildlife forensic genetics (WFG) emerges to provide information on species and individual identification, familial relationships, and geographic origin when the biological samples can provide evidence for law enforcement (Ogden et al. 2009), and that information may contribute to support biological conservation.

Some aspects have already been reviewed on wildlife forensic genetics and three main topics have been focused. The potential use of genetic technologies in wildlife science and their importance for conservation and law enforcement (Alacs et al. 2010; Arif et al. 2011; Bell et al. 2016; Campbell and Godfrey 2010; DeYoung and Brennan 2005; Dormontt et al. 2015; Iyengar 2014; Johnson et al. 2014; Linacre 2008; Mori and Matsumura 2020; Morrison et al. 2018; Ogden 2011; Ogden and Linacre 2015), the contribution of forensic DNA analyses for conservation of specific animal groups (Campbell and Godfrey 2010) and the WFG challenges in the near future (Morrison et al. 2018; Verma and Goswami 2014).

In this chapter, we innovate with a worldwide integrative review including world wildlife crime reports, genetic analyses used, criminal aspects, and conservation significance for the target species, focusing on fauna. We analyzed WFG-related data available in the literature to evaluate which taxonomic groups and crime typologies are more often assessed. We also discussed the main molecular techniques that have been used for the resolution of wildlife crimes. We focused on two main questions: (i) which taxonomic groups have been more prevalent in wildlife forensic genetic studies; and (ii) how forensic genetics has been contributing to wildlife conservation, emphasizing threatened species conservation. We presented several examples of forensic evidence of wildlife crimes in Costa Rica and we added a study case on forensic paternity carried out on a neotropical bird that represents one of the most animal species with illegal hunting notifications in southeastern Brazil (Azevedo 2018).

We produced a picture of the illegal anthropic actions that negatively affect the conservation of biodiversity over the five major geographic regions and concluded that the WFG published cases represent a partial benchmark of the wildlife forensic case work likely due to the deep bias between the potential continuous work carried out in forensic laboratories around the world and the published studies in indexed repositories.

## 14.2 An Overview on the Contributions of Forensic Genetics for Animal Wildlife Conservation

For evaluating the contributions of WFG for animal wildlife conservation, we performed a systematic review of the available literature related to wildlife forensic genetic studies published from January 1991 to July 2020 in indexed repositories. We used three online databases: Web of Science, Springer and Science Direct, employing standard methodologies previously proposed for systematic reviews (Basu 2017; Hunter and Schmidt 2002; Littell et al. 2008; Moher et al. 2016). For data searching, we used the keywords forensic*, genetic* and wildlife*, and then we established a review protocol including three main steps for selection, extraction and data analyses, as summarized in a flowchart (Fig. 14.1).

We recovered a total of 869 documents. In the selection step, we accepted 348 and excluded 516 according to the rejection criteria listed in the Fig. 14.1. At the extraction step, we accepted 63 of the 348 articles. These 63 publications used genetic tools to present evidence in animal wildlife forensic cases subjected to the Justice system and fulfilled the four inclusion criteria of the flowchart (Fig. 14.1). In the last step, we used these 63 accepted articles for data analyses. From each of the 63 accepted articles, we mined information about (i) scientific names of the target species or even its specific common name; papers that mentioned only general names (e.g., whale or fox) were categorized as "without information (WI)", (ii) typology of the crime, following the Oxford Research Encyclopedia of Criminology

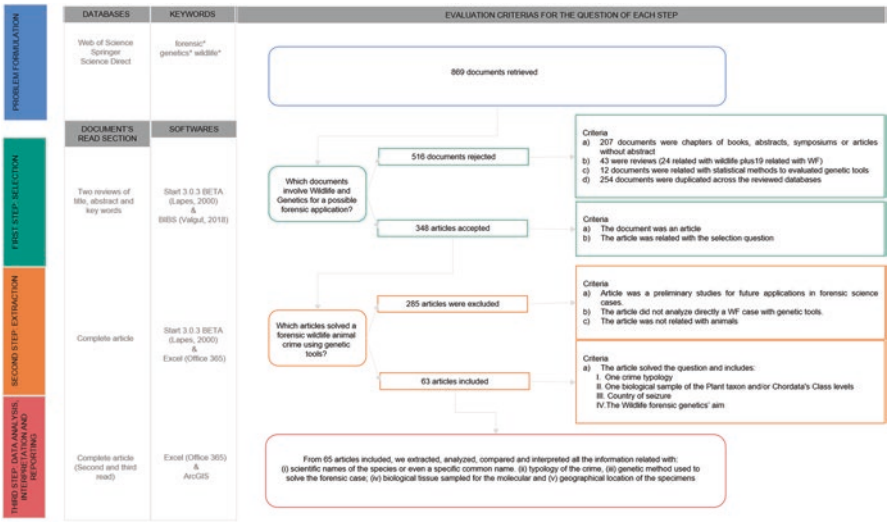


**Fig. 14.1** Flowchart of the methodology carried out for the systematic review and data analyses. Each step is represented within rows that share the same color and includes the question that must be answered and the acceptance and exclusion criteria. The recovered or accepted documents are the result of the documents accepted minus the excluded on each previous step

and Criminal Justice (Boratto and Gibbs 2019), (iii) genetic method used to solve the forensic case; (iv) biological tissue sampled for the molecular analyses (each individual reported in the selected literature was analyzed as one potential victim of a wildlife crime); and (v) geographical location where the crime specimens were analyzed. All the information mined was summarized in a hereafter named WFG dataset (Table 14.1). The countries where the wildlife crimes occurred were grouped into five major geographic regions (Africa, Asia and Pacific, Europe, Latin America, North America), and the number of seized animal samples from each region was represented in a world heat map. Each forensic sample was appropriately grouped into major taxonomic categories (fish, amphibians, reptilians, birds, and mammals). The animal species were classified by their conservation status according to the International Union for Conservation of Nature (IUCN) Red List (IUCN 2019) for evaluating the impact of the reported wildlife crimes on threatened species.

## 14.3 Taxonomic Groups and Species Affected

A total of 4514 animal samples was identified within the 63 accepted articles included in our dataset. The most frequent taxonomic group victim of wildlife crimes reported was mammals, with 66.39% of the total number of the analyzed samples, distributed across all major geographical regions (Fig. 14.2a). Samples from elephants, pangolins, and lions were the most representative analyzed by forensic genetics. Birds were included as the second taxonomic group of proportion of seized samples (17.48%), distributed in only two geographical regions (Europe and Asia-Pacific). China showed the highest number of seized samples (1080) analyzed by forensic genetics, followed by the United Kingdom (601) and South Africa (477) (Fig. 14.2b).

Our WFG dataset retrieved a total of 185 species distributed disproportionately among fish, reptiles, birds and mammals (Fig. 14.3a). Approximately 35% (64) of the species are threatened. Although most assessed species are unthreatened (41%, 75 species) or with an unidentified status of threaten (25%, 46 species), approximately 57% (105 species) of these species are reported as population decreasing or unknown population tendency (IUCN 2020) (Fig. 14.3b, Table 14.1).

We found 134 species of mammals that were distributed in six of the nine threatened IUCN categories, although most of these species appeared at Least Concern (Table 14.1). Pangolin, felines, and elephants were the most affected mammals, and two elephant species appear in the top crime cases among the Endangered and Vulnerable species (Fig. 14.4).

Birds were the second most represented taxonomic group in the WFG dataset (Fig. 14.3a). While most of these bird species are listed at Least Concern, three are Endangered: white-tailed black cockatoo (*Calyptorhynchus baudinii*), saker falcon (*Falco cherrug*), and Egyptian vulture (*Neophron percnopterus*), all with tendency of population decreasing (Table 14.1).

**Table 14.1** Our WFG dataset. The table summarizes the information extracted from the papers of the past 28 years, in addition to the IUCN Red List status and population trend categories

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Chang et al. (2014) | Mammals | Common Bottlenose Dolphin | *Tursiops truncatus* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Harp Seal | *Phoca groenlandica* (*Pagophilus groenlandicus*) | LC | Increasing | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Hooded Seal | *Cystophora cristata* | VU | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Pygmy Killer Whale | *Feresa attenuata* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Pygmy Sperm Whale | *Kogia breviceps* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Risso's Dolphin | *Grampus griseus* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Rough-toothed Dolphin | *Steno bredanensis* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Short-beaked Common Dolphin | *Delphinus delphis* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |
| | Mammals | Wild Boar | *Sus scrofa* | LC | Unknown | Trade and poaching | Meat | Species identification | COI |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Morf et al. (2013) | Mammals | African brush-tailed porcupine | *Atherurus africanus* | LC | Unknown | Trade | Meat | Species identification | Cyt b |
| | Mammals | African clawless otter | *Aonyx capensis* | NT | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Bay duiker | *Cephalophus dorsalis* | NT | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Blue duiker | *Philantomba monticola* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Brush-tailed porcupine spp | *Atherurus spp* | LC | WI | Trade | Meat | Species identification | Cyt b |
| | Mammals | Common duiker | *Sylvicapra grimmia* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Greater cane rat | *Thryonomys swinderianus* | LC | Unknown | Trade | Meat | Species identification | Cyt b |
| | Mammals | Guenon spp. | Unidentified | WI | WI | Trade | Meat | Species identification | Cyt b |
| | Mammals | Marshbuck (Sitatunga) | *Tragelaphus spekii* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Maxwell's Duiker | *Philantomba maxwellii* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Pangolin spp | Unidentified | WI | WI | Trade | Meat | Species identification | Cyt b |
| | Mammals | Peters' Duiker | *Cephalophus callipygus* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Red river hog | *Potamochoerus porcus* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Red-flanked Duiker | *Cephalophus rufilatus* | LC | Decreasing | Trade | Meat | Species identification | Cyt b |
| | Mammals | Walter's Duiker | *Philantomba walteri* | DD | Unknown | Trade | Meat | Species identification | Cyt b |
| | Reptiles | Forest hingeback tortoise | *Kinixys erosa* | DD | WI | Trade | Meat | Species identification | Cyt b |
| | Reptiles | Gaboon viper | *Bitis gabonica* | A | WI | Trade | Meat | Species identification | Cyt b |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Caratti et al. (2010) | Mammals | Wild Boar | *Sus scrofa* | LC | Unknown | Trade | WI | Individualization | STR |
| Wu et al. (2005) | Mammals | Sika deer | *Cervus nippo* | LC | Increasing | Hunting | Skin | Species identification | Cyt b |
| White et al. (2012) | Birds | Red-tailed black-cockatoo | *Calyptorhynchus banksii* | LC | Decreasing | Poaching | Carcasses | Individualization and Parental identification | NA |
| | Birds | White-tailed black-cockatoo | *Calyptorhynchus baudinii* (*Zanda baudinii*) | EN | Decreasing | Poaching | Carcasses | Individualization and Parental identification | NA |
| | Birds | Galahs | *Eolophus roseicapillus* | LC | Increasing | Poaching | Carcasses | Individualization | NA |
| Basu et al. (2019) | Mammals | Oriental House Rat | *Rattus tanezumi* | LC | Increasing | Trade | WI | Species identification | Cyt b |
| Plumer et al. (2018) | Mammals | Grey Wolves | *Canis lupus* | LC | Stable | Destruction of Wildlife | Saliva | Species identification | mtDNA, STR |
| | Mammals | Dog | *Canis lupus familiaris* | WI | WI | Destruction of Wildlife | Saliva | Species identification | mtDNA |
| Cao et al. (2014) | Mammals | Saiga antelope | *Saiga tatarica* | CR | Decreasing | Patent medicine autentification | Bone | Species identification | mtDNA |
| | Mammals | Goat | Unidentified | WI | WI | Patent medicine autentification | WI | Species identification | mtDNA |
| Hadas et al. (2016) | Mammals | Thomson's Gazelle | *Eudorcas thomsonii* | LC | Decreasing | Trade and poaching | Blood, hair and tissue | Species identification, Individualization | STR, 16 s rRNA, 12 s rRNA |
| | Mammals | Mountain Gazelle | *Gazella gazella* | EN | Decreasing | Trade and poaching | Blood, hair and tissue | Species identification, Individualization | STR, 16 s rRNA, 12 s rRNA |
| | Mammals | Przewalski's Gazelle | *Procapra przewalskii* | EN | Increasing | Trade and Poaching | Blood, hair and tissue | Species identification, Individualization | STR; 16 s rRNA; 12 s rRNA |
| | Mammals | Saiga | *Saiga tatarica* | CR | Decreasing | Trade and poaching | Blood, hair and tissue | Species identification, Individualization | STR; 16 s rRNA; 12 s rRNA |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Frosch et al. (2011) | Mammals | Brown Bear | *Ursus arctos* | LC | Stable | Destruction of Wildlife | Tissue and hair samples | Individualization | STR |
| Nash et al. (2018) | Mammals | Sunda pangolin | *Manis javanica* | CR | Decreasing | Trade | Blood and tissue | Species identification and Geographic location | SNPs; COI, Cyt b |
| Barbanera et al. (2012) | Mammals | Sardinian mouflon | *Ovis orientalis ophion* | NT | Unknown | Poaching | Blood | Individualization | STR; Cyt b |
| Szabolcsi et al. (2008) | Mammals | Hungarian Red Deer | *Cervus elaphus hippelaphus* | LC | Increasing | Poaching | Blood, hair, antler and bone | Individualization and Species identification | STR |
| Khedkar et al. (2016) | Mammals | Leo (Lion) | *Panthera leo* | VU | Decreasing | Trade | Claws | Species identification | COI |
| | Mammals | Panthera | *Panthera pardus* | VU | Decreasing | Trade | Claws | Species identification | COI |
| | Mammals | Felidae | Unidentified | WI | WI | Trade | Claws | Species identification | COI |
| | Mammals | Felidae | Unidentified | WI | WI | Trade | Claws | Species identification | COI |
| Dalton and Kotze (2011) | Mammals | Domestic Cattle | *Bos taurus* | A | WI | Poaching | Meat | Species identification | COI |
| | Mammals | Reedbuck (Southern Reedbuck) | *Redunca arundinum* | LC | Stable | Poaching | Carcasses | Species identification | COI |
| | Mammals | Common duiker | *Sylvicapra grimmia* | LC | Decreasing | Poaching | Biltong (dried meat) | Species identification | COI |
| Lorenzini (2005) | Mammals | Wild Boar | *Sus scrofa* | LC | Unknown | Poaching | Meat and blood | Individualization and species identification | D-loop, Cyt b |
| Janjua et al. (2017) | Mammals | Eurasian otter | *Lutra sumatrana* | EN | Decreasing | Trade | WI | Species identification | COI |
| | Mammals | Black bear | *Ursus thibetanus* | VU | Decreasing | Trade | Coat | Species identification | COI |
| | Mammals | Fox | *Vulpes sp* | WI | WI | Trade | Muffler | Species identification | COI |
| | Mammals | Red fox | *Vulpes vulpes* | LC | Stable | Trade | Muffler | Species identification | COI |
| Barrientos et al. (2016) | Mammals | Dog | *Canis lupus familiaris* | WI | WI | Other | Feces | Individualization | WI |

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|---|---|---|---|---|---|---|---|---|---|
| Bollongino et al. (2003) | Mammals | African elephant | *Loxodonta africana* | VU | Increasing | Trade and poaching | Statuettes handcrafted | Species identification | Cyt b |
| | Mammals | Indian rhinoceros (Greater One-horned Rhino) | *Rhinoceros unicornis* | VU | Increasing | Trade and poaching | Statuettes handcrafted | Species identification | Cyt b |
| Gupta et al. (2011) | Mammals | Tigers | *Panthera tigris* | EN | Decreasing | Poaching | Carcass, claw and skin | Individualization and Parental identification | WI |
| Miller et al. (2014) | Mammals | African Lions | *Panthera leo* | VU | Decreasing | Poaching | Blood, skin and hair | Species identification, individualization and sex identification | STR |
| Hagenlund et al. (2015) | Fishes | European smelt | *Osmerus eperlanus L.* | LC | Unknown | Destruction of Wildlife | WI | Identification of geographic origin | STR |
| Stein et al. (2016) | Fishes | Eel | *Anguilla anguilla* | CR | Decreasing | Trade | Muscle | Species identification | COI |
| Mwale et al. (2017) | Mammals | Black-bellied Pangolin | *Phataginus tetradactyla* | VU | Decreasing | Trade | Bags | Species identification | D-loop, Cyt b, COI |
| | Mammals | Giant Ground Pangolin | *Phataginus gigantea* (*Smutsia gigantea*) | EN | Decreasing | Trade | Bags | Species identification | D-loop, Cyt b, COI |
| | Mammals | Pangolin | *Manis spp* | WI | WI | Trade | Bags | Species identification | D-loop, Cyt b, COI |
| | Mammals | Sunda Pangolin | *Phataginus javanica* (*Manis javanica*) | CR | Decreasing | Trade | Bags | Species identification | D-loop, Cyt b, COI |
| | Mammals | White-bellied Pangolin | *Phataginus tricuspis* | EN | Decreasing | Trade | Bags | Species identification | D-loop, Cyt b, COI |
| Caniglia et al. (2010) | Mammals | Italian wolf | Unidentified | WI | WI | Poaching | Tissue and teeth | Individualization and Sex identification | mtDNA, STR |
| Glen et al. (2010) | Mammals | Chuditch (Western Quoll) | *Dasyurus geoffroii* | NT | Stable | Destruction of Wildlife | Tissue and saliva | Species identification | STR |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Fain et al. (2013) | Fishes | Russian Sturgeon | *Acipenser gueldenstaedtii* | CR | Decreasing | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | Chinese Sturgeon | *Acipenser sinensis* | CR | Decreasing | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | Stellate Sturgeon | *Acipenser stellatus* | CR | Decreasing | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | White Sturgeon | *Acipenser transmontanus* | LC | Stable | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | Kalunga | *Huso dauricus* | CR | Decreasing | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | Beluga | *Huso huso* | CR | Decreasing | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | Paddlefish | *Polyodon spathula* | VU | Unknown | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| | Fishes | Shovelnose Sturgeon | *Scaphirhynchus platorynchus* | VU | Decreasing | Trade | Muscle, fin, barbel, blood and eggs | Identification of geographic origin | Cyt b |
| Ogden et al. (2012) | Mammals | Domestic dogs | *Canis lupus familiaris* | A | WI | Without information | Blood | Individualization and Sex identification | WI |

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|---|---|---|---|---|---|---|---|---|---|
| Dawnay et al. (2009) | Birds | Asian Saker Falcon | *Falco cherrug* | EN | Decreasing | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Golden eagle | *Aquila chrysaetos* | LC | Stable | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Gyrfalcon | *Falco rusticolus* | LC | Stable | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Merlin | *Falco columbarius* | LC | Stable | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Northern Goshawk | *Accipiter gentilis* | LC | Unknown | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Peregrine Falcon | *Falco peregrinus* | LC | Stable | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Saker Falcon | *Falco cherrug* | VU | Increasing | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |
| | Birds | Swedish Gyrfalcon | *Falco rusticolus* | LC | Stable | Trade | Blood, tissue, buccal swab and feather | Individualization and Parental identification | STR |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Angom et al. (2015) | Mammals | Hog deer | *Axis porcinus* | EN | Decreasing | Hunting | Tissue | Individualization | mtDNA |
| Boyd et al. (2001) | Mammals | North American Coyotes | Unidentified | WI | WI | Destruction of Wildlife | Blood and tissue | Species identification | STR |
| | Mammals | Domestic dogs | Unidentified | WI | WI | Destruction of Wildlife | Blood and tissue | Species identification | STR |
| | Mammals | North American wolves | Unidentified | WI | WI | Destruction of Wildlife | Blood and tissue | Species identification | STR |
| | Mammals | Rocky Mountain Gray Wolves | Unidentified | WI | WI | Destruction of Wildlife | Blood and tissue | Species identification | STR |
| Chen et al. (2015a) | Mammals | Goat | *Capra hircus* | A | WI | Trade | WI | Species identification | COI |
| | Mammals | Goitered gazelle | *Gazella subgutturosa* | VU | Decreasing | Trade | WI | Species identification | COI |
| | Mammals | Mongolia gazelle | *Procapra gutturosa* | LC | Stable | Trade | WI | Species identification | COI |
| | Mammals | Przewalski's gazelle | *Procapra przewalskii* | EN | Increasing | Trade | WI | Species identification | COI |
| | Mammals | Saiga antelope | *Saiga tatarica* | CR | Decreasing | Trade | WI | Species identification | COI |
| | Mammals | Tibetan Antelope | *Pantholops hodgsonii* | EN | Increasing | Trade | WI | Species identification | COI |
| | Mammals | Tibetan gazelle | *Procapra picticaudata* | NT | Decreasing | Trade | WI | Species identification | COI |
| Khan et al. (2018) | Mammals | Domestic cow | *Bos taurus* | A | WI | Trade | Tissue, skin and hair | Species identification | COI |
| | Mammals | Chinkara | *Gazella bennettii* | LC | Decreasing | Trade | Tissue, skin and hair | Species identification | COI |
| Gentile et al. (2013) | Reptiles | Galápagos Land Iguana (Common Land Iguana) | *Conolophus subcristatus* | VU | Decreasing | Trade | Blood | Identification of geographic origin | Cyt b, tRNAs for Glutamic acid and Threonine |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Wesselink and Kuiper (2011) | Mammals | Red Fox | *Vulpes vulpes* | LC | Stable | Destruction of Wildlife | Blood and tissue | Parental identification | WI |
| Davoli et al. (2018) | Mammals | Brown bear | *Ursus arctos* | LC | Stable | Without information | Blood | Parental identification and Sex identification | STR |
| Mondol et al. (2014) | Mammals | African Elephant | *Loxodonta africana africana* | VU | Increasing | International traffic and poaching | Carcasses and ivory | Sex Identification | WI |
| | Mammals | African Elephant | *Loxodonta africana cyclotis* | VU | Increasing | International traffic and poaching | Carcasses and ivory | Sex Identification | WI |
| Rendo et al. (2011) | Birds | Egyptian vulture | *Neophron percnopterus* | EN | Decreasing | Destruction of Wildlife | Blood | Individualization and Parental identification | STR |
| | Mammals | Sheep | Unidentified | WI | WI | Destruction of Wildlife | Blood | Parental identification and Individualization | STR |
| | Mammals | Sheep | Unidentified | WI | WI | Destruction of Wildlife | Blood | Parental identification and Individualization | STR |
| Kitpipit et al. (2017) | Mammals | African Elephant | *Loxodonta africana africana* | VU | Increasing | Trade and poaching | Tissue | Individualization and Geographical origin | Cyt b, ND5 |
| | Mammals | African Elephant | *Loxodonta africana cyclotis* | VU | Increasing | Trade and poaching | Tissue | Individualization and Geographical origin | Cyt b, ND5 |
| | Mammals | Asian Elephant | *Elephas maximus* | EN | Decreasing | Trade and poaching | Tissue | Individualization and Geographical origin | Cyt b, ND5 |
| Kundu et al. (2019) | Mammals | Greater Hog Badger | *Arctonyx collaris* | VU | Decreasing | Poaching and hunting | Tissue | Species identification | Cyt b |
| Foran and Ray (2016) | Reptiles | Hawksbill Turtle | *Eretmochelys imbricata* | CR | Decreasing | Trade | Guitar picks and jewelry | Species identification | mtDNA |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Ciavaglia et al. (2015a, b) | Reptiles | Scrub python | *Morelia kinghorni* (*Simalia kinghorni*) | A | WI | Trade | Tissue, feathers, buccal swabs, blood, eggshell fragments | Species identification | Cyt b |
| | Reptiles | Carpet python | *Morelia spilota* | LC | Decreasing | Trade | Tissue, feathers, buccal swabs, blood, eggshell fragments | Species identification | Cyt b |
| | Reptiles | Green Tree Python | *Morelia viridis* | LC | Stable | Trade | Tissue, feathers, buccal swabs, blood, eggshell fragments | Species identification | Cyt b |
| Almeron-Souza et al. (2018) | Fishes | Ray orders Rhinopristiformes, Myliobatiformes, Rajiformes, and Torpediniformes | Unidentified | WI | WI | Poaching | Filets | Species identification | COI |
| | Fishes | Shark orders Carcharhiniformes, Squaliformes, and Squatiniformes | Unidentified | WI | WI | Poaching | Filets | Species identification | COI |
| | Fishes | Swordfish | *Genidens barbus* | A | WI | Poaching | Filets | Species identification | COI |
| | Fishes | Swordfish | *Xiphias gladius* | LC | Decreasing | Poaching | Filets | Species identification | COI |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sharma and Gupta (2018) | Mammals | Leopard | *Panthera pardus* | VU | Decreasing | Destruction of Wildlife | Ashes, clot and blood | Species identification and Sex identification | Cyt b, 12S, ZFX/Y; SRY |
| McEwing et al. (2012) | Mammals | Tigers | *Panthera tigris* | EN | Decreasing | Trade and Poaching | Blood, hair and tissue | Sex identification | ZFX/Y |
| Pitra and Lieckfeldt (1999) | Mammals | Bushbuck | *Tragelaphus scriptus* | LC | Stable | Poaching | Tissue | Individualization | Cyt b |
| Parkanyi et al. (2014) | Mammals | Roe deer | *Capreolus capreolus* | LC | Increasing | Hunting | Hair | Species identification | D-loop |
| | Mammals | Red deer | *Cervus elaphus* | LC | Increasing | Hunting | Hair | Species identification | D-loop |
| | Mammals | Fallow deer | *Dama dama* | LC | Unknown | Hunting | Hair | Species identification | D-loop |
| | Mammals | Mouflon | *Ovis aries musimon* (*Ovis gmelini*) | NT | Unknown | Hunting | Hair | Species identification | D-loop |
| | Mammals | Wild boar | *Sus scrofa* | LC | Unknown | Hunting | Hair | Species identification | D-loop |
| Bielikova et al. (2010) | Birds | Golden eagle | *Aquila chrysaetos* | LC | Stable | Destruction of Wildlife | Blood | Parental identification | STR |
| Dubey et al. (2009) | Reptiles | Indian Cobra | *Naja naja* | A | WI | Poaching | Blood | Species identification | 16s rRNA |
| | Reptiles | Rat snake | *Ptyas mucosa* | A | WI | Poaching | Blood | Species identification | 16s rRNA |
| | Reptiles | Indian Rock Python (Burmese Python) | *Python molurus* (*Python bivittatus*) | VU | Decreasing | Poaching | Blood | Species identification | 16s rRNA |
| Vipin et al. (2016) | Mammals | Leopard | *Panthera pardus* | VU | Decreasing | Trade | Claws | Species identification | Cyt b, 16s rRNA |
| | Mammals | Tiger | *Panthera tigris* | EN | Decreasing | Trade | Claws | Species identification | Cyt b, 16s rRNA |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Fumagalli et al. (2009) | Mammals | Cattle | Unidentified | WI | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Cow | *Bos taurus* | A | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Dog | *Canis familiaris* | WI | WI | Poaching and destruction of Wildlife | Swabs | Individualization | mtDNA |
| | Mammals | Donkeys | Unidentified | WI | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Goats | Unidentified | WI | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Grey Wolf | *Canis lupus* | LC | Stable | Poaching and destruction of Wildlife | Swabs | Individualization and Species identification | mtDNA |
| | Mammals | Horses | Unidentified | WI | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Birds | Pelican | *Pelecanus sp* | WI | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Pig | *Sus scrofa* | LC | Unknown | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Red Fox | *Vulpes vulpes* | LC | Stable | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Roe Deer | *Capreolus capreolus* | LC | Increasing | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| | Mammals | Sheep | Unidentified | WI | WI | Poaching and destruction of Wildlife | Swabs | Species identification | mtDNA |
| van Hoppe et al. (2016) | Birds | Hen harrier | *Circus cyaneus* | LC | Decreasing | Poaching | Tissue, feathers, buccal swabs, blood, eggshell fragments | Individualization | WI |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pereira et al. (2019) | Mammals | Unidentified | Unidentified | WI | WI | Poaching | Blood, tissue, buccal swab and feather | Species identification | SPInDel |
| Hogg et al. (2018) | Reptiles | Broad-headed Snake | *Hoplocephalus bungaroides* | VU | Unknown | Trade | Tissue | Individualization | STR, ND4 |
| Chen et al. (2015b) | Mammals | Greater Hog Badger | *Arctonyx collaris* | VU | Decreasing | Poaching and trade | Carcasses | Identification of geographic origin | WI |
| Glover et al. (2012) | Mammals | Common Minke Whale | *Balaenoptera acutorostrata acutorostrata* | LC | Unknown | Trade and harvesting | Meat and blood | Species identification, Sex Identification and Individualization | STR |
| Ramon-Laca and Gleeson (2014) | Mammals | Common Brushtail Possum | *Trichosurus vulpecula* | LC | Decreasing | Destruction of Wildlife | Fur, scat and tissue | Species identification and Sex identification | STR; Cyt b, SRY fragment, GnRH receptor fragment |
| Caniglia et al. (2013) | Mammals | Wolf | Unidentified | WI | WI | Poaching and destruction of Wildlife | Saliva | Species identification, Sex Identification and Individualization | STR |
| Mucci et al. (2014) | Reptiles | Greek tortoises | *Testudo graeca* | VU | WI | Trade | Buccal swabs | Parental identification | NA |
| | Reptiles | Hermann's Tortoise | *Testudo hermanni* | NT | Decreasing | Trade | Buccal swabs | Parental identification | NA |
| | Reptiles | Marginated Tortoise | *Testudo marginata* | LC | Stable | Trade | Buccal swabs | Parental identification | NA |
| Sanches et al. (2011) | Mammals | Lowland tapir | *Tapirus terrestris* | VU | Decreasing | Poaching | Meat | Individualization and species identification | Cyt b |

(continued)

**Table 14.1** (continued)

| Reference | Taxonomic Group | Species Common name | Species Scientific name | IUCN Status | Population Trend | Type of Crime analyzed | Biological evidence | AIM OF STUDY | ANALYSED REGION |
|---|---|---|---|---|---|---|---|---|---|
| Jun et al. (2011) | Mammals | Felidae | Unidentified | WI | WI | Trade and poaching | Leather, hairs, skin and claws | Species identification | COI |
| | Mammals | Wild Korean Asiatic Black Bear | *Ursus thibetanus ussuricus* | VU | Decreasing | Trade and poaching | Leather, hairs, skin and claws | Species identification and Identification of geographic origin | NADH subunits 1, 2, 5 and 6, COI, Cyt b |
| Lorenzini et al. (2011) | Mammals | Mouflon | *Ovis aries musimon* (*Ovis gmelini*) | NT | Unknown | Poaching | Carcass and blood | Species identification | WI |

*CR* CritIcally Endangered, *EN* Endangered, *VU* Vulnerable, *NT* Near Threatened, *LC* Least Concern, *DD* Data Deficient, *WI* Without information, *A* Absent or Not Evaluated by the IUCN Red List. Scientific names include the original name of the article and in parenthesis the name used by the IUCN, when taxonomy has changed. *COI* Cytochrome Oxidase unit 1 mitochondrial gene, *Cyt b* Cytochrome b mitochondrial gene, 12S rRNA mitochondrial gene, 16S rRNA ribosomal gene, *mtDNA* mitochondrial DNA, *STR* Short tandem repeats, *D-loop* mitochondrial DNA, *ZFX/Y* Zinc Finger Protein X or Y linked, protein coding gene, *SRY* sex determining region of the Y gene, *SPInDel* Single polymorphism insertion/deletion, *NADH* reduced nicotinamide adenine dinucleotide coenzyme mitochondrial complex I, *ND4* and *ND5* Mitochondrially Encoded NADH: Ubiquinone Oxidoreductase Core Subunit 4 or 5, protein coding gene, *GnRH* Gonadotropin-Releasing Hormone, *NA* Not apply because the article does not provide the information

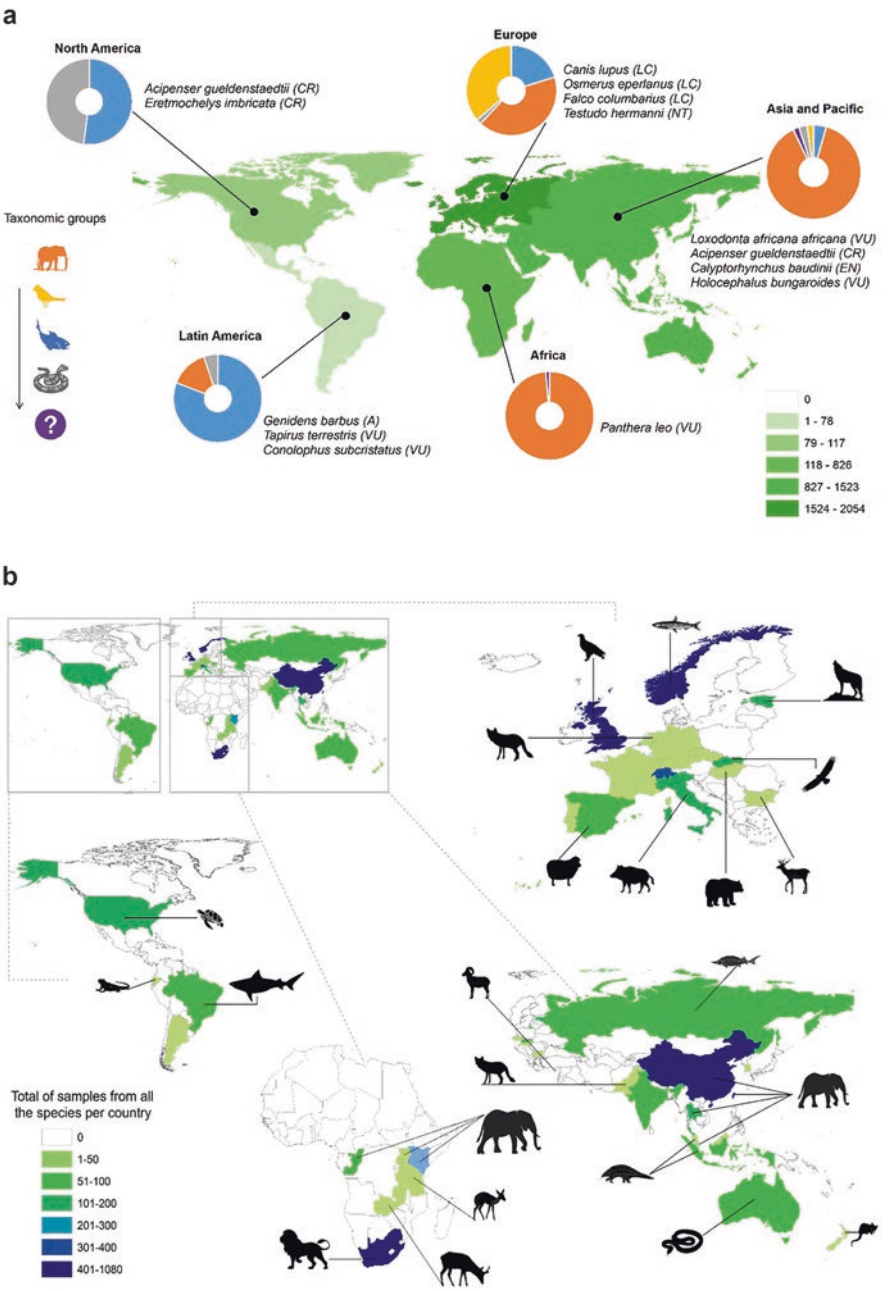


**Fig. 14.2** (**a**) Geographical heat map representing the number of samples analyzed by wildlife forensic genetics (green). Donuts represent the proportion of seized samples analyzed in forensic genetics. Colors represent taxonomic groups. The most frequent species and their conservation status are indicated. *CR* Critically Endangered, *EN* Endangered, *VU* Vulnerable, *NT* Near Threatened, *LC* Least Concern, *NE* Not evaluated. (**b**) Geographical heat map representing the number of samples analyzed by wildlife forensic genetics, shown by country and the most frequent taxa

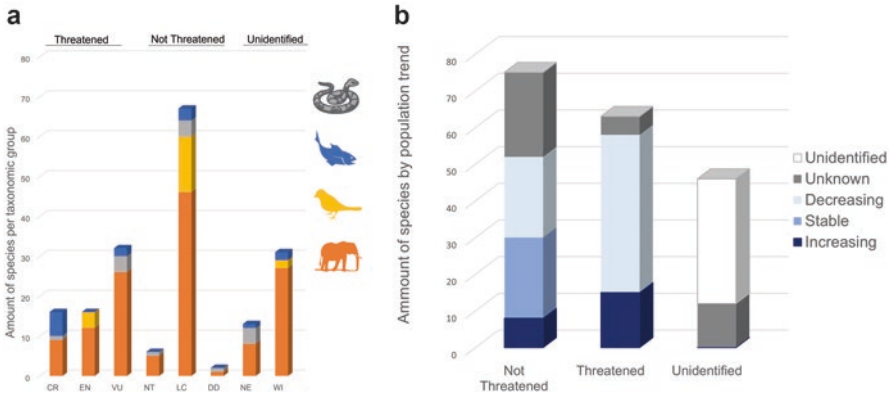


**Fig. 14.3** (**a**) Species affected by taxonomic groups, classified according to the UICN Red List categories. (**b**) Total number of species according to the population trend, grouping by categories of threat. Acronyms meaning: *CR* Critically Endangered, *EN* Endangered, *VU* Vulnerable, *NT* Near Threatened, *LC* Least Concern, *DD* Data Deficient, *NE* Not evaluated, *WI* Without information, the paper used a general name and was not possible to assign a scientific name

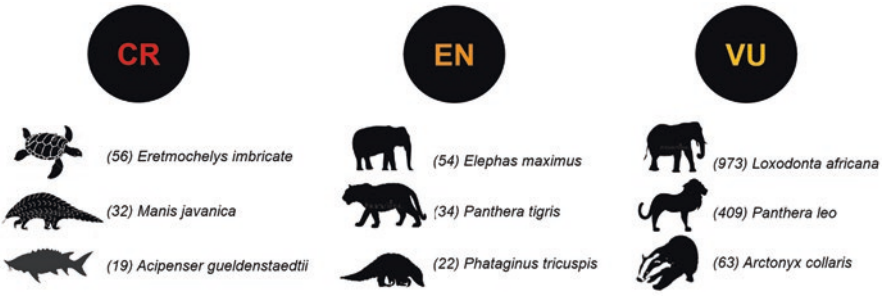


**Fig. 14.4** Threatened species (UICN 2020) more represented in the seized samples analyzed in WFG. In parentheses, the total number of samples of each species. *CR* Critically Endangered, *EN* Endangered, *VU* Vulnerable

Within reptilians, the third group with more cited species among the wildlife crimes, hawksbill turtle (*Eretmochelys imbricate*) was the most represented among the Cryptically Endangered species (Fig. 14.4). Four other species, Galápagos land iguana (*Conolophus subcristatus*), broad-headed snake (*Hoplocephalus bungaroides*), Indian rock python or Burmese python (*Python bivittatus*), and Greek tortoises (*Testudo graeca*), are categorized as Vulnerable (Table 14.1). Four of the six species categorized as not threatened are either decreasing population size or having a population tendency unknown.

## 14.4 Typologies of the Crimes

We identified five different typologies of wildlife crimes in our WFG dataset (Fig. 14.5), where a total of 20 distinct types of biological samples were reported (Table 14.1). Poaching crime includes the animals illegally killed by hunting or through other methods, and this offense was the most represented category among the seized samples (2162 samples), affecting almost all evaluated taxonomic groups. Among the other crime typologies, mammals were the group with more representativeness (Fig. 14.5).

The national and international illegal commerce of parts and products derived from animals extracted from their natural environment or raised under controlled conditions, typified as trade crime, represents the second most common crime (Fig. 14.5). This offense was mainly represented by tissues transformed or processed for consumption and commerce, such as filets, leather, bags, clothes (mufflers and coats), jewelry, guitar picks, and Chinese traditional medicine products. Contrastingly, animal carcasses, fecal vestiges, eggs, spittle, teeth, and other body parts of the animals used in ornaments (tusks and claws), and statues (ivory) were reported in minor quantities and mainly in the illegal international traffic crime using ships, trains, roads or aircraft.

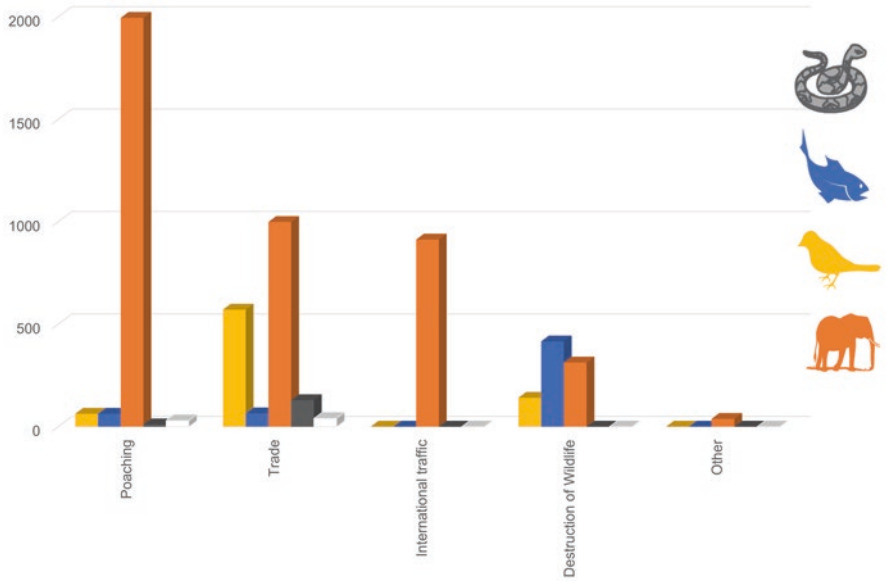


**Fig. 14.5** Typology of crimes and number of seized samples of different taxonomic groups. White columns indicate the samples with an unidentified taxonomic group

### *14.4.1 Examples in Costa Rica*

Wildlife crimes in Costa Rica are growing at a high speed. The Costarrican National Report reported 2576 seizures of wildlife species recorded in 2018, and 59.5% of the total reported between 2014 and 2018 period where birds and fish were the most affected groups (Programa Estado de la Nación and Consejo Nacional de Rectores 2020). Additionally, between 2012 and 2017, inside the judicial field the public Ministry and the Organismo de Investigación Judicial (OIJ) received 9940 complaints related with infractions to special laws that protect flora and fauna in the country (Programa Estado de la Nación and Consejo Nacional de Rectores 2020).

The Costarrican Forensic Department has more judicial requirements for wildlife identification inside the context of illegal traffic of species than in other areas. In 2020, Costa Rica had a significant increase in the number of cases, especially in the number of specimen samples per year sent for identification. The analyses identified mainly hawksbill sea turtles (*Eretmochelys imbricate*), bone remains and skins of mammals, feathers, sea cucumbers, insects, swim bladders and fishes.

The hawksbill sea turtle (*E. imbricate*), classified as critically endangered by CITES Appendix and IUCN Categories (IUCN 2019), is used in Costa Rica to transform the shell tissues on different products as jewelry, souvenirs and spurs for cockfights, which represent evidence of wildlife crime (Fig. 14.6).

Frogs (Fig. 14.7), swim bladders and dehydrated sea cucumbers (*Holothuroidea class*), the two latter related with Chinese medicine, are common evidence in Costa Rica wildlife crimes. The dehydrated sea cucumbers are one of the most confiscated


**Fig. 14.6** Spurs seized made it with the shell scales of the hawksbill turtle (*Eretmochelys imbricata*), these pieces are used in cockfights, an activity that is also prohibited by Costa Rican regulations. (This picture was taken by experts of the Biology section of the Department of Forensic Science, OIJ, Costa Rica)


**Fig. 14.7** Samples of a confiscation of specimens alive. They were recovered from a site dedicated to the illegal breeding and illegal commercialization of frogs and toads (Order Anura). The experts identify the species *Smilisca phaeota* (LC), *Espadarama prosoblepon* and *Aglychnis sp*, the two last are absent from the IUCN Red list. (This picture was taken by experts of the Biology section of the Department of Forensic Science, OIJ, Costa Rica)

pieces of evidence, not only in the number of cases but also in the number of samples per seizure (more than 600 samples in a single case).

The huge number of seizures related to the Insecta class is also of great concern in Costa Rica. Insecta actually has 1217 species registered at IUCN Red List (IUCN 2019), however, if seizures continue on this path, it is very likely that more species of insects will be included in these lists in a few years. Like swim bladders and sea cucumbers, insect seizures are characterized by a large number of samples per seizure that in many cases exceed 500 specimens per seizure (Fig. 14.8).

In these cases, it is common to find complete insects (Fig. 14.9) and mainly groups of insects, usually ensembled for souvenirs as paintings, jewelry boxes, earrings and keychains made of butterfly wings (Fig. 14.10a, b). Ants, beetles and butterflies, and spiders were among the most seized groups at international departure ports (Judicial Investigation Agency of Costa Rica, unpubl. data).

**Fig. 14.8** Example of the volume of specimens seized in a case of organized crime, specifically in an illegal wildlife traffic crime. The Biology section analyzes a total of 2420 specimens of the Coleoptera order in just one seizure. (This picture was taken by experts of the Biology section of the Department of Forensic Science, OIJ, Costa Rica)

**Fig. 14.9** Adult male of the Coleopteran order known as "Cornizuelo" (*Megasoma elephas*) seized by the Costa Rican police in a case of illegal wildlife traffic. (This picture was taken by experts of the Biology section of the Department of Forensic Science, OIJ, Costa Rica)

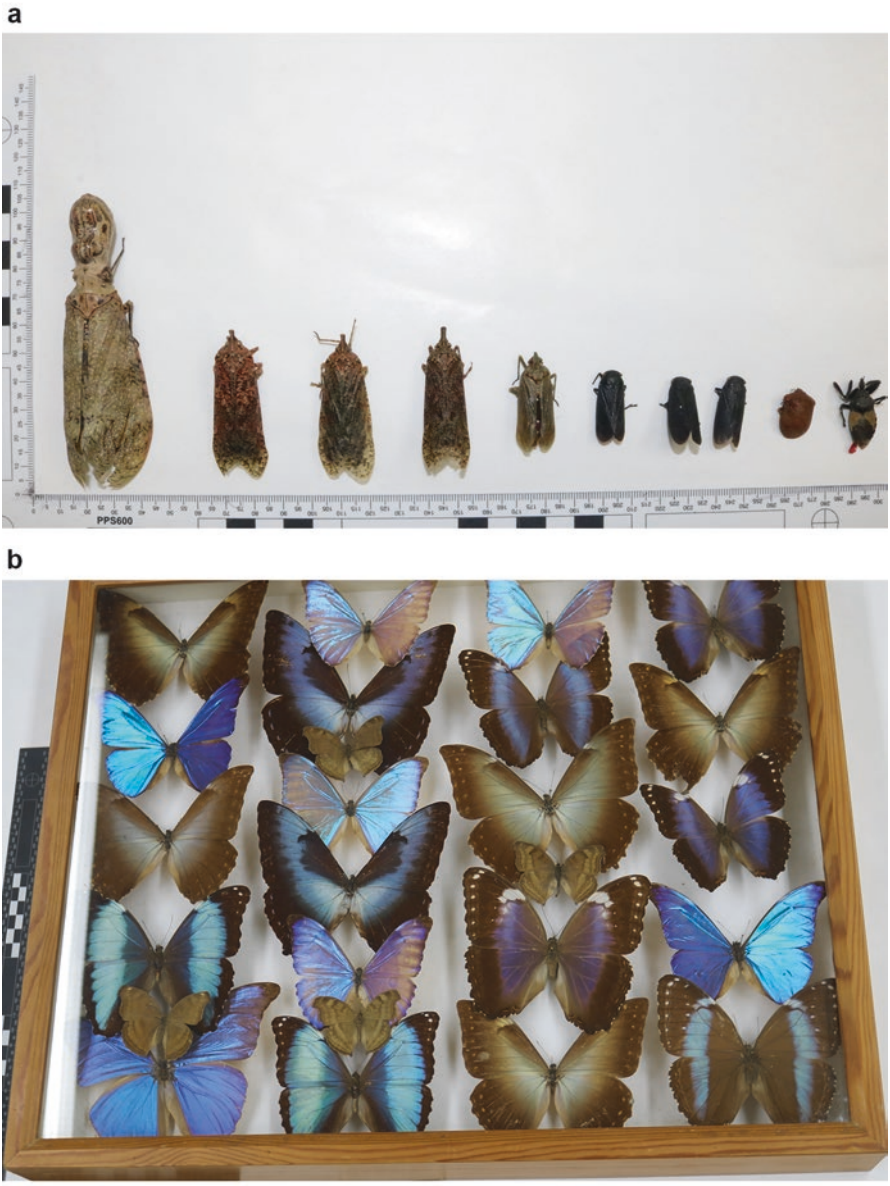


**Fig. 14.10** (**a**) Specimens seized in Costa Rica in a case of illegal wildlife trafficking. Experts identify specimens of the orders Lepidoptera (butterflies and moths), Coleoptera (beetles) and Hemiptera (bugs, cicadas and cicadas). (**b**) Crafts marketed as souvenirs in tourist areas of Costa Rica, confiscated for being made with wild species, that according to the country's law, is a trade and traffic fauna wildlife crime. (These pictures were taken by experts of the Biology section of the Department of Forensic Science, OIJ, Costa Rica)

## 14.5 Molecular Techniques Employed to Solve the Crimes

Protocols based in commercial kits (39) for DNA extraction were frequently used for assessing genetic material from the samples, followed by organic extraction (13) and other DNA extraction methods (11). Different approaches for DNA analyses were used for different goals (Fig. 14.11). In general, DNA sequencing of mitochondrial genes were used for animal species identification (Akasaki et al. 2006; Cao et al. 2014; Ciavaglia et al. 2015a; Dalton and Kotze 2011; Fumagalli et al. 2009; Lorenzini 2005; Lorenzini et al. 2011; Morf et al. 2013). Partial regions of at least six different mitochondrial regions [Cytochrome Oxidase I (COI), 12S and 16S RNA, Cytochrome *b* (Cyt *b*), Nicotinamide Adenine Dinucleotide reduced (NADH) and the control region (D-loop)] have been used for analyzing poaching and animal illegal trade. Some cases used microsatellite markers for species identification (Caniglia et al. 2013; Glen et al. 2010; Glover et al. 2012; Hadas et al. 2016; Lorenzini et al. 2011; McEwing et al. 2012; Miller et al. 2014; Plumer et al. 2018; Ramon-Laca and Gleeson 2014; Szabolcsi et al. 2014).

Sample individualization was mostly used in study cases associated with poaching and hunting (Angom et al. 2015; Barbanera et al. 2012; Caniglia et al. 2010; Fumagalli et al. 2009; Gupta et al. 2011; Hadas et al. 2016; Hogg et al. 2018; Kitpipit et al. 2017; Pitra and Lieckfeldt 1999) while parental identification was used for differentiation between captive-bred and wild-caught individuals to investigate illegal rearing and trade of species with commercial value (Bielikova et al. 2010; Davoli et al. 2018; Dawnay et al. 2009; Gupta et al. 2011; Mucci et al. 2014; Rendo et al. 2011; Wesselink and Kuiper 2011; White et al. 2012). The study cases related to parental identification of animal taxa often are followed by molecular sex identification (Caniglia et al. 2010, 2013; Davoli et al. 2018; Glover et al. 2012;

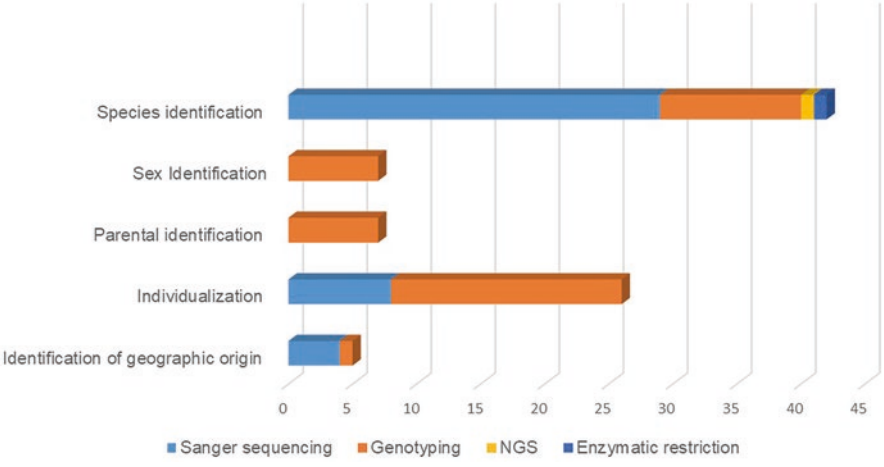


**Fig. 14.11** Issues and methodological approaches used in wildlife forensic genetics

Miller et al. 2014; Mondol et al. 2014; Ogden et al. 2012; Ramon-Laca and Gleeson 2014; Sharma and Gupta 2018; Wictum et al. 2013) (Fig. 14.11).

## 14.6 A Case Study in the Neotropical Bird *Sicalis flaveola* (Linnaeus, 1766)

Brazil is responsible for about 10–15% of the global value of wildlife trafficking. The bird group is among the most affected species, being the Psittaciformes and Passeriformes (such as the saffron finch birds) the groups most commonly apprehended by environmental authorities (Ribeiro and Silva 2007). According to the Brazilian Institute of the Environment and Renewable Natural Resources, IBAMA, the saffron finch (*Sicalis flaveola*) is one of the ten most illegally trafficked bird species in the country and a recent study has shown that this is also the most hunted species in the state of São Paulo, despite being reproduced in countless breeders (Azevedo 2018).

The interest of amateur and commercial breeders in breeding Brazilian species in captivity has expanded as well as illegal hunting and illegal trade (to turn these wild birds into domestic birds). Those facts promoted the creation and application of laws and control systems, such as the "Instrução Normativa, IBAMA, N° 169, N° 3, N° 10/2011" and "Resolução Conama, n° 394", where is explained that all specimen information must be uploaded to the computerized system for the management of breeding of birds named System of Control and Monitoring of the Activity of Amateur Breeding of Birds (SISPASS).

SISPASS includes the breeder owner's personal information and the data of each individual bird present in the breeder, which must be registered after 30 days of birth. These data are: species (including subspecies), common name, sex, date of birth, death, escape, band size and code, transfer to other breeding sites, transfer date, identification of parents and the places where the parents can be located. Inconsistencies between the information registered in SISPASS, would be analyzed as evidence to detect possible individuals taken from nature and kept in captivity as pets victims of trafficking and illegal trade.

In this scenery, we analyzed if individuals of *S. flaveola* kept in breeding are really products of captive breeding or they could be the result of illegal traffic. We used mitochondrial and nuclear molecular markers as tools for identifying the species in captivity and to confirm the parental relationship previously registered in SISPASS.

We analyzed blood samples, including samples from six bird families registered in SISPASS. The samples were carefully collected and preserved as evidence maintaining the properly chain-of-custody to ensure traceability of the evidence from the crime scene to the courtroom (Fig. 14.12).

We used a panel of six microsatellite loci (Corrêa 2009; Ferreira et al. 2015; Medolago et al. 2018) for genotyping the samples and we calculated the maximum


**Fig. 14.12** Ulnar blood birds sampling. Personal photograph collection. (**a**) Materials sterilized for sampling (gloves, masks, headdress, information sheet, FTA paper, nail cutter, ziplock bags, 70% alcohol, DNA Away), (**b**) Canaries caught by the Environmental Military Police, (**c**) Revision of the rings and comparison between the IBAMA number and SISPASS register, (**d**) Bird's leg sterilization with cotton and 70% alcohol (**e**) Cut of the nail in the nail vein, (**f**) Blood collection on FTA paper

exclusion probability when both parents are well known (P1) and the exclusion probability when one of the parents is unknown (P2) using the GenAlex software (Peakall and Smouse 2005). We also calculated the probability of non-exclusion or the probability of identifying a parent when one of the parents is known (NE-1P), when the other is unknown (NE-2P) and the probability of identification of the father and mother together when both are not known (NE-PP) using the Cervus software (Kalinowski et al. 2007).

Our microsatellite panel produced a probability of exclusion higher than the probability of non-exclusion or identification. The average between loci for exclusion P1 was 68% and 53% for P2. On the other hand, the probability of non-exclusion in NE-P1 was 10%, 1.68% for NE-P2, and for NE-PP it was not possible to identify both parents simultaneously with our microsatellite panel (Table 14.2).

Overall, for some families, the molecular results confirmed the information declared in SISPASS, and offspring analyzed were related with the declared parental individuals (sire and dam). In contrast, some declared families showed incongruences between molecular results and the SISPASS information.

In a family analyzed, seven captive individuals of *S. flaveola* registered in SISPASS (29, 30, 31, 32, 36, 35, 40) were molecularly analyzed (Fig. 14.13). Five individuals were registered in SISPASS as belonging to a family (Fig. 14.13a) and two others were registered in SISPASS as not related with the family but with reproductive capacity and possibility of being parents (Fig. 14.13b). A microsatellite panel was used for kinship molecular analyses among the seven individuals. The

**Table 14.2** Maximum probabilities of exclusion and probability of non-exclusion per microsatellite locus, considering blood samples of 45 captive individuals of *Sicalis flaveola*

| | Probability of exclusion | | | Probability of non-exclusion | | |
|---|---|---|---|---|---|---|
| Loci | N | P1 | P2 | NE-1P | NE-2P | NE-PP |
| NF0102 | 45 | 0.795 | 0.657 | 0.546 | 0.371 | 0.190 |
| NF910 | 45 | 0.595 | 0.416 | 0.952 | 0.843 | 0.731 |
| NF2728 | 45 | 0.660 | 0.486 | 0.907 | 0.754 | 0.587 |
| SF01N | 45 | 0.370 | 0.222 | 0.947 | 0.832 | 0.714 |
| NF1516 | 45 | 0.772 | 0.626 | 0.675 | 0.489 | 0.282 |
| Sma29 | 45 | 0.795 | 0.657 | 0.601 | 0.424 | 0.237 |
| PD03 | 45 | 0.743 | 0.588 | 0.594 | 0.415 | 0.228 |
| Mean | 45 | 0.680 | 0.520 | 0.1075 | 0.0168 | 0.0008 |

Number of individuals (N), maximum exclusion probability when both parents are well known (P1) and the exclusion probability when one of the parents is unknown (P2). The probability of non-exclusion or the probability of identifying a parent when one of the parents is known (NE-1P), when the other is unknown (NE-2P) and the probability of identification of both sire and dam together when both are not known (NE-PP)

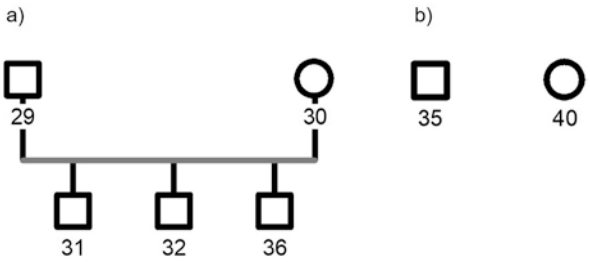


**Fig. 14.13** Heredogram of the samples collected inside the first breeding. (**a**) Birds registered in SISPASS as family. (**b**) Samples of the same breeder included in kinship relation test

results showed a mean value for NE-1P equal to 0.345, 0.117 for NE-2P and 0.0275 for NE-PP. The paternity and maternity molecular analyses identified the best candidates of sire (29) and dam (30). The offspring identification (31, 32 and 36) was obtained with a relaxed confidence level considering paired and screened comparisons. The same parents were identified with a confidence level of less than or equal to 80%. In this case, the molecular test was congruent with the information registered in SISPASS (Fig. 14.14).

In another breeding of *S. flaveola* registered in SISPASS, nine individuals (93–101) were distributed in two families (Fig. 14.15), five (93–97) registered in a family (Fig. 14.15a) and four (98–101) individuals in the second family (Fig. 14.15b). We calculate the maternity and paternity probabilities from the parents of both families related to the sons of the first family. The kinship molecular analyses revealed that the sire 93 is related with offspring 97 (Fig. 14.16a) but there is no relationship with offsprings 95 and 96, these latter were highly related with the sire 98 (Fig. 14.16b), while dam 94 was highly related with the three offspring (95, 96, 97).

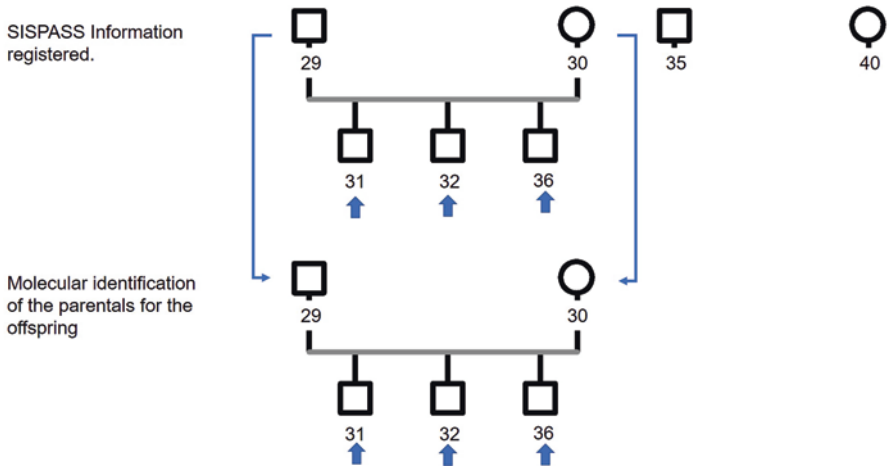


**Fig. 14.14** Comparative pedigrees between parents of the data register at SISPASS and the molecular results

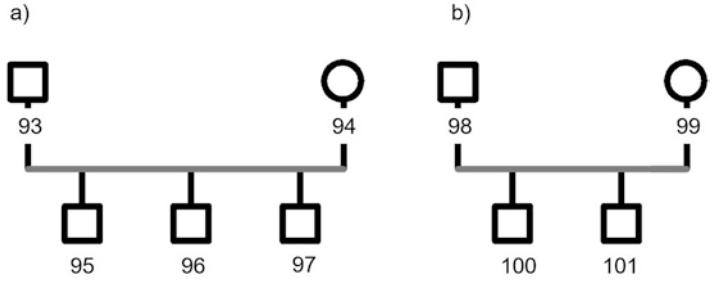


**Fig. 14.15** Pedigrees of the samples collected inside the second breeding. (**a**) Birds registered in SISPASS as family 1. (**b**) Birds registered in SISPASS as family 2

The probabilities of identification in this family have a confidence level equal to or less than 80%.

It is suggested that this breeding has irregularities between molecular analyses and the information registered in SISPASS, which can indicate the presence of wild animals of *S. flaveola* among the captive ones or it represents errors in the SISPASSS registers. However, this conclusion must be seen with caution. The microsatellite panel used can not be able to discriminate parentals with accuracy in breeding if inbreeding occurs. Thus, it is strongly recommended to complement this molecular evidence with a larger number of microsatellites, since in the legal and administrative process the average probability of identification more accepted is at least 90%. This result reinforces that more studies are required to develop more informative molecular markers to support the analyses in WFG investigations.

In a third breeding of *S. flaveola*, seven individuals (70–76) were analyzed (Fig. 14.17). While the individual 75 was registered as sire of all offsprings in

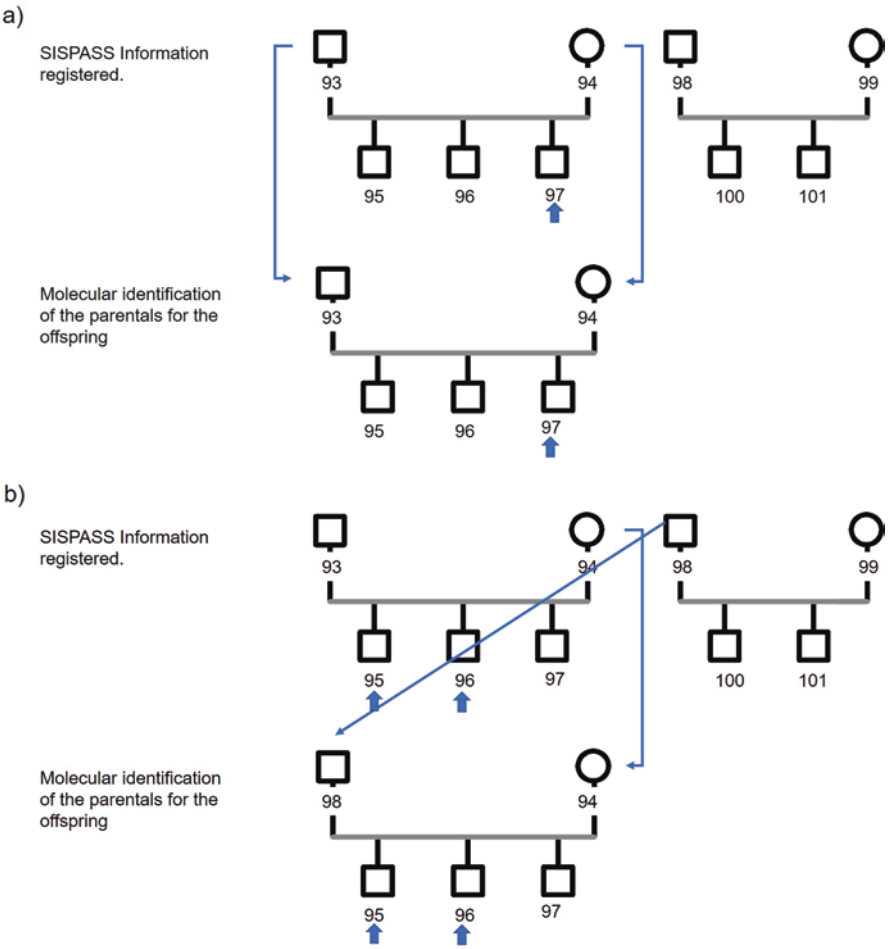


**Fig. 14.16** Comparative pedigrees between parents of the data register at SISPASS and the molecular results. (**a**) Diagram confirmed for the offspring 97, that molecular identification matched with the parents registered in SISPASS. (**b**) Diagram showed that for the offspring 95 and 96 the molecular identification of the sire did not match with the SISPASS information registered

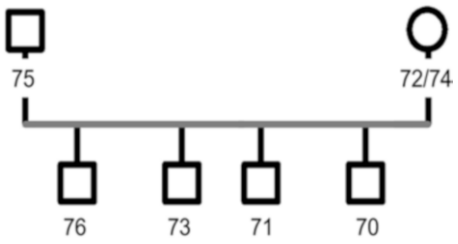


**Fig. 14.17** Pedigree of the samples collected inside the first breeding from birds registered in SISPASS as family

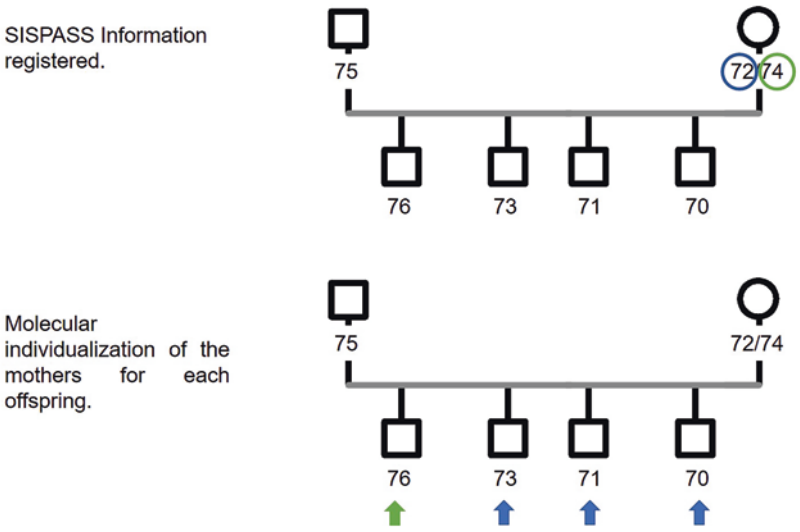


**Fig. 14.18** Comparative pedigrees between parents of the data register at SISPASS and the molecular results. The color of each circle identifies it with its respective progeny

SISPASS, two were indicated as putative dams (72, 74). The maternity analyses suggest that dam 72 is the most probable mother of individuals 70, 71 and 73, while dam 74 is possible mother of individual 76 (Fig. 14.18). The results do not differ from the SISPASS records, but they discriminate more assertively which mother is the most likely for each pup. All identifications were obtained with a confidence level lower than 80% using the paired and screened calculation except the identified maternity of dam 72 with individual 70, with a confidence degree greater than 80% but less than 95%.

## 14.7 Lessons for Conservation

Indeed, there is a quite reduced number of publications in wildlife forensics compared with the potential wildlife forensic work carried out around the world, the seizures of poaching and trade reports (Andimile and Floros 2021; Kitade et al. 2021; Sadé Moneron and Drinkwater 2021; Narang and Watson 2021; Prinsloo et al. 2020) and the international traffic reports (Charity and Ferreira 2020; UNODC 2016, 2020). Several aspects may be contributing to this apparent incipient contribution of these reports for biodiversity conservation. Most cases of forensic analyses are restricted to private or governmental laboratories which have a main purpose of producing molecular evidence against wildlife crimes without the requirement of publications in indexed journals. In addition, forensic genetic experts are focused on the analysis of the crime evidence for court or administrative process, the evidence

can be not exposed, and has a strict chain of custody. Lawsuits can take years limiting publication of these results. Forensic disciplines have the mandatory requirement of use methods previously validated with rigorous standards; this fact would reduce the publications on the innovation areas. Finally, the forensic science process is highly structured and time-consuming that discourages voluntary publication, which is not usually such a fundamental requirement as in the academy or research.

Despite WFG has been worldwide used, its use is geographically unbalanced, suggesting that either the WFG conservation value is not equally recognized among countries or the use of genetic tools is still limited (high laboratory costs, lack of personal specialists) or motivation for publishing study cases in peer reviewed journals is biased. Europe and Asia concentrate the largest amount of WFG reports. Europe appears to be a major destination market and a hub for trafficking in transit to other regions (European Commission 2016; INTERPOOL 2018). In turn, Asia acts as a supplier for the international wildlife illegal trade, including food, pet trade, traditional medicine, and trophies (Petrossian et al. 2010), as well as destination of exotic products, as the deliberate killing of jaguars for their parts (fangs, skulls, bones, skins, paws, meat) in Brazil, destined for the China and southeast Asia markets (Charity and Ferreira 2020). Notwithstanding the Neotropics have few WFG studies, different countries have private and government laboratories dedicated to the production of molecular evidence for the resolution of illegal activities against wildlife.

For instance, the Biology Section of the Department of Forensic Science in Costa Rica is expanding and improving its services, seeking to mitigate the growth rate of environmental crimes especially those related to illegal trafficking. Until now, this institution has been using morphological identification as the standard method for species recognition in biological forensic evidence; however, a new laboratory for molecular wildlife identification was created in 2022 with the aim to improve in a near future the identification of samples in forensic cases. These services against environmental crimes include identification of the presence of wetland or forest in crimes related with the use of land, invasion of protected areas and wetland drainage; identification of zoological or botanical remains in crimes of illegal trafficking of wild flora and fauna; trichology analyses and forensic entomology analyses. It is of note that before offer the service, each laboratory in forensic science must go through a rigorous national and international quality control, which includes an experimentation process, writing of procedures, as well as a complex validation process.

Species identification and sample individualization were the main objectives observed in the WFG case studies recorded, explained by the strong ability of genetic analysis for the identification of samples that have lost identifying morphological characters (Ogden et al. 2009). DNA sequencing was frequently used for species identification and, although the COI mitochondrial gene is universally recognized as a barcode gene among animals (Almeron-Souza et al. 2018; Chang et al. 2014; Chen et al. 2015a; Dalton and Kotze 2011; Janjua et al. 2017; Jun et al. 2011; Khan et al. 2018; Khedkar et al. 2016; Mwale et al. 2017; Nash et al. 2018; Stein et al. 2016), other mitochondrial genes (12S, 16S, Cyt *b*, NADH, D-loop) were also

used in the WFG case studies. Although parental identification is still little investigated among the WFG reports, our case study reinforced its key role to identify potential illegal trade of wild born animals, where trade of captive born animals is allowed.

The use of mitochondrial Cytochrome Oxidase I (COI) gene as DNA barcoding in animals (Hebert et al. 2003a, b), amplify a segment of 500–800 bp and, subsequently, compare the resulting sequences against reference databases as GenBank and BOLD (Barcode of Life Data System) to find the matching species (details in Chap. 2). Nowadays, the concept of barcode is used to obtain taxonomic information about unidentified organisms from at least six different mitochondrial regions (COI, 12S and 16S RNA, Cyt b, NADH and D-loop region) complete or partial segments (Caniglia et al. 2013; Glen et al. 2010; Glover et al. 2012; Hadas et al. 2016; Lorenzini et al. 2011; McEwing et al. 2012; Miller et al. 2014; Plumer et al. 2018; Ramon-Laca and Gleeson 2014; Szabolcsi et al. 2014). In forensic science is even more common the use of mini barcodes (a fragment smaller than 200 bp) than standard barcodes because DNA extracted from forensic samples is commonly degraded or in low quantity.

Microsatellite analysis has been preferentially used for individual identification because it allows to generate unique and individual genotypes (Mitra et al. 2018). These analyses can be useful to monitor the number of animals entering in commercial markets, for instance, even if they are sold as meat or processed products (Panday et al. 2015), and for parental identification, as we showed in the study case of *S. flaveola*.

Mammals were the most affected major taxonomic group present in WFG, represented by the highest number of species and seized samples analyzed. Poaching and illegal trade were associated with the most seized samples of mammals. This fact seems concordant with an unprecedented spike in illegal wildlife trade and poaching in mammals, with a 7.7% increase of rhino poaching in South Africa between 2007 and 2013 (Sade Moneron et al. 2017), the largest seizures of illegal ivory registered in 2011 (with an estimated weight of more than twenty-three metric tons, representing approximately twenty-five hundred elephants) (Global Rights Compliance and WWF-Japan 2019; Scheer et al. 2017), and the poaching of about 3800 elephants and 90 tigers for their skins and bones (World Widelife Fund 2019).

All known elephant species are currently under threat. *Elephas maximus* (Asian elephant) is already Endangered, and both *Loxodonta cyclotis* (African forest elephant) and *Loxodonta africana* (African bush elephant) are categorized as Vulnerable (IUCN 2020). Of note, these both latter species are listed as synonyms species in the Red List (IUCN 2020) although there are evidences that they are two distinct species (Grubb et al. 2000; Murata et al. 2009; Rohland et al. 2010), suggesting that they may be facing different pressures and requiring different conservation strategies. The ivory forensic genetic analyses can have a crucial role in elephant conservation either through the molecular species identification (Santos et al. 2019) or identification of geographical origin (Psonis et al. 2020; Wasser et al. 2004). However, our results showed that most mammal species assessed in the WFG approach were at Least Concern instead of threatened species.

A similar situation was observed among birds which also showed a high number of species and seized samples examined in WFG, probably reflecting the heavy worldwide trade of these animals, smuggled by their colors, for supplying the demand for pets (Raymond et al. 2010). For the three Endangered species appearing in our results (*Calyptorhynchus baudinii, Falco cherrug, Neophron percnopterus*) the use of forensic genetics can give important contributions for conservation. In contrast, for most species which are at Least Concern, there is no evident conservation purpose in using forensic genetics in the reported case studies (van Hoppe et al. 2016; White et al. 2012), although several of these species already show decreasing population.

This scenario is not modified among reptiles and fish. The former animals are mainly poached either for their skins to produce exotic leathers or for meat or for their organs used in the traditional medicine (Almeron-Souza et al. 2018; Foran and Ray 2016; Hogg et al. 2018; Morf et al. 2013). Pythons and small turtles are also illegally traded as pets (UNODC 2020). A small proportion of reptiles is currently cited in the IUCN summary statistics (IUCN 2020), impairing an evaluation on the effective contribution of the recorded forensic genetic study cases to the species conservation. Curiously, fish had the lowest number of species reported in WFG, but the highest number of seized samples analyzed. In only one study case (Hagenlund et al. 2015), 416 samples of a single translocated fish species, *Osmerus eperlanus*, were used to analyze a crime of destruction of wildlife. This crime includes illegal destruction of habitat and illegal translocation or the introduction of exotic species. Approximately 42% of threatened fish species are at risk due to invasive species (IUCN 2019), and the use of genetics for detecting these invasive species can be helpful for conservation of the native species.

## 14.8 Final Considerations

In sum, our analyses showed that wildlife forensic genetics study cases reflect a partial picture of the recorded seizures in the world, suggesting that the use of forensic genetics for solving of wildlife crimes is still worldwide embryonic. However, it is important to point out that this conclusion is limited to only published study cases that were recovered in the literature and potential uses of wildlife forensic genetics that were not reported in the scientific literature may be not considered in the analyses.

Most wildlife forensic genetics study cases involve non-threatened species, suggesting that the conservation status of a given species is not a proxy for these published studies. Nonetheless, our analyses revealed that forensic genetics constitutes a powerful tool for wildlife crimes investigation, allowing us to obtain precise information on species identification, sample individualization, sex, paternity and geographic source identification. We suggest that actions on forensic genetics should be worldwide included for improving wildlife seizures regulation and species conservation policies.

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# Chapter 15
# Environmental Ecogenotoxicity and Conservation


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## 15.1 Introduction

Ecotoxicology is a science that integrates several lines of knowledge and studies the environment and its action on the organisms that live in it. From different biological and trophic levels, these organisms can be subjected to agents from both natural and anthropogenic sources. Therefore, ecotoxicology is a combination of ecology and toxicology, aiming to understand and predict the effects of contaminants on different organisms and communities subjected to different types of pollutants. In the ecological context, we highlight the interactions between species and the environment, the structuring of communities and the trophic relationships between organisms. In the environmental toxicology context, through the use of test organisms, the most critical approach is to assess the effects of one or more unnatural agents (xenobiotics) by conducting laboratory experiments that most closely approximate natural exposure (bioassays). In this way, ecotoxicology comprises a sequence of studies that characterize this science. First, the objective is to study the entry of xenobiotics into the abiotic environment, and how they are distributed throughout the ecosystem. The second step is to assess how these compounds enter organisms and what the consequences are for communities and populations. Finally, we characterize the xenobiotics qualitatively and quantitatively, evaluating the effects from the sub-individual level to ecosystems, being able to extrapolate these conclusions to the effects on human populations (Truhaut 1977).

Other important milestones within ecotoxicology are the discovery of the endocrine-disrupting activity of several compounds (Colborn et al. 2003) and the application of omics techniques from the 2000s onwards. Although in Brazil, ecotoxicology began to be discussed in the 1980s, the first Brazilian scientific meeting in this study field took place only in 1991. Currently, the Brazilian National

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Standards Organization (ABNT) has defined rules for more than 10 different toxicological tests in aquatic organisms, now established as a quality standard for these environments.

It is impossible to separate human activities from the consequences they bring to the environment. Anywhere in the world, even in the most inhospitable environments, it is possible to observe organisms at all levels of the systematic organization influenced by exposure to xenobiotics (Walker et al. 2014). It is believed that the number of contaminants discharged into environments has increased more than 100 times since the beginning of the twentieth century (Nriagu 1996). This increase can be attributed both to the production of new compounds and also to the development of more accurate techniques to detect these compounds in the environment (Geissen et al. 2015).

SETAC (Society of Environmental Toxicology and Chemistry) highlights the importance of information on the sensitivity of native species in order to predict the impacts of xenobiotic agents on local ecosystems and the extrapolation of toxicity test results in species used as regulatory (not native) for local scenarios and rates.

From the twentieth century onwards, toxicologists developed more sensitive and integrated analyzes by grouping data from different levels of biological organization that could represent the health status of organisms and the quality of the environment. These different levels of biological organization provide specific and sensitive responses to the effects of xenobiotics on different tissues of organisms. To this end, hematological, histological, histochemical, metabolic, biochemical and genetic parameters are analyzed in order to assess the damage caused by xenobiotic agents both, when submitting organisms to bioassays (laboratory tests) and when using organisms collected in the field (biomonitoring). For this reason, fish have been widely used in ecotoxicological studies that analyze biomarkers to detect changes in the parameters mentioned above.

## 15.2 Native Fish as Biomonitors, Bioindicators and Test Organisms

As mentioned before, environmental contaminants can exert several harmful effects on exposed organisms and the determination of the danger that these pollutants may exert, requires the performance of studies, using test organisms and biomarkers. Bearing this in mind, several works have been developed worldwide using fish species either as biomonitors or test organisms. Fish can also be used as bioindicators of contaminated environments or environments that have suffered an immediate impact, that is, fish indicates the state of the environment at the time they were collected and processed. Due to the versatility of bioassays that can employ these animals, the analyses that can be performed in an environment over a period of time or at different trophic levels and its high species diversity, fish can be important biomonitors, bioindicators and test organisms in the hyperdiverse Neotropics.

Several species of Brazilian native fish have been used as biomonitors in different regions and environments, such as *Geophagus brasiliensis* (acará) (Benincá et al. 2012; Tincani Osório et al. 2014; Yamamoto et al. 2016), *Hoplias malabaricus* (traíra) and *Serrasalmus brandtii* (piranha) (Jesus et al. 2014), and species of the genus *Astyanax* (lambaris) (Ramsdorf et al. 2012; Katsumiti et al. 2013; Silva et al. 2014; Freire et al. 2015; Yamamoto et al. 2016) used in monitoring studies of estuarine lagoons, lakes and rivers; *Pimelodus maculatus* (bagre) (Brito et al. 2012) and *G. brasiliensis* (Calado et al. 2017) used in water supply reservoirs, and *Atherinella brasiliensis* and *Cathoropis spixii* (Oliveira Ribeiro et al. 2013; Santos et al. 2014; Gusso-Choueri et al. 2016; Pustiglione et al. 2018; Santos et al. 2018) in estuaries.

Fish used as test organisms can be submitted to laboratory bioassays testing different chemical products, either individually or in mixed polls, or even bioassays with water of different origins. Therefore, it is important to use native fish species in the laboratory bioassays, in order to obtain more real responses of the behavior of the local ichthyofauna when exposed to xenobiotic agents in the aquatic environment. Several Brazilian native fishes have already been used as test organisms, such as *H. malabaricus* (Ferraro et al. 2004; Cestari et al. 2004; Silva et al. 2011; Vicari et al. 2012; Cestari 2013; Silva de Assis et al. 2013; Ribas et al. 2014; Sakuragui et al. 2019; Paulino et al. 2020), *Rhamdia quelen* (jundiá) (Ghisi et al. 2011; Pamplona et al. 2011; Klingelfus et al. 2015; Piancini et al. 2015a; Pereira et al. 2016; Guiloski et al. 2017a, b; Mathias et al. 2018; Marques et al. 2019; Perussolo et al. 2019; Bombardelli et al. 2021; Oya-Silva et al. 2021; Bernardi et al. 2022; Kitamura et al. 2022; Vicentini et al. 2022), *G. brasiliensis* (Piancini et al. 2015b; Yamamoto et al. 2016; Disner et al. 2017a; Calado et al. 2018, 2019, 2020), species of the genus *Astyanax* (Erbe et al. 2011; Rossi et al. 2011; Bueno-Krawczyk et al. 2015; Bettim et al. 2016; Galvan et al. 2016; Yamamoto et al. 2016; Disner et al. 2017a; Tincani et al. 2017, 2019; Cimbaluk et al. 2018), *Hoplias intermedius* (trairão) (Disner et al. 2017b; Klingelfus et al. 2017; Vicari et al. 2018; Delmond et al. 2019), *Prochilodus lineatus* (Carmo et al. 2019), *Aequidens tetramerus* (Rocha et al. 2011) and *Corydoras paleatus* (Ghisi and Cestari 2013).

Results from these biomonitoring and bioassay studies using native animals have demonstrated good support for detecting real responses to xenobiotic agents in natural aquatic environments and for testing environmentally relevant concentrations in laboratory assays.

## 15.3 Biomarkers of Genotoxicity and Mutagenicity

### *15.3.1 Piscean Micronucleus Test (Giemsa and Acridine Orange)*

Damage to the structure of the chromosomes causes chromosomal abnormalities that can range from the loss of part of the chromosomes to an entire chromosome (Fenech 2000). In order to quickly and efficiently assess damage to genetic

material, Heddle (1973) and Schmid (1975) proposed a type of assay quantifying cytoplasmic structures known to hematologists as Howell-Joly bodies that are found in dividing cells, which were named micronuclei (MN).

Micronuclei originate from acentric chromosome fragments, chromatids and chromosomes that were not transported by spindle fibers to opposite poles, remaining in the cytoplasm and later being surrounded by a nuclear membrane. Therefore "micronuclei originate from chromosomal fragments or from chromosomes that were not incorporated into the daughter cell nucleus during cell division. Thus, the assay detects both clastogenic events and cell spindle defects which alter the number of (aneugenic) chromosomes" (Ferrari 1991; Albertini et al. 2000). The size of the MN generally corresponds to 1/5–1/20 of the main cell nucleus. In fish, due to the reduced size of the chromosomes, this proportion can vary between 1/10 and 1/30 of the cell nucleus size. In addition, MN must have distinguishable edges and with the same refraction as the main nucleus. During the MN counting process, between 1000 and 2000 cells must be analyzed, with intact cytoplasmic and nuclear membranes, and those that are superimposed or damaged are not analyzed (Al-Sabti and Metcalfe 1995; Ayllon and Garcia–Vazquez 2000; Gustavino et al. 2001).

Cells for the MN analysis can be obtained from the bone marrow, exfoliated cells of the epithelium – buccal (through a simple scraping), bronchi (through the collection of sputum), urinary bladder (through the collection of urine) and peripheral blood (analysis of lymphocytes and nucleated erythrocytes or reticulocytes) (Ferrari 1991).

Hooftman and De Raat (1982) applied the original micronucleus test to blood cells of fish kept in the laboratory and observed that the erythrocyte nuclei, which are normally elliptical, showed morphological changes. Later, Carrasco et al. (1990) described and photographed the morphological alterations found in fish erythrocyte nuclei, classifying them as blebbed, lobed, vacuolated and notched. According to Al-Sabti and Metcalfe (1995), the MN test in fish has the advantage of allowing the prediction of the presence of clastogenic or aneugenic substances in the aquatic environment and yet several studies have shown that the peripheral erythrocytes of teleosts have a high incidence of MN after exposure to different pollutants in the environment or in laboratory conditions (Fig. 15.1).

The Acridine Orange test (AOT) selectively stains immature or young erythrocytes (polychromatic erythrocytes – PCEs), differently from mature erythrocytes (normochromatic erythrocytes – NCEs). The difference in fluorescent colors of the erythrocyte cytoplasm is due to the ability of Acridine Orange (AO) to bind both DNA (greenish yellow color) and RNA (reddish color) (Çavas and Gözükara 2005; McGahon et al. 1995) (Fig. 15.2).

Due to the small size of fish chromosomes and the difficulty of analyzing MN, the use of AO is very common, especially because it differentially stains the DNA, in contrast to the Giemsa dye. This latter dye can be deposited in excess on the slide or even stain clusters of organelles, which are considered artifacts and can be confused with MN. For this reason, the analysis of slides stained with Giemsa must be careful and judicious, maintaining the basic standards that the micronucleus has the same refringence as the main nucleus and with its format delimited by the nuclear membrane.

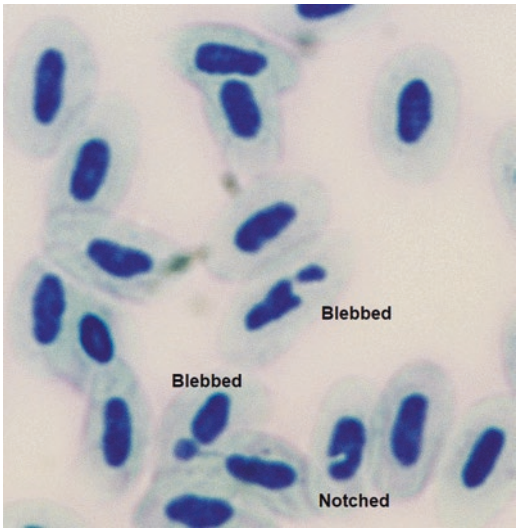


**Fig. 15.1** Nuclear erythrocyte alterations with two blebbed and one notched indicated

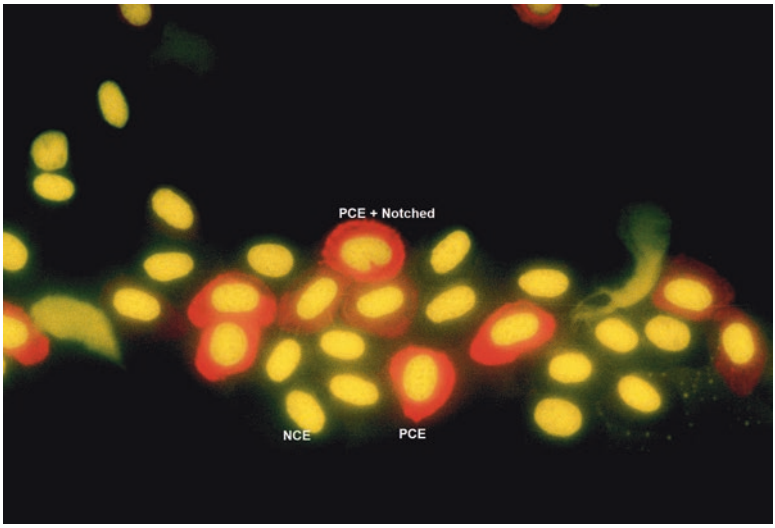


**Fig. 15.2** Acridine Orange (LA) stained erythrocytes. DNA (nucleus) is selectively stained in greenish yellow (NCE) and the RNA-rich cytoplasm appears in reddish color (PCE). A notched nucleus is indicated

Another difficulty found when observing fish micronuclei is the basal frequency, which in rodents is about 3% higher than in fish (Willians and Metcalfe 1992). This low frequency of micronuclei in fish blood erythrocytes may be related to the low mitotic activity of these cells, thus providing no visualization of MN in thousands of analyzed erythrocytes. For this reason, currently, the erythrocyte nuclear alterations are added to the frequencies of the micronuclei, in both biomonitoring and laboratory bioassays. Anyway, the fish MN test still needs refinements, such as the knowledge of the lifetime

of the fish erythrocytes, time for cell maturation and duration of cell cycle, besides preferably taking into account the trophic level of each fish species used as a biomonitor. In fish, the main hematopoietic tissue is the anterior portion of the kidney, but it is not the only one. Furthermore, interspecific differences in the time of production of new erythrocytes must be considered, as well as the lack of understanding of the mechanisms involved in the formation of micronuclei and nuclear morphology abnormalities (Udroiu 2006). Therefore, there are still many gaps that need to be addressed for a faithful interpretation of the fish micronucleus test results.

### *15.3.2 Comet Assay*

DNA of eukaryotic cells is tightly condensed to be accommodated in the nucleus, which is between 5 μm and 10 μm wide. If a DNA molecule suffers enough damage, decondesation will occur, which can be observed using the technique known as Single-Cell Gel Electrophoresis (SCGE) or Comet Assay (Rojas et al. 1999; Collins et al. 2008). This technique detects DNA damage in individual cells and its principle is based on the fact that the DNA of the cell that has no damage will migrate together forming a circle. If DNA damage occurs, fragments of different sizes will be formed that tend to migrate at different speeds, forming a kind of cloud and providing a typical comet image (Olive et al. 1990; Collins et al. 2008).

For assessing the extent of DNA damage, several techniques can be used, and the most used for visual analysis is the one that takes into account the ratio between the radius of the nucleus and the extension of the "tail" formed by the migrating DNA. Damage is classified as Class 0 – no damage, to Class 4 – maximum damage (Collins 2004) (Fig. 15.3). Other ways of measuring damage and the distribution of DNA in the tail are through the cell area, comet coefficient of variance, comet distribution moment, comet extent, comet optical intensity, head optical intensity, tail mode, and tail mean, among others (Kumaravel and Jha 2006; Kumaravel et al. 2007).

Unlike to other types of assays such as micronuclei, chromosomal aberrations (AC) and sister chromatid exchanges (SCE), the Comet Assay does not require proliferating cells for its viability and can be used in nearly any cell type, both in vitro and ex vivo experiments. Therefore, this technique has the advantage of analyzing the action of tissue-specific genotoxic substances, allowing the individual quantification of the damage caused to the DNA of these cells (Monteith and Vanstone 1995; Sasaki et al. 1997; Rojas et al. 1999; Kumaravel et al. 2007).

Briefly, this technique was described for the first time by Ostling and Johanson (1984), whose protocol allowed the identification of damage arising from double-stranded DNA breaks. Later, Singh et al. (1988) modified the original protocol by performing the electrophoresis in alkaline pH condition, which allowed the identification of alkali labile sites, late repair sites and damage from single strand breaks. Speit and Hartmann (1999) improved the technique using slides pre-coated with agarose in combination with drying the gels and fixing the comets. Such enhancements made the assay more sensitive, more versatile, and less expensive.

The sensitivity of this technique is high mainly because it allows the visualization of the comet tail produced by a much lower number of breaks in the DNA

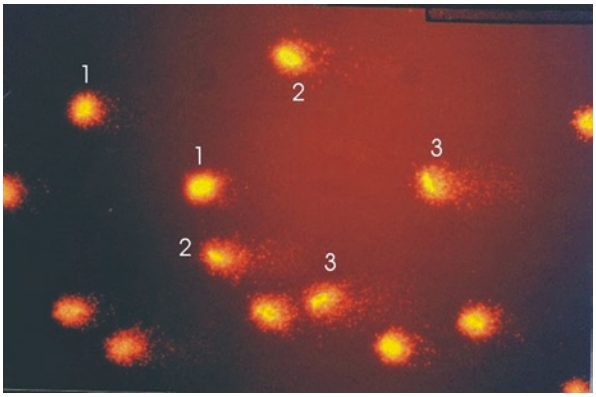


**Fig. 15.3** DNA damage in erythrocytes. Damage classes 1, 2 and 3 are indicated

strand of a cell, than any other method used to detect DNA damage (Rojas et al. 1999). According to Collins et al. (1997, 2008), even after treatment with the lysis solution and the saline electrophoresis solution, the nucleus or nucleoid maintains the nucleosomes in its structure and the comet's tails correspond to a relaxation of the DNA supercoiling caused by damages to its structure.

The Comet Assay can be used in many types of cells, in vitro (cultures of human and animal cells) and ex vivo (cells from different animal and plant tissues), considering that these cells were obtained by means that cause no damage but allow their individualization. In the case of blood cells, the cells can be diluted in fetal bovine serum or in a physiological solution (Ramsdorf et al. 2009b). All cell individualization processing must be performed without additional DNA damage, also the cells must be manipulated in the dark (Ramsdorf et al. 2009a; Ghisi et al. 2011; Benincá et al. 2012).

The Comet Assay was originally developed to detect genotoxic effects in mammals, but turned out to be efficient in detecting genotoxic xenobiotic agents in different aquatic organisms and in DNA repair studies in environmental biomonitoring and human monitoring (e.g. Sasaki et al. 1997; Abd-Allah et al. 1999; Padrangi et al. 1995; Cestari et al. 2004; Ferraro et al. 2004). The comet oxidative assay was used to determine the cytotoxic and genotoxic activity of some emerging contaminants. It was applied to cultured cells of strains of trout (RTG-2) and zebrafish liver (ZFL), and proved to be very effective and resolving (Oliveira et al. 2018; Klingelfus et al. 2019; Rodrigues et al. 2019; Souza et al. 2021; Almeida et al. 2023).

## 15.4 Genotoxicological Responses: Native Fish Versus Exotic Fish

It has been demonstrated that there are differences in responses to various biomarkers between fish species. For instance, *Oreochromis niloticus* (exotic species) and *G. brasiliensis* (native to Brazil), both belonging to the family Cichlidae, responded

differently to the same stressor (Piancini et al. 2015b). In that experiment, both species were submitted to 96 hours of water containing mesotrione at 1.8, 7, 30, 115, and 460 $\mu gL^{-1}$. *O. niloticus* showed significant DNA damage in erythrocytes exposed to mesotrione concentrations of 7, 115 and 460 $\mu gL^{-1}$, and in liver and gill cells at the highest concentrations (115 and 460 $\mu gL^{-1}$). In *G. brasiliensis*, DNA breaks were induced in erythrocytes at mesotrione concentrations of 30, 115, and 460 $\mu gL^{-1}$, in liver cells at concentrations of 30 and 460 $\mu gL^{-1}$ and in gill cells at a concentration of 115 $\mu gL^{-1}$. Yet, differences in the activity of oxidative stress enzymes were shown. In *O. niloticus*, the increase in the activity of enzymes glutathione-peroxidase (GPx) (7, 30, 115, and 460 $\mu gL^{-1}$) and protein thiol/glutathione (GSH) (7, 30, and 460 $\mu gL^{-1}$) were significant in relation to negative control (CN), while for *G. brasiliensis* there was an increase in the activities of glutathione-s-transferase (GST) (7, 30, 115 $\mu gL^{-1}$), superoxide-dismutase (SOD) (115 and 460 $\mu gL^{-1}$) and lipoperoxidation (LPO) (115 $\mu gL^{-1}$) enzymes. These results demonstrate a difference in response between the two species, highlighting that *G. brasiliensis* is more sensitive for the Comet Assay in liver tissue cells while *O. niloticus* is more sensitive in circulating erythrocytes. The enzyme activities showed opposite responses when comparing the two species. In *O. niloticus*, the significant differences in relation to CN were for the GPx and GSH enzymes and in *G. brasiliensis* the enzymes GST, SOD and LPO were altered.

According to Piancini et al. (2015b) the mesotrione-response differences observed between both biomarker species emphasize the need of always using native fish and demonstrate to environmental regulators and decision makers that native fauna may have different sensitivity to xenobiotic concentrations than exotic fauna.

## 15.5 Final Considerations

Environmental contamination represents an enormous threat to the chromosomal structures and to other levels of biological organization of natural populations and may impair long-term species fitness and enhance instability of ecosystems. Genotoxic properties of environmental genotoxicants contribute to the high incidence of genotoxicity and carcinogenicity among human and other species populations (Oberholster et al. 2016; AnvariFar et al. 2018; Angeli et al. 2013).

Conservation efforts to preserve natural populations must be accompanied by constant biomonitoring and risk assessment of compounds that are constantly released in water and soil by anthropogenic activities. At the Norman network website (https://www.norman-network.com/nds/susdat/susdatSearchShow.php) it is possible to access more than 2000 emerging substances with identified genotoxic effects. Considering that emerging pollutants are being identified at high rates, constant monitoring and testing are urgent to prevent human health damage as well as conservation of natural populations.

The growing volume of peer-reviewed literature on ecotoxicology is related to the increasing awareness of the importance of this field of study, which may influence regulatory decisions on the production, use and environmental release of the potentially hazardous substances (Hanson et al. 2017). Considering the outstanding importance of research in ecotoxicology, specially for risk assessment of compounds, it is imperative to develop and use specific and sensitive tools for genotoxicity testing, particularly in the hyperdiverse Neotropics.

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# Part V
# Assessing Molecular Ecology and Communities

# Chapter 16
# Molecular Ecology in Neotropical Mammals: Key Aspects for Conservation

**Bruno H. Saranholi, Carla C. Gestich, and Marina E. de Oliveira**

## 16.1 Introduction

Molecular ecology is a relatively recent interdisciplinary field that has emerged from the advances in molecular techniques which allow researchers to answer ecological questions by comparing genetic information at the individual, species, and community levels. This approach encompasses a broad range of tools aimed at obtaining ecological information such as species confirmation through molecular methods, occurrence and distribution of a given species, demography estimates, relatedness among individuals, inter and intraspecific interactions, dispersal patterns, and sex ratio, among others.

Since it is a DNA-based approach, an investigation in molecular ecology depends on the collection of biological samples for the acquisition of DNA. For mammals, several types of biological samples can be used, such as blood, tissue, skin, hair, and feces (Fig. 16.1). Of these, non-invasive samples, i.e., biological samples which do not require that the animal be captured or handled, such as feces and hair left in the environment (Beja-Pereira et al. 2009), have provided an especially meaningful contribution for many ecological studies of mammals. Feces have been extensively used to assess the DNA of the species that produced them (e.g., Miotto et al. 2007, 2011, 2014; Saranholi et al. 2017, 2022). Because several mammals use their feces for communication and territorial marking (Gorman and Trowbridge 1989), these animals usually defecate on trails or prominent places such as rocks and trunks (Aragona and Setz 2001; Gorman and Trowbridge 1989), which can make the collection of this type of sample easier.

An important second step is the selection of the most appropriate molecular marker to answer the ecological question being investigated. For example,

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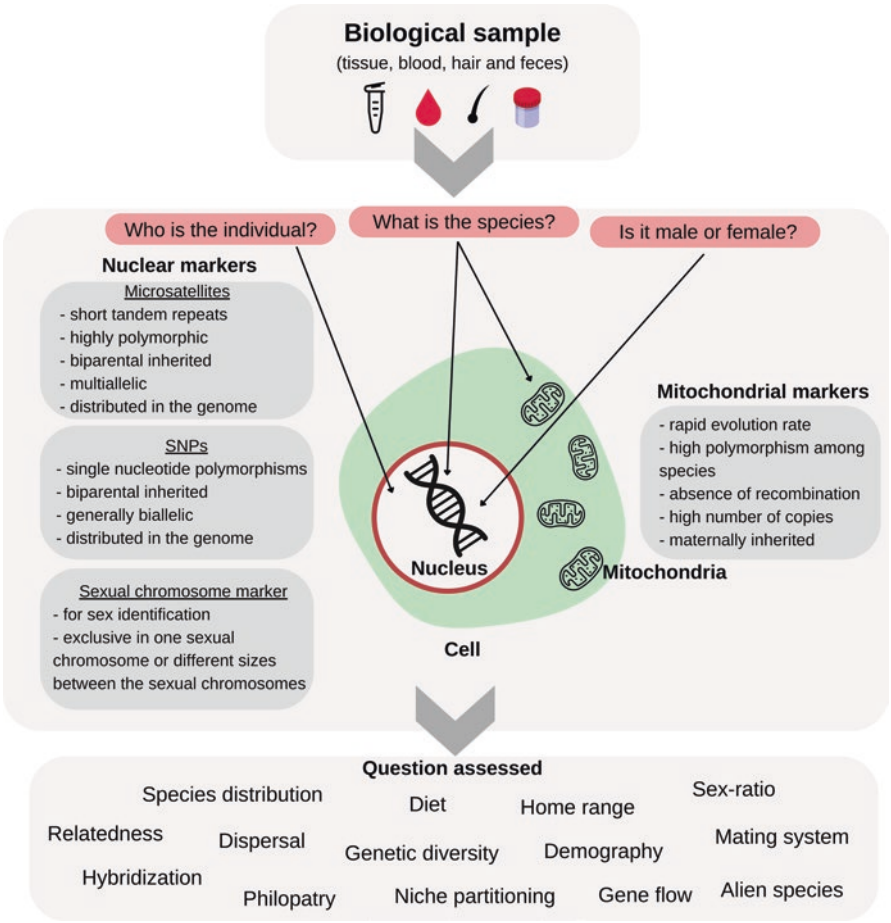


**Fig. 16.1** Overview of the main steps involved in molecular ecology studies, from sampling type, broad questions, molecular markers, and ecological aspects possible to be assessed by molecular tools

mitochondrial DNA (mtDNA) markers are widely used due to certain important characteristics: rapid evolution rate, high polymorphism (even across short sequences), absence of recombination, and a high number of copies per cell if compared to nuclear DNA (Fig. 16.1; Avise 2012). These characteristics allow mtDNA to be used in species identification even if the samples are degraded, making it an effective strategy when working with samples that contain a small amount of DNA and its integrity is poor (Farrell et al. 2000; Chaves et al. 2012; Rodríguez-Castro et al. 2018). The variability in mitochondrial genes is relatively high across different species, but significantly less among individuals of the same species; thus, mtDNA markers can be used to distinguish between species, even those which are closely related (Avise 2012). The huge amount of mtDNA sequences available in public

data repositories, such as the NCBI (National Center for Biotechnology Information, US Government) or BOLD Systems (Barcode of Life Data System), also favors the use of this type of molecular marker for species identification (Galtier et al. 2009).

Concerning the nuclear portion of the genome, highly informative polymorphic markers such as microsatellites – also known as SSRs (short sequence repeats) or STRs (short tandem repeats) – are of great importance for ecological studies (Fig. 16.1; Selkoe and Toonen 2006). These short sequences, which are comprised of one to six base pairs repeated in tandem (Zane et al. 2002), are widely distributed throughout the genome, displaying high levels of polymorphism and, thus, a multiallelic characteristic (Schlotterer and Tautz 1992; Springer et al. 2001). A high degree of polymorphism among individuals allows us to obtain individual genotypes and assess genetic information at population level (Broquet et al. 2006; Haag et al. 2010; Rodgers and Janečka 2013; Figueiredo et al. 2015; Maciel et al. 2019). Microsatellites display codominant inheritance, i.e., it is possible to identify the two alleles of a given locus separately (Sunnucks 2000); also, they are considered to be selectively neutral, which means that, usually, no product is encoded at the loci where they occur. Thus, these markers may be used to compare populations in terms of genetic diversity, since they are not affected by evolutionary pressures (Selkoe and Toonen 2006). Microsatellite regions are generally short and, therefore, their PCR products are also short, which makes them possible to be used with non-invasive samples (Beja-Pereira et al. 2009). Furthermore, with the use of a multilocus panel (Broquet et al. 2006; Beja-Pereira et al. 2009), researchers can identify the individual source of each sample, allowing for the determination of its origin when it is too difficult to obtain such information in the field. More recently, due to advances in next-generation sequencing (NGS) technologies, the use of other nuclear markers, such as SNPs (Single Nucleotide Polymorphisms), has been made easier. These molecular markers can be genotyped through NGS techniques (e.g., GBS – Genotyping by Sequencing), revealing up to thousands of SNP loci, which shows great potential for ecological and conservation studies (Fig. 16.1; Morin et al. 2004). Still, despite such potential, SNPs remain little explored in ecological studies of neotropical mammals.

Nowadays, there are at least 6595 extant mammal species worldwide, and a substantial number of them (~25% of all species) is concentrated in the Neotropics (Burgin et al. 2018). The neotropical region also harbors several endemic mammal groups, such as caviomorph rodents (e.g., capybaras), xenarthrans (armadillos, anteaters, and sloths), and platyrrhine monkeys (Patterson and Costa 2012). Mammals are increasingly threatened by human activities such as poaching, illegal trade, and road killing, in addition to those which result in habitat loss and fragmentation (Ripple et al. 2015; Ceia-Hasse et al. 2017). Consequently, it has been estimated that about 80% of all mammal populations have either been lost or are becoming smaller (Ceballos et al. 2017); this process is occurring predominantly in the Neotropics and southeast Asia (Ceballos et al. 2017). Most threats are still in place and becoming more severe, but their effects on local populations and the long-term survival of the species are still poorly understood. Therefore, the acquisition of ecological data on mammals is critical to guide practical management strategies for the

conservation of these animals, and a molecular ecology approach can offer important contributions to such efforts.

## 16.2 Molecular Species Identification

Determining a species occurrence is a primary task for the acquisition of basic ecological information and implementation of conservation practices. Conservation programs are concerned with establishing the precise distribution of a species and monitoring its occurrence through time (Mace et al. 2008; Sofaer et al. 2019). Moreover, it is important to determine the richness and composition of communities to better establish priorities for the protection of natural environments (Jenkins et al. 2015). Species identification also enables us to retrieve other ecological information, such as home range size as well as overlap and selection of habitat, which are usually helpful to guide conservation practices.

### *16.2.1 Molecular Markers for Species Identification*

Mitochondrial genes have been widely used as informative markers for molecular species identification (Fig. 16.2). Some mtDNA regions can be used in DNA barcoding studies, because certain sequence polymorphisms within the mtDNA are unique for each species (Hebert et al. 2003). In the case of non-invasive samples, when excessive exposure to environmental conditions degrades the DNA in the sample, resulting in lower DNA quality, the primers for the molecular markers used

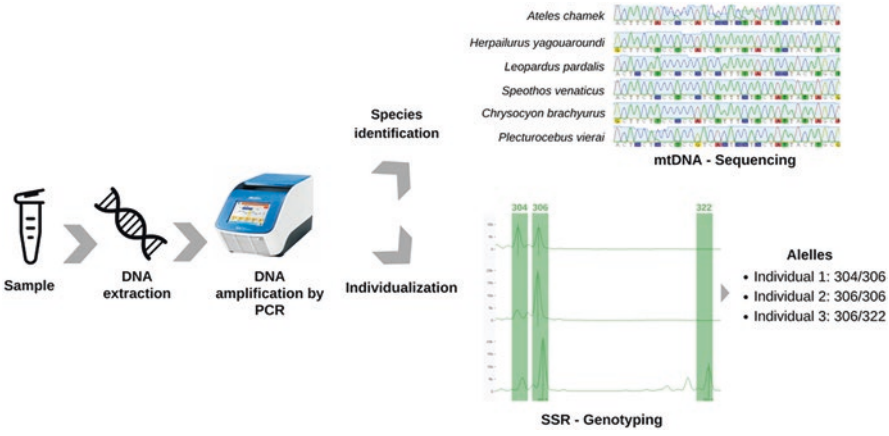


**Fig. 16.2** Steps involved in molecular species identification and individualization based on mtDNA sequences and microsatellite genotyping, respectively

to identify the species must be designed to amplify short sequences located in highly informative regions of the mtDNA, also known as mini barcodes (e.g., Chaves et al. 2012). This strategy has allowed for significant advances in species identification based on non-invasive samples. For instance, due to the elusive habits and naturally low densities of carnivores, the detection of individuals from this group is often difficult, but their territory marking behavior with feces favors the use of a non-invasive sampling approach (e.g., Chaves et al. 2012; Farrell et al. 2000; Saranholi et al. 2017). Various carnivore mtDNA markers have already been identified and evaluated, consisting of regions within the genes for adenosine triphosphate subunit 6 (ATP6), cytochrome oxidase I (COI), cytochrome b (Cyt b), 12S rRNA and 16S rRNA (Farrell et al. 2000; Chaves et al. 2012; Rodríguez-Castro et al. 2018). For instance, the presence of the *Panthera onca*, a rare and highly endangered species, was molecularly confirmed with basis on fecal samples, after an extensive camera trap effort failed to record this animal in a protected area of the Atlantic Forest biome, in Brazil (Souza et al. 2017); molecular species identification was performed based on the amplification of two mini barcode fragments from the mitochondrial genes ATP6 and Cyt b.

Species identification and monitoring based on fecal samples were also successfully concluded for leporids. Rodrigues et al. (2020) used three mtDNA regions (COI, Cyt b and 16S rRNA) to molecularly identify feces from different species of leporids – which would have been challenging if the only information available had been the morphology of the fecal samples, due to the high similarity among them. With this approach, Rodrigues et al. (2020) were able to map the occurrence of the neotropical native *Sylvilagus brasiliensis* and the invasive *Lepus europaeus* throughout an area of approximately 1,500,000 ha in the state of São Paulo, Brazil. This precise identification by molecular fecal analysis represents a powerful tool for effectively monitoring the distribution of both species and supporting future management actions aimed at controlling the growth of the invasive species. Similarly, the community of felids inhabiting a protected area within a tropical rainforest in Mexico was assessed with the molecular identification of hair samples collected from hair snares placed in transects (García-Alaníz et al. 2010). Since an information deficit for carnivore populations in tropical rainforests may be caused by the lack of appropriate, reliable and cost-effective methods, the use of hair-snaring followed by precise molecular identification represents a viable approach for detecting elusive carnivore species (García-Alaníz et al. 2010). The Andean bear (*Tremarctos ornatus*) was also molecularly identified (Cyt b region) from hair and fecal samples collected in the southern areas of its distribution range, leading to the update and expansion of the species range by 150 km in Argentina (Cosse et al. 2014). All these cases highlight the applicability of molecular tools for samples that are not morphologically identifiable, as well as their usefulness in ecological studies and conservation planning.

Despite this being less usual, sequences from nuclear genes can also be applied to species identification. In spite of their lower degree of interspecific divergence and higher homoplasy in relation to mtDNA sequences, nuclear markers are particularly helpful for species identification in cases of hybridization. Combined with

mtDNA markers, nuclear markers may help us understand hybridization processes in natural contact zones between congeneric species, or when human activities result in habitat degradation, promoting a non-natural contact between species. In a study of *Leopardus guttulus* and *Leopardus geoffroyi* at their geographic contact zone, Trigo et al. (2013, 2014) used a combination of nuclear markers (ten microsatellite loci, X chromosome-linked regions – PLP1 and BTK, and Y chromosome-linked regions – ZFY and SMCY3) and mtDNA (NADH dehydrogenase subunit 5), to identify hybrid individuals, mostly when the phenotype of a hybrid was indistinguishable from one of the parental species. The association of different molecular markers, in this case, allowed the construction of a complete scenario of the contact zone between these two species of felids (Trigo et al. 2014). Natural hybridization was also molecularly confirmed in primates, between *Alouatta pigra* and *Alouatta palliata* in Mexico (Cortés-Ortiz et al. 2007). Using microsatellites (eight loci), as well as mtDNA (Cyt b) and Y chromosome-linked genes (SRY gene), the authors were able to trace back the maternal and paternal lineages of hybrid individuals. This approach revealed that only when *A. pigra* females crossed with *A. palliata* males they produced fertile female descendants in the first-generation offspring (F1), whereas their male offspring were infertile (Cortés-Ortiz et al. 2007). Other natural hybridization zones between neotropical mammals (e.g., manatee, Vilaça et al. 2019; camelids, González et al. 2020) were confirmed with the use of molecular tools.

Hybridization is most important when it results from the spread of an invasive species, usually caused by human activity, such as animal trade or habitat disturbance, leading to non-sympatric species being in contact with each other. Among the primates of the *Callithrix* genus, besides causing habitat displacement and engaging in competition for resources (Melo et al. 2020), invasive species threaten native ones due to the possibility of hybridization that produces fertile descendants (Malukiewicz 2019), possibly resulting in the loss of the pure gene pool of the native species. Using the mtDNA control region, Malukiewicz et al. (2014) found different patterns of hybrid formation between anthropogenic and natural zones. In the former, there is a more abrupt removal of the reproductive barrier than in the natural zones, demonstrating that anthropogenic landscape alteration favors hybrid formation.

### *16.2.2 Biodiversity Monitoring Through Molecular Species Identification*

Sampling and detecting mammal species can be a challenging task, because many of them can be rare, elusive, or found in low-density populations. Traditional mammal surveys have involved setting camera traps and line transects for direct or indirect (i.e., feces, footprints) observation. However, species identification based only on the morphology of the feces, for example, is not an easy task, and it can lead to

inaccurate identifications of several mammal groups (leporids: Rodrigues et al. 2020; felids: Farrell et al. 2000; deers: Oliveira et al. 2022). In contrast, the use of molecular identification of biological samples has been employed as an efficient tool for detecting mammal species (e.g., carnivores: Miotto et al. 2014; Saranholi et al. 2017; Souza et al. 2017; Srbek-Araujo et al. 2018; leporids: Rodrigues et al. 2020; bats: Clare et al. 2007; small mammals: Borisenko et al. 2008). For example, the detection of an elusive deer species (*Mazama* spp.) in the Atlantic Forest was conducted by combining camera trap recordings with opportunistic data collection and molecular species identification (Cyt b) of fecal samples (Oliveira et al. 2022). The latter accounted for 72% of the occurrence confirmation data for these deer species, which highlights the potential of this approach for monitoring elusive species. Also, tissue samples from dead animals, e.g., road-killed animals (Balkenhol and Waits 2009; Saranholi et al. 2016), or from forensic apprehensions, e.g., illegally hunted animals (Pun et al. 2009), can also undergo molecular analysis for a very precise species identification.

The molecular species identification of dead specimens is especially important when samples are collected from highly deteriorated carcasses for road ecology studies. The use of DNA barcodes has been proposed as a complementary technique to help understand the impacts of roads on the surrounding biodiversity. For instance, out of a total of 62 vertebrate species molecularly identified (COI gene) among the road-kills of only 25 km of a road crossing an area of Atlantic Forest area in Brazil, 27 were mammal species (Klippel et al. 2015). Molecular tools have also been critical for the identification of small-sized road-killed species (amphibians, reptiles, birds, small mammals), whose carcasses are generally more damaged and degraded than those from large animals (Rodríguez-Castro et al. 2017). In addition to species identification, modeling hotspots where animals are more likely to be road-killed could be useful to elucidate the ability of certain species to move and to explain their relationship with the landscape (Rodríguez-Castro et al. 2017), and this could contribute to guiding mitigation strategies to be implemented along roads (see more in Chap. 17).

The use of DNA barcoding in association with next-generation sequencing technologies has broadened the applicability of species identification through metabarcoding. With this approach, it is possible to recover DNA information pertaining to more than one species from a single biological sample – such as feces, for dietary studies (see below) – or from mixed samples, such as those obtained from environmental DNA (eDNA). Species identification through metabarcoding has greatly enriched eDNA studies that aim to assess a community of species by sampling water (Mena et al. 2021), soil (e.g., Leempoel et al. 2020) and air (e.g., Lynggaard et al. 2022). eDNA has been successfully employed for detecting and monitoring mammals, especially those that are endangered, invasive, or elusive (Bohmann et al. 2014). A similar approach has also been used to identify mammal species with basis on the digestive contents of hematophagous or coprophagous insects, known as iDNA, i.e., the DNA that has been *ingested* by or extracted from *invertebrates* (e.g., Calvignac-Spencer et al. 2013; Lynggaard et al. 2019). eDNA and iDNA have been regarded as powerful tools for rapid biodiversity assessment, constituting a

promising approach that is still little explored in neotropical mammal studies (Cristescu and Hebert 2018; Carvalho et al. 2022; see more in Chap. 18).

### *16.2.3 Molecular Identification of Prey and Diet*

The study of diet is another use for the barcoding approach through species identification from feces or digestive content. By identifying which animals or plants are eaten by the target species, researchers can have a better understanding of their feeding habits, the resources at their disposal, prey densities, and potential competition with sympatric species from the same guild (Janečka et al. 2020). For instance, by amplifying a mtDNA (COI) fragment from the genetic material retrieved from guano, Rolfe et al. (2014) compared the diet of two sympatric species of insectivorous bats (*Mormoops blainvillei* and *Pteronotus quadridens*) in Puerto Rico. The authors were, then, able to identify the families and even the genera of the insects consumed, whereas previously they had reached the order level at best, based only on the morphology of prey remains; this reaffirms the greater accuracy of molecular analyses for the species identification. More recently, metabarcoding has been successfully employed to investigate the diets of top predators (e.g., giant otter *Pteronura brasiliensis*, 12S rRNA and COI, Quéméré et al. 2021), herbivores (e.g., lowland tapir *Tapirus terrestris*, P6 loop region of the chloroplast trnL intron-UAA, and internal transcribed spacer – ITS gene, Hibert et al. 2013), and small mammals (e.g., *Ctenomys* spp., UAA and ITS, Lopes et al. 2020) in the Neotropics, based on their fecal samples. For instance, a study that aimed to analyze the coexistence and niche partitioning by 25 species of bats from various guilds, in the tropical dry forests of Belize, found no case of complete overlap of the feeding items consumed by those species (Ingala et al. 2021). To determine the list of consumed items, the authors collected fecal DNA samples, and used the metabarcoding approach by amplifying mini barcodes of ingested plants (P6 loop region), arthropods (16S rRNA), and vertebrates (12S rRNA) (See more details in Chap. 18).

## 16.3 Sample Individualization and Molecular Sex Identification

After the species is identified, distinguishing between the individuals within the collected samples and determining each one's sex is also possible with the use of molecular tools. Individualizing samples with the use of molecular markers, that is, determining which individual corresponds to a given sample, represents a significant development for ecological studies, especially those that aim to make demographic estimates, such as density and abundance, but also for studies interested in estimating distribution as well as extension and overlap of home ranges for different

individuals (Selkoe and Toonen 2006; Rodgers and Janečka 2013). This approach is especially useful when the sampling method does not involve collecting a biological sample directly from the animal, but rather using traces left in the environment – such as feces, regurgitates and hair. In addition, the knowledge of sex ratios within a population enhances our understanding of the population's demography, and sex identification can be used to investigate behavioral differences between sexes (Rodgers and Janečka 2013). The combination of these basic – but critical – information sets (molecular identification of species, sex, and individualization) can enhance the quality of a species' ecological data.

### *16.3.1 Molecular Markers Used in Sample Individualization*

In order to obtain genotypes that differentiate samples at an individual level, the molecular markers used in the individualization of samples must exhibit a high degree of polymorphism, and among the most used and important markers are microsatellites (Fig. 16.2). As they are codominant and display a high degree of polymorphism, the genotypes obtained from them allow us to differentiate individuals with great success, even in the case of related individuals. Furthermore, microsatellites are usually sequences that are short enough to be amplified by PCR even in samples containing degraded DNA, such as non-invasive samples or the carcass of a road-killed animal. From a microsatellite panel, it is possible to calculate the Probability of Identity ($P_{ID}$) value, which estimates the likelihood that two unrelated individuals have the same genotype profile by chance (Waits et al. 2001). Low $P_{ID}$ values indicate better assignment at individual level.

Other nuclear markers can be used for individual identification. SNPs are promising candidates for that, although these molecular markers are generally biallelic, which could entail a reduction in individualization power when compared with multiallelic molecular markers (e.g., microsatellites). Next-generation sequencing (NGS) technology advancements now make it possible to obtain and genotype hundreds or even thousands of SNP loci, and this extensive genomic coverage enables robust sample individualization (e.g., Buchalski et al. 2022).

### *16.3.2 Applicability of Individualization in Ecological Studies*

The molecular individualization of samples provides a wealth of information for ecological studies. Miotto et al. (2007, 2012) collected feces to identify the minimum number of *Puma concolor* individuals inhabiting conservation units in southeastern Brazil. By conducting systematic sampling over the years, it was possible to recapture some of those individuals, i.e., collecting fecal samples of the same individual several times within the study period allowed for the identification of

residents and new individuals inhabiting the area. The same approach was used by Ramalho et al. (2014) to obtain demographic estimates for the *Chrysocyon brachyurus* in a conservation unit of the Cerrado biome, in Brazil. Also using fecal samples in their work, Trinca et al. (2013) determined the number of individuals and the population density of *Lontra longicaudis*, a semi-aquatic mammal, inhabiting an Atlantic Forest area in Brazil. In addition to aiding in the production of demographic estimates, individualization through fecal samples may be helpful to understanding aspects of a species' behavior. For example, by individualizing fecal samples from *Leopardus pardalis* inhabiting Barro Colorado Island, in Panama, Rodgers et al. (2015) found that communal latrines can constitute scent communication centers, where one *L. pardalis* could establish communications with up to fourteen others.

Individualization is also used for estimating genetic parameters of a population, such as genetic diversity, gene flow, and effective population size, since obtaining the genotypes of the individuals is necessary for such studies. Therefore, when using non-invasive samples, or when the origin of a biological sample is uncertain, molecular individualization is a mandatory step before performing population genetic analyses. By molecularly individualizing fecal samples, several studies have obtained information on effective population size, inbreeding, and relatedness for several neotropical mammals, such as *P. concolor* (Miotto et al. 2011; Saranholi et al. 2017), *T. terrestris* (Saranholi et al. 2022), *Panthera onca* (Wultsch et al. 2016); *C. brachyurus* (Ramalho et al. 2014), *L. longicaudis* (Trinca et al. 2013). All these studies relied on microsatellites for sample individualization, which supports the applicability of this molecular marker even for samples containing degraded DNA.

Despite the potential of SNPs for sample individualization above mentioned, to the best of our knowledge, only two studies have focused on establishing an informative SNP panel for the individualization of samples from a neotropical mammal, thus far. *P. concolor* was investigated in both studies (Fitak et al. 2016; Buchalski et al. 2022), in which 25 and 95 SNP loci were used to differentiate between individuals, respectively.

### *16.3.3 Molecular Sexing*

The molecular markers commonly used for sex identification are located in sexual chromosomes. The primers that amplify a segment of the SRY (sex-determining region Y) gene, located in the Y chromosome, are widely used in sex determination for several mammalian species, such as primates (Di Fiore 2005), tapirs (*T. terrestris,* Pelizzon et al. 2017), mustelids (*L. longicaudis,* Trinca et al. 2013) and carnivores (DeCandia et al. 2016). In these cases, the successful PCR amplification of the SRY fragment indicates that the analyzed individual is a male. Furthermore, it is not necessary to sequence the amplified fragment, as one can observe its presence or absence directly through agarose gel electrophoresis, which makes this test relatively inexpensive. In other cases, molecular markers present in both X and Y chromosomes can be used. In the amelogenin (AMELX and AMELY) (e.g., felids,

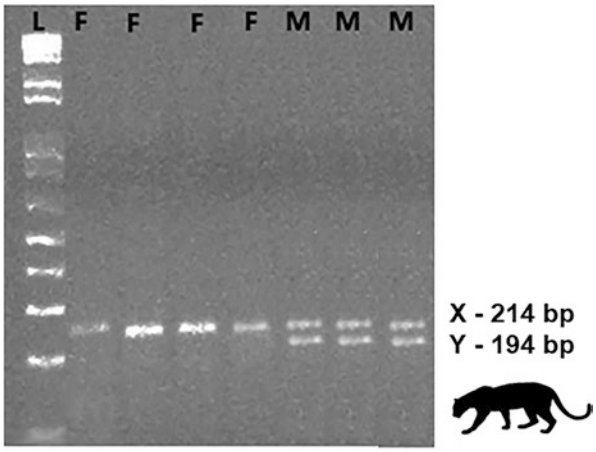


**Fig. 16.3** Molecular sexing of fecal samples from *Puma concolor* in agarose gel 3% based on the amelogenin gene region polymorphism. Female individuals are represented by a single band in the agarose gel, because the amelogenin gene has the same size in both X chromosomes. Male individuals are represented by two bands in the agarose gel, because there is a deletion of 20 base pairs (bp) in the Y chromosome. *L* Ladder (1 Kb Plus DNA, Invitrogen), *F* Female, *M* Male. Gene sizes: 214 bp in X chromosomes and 194 bp in Y chromosome

Pilgrim et al. 2005) (Fig. 16.3) or zinc-finger (ZFX and ZFY) (e.g., *Tayassu pecari*; Rufo et al. 2015) genes, the existing polymorphisms between the X and Y chromosomes lead to variations in fragment sizes because of nucleotide deletions in the Y chromosome. Thus, two same-sized fragments are amplified for females, resulting in a single visible band on the agarose gel, whereas two fragments of distinct sizes are visible for males, since they possess the X and Y copies of the genes (Fig. 16.3). Particularly for methods based on a single gene, such as the SRY gene, it is important to include the amplification of other genes present in both sexual chromosomes in the same PCR (multiplex PCR), such as the zinc finger gene, as a positive control, in order to prevent the false identification of males as females, since amplification of the Y chromosome gene may fail in samples with low-quality DNA.

Obtaining sex-related data from individuals can help us to better understand the ecology of the species. For example, in Souza et al. (2017), the presence of *P. onca* in an area where it was believed already extinct was revealed by fecal molecular identification, and the individualization of the samples counted three different individuals; in addition, molecular sexing revealed that two individuals were females, and one male. These results not only confirmed the presence of a rare and elusive species in the area, but also provided more detailed information on the number and sex of the individuals, which can be valuable for monitoring the populations of this threatened felid species. Molecular sexing can also be useful for studying behavioral patterns in mammals. In the studies of Trinca et al. (2013) and Miotto et al. (2014), the female *L. longicaudis* and *P. concolor* individuals, respectively, were considered philopatric, whereas males of both species tended to disperse. In the

study on scent communication in communal latrines used by *L. pardalis*, molecular sexing of the fecal samples revealed that males had the potential to communicate with more individuals than females by marking the territory with their feces (Rodgers et al. 2015). Sex information obtained through molecular methods in *Myrmecophaga tridactyla* was used to test the existence of sex-biased dispersal in the species, which was not corroborated by the authors within the studied region (Barragán-Ruiz et al. 2021).

## 16.4 Behavioral Ecology

The development of molecular techniques has allowed us to better understand species behavior, and its evolution under different ecological circumstances. The study of relatedness among individuals within a population is of great interest to biologists, from classical geneticists to conservation biologists and molecular ecologists. Dispersal and philopatry are two of the main behaviors that can shape relatedness and other genetic characteristics of populations. Relatedness is central in quantitative genetic studies aimed at investigating the inheritability of a given quantitative trait, the mating system of a wild population, dispersal patterns that shape genetic diversity, and predictions on the best mating pairs in captive breeding programs, among many other applications (Lynch and Walsh 1998; Prugnolle and De Meeûs 2002; Jones and Wang 2010; Fienieg and Galbusera 2013). Some of these aspects will be presented in the following sections.

### *16.4.1 Relatedness and Mating Systems*

In wild populations, the observation of genealogy or pedigree structure is often unfeasible; thus, researchers can only rely on DNA-based methods to estimate relatedness. Since sample individualization is based on genotyping, typically with the use of biparentally inherited and independent nuclear markers such as microsatellites or SNPs, this genetic data can be used to assess relatedness between individuals, groups, or within populations. Methods for relatedness analysis can be divided into two categories, relatedness estimators and assignment of individuals to relationship categories (Blouin 2003). Relatedness estimators calculate the probability of identity by descent (IBD), which is the probability that two alleles at a given locus, one from each individual, are recently descended from a common ancestral allele within a reference population (Blouin 2003). At any locus, two individuals may share zero, one or two alleles that are identical by descent, and the probabilities of these events (also known as $k_m$, where $m$ is the number of IBD alleles) depend on their true relationship (Table 16.1). For example, the probability that parent and offspring share one allele that is IBD at any locus ($k_1$) is 1. The estimate takes the form of a range of values usually between −1 and 1 or 0 and 1, depending on how

**Table 16.1** Probabilities $k_m$ that two individuals share $m$ alleles (zero, one or two) which are identical by descent, given their relationship

| Relationship | $k_0$ | $k_1$ | $k_2$ |
|---|---|---|---|
| Parent-offspring | 0 | 1 | 0 |
| Full-siblings | 0.25 | 0.5 | 0.25 |
| Half-siblings/grandchild-grandparent/niece or nephew-uncle or aunt | 0.5 | 0.5 | 0 |
| First cousins | 0.75 | 0.25 | 0 |
| Unrelated | 1 | 0 | 0 |

the estimator fits the $k$ probabilities into its algorithm (Milligan 2003). The categories of genealogical relationships between individuals, such as full siblings, parent-offspring, half-siblings, etc., are inferred from the probabilities of alleles being shared (Kalinowski et al. 2006) (Table 16.1). Parentage analysis is a special class of analysis in which one aims to assign an offspring to its true mother and/or father by using likelihood ratios, which compare the probabilities that the observed genotypes are parent-offspring under alternative hypotheses concerning their relationship category (Jones and Ardren 2003; Weir et al. 2006).

There are many relatedness estimators available (e.g., Queller and Goodnight 1989; Lynch and Ritland 1999; Wang 2007; Milligan 2003), and each estimator has its advantages and limitations (van de Casteele et al. 2001; Blouin 2003; Oliehoek et al. 2006). There are several softwares that implement more than one estimator simultaneously, so the most suitable to the input data may be chosen (Wang 2011). Estimates of relatedness and relationship are strongly affected by the number of loci and the number of genetic marker alleles chosen, the distribution of the alleles within their loci, rates of allelic dropout, presence of null alleles (i.e., alleles that fail to amplify), and allele frequencies within the reference population (Oliehoek et al. 2006; Weir et al. 2006; Wagner et al. 2006). For example, if only two alleles (*i* and *j*) are present in a population for a given locus, then all individuals in this population are either heterozygous (*ij*) or homozygous (either *ii* or *jj*) for these alleles. Considering this locus alone, if two individuals present the same genotype, it will be impossible to distinguish between alleles that are identical by descent or identical by state (IBS, i.e., same nucleotide sequence, but not necessarily inherited from a common ancestor), and thus relatedness cannot be estimated. However, with the use of more loci, it is possible to study relatedness even in populations that display low genetic diversity, including inbred individuals (Wang 2011). Relatedness between individuals can also be influenced by numerous ecological and behavioral factors, such as their mating system, overlapping of generations (Kopps et al. 2015), sexual selection (Young and Bennett 2013), patterns of dispersal behavior (Prugnolle and De Meeûs 2002), breeding success (Amos et al. 2001), kin selection (Aronsson et al. 2020), genetic diversity, bottleneck events (Robinson et al. 2013), and inbreeding avoidance (Cohas et al. 2008).

Population, group or individual kinships are not static throughout space and time (Croft et al. 2021) and understanding all ecological and behavioral factors influencing relatedness is not a trivial task. The case of the vulnerable white-lipped peccary

(*T. pecari*) is an interesting example of relatedness being used to study their mating system and the relationship with competition, sex ratio and dispersal behavior (Leite et al. 2018). This species presents no apparent sexual dimorphism (Keuroghlian and Desbiez 2010). A monogamous mating system is usually expected among species without sexual dimorphism and for which the operational sex ratio (the average ratio of sexually active males to receptive females) is not skewed (Clutton-Brock 2007). However, through parentage tests, Leite et al. (2018) observed males and females having offspring with more than one partner, which is consistent with a promiscuous mating system. The authors suggested that the observed pattern could have resulted from intrasexual competition as well as the influence of natural and/or sexual selection for both sexes (Biondo et al. 2011; Leite et al. 2018).

In captive populations of ongoing breeding programs, minimal relatedness between mating pairs is desired in order to maintain genetic variability and avoid the effects of endogamy and genetic drift (Montgomery et al. 1997; Rudnick and Lacy 2008). The endangered black-lion-tamarin (*Leontopithecus chrysopygus*) is an example of how relatedness can be used in ex-situ management. This rare primate is endemic to the Atlantic Forest of the state of São Paulo in southeast Brazil (Kierulff et al. 2008), and only 1600 individuals are estimated to remain in the wild (Rezende et al. 2020). After a few dozen individuals were moved to zoos and conservation facilities, the studbook for black-lion-tamarins was created in 1987 to keep track of the genealogy of captive individuals (Simon 1988). Despite the intense efforts to maintain records on the genealogy of the black-lion-tamarins (as well as other captive species), this information is often incomplete, and thus molecular methods are useful to estimate the relatedness and relationships among contemporary and founder individuals (Russello and Amato 2004). Ayala-Burbano et al. (2020) analyzed the whole ex-situ population of black-lion-tamarins using microsatellite markers and found an average of two alleles per locus in addition to a high level of relatedness among captive individuals. The authors proposed an integrative approach for the ex-situ conservation of this species, which could be applied to other captive populations as well, conducting analyses of nuclear markers – as presented in this chapter – to monitor expected heterozygosity, individual heterozygosity, allele richness, private alleles, population structure, inbreeding, and relatedness.

For lowland tapirs (*T. terrestris*), a combination of relatedness analysis (relationship categories and relatedness estimators) was used to test the hypothesis that this species exhibits relatedness-based social behaviors (Pinho et al. 2014). In this study, the authors collected non-invasive samples (feces) from an island complex formed by the flooding of an area after the construction of the Balbina hydroelectric dam in central Amazon, Brazil, and they genotyped individuals using five sufficiently informative microsatellite loci to discriminate between individuals. The authors found no statistical difference between distances separating related and unrelated pairs of individuals, concluding that tapirs in this region have no preference for being close to relatives of either sex, which may suggest that both sexes are prone to dispersing. The opposite was found for the neotropical otters (*L. longicaudis*) in the Atlantic Forest in southern Brazil, where the social organization of this species appears to be highly influenced by relatedness, since relatives were usually found in

proximity, with this organization driven by female philopatry (Trinca et al. 2013). Otters are assumed to occur at low densities and show an elusive behavior, but they also usually defecate in latrines along the margins of rivers (Kruuk 2006), which facilitates the use of non-invasive sampling for this species. Trinca et al. (2013) employed a non-invasive approach combined with the amplification of ten microsatellite loci to assess demographic parameters, spatial organization and relatedness within this neotropical otter population.

Biologists are frequently limited to a small number of already available microsatellite loci to study relatedness in captive and wild populations. The development of next-generation sequencing has made it possible to identify thousands of single polymorphism nucleotides (SNP), which increases our ability to distinguish between individuals and their relatedness. However, the studies of neotropical mammals still make poor use of this methodology. Although relatedness analysis has many applications, studies would generally benefit from sampling as many individuals as possible within and across populations of interest, as well as from using a panel of markers that is sufficiently informative (either microsatellites or SNPs) (Pemberton 2008).

### *16.4.2 Dispersal and Philopatry*

Dispersal can be defined as "the movement the animal makes from its point of origin to the place where it reproduces or would have reproduced if it had survived and found a mate" (Howard 1960). On the other hand, philopatry is the opposite behavior, and can be defined as "the faithfulness of an individual to its natal and breeding site or group" (Greenwood 1980). Philopatry causes related individuals to remain in proximity, whereas dispersal promotes the geographical separation of related individuals, directly influencing the genetic structure pattern of a species. The clustering of related individuals due to philopatry is found not only in gregarious species, but also in solitary ones (Waser and Jones 1983), which is generally expressed in the form of overlapping home ranges (Ratnayeke et al. 2002; Quaglietta et al. 2013). Due to the differences in constraints and advantages experienced by individuals of each sex, behavior can differ between sexes (Greenwood 1980; Dobson 1982). Dispersal is often sex-asymmetrical, and the tendency for each sex to disperse or remain philopatric has been strongly correlated with the species mating system.

Dispersal is commonly associated with three main causes, which are not mutually exclusive: competition for mates, competition for resources and inbreeding avoidance (Packer 1979; Greenwood 1980; Dobson 1982; Moore and Ali 1984; Pusey 1987). In polygamous mammals, dispersal is generally male-biased, whereas females are prone to philopatry (Greenwood 1980). In this case, males can benefit from dispersal because this is likely to increase their access to females, decrease competition with resident dominant males, and avoid inbreeding with related females. In polygamous species where females are primarily responsible for parental care, not dispersing allows them to take advantage of their knowledge of local

resources; when resources are available, they are more likely to share their home ranges with daughters, but they are less likely to allow the permanence of male offspring in order to avoid inbreeding (Waser and Jones 1983; Pusey and Packer 1987; Sandell 1989). In monogamous mammals, dispersal should be equally frequent in both sexes, and parents do not evict either sex, because fathers do not have to compete with their sons for the breeding female, and the breeding of female offspring does not incur any cost to the mother in populations that are not at carrying capacity (Dobson 1982; Liberg and von Schantz 1985). It is important to highlight that there are exceptions to these predictions (e.g., Dechmann et al. 2007; Nagy et al. 2007; Blair and Melnick 2012).

There are two main approaches to inferring sex-biased dispersal from genetic data: population-level analysis and individual-level analysis (Banks and Peakall 2012). The first considers the set of samples (either a population or group of individuals) as the unit of analysis. This approach includes analyses such as F-statistics and assignment tests, which are based on the expected genetic signature of male and female individuals within populations (Goudet et al. 2002). At the individual level, multilocus genotypes of individuals are the units of analysis, for example, when doing correlation (i.e., Mantel test and spatial autocorrelation analysis) and relatedness analyses (Prugnolle and De Meeûs 2002; Banks and Peakall 2012). Population genetics methods (e.g., F-statistics and assignment tests) are better explored elsewhere, as in Templeton (2021).

One way of inferring sex-specific dispersal is through spatial autocorrelation analysis. Spatial autocorrelation analysis (Fig. 16.4) is constructed on the basis of two matrices (genetic and geographic distances between individuals). Pairs of individuals are categorized according to classes of distances between them in order to test, at each distance class, if the individuals are more or less genetically distant than

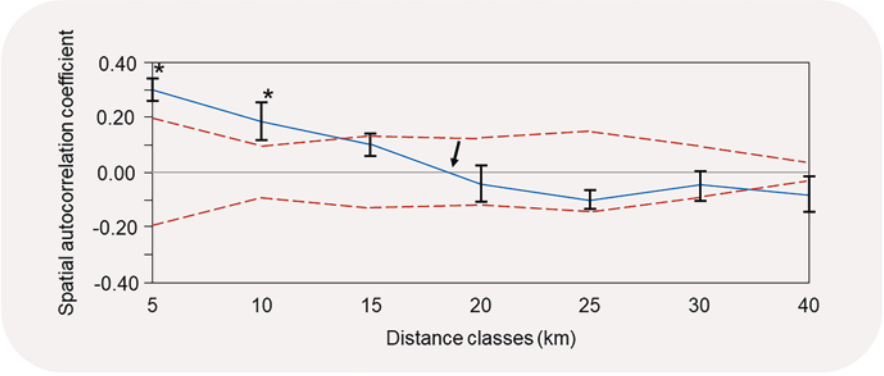


**Fig. 16.4** Example of a spatial autocorrelogram of a species where individuals that are nearby (up to 10 km apart) are more genetically similar than expected, which could be an indication of philopatry or restricted dispersal. Blue lines connect the autocorrelation values of each distance class. Dashed red lines represent the upper and lower limits of the null distribution. Confidence error bars (usually 95%) are shown in black. Asterisks indicate significant spatial autocorrelation values (usually considering a p-value of <0.05). Arrow indicates the intercept with x-axis

what would be expected by chance (no spatial genetic pattern) (Smouse and Peakall 1999). This analysis can be performed for each sex separately in order to assess sex-specific patterns of dispersal (Gour et al. 2013), or with both sexes pooled together to assess the general spatial organization of the population (Schmidt et al. 2016; Wultsch et al. 2016).

Wultsch et al. (2016) assessed the dispersal of pumas (*P. concolor*), jaguars (*P. onca*) and ocelots (*L. pardalis*) in Belize, Central America. The authors opportunistically collected fecal samples from protected and unprotected areas to assess the human impact on the genetic structure of these species. After the species were identified through mitochondrial DNA sequencing, 14 species-specific microsatellites were amplified in order to individualize the samples, and the sex of the individuals was determined through the amplification of two genes that are only present in the Y chromosome. The authors used assignment tests and spatial autocorrelation analysis (SAA) to examine the spatial extent of the genetic structure (Fig. 16.4), modulated by dispersal, and to determine if dispersal was sex-biased in each of the three species, as predicted for other polygamous felids (de Oliveira et al. 2021). Due to the small number of samples, female jaguars and male ocelots were not analyzed separately. Genetic association was shown to occur between the jaguars (mostly males) that were less than 20 km apart from each other. Concerning the male pumas, no spatial autocorrelation was verified across all distance classes, suggesting an absence of spatial structure caused by dispersal; on the other hand, female pumas showed positive autocorrelation up to 23 km apart, indicating female philopatry. Female ocelots that were less than 83 km apart from one another showed genetic association. These results suggested female philopatry, and also that ocelots could be more successful moving through human-dominated landscapes than the other two species. Jaguars are bigger than pumas, but the latter are known to travel longer distances in their dispersal movements, even when moving through fragmented areas (Stoner et al. 2008), whereas the former typically prefers forested areas (Crawshaw and Quigley 1991). Results suggested subdivisions in the genetic structure of male jaguars, but not in male pumas, likely because jaguars are more sensitive to disturbed areas.

Species differ markedly in their dispersal distances (Whitmee and Orme 2013) and abilities to overcome human disturbances in the landscape. Less mobile species that rely on forest cover may be more affected than generalist and highly mobile carnivores. Groups of reintroduced golden lion tamarins (*Leontopithecus rosalia*) were monitored and the dispersal pattern (distance and sex bias) could be investigated in the Atlantic Forest in southeastern Brazil (Moraes et al. 2018). Hair samples were collected, and 14 microsatellite loci were used to generate individual genotypes. Dispersal potential was assessed with basis on the distance between locations where shared alleles were found, whereas sex bias was assessed through spatial autocorrelation analysis. Golden lion tamarins were found to effectively disperse up to 8 km, but gene flow was high only within a two km radius. The authors also observed no sexual bias in the frequency of effective dispersal, which is expected for monogamous mammals, as is the case of golden lion tamarins, although they found evidence of sexual bias in dispersal distances. The absence of sex bias in

effective dispersal is relevant for the conservation of the species, because it promotes a higher gene flow and mitigates the effects of reproductive skew in monogamous mating systems.

Although the behavior of dispersal has been strongly correlated to mating, there are exceptions. The greater sac-winged bat (*Saccopteryx bilineata*) in Costa Rica, for instance, has a mating system described as resource-defense polygyny, in which dispersal was found to be female-biased and males form a patrilocal colony structure (Nagy et al. 2007). These results were based on a panel of 11 microsatellites that were used to conduct paternity and relatedness analysis on a colony monitored for eight years. For this species, it was proposed that inbreeding avoidance was the main force driving the dispersal of females, since there was a generational overlap between philopatric fathers and their female offspring. The authors suggested that this behavior evolved from a state of complete offspring dispersal, as both the male and female offspring of different species within the same genus are prone to dispersing.

Local circumstances can also influence the propensity of individuals to disperse or remain philopatric. For instance, changes in the social organization pattern of primates have already been suggested to be the result of anthropogenic impacts (Di Fiore et al. 2009). Oklander and Corach (2013), working with *Alouatta caraya*, used a panel of eleven polymorphic DNA microsatellite markers to estimate kinship and maternity/paternity relations of juvenile and subadult individuals in eleven social groups dwelling in fragmented areas, and seven social groups in a continuous forest, in Argentina. Based on the obtained data, they found that both males and females from the groups living in the continuous forest dispersed, whereas dispersal was male-biased in the groups dwelling in fragmented forests, and this affected the relatedness among individuals within their respective social groups. In the groups dwelling in the continuous forest, adults were not closely related – whereas, in the fragmented forests, most adult females were related (Oklander and Corach 2013). These findings suggest that habitat fragmentation alters the ability of *A. caraya* to disperse, thus increasing the occurrence of inbreeding, which, in the long term, threatens the populations living in modified landscapes (Oklander and Corach 2013). For the guigna (*Leopardus guigna*) from Chile, increased dispersal distances were correlated with increased fragmentation (Napolitano et al. 2015). In Chloé Island (Chile), Napolitano et al. (2015) used a combination of biological samples (blood from captured animals, feces and tissue from road-kills and retaliatory kills) and molecular markers (mitochondrial DNA sequences, 15 microsatellite loci and two sex chromosome genes) to investigate the influence of fragmentation on the genetic diversity, kinship, inbreeding, and dispersal of guigna. The authors utilized a combination of relatedness and spatial autocorrelation analyses to infer on dispersal. In more pristine areas, dispersal was lower, probably because of a greater abundance of resources, whereas in the more fragmented areas dispersal rates were higher, which may reflect a strategy aimed at reducing competition over scarce resources. Therefore, in general, dispersal and philopatry are dynamic processes that can be shaped by the costs and advantages of dispersing or remaining

philopatric, and these processes have a significant impact on the genetic structure of populations.

## 16.5 Concluding Remarks

The investigation of ecological questions and the assessment of species with cryptic behaviors have been greatly advanced thanks to the use of molecular tools. Utilizing mitochondrial and nuclear markers, we can describe aspects of biodiversity even when working with low-quality biological samples. We can obtain valuable data as simple as which species occur within an area and their individualized information, which may be used to infer spatial distribution, individual behavior (e.g., use of space) and interspecific interactions (e.g., territoriality), to more complex relationships concerning individuals and populations (Fig. 16.1). All this information, associated with the ecological data gathered through traditional methods, can be very useful to assess biological patterns and processes, and for implementation of conservation efforts, which are especially needed in the current scenario of biodiversity loss promoted by human activities.

The conservation status of mammals around the world is worrisome. Given the profound impact that humans have had on the environment during the Anthropocene, it is imperative that we understand which ecological processes are being affected, as well as the original states of these processes, which can still be detected in well-preserved areas. The molecular ecology tools can be very helpful in achieving that. However, the application of genetic data for answering ecological questions and supporting strategies for the conservation of neotropical mammals remains underdeveloped or employed with a focus limited to certain groups (Torres-Florez et al. 2018). Therefore, the number of molecular ecology studies must be increased and combined with other disciplines, as to enrich our knowledge of neotropical mammals and strengthen biodiversity conservation efforts.

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# Chapter 17
# Molecular Tools to Analyze the Effects of Roads on Wildlife in the Neotropics


**Carmen E. Barragán-Ruiz, Carla C. Gestich, Clarissa Rosa, and Clara Grilo**

## 17.1 Introduction

Transport infrastructures, whether by land, water, or air, are critical for economic development and people movement. Roads are predominant in some regions of the world as they are the principal way of transportation among cities and countries (Torres et al. 2016). This transportation infrastructure has expanded in recent decades, which has raised conservationists' concerns about its impact on biodiversity (Van Der Ree et al. 2015; Kent et al. 2021; Toth et al. 2022).

An additional 25 million kilometers of roads are expected by 2050 in the world, especially in developing countries that host high biodiversity (Alamgir et al. 2017; Meijer et al. 2018). Roads are shaping the landscapes (Iuell et al. 2003) and their implications on biodiversity are largely unknown. It is well documented that roads contribute to habitat loss and fragmentation, but also to habitat degradation, due to increased light, noise, and atmospheric pollution (Balkenhol and Waits 2009; Jackson and Fahrig 2011). Mortality from collisions with vehicles is another

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principal negative effect of roads on several species, and all these treats can increase the risk of extinction (Grilo et al. 2021). In addition, collisions pose a threat to human safety and incur an economic cost to society, especially when occurring with medium and large animals (Abra et al. 2019).

Understanding how roads affect wildlife and proposing mitigation measures to maintain long-term viable populations are the central issue of road ecology studies, in which molecular tools have been promoted as a great advance to quantify the impacts on populations and to estimate extinction risk (Balkenhol and Waits 2009).

## 17.2 Road Impacts on Wildlife

Roads impact individuals, populations, species, ecosystems, and landscapes in various ways, encompassing ecological and genetic aspects (see Van Der Ree et al. 2015). Mortality due to collision with vehicles, habitat loss and fragmentation, barrier to animal movement, pollution, and human proximity to natural areas are the main negative effects of roads on wildlife (Fig. 17.1). For many species, the negative impacts of roads on wildlife may include a drastic reduction in population size

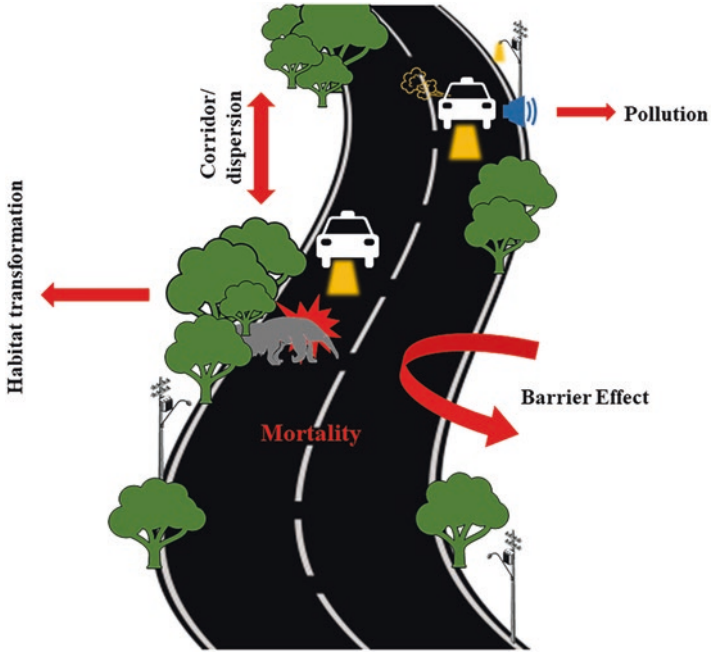


**Fig. 17.1** Cartoon representation of the principal consequences of road transport infrastructure on wildlife. (Author: Barragán-Ruiz 2023)

and change in the dynamic of populations which can cause a reduction in population viability and increase the risk of local extinctions in the long term.

Every year, millions of animals are killed on roads worldwide (González-Suárez et al. 2018; Grilo et al. 2018; Schwartz et al. 2020). The individual loss due to additional mortality from collision with vehicles may decrease the genetic diversity in natural populations due to the reduction in the effective population size (Frankham 2005). However, high roadkill rates do not necessarily imply population reduction, as long as the species is abundant and has high birth rates, which would overcome this additional source of mortality (Grilo et al. 2021). But even low roadkill rates in small populations may have a major impact on long-term persistence. For example, populations of maned wolf *Chrysocyon brachyurus* in Uberlândia-Uberaba region (Brazil) and southern tiger cat *Leopardus guttulus* in western Santa Catarina (Brazil) may be at risk of extinction if observed roadkill rates persist in the surveyed regions (Grilo et al. 2021).

Another effect of collision of vehicle and wildlife is the gender bias when members of one sex from a species are more prone to be roadkilled due to the greater tendency to disperse and move than members of the opposite sex, as is the case of several species of mammals and birds (Greenwood 1980; Pusey 1987; Kuijper and Johnstone 2017; Oliveira et al. 2022). Evolutionary explanations of sex-biased dispersal have been the subject of persistent debate, but it is suggested to be a mechanism to avoid inbreeding (Reed and Frankham 2003; Pike et al. 2021). In such cases, individuals dispersing long distances are more susceptible to the negative effects of roads. Sex bias on road mortality also affects the population structure when movements are associated mainly with foraging, reproduction, or dispersal to establish new groups (Aresco 2005; Shepard et al. 2008; Fensome and Mathews 2016). For example, Moore and Mangel (1996) predicted population declines for barn owls *Tyto alba* due to higher road mortality rates for females in California, concluding that species with long generation times and low reproductive rates may exhibit higher population declines. In the Neotropics, it was also observed higher roadkill mortality of male southern tamanduas, *Tamandua tetradactyla*, inhabiting the Brazilian savanna (Barragán-Ruiz et al. 2021a). A similar pattern was also verified in cougars, *Puma concolor*, showing male-biased mortality in roads crossing the Atlantic Forest, possibly as a result of the philopatric behavior of females and the dispersion of males to new territories (Saranholi 2018). The loss of individuals may represent a reduction of genetic diversity and may result in drastic changes in the effective population size over time (Forman and Alexander 1998; Reed and Frankham 2003).

Habitat loss and fragmentation are anthropogenic processes that are also modulated by roads and can influence species richness and population size (Rytwinski and Fahrig 2007; Holderegger and Di Giulio 2010). Road networks can fragment the habitat for several species leading to the isolation of populations (Andrén 1994; Canters et al. 1997; Iuell et al. 2003). The lack of habitat connectivity caused by road-induced fragmentation (Fig. 17.2) may decrease the individual likelihood of moving between patches which can modify the dispersal dynamics of species, altering their behavior, and reducing the interchange of individuals among populations

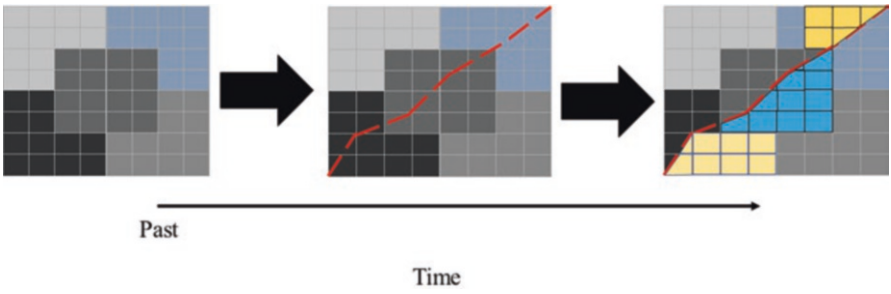


**Fig. 17.2** Schematic representation of the effects caused by roads, promoting fragmentation on local populations (colors) in time. A road is represented by the red line. (Adapted from Barbosa et al. 2020)

which will affect the population genetic diversity (Riley et al. 2006; Holderegger and Di Giulio 2010; Esperandio et al. 2019). Furthermore, certain alleles might get arbitrarily fixed or lost by genetic drift across generations. The increased inbreeding may result in harmful mutations and lower fitness, risking long-term population survival (Frankham 2010).

The barrier effect are also determined by the characteristics of the road and the surrounding environment (e.g., high slopes, presence of guard rail, number of lanes, noise, light and traffic rate) that, associated with morphological characteristics of individuals, risk perception, feeding and mating behavior will determine the ability of species to cross a physical barrier (Forman and Alexander 1998; Esperandio et al. 2019). The pattern of mortality according to the type of road surface and traffic noise pollution is modulated by morphological limitation and behavior of species, as demonstrated in North American mammals in which collision with vehicles increases with body size and for herbivores and omnivores (Ford and Fahrig 2007). In addition at night, vehicle collisions with wildlife are more predictable because of the activity pattern of some species, and because the vehicles' lights can lure several animals to the road (Mendes et al. 2020). Also, nocturnal insects are attracted to roads, which in turn can increase the number of their predators such as birds and flying mammals on the road (Benítez-López et al. 2010; Fensome and Mathews 2016; Damásio et al. 2021).

In sum, the most important effects of roads on wildlife may be divided into two major consequences (ecological and genetic) over time, starting at time zero (state A in Fig. 17.3), when road structure was non-existent, to a time when road infrastructure is the cause of changes in gene flow and genetic diversity (state C in Fig. 17.3), causing impacts from individual losses to local extinctions (Fig. 17.3). Although building a road can immediately affect local population, for example by individual loss by roadkill, detecting genetic signs on the populations may take some years or up to long generations. In this sense, monitoring the potential negative consequences of the presence of roads on genetic diversity is crucial to measure the impact of roads on species (Fig. 17.2).

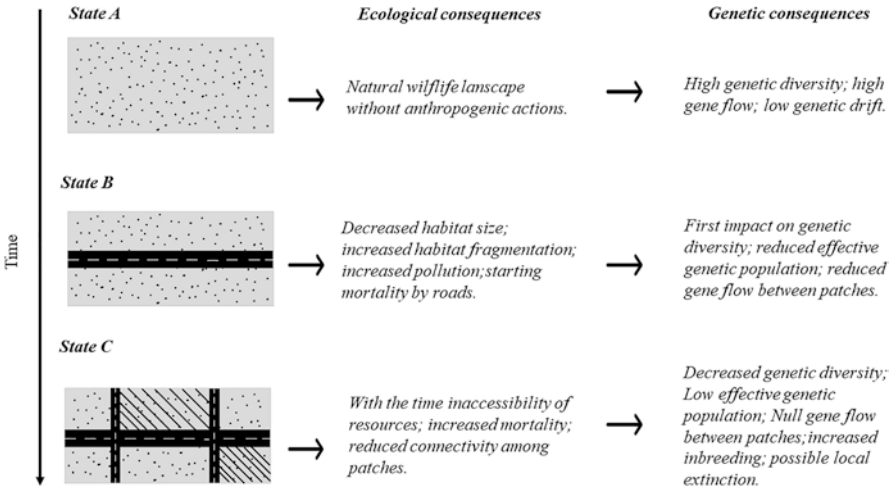


**Fig. 17.3** Representation of the effects of roads on ecological and genetic aspects of wildlife populations. (Adapted from Fahrig 2003; Balkenhol and Waits 2009)

## 17.3 Molecular Road Ecology in the Neotropics

Studies on road effects that use genetic analysis are scarce in Neotropics. A literature review was conducted on the databases Web of Knowledge, Science Direct and Google Scholar using combinations of the following search terms: ("road OR highway & molecular analysis" OR "road OR highway & genetic diversity" OR "gene flow & roads OR highway") & Neotropic* in three languages (Portuguese, Spanish and English). We only found 13 studies that performed a molecular approach using neotropical road-killed vertebrate's samples; nine were with anteaters, felids, and canids (Miotto et al. 2011; Klippel et al. 2015; Saranholi et al. 2016; Cosse et al. 2017; Rodríguez-Castro et al. 2017; Esperandio et al. 2019; Barragán-Ruiz et al. 2021a, b; Pizzano et al. 2021).

Molecular analyses for road ecology studies in Neotropics have been mainly used to identify species (e.g., Klippel et al. 2015; Saranholi et al. 2016; Rodríguez-Castro et al. 2017; Damásio et al. 2021) and two studies assessed genetic diversity parameters evaluating the consequences of roads on populations (Esperandio et al. 2019; Barragán-Ruiz et al. 2021b). Only one used roadkill samples to assess the gender of individuals (Browett et al. 2020) and another investigated phylogenetic relations among sampled individuals (Cosse et al. 2017). From all of them, five studies (Miotto et al. 2011; Bou et al. 2021; Pizzano et al. 2021; Rodríguez-Castro et al. 2022; Salom-Pérez et al. 2022) opportunistically used roadkill vertebrate samples to analyze population parameters (Table 17.1).

A diverse set of biological samples can be found in these surveys to obtain DNA to perform genetic analysis, such as non-invasive (e.g., feces) or tissue samples

**Table 17.1** List of articles that used roadkill vertebrate samples in a road ecology or an opportunistic way

| Reference | Country | Group/ Species | Molecular approach | Analysis | Study aims |
|---|---|---|---|---|---|
| Miotto et al. (2011) | Brazil | Felids | Microsatellites | Sample identification | Population genetics |
| Klippel et al. (2015) | Brazil | Vertebrates | DNA barcode | Sample identification | Road ecology |
| Saranholi et al. (2016) | Brazil | Vertebrates | DNA barcode | Sample identification | Road ecology |
| Cosse et al. (2017) | Uruguay | Fox | DNA barcode | Phylogenetic analyses | Phylogeography |
| Rodriguez-Castro et al. (2017) | Brazil | Vertebrates | DNA barcode | Sample identification | Road ecology |
| Esperandio et al. (2019) | Brazil | Rodent | Microsatellites | Population analyses | Road ecology |
| Barragán-Ruiz et al. (2021b) | Brazil | Xenarthras | Microsatellites | Population analyses | Road ecology |
| Barragán-Ruiz et al. (2021a) | Brazil | Xenarthras | Molecular sex | Sex identification | Road ecology |
| Bou et al. (2021) | Uruguay | Felids | Microsatellites | Population analyses | Population genetics |
| Pizzano et al. (2021) | Argentina | Canids | Microsatellites | Population analyses | Population genetics |
| Damasio et al. (2021) | Brazil | Bats | DNA barcode | Sample identification | Road ecology |
| Rodriguez-Castro et al. (2022) | Brazil | Canids | Microsatellites | Population analyses | Population genetics |
| Salom-Pérez et al. (2022) | Cosa Rica | Felids | Microsatellites | Population analyses | Population genetics |

Country, group/species, molecular approach, analysis, and study aims of each of one

(hair, bone, blood, skin, and muscle). When working with roadkilled animals, the carcasses from collisions can provide a wide range of samples from muscle to nails, depending on the state of the carcass (Barragán-Ruiz et al. 2021a). The major challenge for the molecular approach is to find samples on roads that are in good condition for high-quality DNA extraction because the quality of carcasses is dependent on various environmental factors such as temperature, precipitation, scavenging, traffic, size of the animal, and time till detection (Slater 2002).

### *17.3.1 Molecular Species Identification*

Species identification is pivotal in road ecology studies to a better understanding of which species are most roadkilled and where roadkillings occur. Traditional methods for species identification of roadkill carcasses are usually based on morphological traits and for some taxa identification is dependent on the expertise of specialists. Nonetheless, with molecular techniques for species identification in road ecology, just a piece of a biological sample of a dead individual's body is enough to precisely identify species (e.g., Klippel et al. 2015; Rodríguez-Castro et al. 2017).

Species identification using the barcode approach is based on the amplification of specific fragments from the mitochondria DNA (Hebert et al. 2003). With this approach, it is possible to molecularly distinguish one species from a unique sequence of about 600 base pairs (bp) (e.g., COI gene, Folmer et al. 1994). However, obtaining this unique sequence with specific size and informative regions depends mainly on the quality of DNA sample and the accurate identification depends on the availability of reference sequences in databases (e.g., GenBank and BoldSystem).

Molecular species identification in Neotropics has been helpful for a variety of species, especially small vertebrates. Rodriguez-Castro et al. (2017) were able to compare the landscape structural elements at roadkill locations between functional groups of small vertebrates categorized according to their mobility. The authors identified species by amplifying two mtDNA regions (COI and 16S). DNA Barcodes technique using the mtDNA (COI gene) amplification was also used to identify 222 roadkilled vertebrate samples that often cannot be morphologically determined in Espirito Santo, Brazil (Klippel et al. 2015). The authors used a positive control of 23 species that have been identified by morphological characteristics and tested the barcode technique in all roadkill samples. They successfully amplified 138 (62.16%) samples, from which they identified 62 different species.

Another example of this application is demonstrated by Damásio et al. (2021) that used the same technique to identify the diversity and abundance of roadkilled bats in the Brazilian Atlantic Forest. In this case, the authors collected a total of 773 individuals over five years and used DNA barcoding to identify 14.15% of the samples. However, Klippel et al. (2015) and Damásio et al. (2021) showed that the absence of reference sequences in different molecular databases as BoldSystem and GeneBank impairs the identification of many species, especially reptiles.

### *17.3.2 Molecular Sex Identification*

Although for most species sex determination relies on the observation of sexual morphological features in the field, for some groups, such as Xenarthrans, Anura, and Squamata, sex identification is only possible by the observation of the ventral region, which is a rare event *in situ*. Morphological sex identification of dead individuals from the collision with vehicles, depends initially on the degree of carcass

degradation that in turn depends on the size of the animal and local temperature, precipitation and quantity of pollution (Santos et al. 2011). In this sense, molecular analysis can fill this gap and has become a critical and easier tool for the sex identification of carcasses (Prithiviraj and Melnick 2001; Shaw et al. 2003; Barragán-Ruiz et al. 2021a).

The most used molecular markers for molecular sex identification are based on the SRY, Zinc Finger (*ZF*) and amelogenin (AMELX and AMELY) genes. When using sex identification based on the SRY marker, the results can indicate the sample as male when amplifying the SRY region because it is present in the Y chromosome, while no amplification indicates the sample as from a female. However, using a unique fragment for molecular identification may be problematic, especially in degraded samples, because it can indicate a false female result due to non amplification of the target region. Therefore, the use of molecular markers for sex identification based on regions present in both chromosomes is more adequate. This approach has been implemented for different types of samples and many species including amphibians, reptiles, birds, canids, xenarthrans, and primates with primers and protocols have been described specifically for each species (DeMatteo et al. 2009; Martinelli et al. 2010; Morinha et al. 2013; González et al. 2015; Hrovatin and Kunej 2018). Unfortunately, it has not been widely used to address Road Molecular Ecology questions. We only found one study in the Neotropics that identified the sex of 601 roadkill samples from different Xenarthrans species (Barragán-Ruiz et al. 2021a).

### *17.3.3 Population Analyzes*

With an appropriate sampling scheme, molecular tools and genetics can provide information on population structure, functional connectivity, and effective population sizes (Frankham et al. 2010; Akemi et al. 2012). To analyze the barrier effect, we need to quantify the gene flow between the sides of the road. It is also important to collect samples from live animals in natural areas, to identify the population of origin of the dead animal, as well as estimate genetic parameters on natural populations (e.g., Esperandio et al. 2019).

Additionally, a diverse set of approaches, such as spatial autocorrelation (Mantel test and Spatial correlation analysis) and landscape analysis combined with genetic data can be used to address a high diversity of questions related to the impact of roads on wildlife (e.g., Reh and Seitz 1990; Barragán-Ruiz et al. 2021b). For this, nuclear markers, such as microsatellites (SSR) (see more Chap. 16), are used in population analyses to assess values of genetic diversity to make inferences on population structure, generally based on fixation index ($F_{ST}$) to estimate the effectiveness of gene flow and the movement of individuals among patches (Weir and Cockerham 1984; Yumnam et al. 2014; Grilo et al. 2016). Furthermore, the allele

frequencies can be used to estimate demographic changes in the future and to estimate the relatedness among individuals, although few studies have attempted it so far (Cabrera and Palsbøll 2017; Lowe et al. 2017).

Although some surveys have been developed in the Neotropics to integrate genetic data with road ecology, there are still few studies reported, most of them in Brazil (Rodríguez-Castro et al. 2017; Esperandio et al. 2019; Damásio et al. 2021; Barragán-Ruiz et al. 2021b). For example, for a subterranean small rodent, the tiny tuco-tuco *Ctenomys minutus*, Esperandio et al. (2019) analyzed 80 tissue samples, from four different colonies to test whether the patterns of differentiation and genetic structuring differ between pair of colonies separated by a road, comparing that with a pair separated by a similar distance of only grassland. In this case, Esperandio et al. (2019) used 14 microsatellites and assessed the diversity index as well as the fixation index ($F_{IS}$) and concluded that the road does not seem to act as a barrier for the gene flow between the roadside colonies, but it can represent the beginning stage of population structure (Fig. 17.2). Another study assessed the genetic diversity and population structure of 107 roadkilled giant anteaters, *Mymercophaga tridactyla* using a total of 10 microsatellite loci (Barragán-Ruiz et al. 2021b). In this particular case, they evaluated gene flow and population structuring by roads using a wide variety of genetic analyses: Bayesian assignment and population spatial clustering models and multivariate approach to define the genetic structure, Approximate Bayesian Computation (ABC) to investigate population reduction and demographics approaches to find the sign of effective population size variation. Contrary to their expectations, the researchers did not find a signal of a lack of gene flow or population structuring. However, it was possible to find signs of population decline in the next 100 years, and the individual loss caused by road mortality can have a great contribution to this negative scenario.

Roadkill samples can also be used in an opportunistic way to study population dynamics by using samples obtained from carcasses of dead animals on the road. For instance, Rodríguez-Castro et al. (2022) and Miotto et al. (2011) showed the importance of using roadkill samples as a source of genetic material of large mammals to increase sampling and obtain more resolution in genetic analyses. In the study of Miotto et al. (2011), 10% of all analyzed samples were from roadkill animals. The authors used 12 species-specific microsatellite loci to demonstrate that the population of cougars (*Puma concolor*) in the northeast of São Paulo state does not suffer from endogamy or population fragmentation but show evidence of decreased effective population size due to human expansion. Also using microsatellite genotyping, Rodríguez-Castro et al. (2022) were able to estimate genetic diversity, population structure, identify migrants and population size of the maned wolf *Chrysocyon brachyurus*, and 17% of all analyzed samples came from roadkill animals. Thus, as a result, they suggested isolation by distance (IBD), spatial genetic population structuring, and bottleneck signatures for the maned wolf population.

## 17.4 Concluding Remarks – Implications to Neotropical Wildlife Conservation

Over the last decade, conservation initiatives and research organizations have recognized that biodiversity is more than the number of species and that genetic variation is an important component of it. Similarly, molecular techniques have revealed that methods to study road impacts, such as genetic analyses, are valuable to identify not only species but also for evaluating population structure, estimating barrier effect, assessing functional connectivity, and estimating effective population size. However, when considering the road ecology and the effects of road networks on neotropical biodiversity, the number of research projects being developed to study the effects of roads using molecular tools is still scarce. Considering the huge number of animals killed by roads annually, an immeasurable amount of genetic information is wasted when such carcasses are just discarded. Fortunately, several studies have been collecting biological samples opportunistically from dead animals on roads, even though not aiming to answer road ecology questions (Bou et al. 2021; Pizzano et al. 2021; Salom-Pérez et al. 2022). Extracting all kinds of biological and ecological data from dead animals is a small contribution to science. However, it is important to use molecular studies that provide valuable and accurate insights into the impacts of roads on wildlife and therefore guidance on where to mitigate those impacts. It is, therefore, critical to establish multidisciplinary teams with geneticists, ecologists, and road planners in road studies to provide the best science-based information to guide stakeholders and to avoid or minimize the impact of roads on wildlife. Future research must also consider evaluations before and after building transport infrastructures to design effective control mechanisms to prevent the negative effects of roads on the neotropical wildlife.

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# Chapter 18
# Environmental and Invertebrate-Derived DNA: A Powerful Approach for Surveying and Monitoring Biodiversity

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## 18.1 Introduction – eDNA and iDNA for Monitoring Biodiversity

Biodiversity is facing a worldwide crisis (Brooks et al. 2002; Bellard et al. 2012; Dirzo et al. 2014; Haddad et al. 2015). Thus, species monitoring is currently a top priority for biodiversity protection. However, owing to various limitations, traditional biodiversity monitoring methods may not always achieve the given monitoring purpose, raising the urge for additional methods capable of reliably monitoring species on wider spatio-temporal scales. Aiming to fill this gap, the advances in high-throughput sequencing paved the way for a new era in the biodiversity monitoring field, with recent advancements now opening new opportunities for studying biodiversity by sequencing DNA retrieved from a plethora of sampling media. Some of the main emerging techniques rely on the collection of traces of DNA present in the environment, the so-called "environmental DNA" (eDNA). Since all organisms release DNA continuously into the surrounding environment (e.g., shed skin, excretes, gametes, saliva, hair, feathers, scats), eDNA surveys aim to obtain such DNA remnants from environmental samples (e.g., water, sediments, soil, air,

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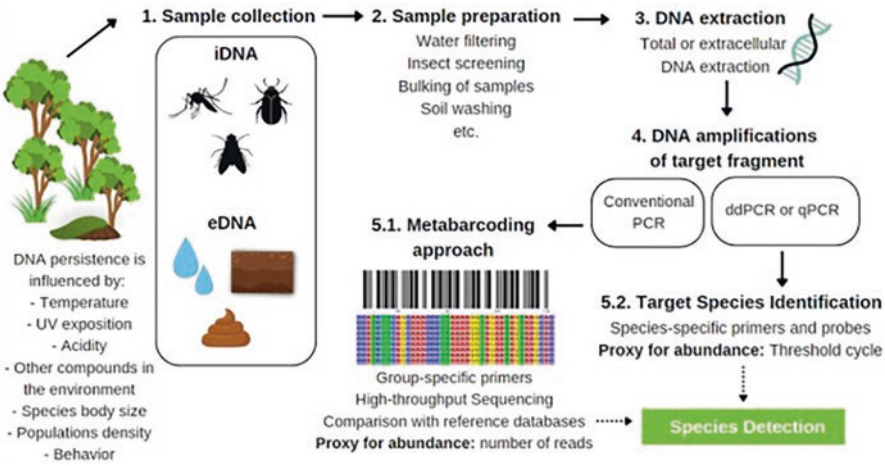


**Fig. 18.1** Diagram of environmental DNA (eDNA) and invertebrate-derived DNA (iDNA) applications for surveying and monitoring biodiversity

lake and ice cores) and identify the taxa through the use of specific molecular markers (Fig. 18.1).

Also, as in a science fiction movie, invertebrates that feed on vertebrates or use them to fulfill vital functions of their cycle (e.g., oviposition) have been used as a source of vertebrate DNA. Ingested DNA or invertebrate-derived DNA (iDNA) studies (Calvignac-Spencer et al. 2013; Schnell et al. 2015; Drinkwater et al. 2021) have previously investigated leeches, mosquitoes, flies and beetles to successfully survey wildlife and in some situations to conduct ecological and biodiversity assessments (Fig. 18.1).

Species detection studies can be focused on targeting a single species or the whole community within an area. Single-taxon approaches use specific narrow-target molecular markers and PCR, ddPCR, qPCR amplification (Ficetola et al. 2008; Takahara et al. 2013; Piaggio et al. 2014), while community-level approaches use universal markers and parallel sequencing to detect a broad range of taxa (i.e., "metabarcoding") (Wilcox et al. 2013; Rees et al. 2014; Thomsen and Willerslev 2015; Turner et al. 2015). Species-specific essays have been proven as the most suitable method when targeting one or few species, especially when considering rare and difficult-to-detect taxa (e.g., Hernandez et al. 2020), whilst the metabarcoding approach has been shown as a more efficient and cost-effective option for a broad characterization of the ecosystem, also allowing the detection of unexpected species or hidden diversity (Gillet et al. 2018).

Genes COI for animals, and rbcL and matk for plants were previously chosen for species identification by sequencing biological samples of individual specimens in the DNA barcoding approach (Hebert et al. 2003; Hollingsworth 2011). However, for these emerging approaches, the development of new molecular markers was and still is necessary, especially because the DNA obtained from eDNA and iDNA samples is often degraded. The need to choose smaller fragments (mini-barcodes) for the amplification of degraded genetic material, which is expected in eDNA and

iDNA samples, led to the use of new genes and new primers to be targeting these shorter gene regions. For animals, the use of primers targeting small fragments, about 50–170 bp long, from the genes 12SrRNA (targeting vertebrates) (e.g., Riaz et al. 2011; Miya et al. 2015) and 16SrRNA (mammals, e.g., Taylor 1996, and frogs e.g., Bálint et al. 2018) have provided satisfactory results for the identification of the biodiversity when working with mixed DNA samples. For plant identification, the use of the genes matK and rbcL was complemented with ITS (Hollingsworth 2011; Song et al. 2012) and trnL fragments (Riaz et al. 2011; Fahner et al. 2016). The matK has provided better results when used for invasive species identification due to the presence of specific regions that amplify only in target species (Scriver et al. 2015). ITS2 and rbcL were more efficient in general studies with vascular plants, when not using customized reference databases or local surveys (Fahner et al. 2016). However, the chloroplast trnL (UAA) intron is proposed as the most suitable marker for plant eDNA metabarcoding (Riaz et al. 2011; Coissac et al. 2012), although the reference database must be supplemented (Taberlet et al. 2007).

Since its onset, the number of studies that have addressed different aspects of the application of environmental DNA towards the detection of species has increased almost exponentially. This has led to a recent and broad knowledge gathered in this field, mainly in temperate and already well-studied ecosystems where eDNA and iDNA have been used especially for species detection. However, the combination of eDNA and iDNA samples with NGS technologies has great potential for population genetic studies, as shown by Adams et al. (2019). Still, eDNA and iDNA-based surveys in the neotropical realm, a high biodiversity region that is of great conservation concern, are still incipient, and a boost of studies is foreseen for the next few years. In the following sections, we will focus on demonstrating how eDNA/iDNA approaches have been used to study vertebrate and plant species, with a greater focus on the neotropical region.

## 18.2 Environmental Samples

In biodiversity and conservation surveys, a myriad of biological samples can be obtained in the environment, including scats or gut content to assess the microbiota, understand the species' food preferences or ultimately, conduct biodiversity assessment by analyzing the species which the organisms have fed upon through the identification of gut content, and build interaction networks. Samples collected from the environment without the requirement of handling and/or seeing the animal or its traces are collectively categorized as environmental samples (Lacoursière-Roussel and Deiner 2021). In eDNA studies, some examples of sampling media include soil and sediments to study both micro and macro-organisms (e.g., from bacteria to large mammals) (Kestel et al. 2022); permafrost to detect ancient DNA (aDNA) and investigate past biodiversity history (Willerslev et al. 2003); air for pathogen detection or even in detecting the presence of terrestrial vertebrates such as mammals (Klepke et al. 2022); and water, that is widely used to study micro and macro-organisms, allowing the detection of both aquatic and terrestrial species.

### *18.2.1 Water Samples*

One of the most popular media used in eDNA studies is water. Taking a few millimeters of water from an aquatic environment has already proven to be sufficient to reveal the occurrence of aquatic and terrestrial species present in a given area and its surroundings (Deiner et al. 2017). In this context, water eDNA has been recognized as providing a more complete picture of biodiversity composition when compared to traditional surveys, as well as allowing for multi-trophic analysis in metabarcoding studies (Blackman et al. 2022).

So far, the majority of eDNA studies conducted in neotropical realms used water as the main sampling media. Water eDNA samples have been used to detect the presence of invasive species such as the freshwater dinoflagellate *Ceratium furcoides* in Argentina (Accattatis et al. 2020), the North American crayfish *Procambarus clarkii* in Ecuador (Riascos et al. 2019) and the golden mussel *Limnoperna fortunei* in Brazil (Pie et al. 2017); to study the fish diversity in Argentina (Chalde et al. 2019; Nardi et al. 2020), Brazil (Sales et al. 2019, 2021; Dal Pont et al. 2021; Jackman et al. 2021) and French Guiana (Cantera et al. 2019; Cilleros et al. 2019); and also, to detect the presence of terrestrial vertebrates in Brazil (Sales et al. 2020) and Colombia (Mojica and Caballero 2021; Polanco et al. 2021).

Considering that approximately 71% of the Earth's surface is covered by water and thousands of taxa are expected to inhabit the aquatic ecosystem, retrieving genetic data from water samples opens up more opportunities to better detect and monitor species, mainly the rare, elusive and often neglected ones, enabling researchers to better understand the relationship between species and their habitat. As an example, ecological indices gathered from an eDNA study conducted in Curaçao revealed the contrasting anthropogenic pressure on functional diversity and species richness of reef fish (Polanco et al. 2022). Moreover, with the new and future improvements in the field, eDNA surveys are expected to move forward from the biodiversity inventories (e.g., species list) to provide more in-depth ecological data, even being able to provide additional population genetics information.

To some extent, eDNA is expected to be more widespread in aquatic environments and easier to capture species' presence when compared to soil samples. For instance, an integrative biodiversity assessment can be obtained across the land-water interface through the analysis of eDNA transported in river networks (Deiner et al. 2016). The persistence of eDNA molecules can vary greatly depending on the sampling media. Water samples are often used to retrieve eDNA signals representing a shorter time span (e.g., days), in comparison to other media (e.g., soil – days to years, permafrost – thousands of years). The effect of eDNA ecology in distinct environments is noteworthy, and the short persistence of eDNA in water is linked to several factors that are usually intertwined, such as eDNA origin, state, transport, and fate, with the latter being impacted by UV exposure, temperature and other environmental factors (Barnes and Turner 2016). However, UV exposure, temperature, and other environmental factors' effects on eDNA persistence remain a puzzle for neotropical areas.

The lack of knowledge regarding the effect of these synergistic environmental factors on DNA degradation rates, coupled with a largely restricted understanding of eDNA transport in neotropical water bodies (e.g., streams, rivers) represents one of the key challenges in disentangling signals associated with eDNA *vs* species ecology. For instance, Sales et al. (2021) demonstrated the potential of using water samples to infer spatiotemporal changes in fish assemblages, nevertheless, the effect of eDNA transport and degradation on species detection could not be assessed. Studies aiming to investigate the eDNA ecology in neotropical realms are expected to increase in the forthcoming years, aiming to address these aforementioned limitations in providing a fine-scale spatiotemporal resolution of species detection.

In order to decrease its degradation and optimize eDNA yield, samples must be collected, stored and processed properly (Fig. 18.2). After collection, water eDNA samples require the shed DNA to be captured and/or concentrated, a process often conducted via centrifugation, precipitation (Fig. 18.2b) or filtration (Fig. 18.2c). Precipitation refers to a chemical process using ethanol to precipitate and isolate the nucleic acids, whereas, filtration is employed to retain DNA molecules using a filter of fine mesh, whilst allowing the passage of water (Jerde et al. 2011; Eichmiller et al. 2016). Filtration has been widely used and considered a better option when retrieving eDNA from water samples. Still, a broad range of filters of different compositions and mesh sizes is available and so far, no consensus has been reached


**Fig. 18.2** Field and laboratory procedures for water eDNA samples. (**a**) collection of the water sample; (**b**) filtration of water samples in a filter using a manual vacuum pump and (**c**) precipitation of the nucleic acids from eDNA water samples

regarding the best filter pore size and material to be used in eDNA surveys. In neotropical areas, it might be even more challenging, as the ideal total volume of water to be filtered has not been analyzed across different ecosystems yet. As an example, Lopes et al. (2021a) filtered 2–30 L of water per sample to investigate the presence of frogs, Cilleros et al. (2019) collected approximately 50 L of water in each sample to survey fishes, and Sales et al. (2020, 2021) used 500 mL to 1 L to recover eDNA from both fish and mammals. Considering the vast distinctiveness of habitats to be sampled in the Neotropics, it is important to conduct pilot tests to evaluate the best filter pore size and composition to be employed. As an example, in very turbid and sediment-rich water bodies, the filtration of large volumes of water might be impossible if using a fine mesh because the filters can get easily clogged preventing the water to go through the filter.

### *18.2.2 Soil Samples*

Soil is also a promising source of DNA to study vertebrate, invertebrate, plant and microorganism biodiversity and the interactions of these organisms with the environment (e.g., Oliverio et al. 2018; Zinger et al. 2019; Andersen et al. 2012; Nuñez et al. 2021; Ariza et al. 2022). Unlike in water, DNA can persist in the soil for thousands of years (Haile et al. 2007; Barnes and Turner 2016) as it can bind to environmental compounds, such as clay minerals or organic compounds, that protects DNA from total degradation (Blum et al. 1997; Crecchio and Stotzky 1998). Soil samples can be collected from the surface or from more deep soil profiles, if recent or ancient biodiversity is to be assessed, respectively. It is also generally assumed that the DNA recovered is the same age as the soil in which it was collected. Although this is a reasonable assumption, leaching of DNA to lower strata of soil must be taken into account in non-frozen areas, especially in the Neotropics where rain is more frequent and abundant (Andersen et al. 2012; Haile et al. 2007).

In the case of vertebrates, the identification of species presence through eDNA in soil was first applied in areas of permafrost, where DNA is better preserved due to the very low temperatures and leaching is expected not to occur (Willerslev et al. 2003; Pedersen et al. 2015). The quantity and deepness of DNA in the environment can be influenced by a number of biological (animal movement, defecation and other behaviors, demography, rate of cell shedding, etc.), edaphic (pH, particle size, organic matter content, etc.) and climatic factors (precipitation, temperature, UV exposure, etc.) (Levy-Booth et al. 2007; Andersen et al. 2012; Leempoel et al. 2020; Ryan et al. 2022). When these factors are accounted for, eDNA has been pointed out to reflect vertebrate abundance and richness from only a few grams of soil (Andersen et al. 2012). Soil has already been successfully applied to study a variety of vertebrate species (e.g., Kucherenko et al. 2018; Leempoel et al. 2020; Ryan et al. 2022). Although microorganisms have been studied through soil DNA in the Neotropics (Câmara et al. 2022), the potential of this DNA source for neotropical vertebrates has yet to be explored (Carvalho et al. 2022). To our knowledge, only the studies of Ritter et al. (2019) and Lopes et al. (2020) have applied this method in soils from the Neotropics.

Ritter et al. (2019) used soil, litter (the organic portion above the mineral soil) and insects to test if the east-to-west biodiversity gradient known to occur in the Amazon forest for birds and trees could be recovered from eDNA and iDNA data. The authors collected 40 soil and 40 litter samples from each of the 39 plots and analyzed the total DNA extracted from the samples. A metabarcoding approach was used by amplifying portions of the genes 16SrRNA for prokaryotes, and 18SrRNA and COI for eukaryotes. There was no relationship between the operational taxonomic units retrieved and the richness of both birds and trees from previous field studies. Furthermore, the west-to-east biodiversity gradient was only partially reflected in the metabarcoding data, due to the effect of outliers in the dataset, which was pointed to be a result of the particularities of each studied area.

Lopes et al. (2020) analyzed litter as a source of eDNA to study the biodiversity of vertebrates, with a special focus on anurans, in an area of the Atlantic Forest in Brazil. Authors collected 32 samples of litter that were later combined into two bulks of 1 kg each. Litter was washed with a mixture of water and buffer and filtered in a fine cellulose membrane. Total and extracellular DNA was extracted from the membrane. A portion of the genes 18SrRNA for eukaryotes and 12SrRNA for vertebrates and anurans was analyzed. The authors were able to retrieve a large eukaryotic diversity with the 18SrRNA gene, but only two sequences corresponding to anuran species with the 12SrRNA gene. They considered the analysis of eDNA obtained from litter a successful method to characterize the eukaryotic community. Low rate of shedding by amphibians, low number of sampling replicates, low volume of litter collected may have affected the detection of anurans in the area, since these species were observed by researchers in the litter during sampling.

As seen, soil is still beginning to be used and understood as a source of environmental DNA from vertebrates, especially in the Neotropics. A few peculiarities of each study area must be taken into account when planning future eDNA studies from soil samples. For example, soils that are acidic or go through a process of acidification could reduce DNA absorption by the soil, as well as higher precipitation can contribute to a higher leaching of DNA (Allemand et al. 1997). Furthermore, the presence of DNA traces in the soil depends heavily on species abundance and soil use (Leempoel et al. 2020; Lopes et al. 2021b; Ryan et al. 2022). Unlike microorganisms that are spread across the soil, vertebrates move, defecate, urinate, shed cells and perform other behaviors not uniformly on the soil, depending on complex biotic and abiotic interactions. Thus, the amount of soil needed to detect biodiversity also varies, from a few grams in a controlled environment (Andersen et al. 2012) to several liters or kilograms in a natural environment (Leempoel et al. 2020; Lopes et al. 2020). This method can also benefit from a more targeted sampling in trails of frequent use or with the use of fences that direct species movement to where soil will be collected (Burns et al. 2020). The advantages of using soil to sample vertebrate DNA are that (i) due to the role of soil as a source of DNA for microorganism studies, there are highly efficient commercial kits available that are specially designed to extract DNA from soil, that can be applied to studies focusing on higher taxonomic groups, and (ii) soil samples require low maintenance and processing in the field.

### *18.2.3 Alternative Sources of DNA to Assess the Biodiversity and Species Ecology*

Scat samples, especially those from species that have a generalist feeding habit, are also a powerful source of genetic material. Scats can provide data to assess biodiversity through the identification of the species that deposited the scats and the species that were fed on, thus allowing researchers to obtain information on species presence, distribution and diet. From these data, it is also possible to make inferences about species' ecological aspects such as networks, and spatial and temporal interactions. Recent studies with rodents (Lopes et al. 2020), bats (Ingala et al. 2021; Martínez-Fonseca et al. 2022) and large carnivores (Quéméré et al. 2021) have made it possible to corroborate or question the knowledge we currently have about the diet of these species in neotropical environments.

Using an indirect and combined approach, Lopes et al. (2020) were able to study the niche overlap of seven rodent species of the *Ctenomys* genus that live in South America. Through the metabarcoding approach, the authors used rodent scats and soil to investigate plant consumption and plant availability, respectively. Scats were obtained from captured rodents and in burrows used by the species. Authors amplified regions of the P6 loop of the chloroplast trnL (UAA) intron and the first internal transcribed spacer (ITS1) of nuclear ribosomal genes to detect plant species in scat and soil samples. They found that the rodent species consumed 60% of the plant species detected in the soil samples, indicating that these species present a generalist feeding habitat. This result not only revealed the feeding habitat, but also that the allopatric distribution of the rodents reduces interspecific competition for the same resources.

Elusive species such as bats, which are characterized by their small size and nocturnal and volant behavior, are another good example of a group that can benefit from the use of molecular methods to study diet, because it is difficult to observe their feeding habits. In the study of Martínez-Fonseca et al. (2022), the metabarcoding approach was used to investigate the diet of the *Vampyrum spectrum*, a carnivorous bat, in Nicaragua. Scat samples from this species were collected directly under bats in roots and fragments of the COI, 12SrRNA and 18SrRNA genes targeting vertebrate and arthropod DNA were sequenced using the metabarcoding approach. This study revealed a total of 27 different vertebrate species in the bat diet, including birds, rodents and other bat species, besides arthropods, indicating that *V. spectrum* forages opportunistically. In another study, Ingala et al. (2021) investigated the diet of 25 bat species that co-occur in Belize, using DNA metabarcoding for the detection of vertebrates, invertebrates and plants. Bats were captured and placed into cloth bags, where they defecated, and scats were collected. This effort allowed the authors to document bat diet at a multi-trophic level and fine-scale association between bat species and dietary items, showing that most of the studied species do not have restricted diets and that their habits are rather opportunistic. Although Ingala et al. (2021) did not propose to provide a full dietary niche breadth for the 25

species, because it would require many replicates, this study paved the way for future studies that aim to understand the coexistence and niche partition in bat assemblages.

Carnivores are also known to be generally elusive, rare (occur at low densities either because of natural or anthropogenic causes) and difficult to capture. This group plays an important role in ecosystems by regulating the population of other trophic levels, therefore, having a better comprehension of carnivore species diet is a pivotal concern that also supports the verification of ecosystems health. For example, the diet of the Endangered riverine *Pteronura brasiliensis,* the giant otter, was assessed by collecting scats deposited in communal latrines along river banks or on small islands, in French Guiana (Quéméré et al. 2021). Authors also used a metabarcoding approach based on portions of the 12S and COI genes targeting vertebrate and invertebrate species, respectively. In this study, scat DNA-based metabarcoding was more efficient than conventional methods to study otter diet. It revealed the presence of species from several groups in the giant otter diet, including fishes, amphibians, snakes, birds and earthworms, and provided a basis to better understand possible human-otter conflict due to predation on species that are valuable as resources for human populations.

Some of the more elusive species are difficult to study. For example, little is known about the plant-animal interactions of the lowland tapir *Tapirus terrestris* despite their known role as engineers of the ecosystem. Hibert et al. (2013) studying the scats of the lowland tapir using metabarcoding succeeded in establishing the diet of this large mammal with great taxonomic resolution, increasing in two new families and eight genera the list of plants consumed by the species.

All these examples highlight the potential of scat DNA-based metabarcoding to investigate species diet with high accuracy, also supporting the exploration of ecological implications from that. Moreover, the information obtained from diet studies can be interpreted as a biodiversity assessment, especially when studying the diet of generalist species. Thus, diet metabarcoding can be an effective, noninvasive, and economically viable method for biodiversity monitoring, supporting management decisions (Nørgaard et al. 2021; Shao et al. 2021).

## 18.3 Invertebrate-Derived DNA (iDNA)

Another complementary and more recent approach to biodiversity inventories is the detection of vertebrate species from DNA obtained through the gut content of invertebrates that feed on vertebrates or from the insects that use the vertebrates to fulfill vital functions of their cycle (ingested-derived DNA or invertebrate-derived DNA, iDNA) (Calvignac-Spencer et al. 2013; Rodgers et al. 2017). Using invertebrates, such as carrion flies and mosquitoes for sampling DNA of vertebrates is advantageous because these insects are cosmopolites, can be easily sampled using

commercial and handmade traps (Fig. 18.3), and can feed on all terrestrial vertebrates (Norris 1965; Lynggaard et al. 2019). Other invertebrates that feed on vertebrates and can be used as samplers include dung beetles (Drinkwater et al. 2021) (Fig. 18.4), leeches (Schnell et al. 2015), sandflies (Massey et al. 2021). The iDNA approach can aid the biomonitoring of vertebrates that are elusive, rare or present in low population density, and in regions of high biodiversity, such as the Neotropics, that are notably areas where a large portion of the local biodiversity remains unknown. However, the use of this approach in the Neotropics has been narrowly explored (Carvalho et al. 2022). To date, only six studies used iDNA focused to obtain information about vertebrate communities in the neotropical region. Rodgers et al. (2017) compared the effectiveness of iDNA obtained from carrion flies and traditional methods to survey a well-documented mammal community in Barro Colorado Island, Panama. Although the authors focused on detecting mammal species, other vertebrate species were also recorded, because a mammal-specific (16SrRNA, Taylor 1996) and a broader vertebrate-specific mini-barcodes (12SrRNA, Riaz et al. 2011) were used. A total of 20 mammal species, four birds and one lizard were detected by carrion fly iDNA, a larger number of species than that obtained by the traditional methods (transect court = 13 species; camera-trap = 17 species; iDNA = 25 species) (Rodgers et al. 2017).

Lynggaard et al. (2019) asked if it would be possible to detect vertebrate DNA without targeting a specific vertebrate-feeding invertebrate but using arthropod bulk

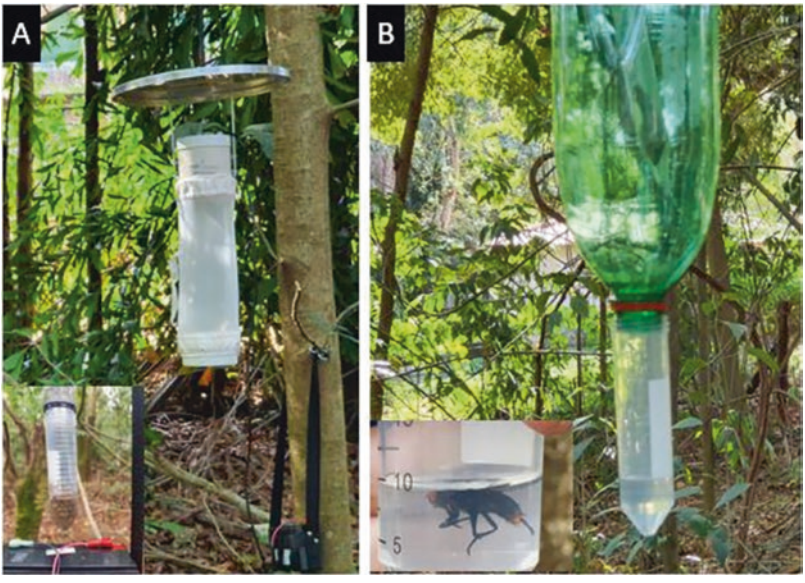


**Fig. 18.3** Traps used to capture insects, modified to study iDNA, preserving the captured insects immediately in alcohol and avoiding the degradation of the genetic material. (**a**) CDC-type trap used to capture mosquitoes; (**b**) Trap made with a plastic bottle baited with a piece of meat to attract flies


**Fig. 18.4** Field and laboratory procedures for iDNA analyses using the dung beetles as samplers. (**a**) Collection of the dung beetles using pitfall traps; (**b**) Dissecting the beetles' gut; (**c**) Digesting the beetles' gut for DNA extraction; (**d**) Performing PCRs in a sterile room for mini-barcodes amplification

samples. The authors investigated this question by collecting bulk arthropod samples in two regions, including a neotropical area in Brazil and amplifying the same mini-barcodes as Rodgers et al. (2017). Fourteen vertebrate species were recovered in the neotropical region, including anurans, carnivores, chiropterans, primates, artiodactyls and other mammals. This method was efficient in recovering vertebrate biodiversity, as it does not require prior taxonomic knowledge of the collected arthropod taxa and reduces laboratory procedures because arthropods do not need to be processed individually (e.g., preparation and DNA extraction). Authors pointed to the need for a detailed assessment of the number of replicates required to comprehensively assess vertebrate diversity, but also indicated that iDNA from arthropod bulks paired with metabarcoding could serve as a supplementary method to vertebrate monitoring.

In the study of Massey et al. (2021), the authors compared the effectiveness of three invertebrates (carrion flies, sandflies and mosquitoes) as iDNA samplers to

survey vertebrates in the Amazon region, Brazil. In this area, carrion fly DNA was the best method for landscape-scale biodiversity surveillance as it retrieved higher vertebrate richness than mosquitoes and sandflies (gamma diversity). Also, mosquitoes and sandflies showed a feeding preference for humans and armadillos (Dasypodidae family), respectively. In the same study, iDNA results were compared to camera trapping surveys and, although camera trapping showed the highest mean species richness at site-level, it also showed a detection bias towards carnivore and ungulate species (alpha diversity). Much of the biodiversity detected by the iDNA method was not evidenced by the camera-traps, as this latter method was biased towards large-bodied mammals. These results highlight that the combination of different iDNA samplers can provide better representativeness of the biodiversity.

Considering that iDNA is still narrowly explored in the neotropical region for biodiversity assessment, Saranholi et al. (2023) aimed to assess the effectiveness of the iDNA approach for surveying terrestrial mammals in a semi-controlled area, a zoo that houses several mammal species. The effectiveness of mosquitoes and flies as iDNA samplers were compared by the number of mammal species detected and by the distance between the trap where an insect was captured and the enclosure of the mammal whose DNA was ingested by the insect. To achieve this, differently from the previous studies, each insect captured was analyzed individually. A total of 45 OTUs were recovered. There was no difference between the number of mammal species recovered per individual insect, but the number of flies captured was higher than that of ingurgitated female mosquitoes, resulting in more mammal species recovered by flies. Eight and twenty mammals were recorded exclusively by mosquitoes and flies, respectively, suggesting that the use of both samplers allowed a more comprehensive screening of the biodiversity. The maximum distance recorded between an insect and the enclosure of the mammal that were fed upon was 337 m for flies and 289 m for mosquitoes, not differing significantly between groups. These results are helpful to raise insights to guide further sampling design and calibrate efforts for surveying mammals in high biodiversity areas, such as the Neotropics.

Besides the examples of iDNA applications cited above that have focused on surveying vertebrate communities and used a metabarcoding approach, iDNA can also be used to study host-vector interactions. Araujo-Pereira et al. (2020) and Rodrigues et al. (2021) investigated the mammal DNA presents in the blood meal of sandflies from the Psychodidae family, a Diptera that can transmit the protozoa that causes leishmaniasis. The identification of blood meal sources of the sandflies and other insects that serve as disease vectors is an important step for vertebrate host identification, supporting the control of vector-borne diseases. Both studies captured sandflies in the Amazonian region, Brazil, and identified the vertebrate species from the insect blood meal by sequencing (Sanger) a mini-barcode of the Cytb mitochondrial gene. Humans, the nine-banded armadillo (*Dasypus novemcinctus*) and the lesser anteater (*Tamandua tetradactyla*) were the most frequent mammal species detected in the study of Araujo-Pereira et al. (2020), while the nine-banded armadillo was the most

common species detected by Rodrigues et al. (2021) in the blood meal iDNA. Of note, both studies reported many sequences lacking sufficient quality for species identification. This could be explained by the use of the Sanger-based sequencing that does not allow the precise analysis of mixed sources of DNA. In this sense, the use of metabarcoding sequencing must be preferred in future studies that aim to investigate host-vector interactions in the neotropical region.

## 18.4 Challenges and Perspectives

### *18.4.1 Methodological Concerns*

The incompleteness of the reference databases is often assumed as one of the main limitations to eDNA-based surveys reaching their full potential in megadiverse areas (Jackman et al. 2021). Metabarcoding analyses are usually heavily impacted by the quality of databases as it relies on the taxonomic assignment obtained through a comparison between the DNA data retrieved metabarcoding sequencing and the reference sequences available. In the absence of such reference data, a massive amount of data might be lost, consequently leading to an underestimation of the total biodiversity recovered. Particularly in the hyper-diverse Neotropics, the lack of reference sequences in public databases has been reported as a critical aspect that limits the use of metabarcoding (Kocher et al. 2017; Rodgers et al. 2017; Banerjee et al. 2022) and efforts to produce such sequences are still needed, as already pointed by Carvalho et al. (2022).

The impact of reference databases is not limited to metabarcoding applications and can also affect single-species essays (e.g., qPCR, ddPCR studies). The establishment of sound eDNA detection through qPCR essays relies on the quality and suitability of genes, primers and probes specificity used. To ensure the robustness of essays, previous analyses should be conducted on the search for potential confounding taxa, including information about closely related taxa (Langlois et al. 2019). The urge for increasing the reference data for a broader range of species is therefore shared across all aforementioned eDNA/iDNA methods. Many researchers agree with this need and therefore there are initiatives to identify the major gaps in the genetic basis (e.g., Marques et al. 2021).

Completing the databases and having genes that complement the identification of all species will not be enough to perform reliable surveys, since all species are not sampled with the same ease, probability or sampling technique (e.g., Massey et al. 2021; Saranholi et al. 2023). Thus, the use of traditional methodologies combined with eDNA and metabarcoding can reach more reliable and faster results, improving cost-benefit ratios (Carvalho et al. 2022). Also, techniques such as capture enrichment are being developed to increase DNA yield and reduce bias in species detection (Wilcox et al. 2018).

Exogenous contamination also represents an important concern in the eDNA/iDNA approach, which can occur at any step (sampling, DNA extraction, PCR, and sequencing). To avoid this, it is important to conduct the studies in an eDNA-dedicated laboratory using UV-sterilized room (Fig. 18.4d) and exclusive equipment for such purposes (centrifuges, thermocycler, pipettes, lab coats, etc.). Since human DNA contamination is almost imminent, the use of blockers during PCR has shown successful results (e.g., Boessenkool et al. 2012) and is highly accepted among the scientific community. However, the use of these tools should be treated with caution, since the identification of some taxonomic groups may be affected by using blockers of phylogenetic related species (for example, humans and primates).

With reduced costs of sequencing, high throughput sequencing is becoming more available. However, the potential of this type of sequencing is still deeply underexplored in the neotropical region, mainly because reagents and equipment are imported, making services being charged in dollars or euros or other currency, which incurs comparatively high costs of this type of sequencing. Besides the decrease in sequencing costs, new possibilities of types of sequencing and applications for eDNA/iDNA are emerging, such as genomic and metagenomic analyses (Seeber and Epp 2022).

Different methods to collect samples that can decrease contamination and improve eDNA sampling, such as automated water collection, are still being developed, tested and improved (Sepulveda et al. 2020, 2021; Wandhekar et al. 2021). One of the approaches that can present a high cost-benefit relationship is what has utilized air samples to collect pollen and survey plant species (i.e., Kraaijeveld et al. 2015). The methodology has proven to be very efficient to identify the species and, to our knowledge, it has not been used to sample high biodiversity regions such as the Neotropics. With improved collection, sequencing and analyzing tools for eDNA, we also expect to be able to use environmental DNA data for other applications, such as a source of information for population genetics. Although some studies have been able to study aspects of population genetics from eDNA (Sigsgaard et al. 2017; Baker et al. 2018), this field is still in its onset as the identification of individuals is still an obstacle because it relies mostly on nuclear markers that can be lost more easily to environmental degradation (Adams et al. 2019).

### *18.4.2 Conservation Remarks*

The eDNA and iDNA sampling combined with the molecular tools and the advances in the NGS technologies present a great sensitivity for species detection along with reduced costs in comparison to traditional methods, which creates a remarkable opportunity to advance biodiversity monitoring (Sutherland et al. 2013; Cristescu 2014; Kelly et al. 2014b). Also, eDNA and iDNA approaches permit noninvasive

monitoring, reducing the risks involved in using methods based on species capture, which is critical especially for threatened species.

The number of environmental DNA surveys has seen an exponential increase in the past decade. However, there is a heavy bias on the proportion of studies towards the global North, with this application still remaining incipient in neotropical countries (Carvalho et al. 2022; Schenekar 2022). Although there are clear advantages with the eDNA and iDNA approaches, it is still little explored in the Neotropics (Carvalho et al. 2022).

Despite the potential highlighted to survey biodiversity through environmental DNA, there are still few official monitoring initiatives using this methodology. It is imperative for eDNA/iDNA to be acknowledged by government agencies as a valid monitoring tool so that standards (e.g., sampling protocols, minimum replicates, data processing) and maximum error values can be defined (Kelly et al. 2014a). The conduction of experiments in controlled situations is expected to generate the necessary data, but this is still deficient in the highly diverse neotropical region where protocols and standards from temperate regions do not apply completely.

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# Part VI
# Conservation Genomics

# Chapter 19
# Conservation Genomics of Neotropical Carnivores


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## 19.1 Introduction

Genetic concepts and methods have been part of conservation biology since the origin of the field, and have grown during the 1970s–1980s to create the vibrant discipline of conservation genetics (Avise and Hamrick 1996). In the early 2000s, the dramatic development of methods to characterize genome-wide variation and

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the growing feasibility of applying them to non-model organisms allowed the expansion of the field, giving rise to the young and burgeoning area of conservation genomics (Ryder 2005; Shafer et al. 2015; Allendorf et al. 2010). Throughout these developments, carnivoran species have been the focus of numerous studies and often the first taxa on which methodological advances were applied (e.g. O'Brien et al. 1983; Gilbert et al. 1990; Wayne and O'Brien 1987). This was likely driven by the charismatic nature of these species, which attracts interest from scientists and the general public alike, but also because they tend to be more severely threatened than many other groups due to their naturally lower densities, large area requirements and frequent conflicts with humans (Gittleman et al. 2001). The early use of genetic and genomic approaches on several carnivoran species and the application of the resulting data in the context of conservation planning and actions, make this an interesting group for the study of the evolution of the conservation genomics field among non-model organisms.

In the earliest days of conservation genetics, carnivorans (specifically the northern elephant seal, *Mirounga angustirostris*) were arguably the first wild organisms to be typed with molecular markers in the context of assessing the implications of past population declines (Bonnell and Selander 1974). Subsequent studies in the 1980s applied molecular markers to reveal low levels of genetic diversity in wild felid populations, with implications for their conservation (O'Brien et al. 1983, 1985; Wildt et al. 1987; see O'Brien et al. 1996; Culver et al. 2010 for reviews). Soon afterwards, molecular techniques were employed to survey genetic diversity and address conservation issues in canids (e.g. Wayne et al. 1991, 1992; see Wayne 1996 for a review) and bears (e.g. Taberlet and Bouvet 1992, 1994), and a surge of similar studies targeting several carnivoran species ensued.

In the last three decades, a robust literature has accumulated on carnivoran conservations genetics, addressing topics ranging from basic surveys of molecular diversity to resolution of taxonomic issues, characterization of phylogeographic patterns and population structure and history (which are required to delimit geographic units for conservation planning), along with insights on social structure, dispersal patterns and other topics in molecular ecology relevant to countering anthropogenic threats. At the same time, there are important skews in this literature, stemming from varying amounts of effort placed on different geographic regions, taxonomic groups, and types of research questions. As a consequence, while for some species the field is very advanced, for others there are scarce or even no studies conducted so far. This skew can be observed among neotropical carnivorans, some of which have been the focus of advanced conservation genetic studies, while others still lack basic assessments of their genetic diversity or even clear-cut taxonomic delimitation (see below).

While the field of conservation genetics continued to advance in the early 2000s, dramatic improvements in DNA sequencing technologies and computational capabilities led to the possibility of characterizing large genomic segments, or even complete genomes, from non-model species (Ekblom and Galindo 2011). While in 2001, the year in which the human genome sequence was first reported (International Human Genome Consortium 2001; Venter et al. 2001), only two other mammals

(mouse and rat) had ongoing genome sequencing projects (O'Brien et al. 2001), the situation changed rapidly in subsequent years. Again, carnivorans were at the forefront of this development, given the relevance of domestic dogs and cats as companion animals as well as model systems for biomedical research. This led to the first reports of the sequences of the dog genome (Lindblad-Toh et al. 2005) and the cat genome (Pontius et al. 2007), which provided important resources for comparative analyses of non-model species and ushered in an initial phase of genomic characterization of other carnivorans.

Among wild non-model organisms, the first carnivorans to have their genome sequenced were the giant panda (Li et al. 2010; Zhao et al. 2013), polar and brown bears (Miller et al. 2012), and the tiger (Cho et al. 2013). The latter study also reported draft genome sequences of two other big cats, the lion and snow leopard, and included some comparative analyses among them. These initial studies tended to target endangered and/or charismatic species, and their genome sequences, along with comparisons with those of other mammals, were employed in some cases to make inferences with potential relevance to their conservation. Soon afterwards, whole-genome sequences of other carnivores were reported, including wolves and a jackal (Freedman et al. 2014), cheetahs (Dobrynin et al. 2015), leopards (Kim et al. 2016) and small wild cats (Li et al. 2016).

From then onward, a plethora of carnivoran genomes have been sequenced (Fig. 19.1), initially focusing on basic characterization of genomic diversity and broader comparative analyses, and subsequently including more in-depth studies of genomic architecture, phylogenomics and population genomics. For example, a study analyzed 10 Scandinavian wolverine (*Gulo gulo*) individuals using whole genome sequencing (Ekblom et al. 2018), showing that it had one of the lowest levels of genetic diversity among threatened species, and that its demographic history indicated a strong trend of decline in effective population size. Similar studies have now been published on a variety of carnivorans, including neotropical taxa (see below).

## 19.2 Neotropical Carnivores

The neotropical region harbors a highly diverse carnivoran assemblage, encompassing 84 currently recognized species (out of 286 worldwide), representing eight different families (out of 16 worldwide): Canidae (dogs and foxes), Felidae (cats), Mephitidae (skunks), Mustelidae (otters, weasels and relatives), Otariidae (sea lions and fur seals), Phocidae (true seals), Procyonidae (raccoons, coatis and relatives), and Ursidae (bears). The two pinniped families, Otariidae and Phocidae, are represented by breeding colonies as well as occasional dispersing individuals found along the Atlantic and Pacific coasts of South America. These species mostly derive from southern-hemisphere radiations that led to colonization as well as cases of autochthonous divergences in neotropical coastal waters (e.g. Lopes et al. 2021). The terrestrial families that inhabit the region are the results of multiple episodes of

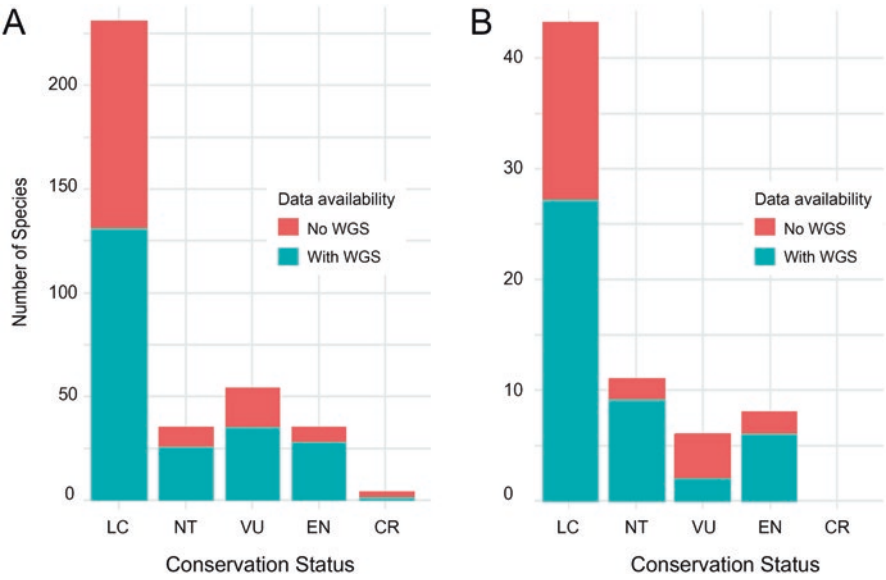


**Fig. 19.1** Present availability of whole-genome sequence (WGS) data for species of the mammalian order Carnivora on the NCBI archive. Each panel depicts the number of carnivoran species with or without WGS data at different geographic scopes: (**a**) worldwide; (**b**) Neotropical region. Species are separated based on their IUCN threat category: *LC* least concern, *NT* near threatened, *VU* vulnerable, *EN* endangered, *CR* critically endangered

invasion from North America, most of which occurred during or after the Great American Biotic Interchange (GABI) in the Late Pliocene, including several cases of endemic radiations (Eizirik 2012). Each of the families will be briefly described below.

The neotropical Canidae are represented by 10 endemic South American species, as well as two North American taxa (coyote [*Canis latrans*] and gray fox [*Urocyon cinereoargenteus*]) whose ranges include Central and South America. The origin and evolutionary history of South American endemic canids have been the focus of many studies (e.g. Tchaicka et al. 2016; Favarini et al. 2022), and the most recent evidence, based on analyses of complete genomes (Chavez et al. 2022), indicates that they have radiated in South America after the invasion of a single ancestor during the GABI (see Sect. 19.4.5 below). This autochthonous radiation gave rise to the most speciose canid assemblage worldwide, including several fox-like species along with disparate specialists such as the long-legged, omnivorous maned wolf (*Chrysocyon brachyurus*) and the short-legged, hypercarnivorous bush dog (*Speothos venaticus*).

Neotropical felids comprise 11 currently recognized extant species (Kitchener et al. 2017) whose ancestors have also colonized the region during the GABI. Eight of them, comprising genus *Leopardus* (the most speciose genus in the Felidae), derive from an autochthonous radiation following a single invasion by their

common ancestor (Eizirik 2012; Li et al. 2016; Trindade et al. 2021). In addition to the eight currently recognized species, recent morpho-ecological and/or molecular analyses have indicated that the genus may actually comprise several additional species which are currently contained in the tigrina (*Leopardus tigrinus*) and pampas cat (*Leopardus colocola*) species complexes (Trigo et al. 2013; Nascimento and Feijó 2017; Nascimento et al. 2020; Trindade et al. 2021). Finally, the three other neotropical species are the sister-pair puma (*Puma concolor*) and jaguarundi (*Herpailurus yagouaroundi*), whose divergence may have occurred prior to or during the GABI, and the jaguar (*Panthera onca*), which entered the region more recently (Johnson et al. 2006; Li et al. 2016).

The neotropical Mustelidae comprise four species of otters (see Sect. 19.4.4 below), two grisons (*Galictis cuja* and *G. vittata*), the tayra (*Eira barbara*) and four weasel-like species (three placed in genus *Neogale* and one in *Lyncodon*). The family Mephitidae is represented in the region by at least three species of hog-nosed skunks (genus *Conepatus*) whose taxonomy is still poorly resolved. The marine (pinniped) families Otariidae and Phocidae are represented in the region by at least eight and five species, respectively. The Procyonidae comprises at least 15 species, all of which occur either completely or partially in the Neotropics. Finally, the Ursidae is represented in the region by a single species, the spectacled bear (*Tremarctos ornatus*).

Overall, these neotropical species comprise a very diverse array of carnivorans, adapted to a variety of habitats and life histories, and exhibiting distinct evolutionary processes (e.g. recent and rapid autochthonous radiations *vs.* ancient lineages). Many of these species are threatened by anthropogenic processes such as habitat loss, modification, and fragmentation, along with hunting, depletion of the prey base, diseases transmitted by domestic animals and/or direct human-wildlife conflict. It is therefore important to design and implement adequate conservation and management strategies on their behalf, which must be enabled by sufficient knowledge on their taxonomy (e.g. species delimitation), biology, ecology, genetics, behavior and health. All of these areas can benefit from the use of molecular approaches, and, more recently, from the analysis of genomic data. The recent developments in this field, and the genetic and genomic studies that have already been conducted on neotropical carnivores, will be the focus on the following sections.

## 19.3 Genomic Resources for Neotropical Carnivores

In the last decades, due to technological advances such as the improvement of molecular techniques and bioinformatic tools, the sequencing of complete genomes became much more accessible, allowing its application to answer ecological and evolutionary questions of natural populations (Ekblom and Galindo 2011). Along with that, another important step for the study of non-model species was the use of non-conventional biological samples (e.g. museum specimens and non-invasive

samples) enabled by these technologies, which facilitated the study of rare and/or elusive species. However, even though the prices for whole-genome sequencing have been decreasing, this is still one of the challenges for data collection, especially for researchers from underfunded countries. Overall important things to note and take into account when planning a study that will sequence and/or use available genomes are: (i) sequencing depth of coverage, which is how many times the same region of the genome has been sequenced; (ii) existence of one or more reference genomes (since for some approaches a genome reference is required for mapping the data against) and their contiguity, i.e. how fragmented the genome assemblies are; and (iii) availability of a chromosome-level assembly to be used as the reference, as for some projects a highly contiguous and well-assembled genome is important.

Currently, there is already a substantial amount of genomic data generated for neotropical carnivores (Fig. 19.1). Over half of the extant species have publicly available genomic data for at least one individual, which is a similar proportion to the one observed for carnivores globally. Therefore, the neotropical region is overall well represented in carnivoran genomic studies, including species belonging to different IUCN threat categories. The fact that critically endangered species are underrepresented could be influenced by their small number (only four worldwide) and also caused by their rarity and/or elusive behavior, making them harder to sample. Overall, this assessment shows a trend of prioritizing endangered species, which is expected, considering the more urgent need to implement conservation measures on their behalf, the various gaps in knowledge that can be bridged with genomic data, and the limited financial resources available. It is expected that the rate of generation of genomic resources for neotropical carnivores (as well as carnivoran species from other regions) will increase in the coming years, as more species are sampled and included in genome-scale studies. Furthermore, it is expected that more studies will begin to focus on multi-individual datasets, targeting phylogeographic and population-level questions, which are very relevant for conservation-oriented projects.

In this context, and given that sequencing costs may remain a limiting factor in the foreseeable future, several alternative approaches may be contemplated, especially for projects with the need to sequence many individuals and/or in which high depth of coverage is not mandatory. One of the options is sequencing several individuals at once, from which one can extract population statistics (e.g. Hivert et al. 2018; Taus et al. 2017; Gautier et al. 2022), but losing individual-level information. This approach is called Pool-Seq and has been used as a very cost-effective option for population studies; however, it can have some disadvantages, such as the impossibility of filtering out biased data at the individual level (Kurland et al. 2019; Schlötterer et al. 2014). Another option is the use of low-coverage whole genome sequences (lcWGS; *ca.* 5× depth), in which the individual-level information is kept, but requiring larger samples (depending on the analyses) to reliably estimate population parameters (Lou et al. 2021). Furthermore, even ultra low-coverage whole genome sequences (ulcWGS; less than 0.5× depth) are currently been used to estimate population structure using imputed genotypes with the help of new

bioinformatic techniques. As these approaches mature and new ones emerge, it will be important to assess their potential use when planning a population-level genomics project targeting carnivores or any other non-model species.

In addition to projects generating novel genomic resources to directly address questions pertaining to neotropical carnivores, it will remain important to constantly monitor and incorporate the data on these species that are produced and published by large genomics consortia. For example, the Earth Biogenome Project (EBP) includes several affiliated projects, such as the Vertebrate Genomes Project (VGP), that aim to produce high-quality whole-genome sequences of many species, including carnivores worldwide. Other projects (e.g. DNAZoo, Zoonomia) have already produced genomic resources for neotropical carnivores, and will tend to continue to do so in the future. These publicly available genomic data have already been incorporated in recent studies targeting these taxa (e.g. de Ferran et al. 2022; Lorenzana et al. 2022), a trend that is likely to be maintained. The next section will describe the studies that have been conducted so far on the genomics of neotropical carnivores, along with some of the background of genetic analyses that have set the stage for the most current developments.

## 19.4 Genomic Studies of Neotropical Carnivores and Their Implications for Conservation

Neotropical carnivores have been the focus of genomic studies since 2011, which have led to several discoveries regarding their evolutionary history and current levels of diversity. In many cases, the resulting data have had implications for the conservation of the target species, and have been incorporated in management efforts on their behalf. The studies conducted so far have addressed species belonging to the families Felidae (cats), Mustelidae (especially the otter subfamily, Lutrinae) and Canidae (dogs, wolves and foxes). Each of these groups will be discussed in detail below.

### *19.4.1 Jaguars (*Panthera onca*)*

The jaguar (*Panthera onca*) is the largest felid from the Americas and the only representative of the *Panthera* genus in the continent. The species once ranged from the southern United States of America (USA) to central Argentina, occupying a variety of biomes (Seymour 1989; Sanderson et al. 2002). However, due mainly to habitat loss and human persecution, the species was extirpated from over 50% of its distribution and, in some areas, the remaining populations are highly fragmented and isolated (Sanderson et al. 2002; Jędrzejewski et al. 2018; Quigley et al. 2017). As a large predator, the species is invaluable for the conservation of ecosystems, as it

regulates the populations of prey species and influences habitat health as a whole (Crooks 2002; Ripple et al. 2014; Wolf and Ripple 2017). The jaguar is also considered a charismatic species and can thus be used as an umbrella taxon to increase public awareness for the protection of natural areas and the conservation of other groups with which it coexists (Karanth and Chellam 2009; Macdonald et al. 2015).

Due to its functional importance and high public profile, the jaguar has been the focus of several genetic studies for over 20 years. The first genetic study focusing on this species reported its genetic diversity, population structure and demographic history across most of its geographic range (Eizirik et al. 2001). Based on mitochondrial DNA (mtDNA) control region sequences and microsatellite loci, those authors observed that jaguars present moderate to high levels of genetic diversity and weak geographic structure, indicating that it has maintained considerable historical gene flow across its range. However, some degree of genetic differentiation could be detected between populations on opposite sides of the Amazon river and also between Central and South America. In that same year, Ruiz-García (2001) reported an assessment of genetic diversity in primates and felids from Colombia, based on microsatellite data, and estimated moderate to high levels of genetic diversity for jaguars in that country. In the following years, a series of studies using microsatellite data were performed, analyzing the genetic structure and diversity of populations, evolutionary history, effective population size and/or kinship in Colombia and nearby countries (Ruiz-García et al. 2003, 2006, 2007, 2013), in the Cerrado (Soares et al. 2006), Amazon (Pinho et al. 2014), Caatinga (Roques et al. 2014), Paraguayan Dry Chaco (Giordano 2015) and Pantanal (Eizirik et al. 2008; Roques et al. 2014; Giordano 2015; Valdez et al. 2015; Kantek et al. 2021). In addition to demonstrating the high genetic variability and small differentiation between the northern and southern Pantanal, this latter study also generated the first timeline pedigree and direct estimate of the jaguars' generation time, improving our understanding on the population dynamics of the species (Kantek et al. 2021). Overall, despite using different sets of microsatellite loci, these studies found generally the same trend reported by Eizirik et al. (2001) of moderate to high genetic diversity and considerable connectivity across the species' range.

However, a distinct pattern was reported by Haag et al. (2010) when analyzing jaguars from fragmented areas of the Atlantic Forest biome, indicating what will probably happen in other biomes suffering with habitat loss unless effective conservation measures are applied. Using 13 microsatellite loci, Haag et al. (2010) demonstrated that the extreme habitat conversion suffered by this biome over the last decades has isolated jaguar populations in small patches, with the landscape matrix surrounding them precluding individuals from moving among these fragments. As a result, populations are already genetically differentiated from one another and at least some of them present signs of inbreeding and intense genetic drift, decreasing their genetic diversity. This was the first study that demonstrated the impact of habitat alteration on jaguar genetic diversity and connectivity. Later, DeMatteo et al. (2014) found similar results for one of the fragments studied by Haag et al. (2010), in Iguazú National Park, in Argentina (including more individuals). Subsequently, Souza (2015) and Srbek-Araujo et al. (2018) estimated even lower values of genetic

diversity for three other fragments of the coastal Atlantic Forest, in Southeastern Brazil (Parque Estadual da Serra do Mar and Parque Estadual de Intervales, in São Paulo state, and Reserva Natural Vale, in Espírito Santo state). Similar levels of genetic diversity and structure were also found for Central American and Mexican jaguars, where habitat loss has increased severely in the last decades (Wultsch et al. 2014, 2016a, b; Menchaca et al. 2019).

It was only more recently that other large-scale assessments of jaguar genetic diversity were performed, including areas not sampled by Eizirik et al. (2001) and a larger number of individuals (71, 102 and 190 samples in Zanin et al. (2021), Roques et al. (2016) and Lorenzana et al. (2020), respectively). These studies strengthened the view that jaguar populations originally presented high genetic diversity and connectivity across its range, but habitat loss has led to the reduction and isolation of some populations, as seen in the Atlantic Forest and Central America, and highlighted the importance of the Amazon (which currently presents the highest levels of genetic diversity and may consist of a single panmictic population) as a stronghold for the species' conservation.

In addition to the continued assessment of jaguar populations with traditional molecular markers, this felid was the first neotropical wild mammal to have its whole genome sequenced, in 2014, ushering in new possibilities of genomic analyses targeting this species. The genome was reported in a publication that focused on its evolutionary history, regarding the phylogenetic relationships with other big cats, along with inferences of demographic history and signatures of selection (Figueiró et al. 2017). The main results included the identification of strong evidence of historical inter-species hybridization in the genus *Panthera* (including inferred cases of potentially adaptive introgression) and multiple signatures of positive selection in the lineages leading up to present-day species.

This was the first publication of a broader initiative called "The jaguar genome project", initiated in 2011, which focuses on characterizing this species' genome, including studies of intra-specific diversity and the generation of the necessary resources for enabling improvements in conservation and management strategies targeting both wild and *ex-situ* populations. After the first report, more jaguars had their genomes sequenced, which enabled additional analyses focusing on the genomic diversity, demographic history and inbreeding evaluation for 13 individuals representing all Brazilian biomes where the species still occurs and also including one individual from the USA and another from Guatemala (Lorenzana et al. 2022).

The ongoing and future steps of this initiative include the addition of more individuals from each sampled population, focusing on detailed assessments of local inbreeding and its consequences, along with estimates of historical and current levels of gene flow within and among biomes across the entire species' range. These steps will provide the necessary genomic resources for the development of effective genetic tools for surveying and monitoring jaguar populations, understanding their demography and behavior in more detail, enabling fine-tuned *in situ* and *ex situ* management strategies, and performing accurate and agile forensic analyses to curb the illegal traffic affecting this species.

### *19.4.2 Pumas (*Puma concolor*)*

The puma is the most broadly distributed carnivore of the Americas, a highly charismatic species and the top predator of many habitats in which it occurs. It has remarkable ecological flexibility, thriving in a wide variety of ecosystems, from equatorial forests to cold mountainous habitats, from the southern tip of South America to Canada. It has been widely persecuted by humans, and extirpated from some areas (e.g. eastern USA except for a remnant population in Florida), but maintains healthy populations in other regions. It has been the subject of broad phylogeographic analyses using molecular markers (e.g. Culver et al. 2000; Matte et al. 2013) and many genetic studies focusing on Nearctic populations (e.g. Loxterman 2011), while those in the Neotropics have so far received comparatively less attention, although several local and regional studies have been conducted in the last 12 years (e.g. Miotto et al. 2011, 2012; Castilho et al. 2012; Wultsch et al. 2016b; Saranholi et al. 2017; Gallo et al. 2021). Pumas have also been the focus of an early effort to generate and apply genomic resources to develop a single nucleotide polymorphism (SNP)-based genotyping assay for use in population genetic, molecular ecology and forensic analyses (Fitak et al. 2016).

More recently, pumas have been the focus of two studies reporting whole-genome sequences for this species (Ochoa et al. 2019; Saremi et al. 2019). The study of Ochoa et al. (2019) reported the assembly and annotation of a Florida puma genome, while also sequencing nine additional individuals to investigate genes under selection and to assess genetic diversity. More specifically, they compared diversity levels between animals sampled the highly threatened and inbred Florida population (which received translocated animals in the mid-1990s in a genetic rescue operation) and the source population (Texas) of the introduced individuals. They found that a large proportion of the identified variants was unique to the Texas population, and were likely incorporated in post-translocation Florida individuals, which exhibited much higher levels of diversity compared to the original individuals from that area. The highest estimated diversity was observed in an individual from the Everglades National Park (also used to assemble the reference genome), a site in which anthropogenic releases in the 1950s and 1960s has resulted in the input of Central American genomic material (Roelke et al. 1993; Johnson et al. 2010).

The study of Saremi et al. (2019) reported the assembly and annotation of a highly contiguous genome from a California puma, along with additional genomes from seven individuals sampled at different locations in North America (including Florida) and two individuals from Brazil. They observed that Brazilian pumas exhibited higher levels of diversity, a much smaller load of Runs of Homozygosity (RoH), and a distinct demographic history relative to North American ones. They estimated the divergence between South and North American lineages to have occurred between 300 and 100 thousand years ago (KYA), and showed that the sampled Everglades individual (a different animal relative to the one sequenced in the Ochoa et al. 2019 study) did possess genomic evidence of admixed ancestry with lineages related to South America (no Central America lineage was included).

There was genetic structure among the sampled North American populations, which also differed in their levels of heterozygosity and RoH burden. The lowest levels of diversity and largest RoH burden were observed in the non-admixed (and highly inbred) Florida individuals, while the Everglades sample exhibited an unusual combination of relatively high heterozygosity with high RoH burden. As this pattern is likely the consequence of its unique history of admixed ancestry followed by local inbreeding, it led to a discussion on genetic rescue programs, which require more than a single outbreeding event to maintain higher diversity levels in the long term.

These two initial studies reporting puma genomes led to congruent results pertaining to present-day diversity and the underlying demographic processes in Florida populations, as well as comparisons with individuals from other regions. A recent follow-up study (Ochoa et al. 2022) re-analyzed the genomic data from both of them to focus in more detail on the mutation load of these Florida populations. Their results indicate that rescued populations whose effective size remains small may suffer from the effect of deleterious alleles that were introduced during the rescue itself, highlighting the need for long-term monitoring and management of this problem.

As a consequence of these initial studies, puma genomics can now hopefully advance at an accelerated pace. In addition to expanding the genomic assessment of Nearctic populations, which is certainly necessary, it is noteworthy that neotropical pumas remain largely unstudied, except for the two Brazilian individuals included in the Saremi et al. (2019) article. Given their diversity, the breadth of their distribution, the variety of habitats in which they dwell, and the anthropogenic threats that they face, they should undoubtedly be the focus of increased attention from genomic studies in the near future.

### *19.4.3 Small Cats of the Genus* Leopardus

The neotropical genus *Leopardus* comprises one of the eight main extant lineages of the family Felidae (Johnson et al. 2006). It encompasses at least eight living species of small to medium-sized cats, being the most speciose genus of the family (Kitchener et al. 2017). The eight recognized species are: ocelot (*L. pardalis*), margay (*L. wiedii*), Andean mountain cat (*L. jacobita*), pampas cat (*L. colocola*), Geoffroy's cat (*L. geoffroyi*), huiña (*L. guigna*), northern tigrina (*L. tigrinus*) and southern tigrina (*L. guttulus*) (Kitchener et al. 2017). Having derived from a recent and rapid radiation, these cats have interesting differences concerning ecoregions of occurrence, habitat usage, behavior, and morphology (Sunquist and Sunquist 2017). So far little is still known about the genetic bases of these striking adaptations, but some progress has been in this regard using genomic data. Further, considering that at least four *Leopardus* species are considered Vulnerable or Endangered by the IUCN (de Oliveira et al. 2016; Napolitano et al. 2015; Payan and de Oliveira 2016; Villalba et al. 2016), and that recent studies using genome-wide data shown that the

number of species is actually larger (see below), the relevance of employing genomic approaches to study the group's genetic structure and phylogeny increases.

Molecular data suggested that the genus began its diversification *ca.* 2.8 million years ago (MYA) (Johnson et al. 1999, 2006), but more recent analyses indicate that this process may have begun earlier, *ca.* 3–4.6 MYA (Li et al. 2016; Trindade et al. 2021). From a phylogenomic perspective, using complete genomes of four *Leopardus* species, the genus was shown to have high levels of genealogical discordance (i.e. distinct topologies across different genomic regions), with very similar proportions of three different topologies for the relationship of *L. pardalis* and *L. colocola*. This is an intriguing result, especially because, except for the ocelot lineage, for all Felidae lineages there is a predominant topology (Li et al. 2019). The two main processes that can cause such discordance are incomplete lineage sorting (ILS, when a polymorphism is maintained through a speciation event, leading to variable segregation of allelic lineages in a subsequent divergence) and hybridization (leading to interspecies introgression). These processes can currently be dissected more effectively by using complete genomes of the related species and sophisticated statistical methods (e.g. Bjorner et al. 2022; Komarova and Lavrenchenko 2022; Payseur and Rieseberg 2016; Peter 2016). Indeed, genetic studies of *Leopardus* have shown that this genus presents a very complex evolutionary history, with past and ongoing hybridization events (see below), which is potentially related to its recent diversification.

Molecular studies performed by Johnson et al. (1999), Trigo et al. (2008) and da Silva Santos et al. (2018) identified hybrids between *L. colocola* and *L. tigrinus* in central and northeastern Brazil (NE tigrina), where the two species coexist. This process led to the complete introgression of the mtDNA from *L. colocola* into this *L. tigrinus* population (Trigo et al. 2013). However, this hybridization seems to be very ancient, as no shared mtDNA haplotypes were found between the two species (da Silva Santos et al. 2018). Further, there were no nuclear genomic signatures of introgression between these species (Trindade et al. 2021), and no introgression analyses using complete genomes have been performed yet to explore this question. In contrast, in the southern portion of Brazil, in Rio Grande do Sul state, there is a hybrid zone between *L. guttulus* and *L. geoffroyi*. These species are mainly allopatric but coexist and hybridize in the transitional area between the Atlantic Forest, a forested biome, and the Pampa, a savanna habitat (Trigo et al. 2013, 2014; Sartor et al. 2021). In this region, hybridization is extensive and bidirectional, with hybrids from the F1 and F2 generations and backcrosses being found, in addition to a great genetic similarity between the parental species (Trigo et al. 2013). Although this hybridization process has already been studied with molecular data, questions regarding the origin of the current hybrid zone (if natural or anthropogenic), for how long this admixture has been occurring and what are its genomic consequences for both parentals and hybrids, remain open. By using complete genomes from a population perspective, local ancestry inference methods can be applied to identify, at each region of the entire hybrid genome, its parental origin. This can provide valuable information, including the measurement of genomic segments from one or another parental, and the use of their length to estimate if the admixture has been

happening for a few (e.g. large segments not disrupted by recombination) or several generations (Pool and Nielsen 2009; Corbett-Detig and Nielsen 2017). This example illustrates how only genomic analyses can shed light on the origin and the outcomes of some important evolutionary processes such as hybridization.

Interestingly, three of the four species involved in the above-mentioned hybridization events belong to two species complexes (i.e. two or more species which are taxonomically difficult to delimit due to their morphological similarity) within this genus: the tigrina complex and the colocola complex (García-Perea 1994; Trigo et al. 2013; Kitchener et al. 2017; Ruiz-García et al. 2018; Nascimento et al. 2020; Trindade et al. 2021). The latter complex was proposed based mainly on morphological and ecological analysis and may comprise five distinct species that may present mainly allopatric distributions across different South American regions: *L. colocola* (western slope of Andes, central Chile), *L. pajeros* (southern Chile to north-western Argentina), *L. braccatus* (central Brazil to north Argentina), *L. garleppi* (both sides of Andes, from Ecuador to Argentina) and *L. munoai* (southern Brazil to northeastern Argentina) (Nascimento et al. 2020). Genetic analysis with this complex has been performed so far with mitochondrial markers and microsatellite data, which have not yet provided a complete answer regarding the depth of differentiation among these geographic units (Johnson et al. 1999; Napolitano et al. 2008; Cossíos et al. 2009; da Silva Santos et al. 2018; Nascimento et al. 2020). Therefore, the use of genomic data should be a priority to assess more conclusively the evolutionary history of the colocola complex, enabling a better understanding of the morphological and ecological differences among units, and assisting in the definition of its taxonomic composition.

The tigrina complex, in its turn, has already been separated into at least three distinct species on the basis of molecular evidence. Trigo et al. (2013) used several molecular markers to identify a strong genetic differentiation between *L. tigrinus* populations from south-southeastern and those from central and northeastern (NE) Brazil, recognizing the southern unit as a distinct species, *L. guttulus*. In addition, Johnson et al. (1999) and Trigo et al. (2008) had already demonstrated, with mtDNA data, that Central American tigrinas were very divergent from *L. guttulus* populations. Later, employing genome-wide SNP data, Li et al. (2016) demonstrated that the Central American tigrinas were also very distinct from northeastern tigrina. Finally, Trindade et al. (2021) analyzed these three tigrina populations together, also with genome-wide SNP data, and showed that *L. guttulus* and NE tigrina are in fact sister species, with a depth of genetic differentiation similar to the one found between *L. geoffroyi* and *L. guigna*, reassuring the recognition of *L. guttulus* as a separate species. Further, this study strongly indicated that the Central American tigrinas correspond to a distinct species from *L. guttulus* as well as the NE tigrina, and that the tigrina complex is actually paraphyletic, since phylogenetic analyses support the allocation of Central American tigrinas at a more external position than two other *Leopardus* species. Analyzing the remaining populations of the tigrina complex is urgently needed to understand the evolution of this complex and to assign the appropriate taxonomic name to each species. This definition hinges upon

the identification of the evolutionary affinities of the populations from the Guiana shield, since this corresponds to the type locality for *L. tigrinus*.

Another very interesting trio is *L. wiedii, L. pardalis* and *L. jocobita*. *Leopardus wiedii* and *L. pardalis* have been characterized as sister-species, sharing a wide geographic distribution and similar coat patterns, but also showing very striking ecological and phenotypic differences, such as body length and cranial measures (Li et al. 2016; Sunquist and Sunquist 2017). By performing natural selection scans over complete genomes from both species, it was observed a signature of positive selection in the gene *POU4F2* in the *L. wiedii* lineage, which was further shown to be a fixed mutation in multiple South American samples of this species. This gene is related to retinal development and response to light, which correlates with the species' nocturnal activity and large eyes (Ramirez et al. 2022). Additionally, regarding phylogenetic relationships among these three related species, genome-wide data have shown considerable uncertainty regarding the support for the sister-species pair, although it was suggested that missing data may be the reason for such low resolution (Li et al. 2016; Trindade et al. 2021). Therefore, a deeper investigation on this trio is required, aiming to explore their relationship by using complete genomes to conclusively disentangle their evolutionary history.

Interestingly, despite sharing a great portion of their distribution, *L. pardalis* seems to have significantly higher levels of genetic diversity than *L. wiedii* (Ramirez et al. 2022). In fact, that species presents the highest heterozygosity values for any felid studied so far, probably due to the combination of a large geographic range, low population structure across large areas and higher density than most small neotropical cats. In addition, of the five species analyzed by Ramirez et al. (2022), *L. tigrinus* presented the lowest levels of heterozygosity for the genus, followed by *L. colocola* and then *L. wiedii* and *L. geoffroyi*. That study also demonstrated a curious trend of an opposite historical trajectory of the effective population size between *L. geoffroyi* and *L. tigrinus* and *L. colocola* and *L. wiedii*, probably related to their ecology (generalist *vs.* more specialized species) and how they responded to the climatic changes between glacial and interglacial periods. Although that study presented new insights about the genetic diversity and evolutionary history of the genus, it also highlighted how much is still unknown about these species and how powerful genomics tools can be to address these questions.

### *19.4.4 Otters (Mustelidae: Lutrinae)*

The Lutrinae subfamily includes the 13 currently recognized otter species, which are widely distributed around the globe, occurring on all continents except for Antarctica (Nowak 1999). They are a group of semi-aquatic carnivores that occupy a great diversity of habitats. Four species are distributed in the Neotropics: *Lontra longicaudis* (neotropical river otter), classified as Near Threatened by the IUCN, is distributed from Mexico to Northern Argentina (Macdonald and Mason 1990); *Lontra felina* (marine otter), classified as endangered, is distributed in the southern

Pacific Coast of South America from northern Peru to southern Chile and eastwards into Argentina; *Lontra provocax* (Southern river otter), classified as endangered, occurs in Chile, south of the Imperial River, and in Argentina mainly on the Limay watershed; and *Pteronura brasiliensis* (giant otter), classified as endangered, is distributed east of the Andes mostly in the Orinoco, Amazon, and Parana basins.

Despite their worrisome conservation status and charisma, there are few genetic studies of otters, most of which used small segments of mitochondrial DNA and/or microsatellite data. Just recently, genomic data started being published for otters (Jones et al. 2017; Beichman et al. 2019, 2021; Mead et al. 2020; de Ferran et al. 2022) but there is still no population-level genomic data for any of the neotropical species. Previous genetic studies encompassing these species focused mostly on their phylogeny or phylogeography, with population-level studies restricted to small areas of their ranges (e.g. Trinca et al. 2013; Latorre-Cardenas et al. 2020).

For *Lontra felina*, studies using mitochondrial DNA segments found no population structure within the Peruvian population (Valqui et al. 2010), but differences between this and the Chilean population (Vianna et al. 2010). These studies reported low nucleotide diversity but high haplotype diversity, which declined towards the extremes of its distribution (Vianna et al. 2010). A recent study using whole-genome data found a very low heterozygosity and a large number of Runs of Homozygosity (RoH) in this species, the second among otters after *Lontra provocax* (de Ferran et al. 2022). The reconstruction of past effective population size (Ne) showed a strong decline between 200 and 100 thousand years ago (KYA), leading to very low values towards the present (de Ferran et al. 2022).

Studies with *Lontra provocax* identified two subpopulations with striking differences between the freshwater and the marine populations, with the latter presenting higher diversity (Centron et al. 2008; Vianna et al. 2011). As mentioned above, genomic data identified extremely low levels of heterozygosity in this species, the lowest estimated Ne, and a large number of RoH, all of which presented the most striking values among otters globally (de Ferran et al. 2022). Furthermore, genomic data estimated the divergence time between *Lontra felina* and *L. provocax* as 0.43 MYA (de Ferran et al. 2022), more recent than previous estimates of 0.88 MYA based on mitochondrial DNA segments (Vianna et al. 2010).

Most studies that address *Lontra longicaudis* genetics focused on the phylogeography of the species and used mitochondrial DNA segments (Trinca et al. 2007, 2012; Guerrero et al. 2015; Ruiz-García et al. 2018). These studies found results that were partially congruent with previously described subspecies, with the identification of four well-delimited groups: Mexico, Colombia and Costa Rica, corresponding to *L. l. annectens*; Bolivia, with no subspecies defined; Amazon and French Guiana, corresponding to *L. l. enudris*; and eastern South America, corresponding to *L. l. longicaudis* (Trinca et al. 2012; Guerrero et al. 2015; Hernández-Romero et al. 2018). It was observed that there is a deep separation between *L. l. annectens* and the other phylogroups (Trinca et al. 2012), with divergence times estimations being close or even greater than those between *L. felina* e *L. provocax* (Trinca et al. 2012; Hernández-Romero et al. 2018). The striking divergence between cis- and trans-Andean groups is also found in studies using morphological

characters (Hernández-Romero et al. 2015), and niche and geographic barrier analyses (Hernández-Romero et al. 2018). However, a study focused on northwestern South America, especially Colombia, Peru and Bolivia, found no difference between the Orinoco, Magdalena and west Amazon basins (Ruiz-García et al. 2018). This study, however, only included one sample from the Parana river and none from Brazil, which limits further comparisons with other studies. The only genomic study reporting data on this species is that of de Ferran et al. (2022), which included a single individual bearing high levels of diversity and a demographic history dominated by a graduate expansion followed by a recent decline, distinct from the patterns observed in other otters.

Finally, most studies focused on *Pteronura brasiliensis* genetic diversity used mitochondrial and nuclear (microsatellites) markers (Garcia et al. 2007; Pickles et al. 2011, 2012; Caballero et al. 2015). They reported a complex phylogeographic structure with at least four biogeographic groups which differed from previously described subspecies: Uruá and Iténez rivers; Madre de Dios and Madeira rivers; Pantanal; Amazon, Guianas and Orinoco, with one study finding a new phylogroup as a subdivision of the latter (Puerto Carreño; Caballero et al. 2015). The divergence between giant otter populations would have occurred in two stages, the first split between the Iténez and Madre de Dios/Madeira groups around 1.24–1.69 MYA, followed by the separation of Pantanal and Amazon/Orinoco/Guianas, 0.88 MYA (Pickles et al. 2011). Despite this geographic structure, there was an inference of gene flow among the populations of northern South America (Pickles et al. 2012; Caballero et al. 2015). Two studies have reported genomic data on this species: Beichman et al. (2019) and de Ferran et al. (2022). The latter study assessed genomes from two giant otter individuals that exhibited a very congruent trajectory in their demographic history, comprising an expansion phase followed by a substantial population decline in the last 80 thousand years.

### *19.4.5 Canids*

The family Canidae comprises 37 currently recognized species distributed in 13 genera. They are one of the most widespread carnivorans, occurring throughout the world except Antarctica (Castelló 2018). In the Neotropics, there are 12 extant species: *Speothos venaticus* (bush dog), *Chrysocyon brachyurus* (maned wolf), *Cerdocyon thous* (crab-eating Fox), *Atelocynus microtis* (short-eared dog), *Lycalopex vetulus* (hoary fox), *L. fulvipes* (Darwin's fox), *L. griseus* (South American gray fox, or 'chilla'), *L. culpaeus* (culpeo fox), *L. gymnocercus* (pampas fox), *L. sechurae* (sechuran fox), *Urocyon cinereoargenteus* (gray fox) and *Canis latrans* (coyote). These species vary in size and overall morphology, with interesting cases of very unique phenotypes (e.g. mane and long legs in *Chrysocyon brachyurus*, and short legs in *S. venaticus*). Most of them occur only within South America (the geographic region with the greatest canid diversity), except for *Urocyon*

*cinereoargenteus* and *Canis latrans*, which are essentially Nearctic and are not included in the monophyletic South American group (see below) (Castelló 2018).

One very interesting characteristic of canids, which is not common in other carnivores, is their variation in chromosome number and organization. Almost all South American canids, except for *Chrysocyon brachyurus* and *S. venaticus* (2n = 76), have 2n = 74 chromosomes, while *Urocyon cinereoargenteus* has 2n = 66 and *Canis latrans* has 2n = 39 (Castelló 2018). The canid chromosome arms seem to be a mosaic of conserved carnivoran segments, probably organized in the ancestor of the family, with posterior fusions and fissions occurring in some species (Nash et al. 2001). Conserved 3D chromatin conformation can be a response to evolutionary forces acting on gene regulation, adaptation of phenotypes and speciation. By using cutting-edge genomic approaches, it was observed that canids present some conserved regions, but the highly variable number of chromosomes within the family also resulted in differences in chromatin structure (Corbo et al. 2022). However, this perspective has never been explored in neotropical canids, and knowing this can be potentially valuable to help understand the adaptations and specialized traits observed in some of them. Furthermore, it could also help to understand more about the viability of hybridization in this group.

The South American endemic genera (*Chrysocyon, Speothos, Cerdocyon, Atelocynus* and *Lycalopex*) comprise a monophyletic group (Chavez et al. 2022; Lindblad-Toh et al. 2005; Wayne et al. 1997), with especially complex phylogenetic resolution in the genus *Lycalopex* due to its rapid diversification and occurrence of inter-species gene flow during this period (see below). The invasion of canids into the neotropical region has been a difficult problem to investigate, with fossils and molecular data suggesting at least three different events of dispersion and supporting a North American origin of the neotropical (extinct and current) lineages (e.g. Perini et al. 2010; Wayne et al. 1997). Recently, by using ~30 complete genomes from neotropical canids, it was inferred that South American canids may have entered the region once, with the ancestor occurring 3.9–3.5 MYA, first dispersing east of the Andes (Chavez et al. 2022). The arrival in South America is tightly associated with the Panama Bridge, during the Pliocene, while *Urocyon* and *Canis* may have occupied the Neotropics later during the Pleistocene (Castelló 2018). *Urocyon cinereoargenteus* belongs to an older, divergent canid group, while *Canis latrans* belongs to the wolf-like canid lineage and seems to have had a very important role in the evolution of other *Canis* species due to hybridization and introgression (vonHoldt et al. 2016). Knowing that hybridization between species has already happened in this group, and that rapidly diversified groups are also prone to admixture due to the lack of properly established reproduction barriers, hybridization also seems to be present among the neotropical species (Chavez et al. 2022; Favarini et al. 2022; Garcez 2019) and should be a focus of increased research attention in the future.

*Lycalopex* is a genus comprising six very recently diverged species which occur in several regions of South America, mostly in open habitats (Favarini et al. 2022). Based on both traditional molecular markers and more recently with genomic data, it was observed the occurrence of older and current processes of inter-species

admixture (Tchaicka et al. 2016; Garcez 2019; Chavez et al. 2022). Signatures of older gene flow within *Lycalopex* seem to be intense, especially involving *L. gymnocercus*. Genetic analyses support two current and/or recent admixture processes between *L. gymnocercus* and *L. vetulus*, and between *L. gymnocercus* and *L. griseus*. The use of genomic data showed that the hybridization between the first pair is very recent, potentially associated with human disturbances on the landscape which may have broken the barriers between them (Garcez 2019). Knowing that gene flow has had an important role in canid evolution, and that genomic explorations can help to better visualize hidden patterns of species' evolution, by for example exploring the effects of recombination rates on phylogenetic reconstruction (Hennelly et al. 2021; Li et al. 2019), a deep characterization of their evolutionary history using population genomics can be very valuable. In canids, this has already been explored for gray wolves, for example, showing that populations from Southern Asia can be potentially a separate, older lineage than the North American ones (Hennelly et al. 2021), supporting the protection of unique regional units in the context of wolf conservation. In this regard, a similar understanding for neotropical canids with known processes of current and older processes of divergence and/or gene flow is required. Such an improved knowledge, enabled by genomic analyses, will be important to guide paths of conservation planning and management actions targeting these species.

## 19.5 Current Challenges and Future Directions

Even though carnivorans are one of the most studied mammalian orders, and have already been the focus of several genomic studies, many knowledge gaps remain for all the species contained in this group, including those that occur in the Neotropics. Moreover, while some families (e.g. Felidae, Canidae, Ursidae) have received more attention, others have scarcely begun to be investigated using genomic approaches. Such skews in the literature, which follow similar patterns to the ones observed previously with studies employing traditional molecular markers, should be ameliorated by fostering more interest in conducting studies targeting the other groups.

If we only consider the neotropical terrestrial carnivorans, resources are currently most developed for jaguars and some small cats, with multiple individuals sequenced from across the species' ranges, and population-level analyses underway. In addition, genome sequences have been generated for at least one individual of all the species of neotropical felids (Li et al. 2019; Ochoa et al. 2019; Saremi et al. 2019; Ramirez et al. 2022; Lescroart et al. [in prep.]) and likewise for all neotropical canids (Chavez et al. 2022). Whole-genome sequence data have also been reported for all four currently recognized neotropical otter species (de Ferran et al. 2022). These studies have led to a variety of insights on the phylogenomics, demographic history and adaptive evolution of these carnivoran groups, as described in the sections above.

While these and other studies have generated whole-genome sequence (WGS) data from many neotropical carnivorans (Fig. 19.1), there are still no high-quality, chromosomal scale genomic assemblies for these taxa, precluding analyses focused on questions related to genome architecture. Such questions thus remain open for neotropical carnivores, and should be the focus of in-depth studies to be conducted in the future. To accomplish this, more effort should be placed on obtaining and storing high-quality biological samples suitable for long-read DNA sequencing approaches, which is a substantial challenge in the case of rare and elusive species. In addition, for most species in these better-studied families, no population-level genomic data have been collected, with only one or a few individuals having been sequenced so far. This constitutes another frontier in the study of neotropical carnivoran genomics, since many questions regarding their taxonomy, evolutionary history, population structure and present-day diversity (including assessments of anthropogenic impacts) remain unanswered and will benefit from the use of genome sequences. Furthermore, other types of studies, e.g. focusing on epigenomics, transcriptomics and hologenomics, have not even begun for this group, and are expected to emerge as the field matures, these techniques become more accessible, and suitable samples are procured.

While these advanced topics represent the frontiers for evolutionary and conservation genomics of these better-studied carnivoran families, for the others the field is in its complete infancy. For several poorly known species in the Procyonidae, Mustelidae and Mephitidae, even basic studies describing their genetic diversity and clarifying their taxonomy with traditional molecular markers are lacking. Is therefore a priority to address these knowledge gaps as quickly as possible, and to simultaneously include these taxa in the era of genomic data. It is expected that in the coming years genomic studies will be conducted on several of these poorly-known species, leveraging the expertise developed for other carnivores to more rapidly investigate and clarify questions pertaining to their taxonomy and evolutionary history. Moreover, it is likely that regional genomic consortia focused on neotropical biodiversity will emerge and gain momentum in the near future, fostering the acceleration of data collection, the sharing of resources, and the incorporation of genome-scale information in the context of conservation assessment and planning.

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# Chapter 20
# Challenges with Conservation Genetics and Genomics in Neotropical Forest


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## 20.1 Brief History of Deforestation in the Neotropics and Loss of Biodiversity

From the beginning of the territorial expansions of civilizations to the present, forests have always been threatened. In the Neotropics, especially in Brazil it was no different. Its colonization was carried out with the premise of exploiting its natural resources, such as the Brazil-wood (*Caesalpinia echinata*), which culminated in the devastation of the Atlantic Forest. Then, the exploration of rubber tree (*Hevea brasiliensis*) led to the felling of the Amazon Forest. With the expansion of the

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Brazilian territory, the other biomes lost space with the development of cities and especially agriculture and pastures.

The neotropical region is characterized as one of the areas that retains the largest vegetation cover in the world. On the other hand, since 2017 there was an increase in deforestation rates, especially in countries that have the Amazon Forest (Brazil, Colombia and Peru) (Betts et al. 2017; Davis et al. 2020). In order to get this magnitude, Granthan et al. (2020) estimated the vegetation cover of this region, via satellite monitoring in the period 2001–2018. The authors used the Forest Landscape Integrity Index (FLII), which ranges from 0 to 10, where 10 means that the vegetation has not changed. The neotropical region presented an average of 7.81, with a negative highlight for Paraguay, Argentina, Chile and Brazil; and a positive highlight for Suriname, Guyana, Peru and Venezuela. Another worrying information in this study was that the proportion of forest remnants in the neotropical region is 68%, that is, 10,271,519 km$^2$ (initially 14,965,342 km$^2$).

It has been reported that the present biota is entering a "sixth" mass extinction, because of chronic exposure to anthropogenic activities (Khan et al. 2016). Currently, the report made by the IPCC (Intergovernmental Panel on Climate Change) showed that there was an increase of 1.1 °C in the pre-industrial period, causing a decrease in glaciers and high sea level, due to the presence of high concentrations of greenhouse gases. Consequently, in that period it was registered as the highest temperature in history. Species may respond to climate change in different ways, some can acclimatize, suffer evolutionary adaptation and/or shift their distribution to more suitable areas. However, neotropical plants area adapted to limited seasonal variation in temperature (Zwiener et al. 2017). To reverse this scenario, it is necessary to reduce emissions of these gases by 7.6% each year, until 2030 (IPCC 2018).

Unfortunately, today we do not have the knowledge and appreciation for our "green treasures". Neotropical forests are incredibly biodiverse; they support at least two-thirds of the world's biodiversity despite covering less than 10% of Earth's land surface (Giam 2017). Beech et al. (2017) reported that there are 60,065 tree species recorded worldwide. The country with the most diverse tree flora is Brazil (8715 spp.), followed by Colombia (5776 spp.) and Indonesia (5142 spp.). Almost 58% of all tree species are endemic to a single country, with Brazil (4333 spp.) with the largest number of species. In Brazil, the biomes that retain the greatest biodiversity of the flora as a whole are the Atlantic Forest, Amazon and Cerrado. However, the Atlantic Forest, Cerrado and Caatinga are the regions with the highest number of endangered species (Table 20.1). This information is worrying because, unfortunately, prospects for neotropical forests and the biodiversity therein are becoming increasingly bleak owing to unabated deforestation and forest alteration (Giam 2017).

This information contextualized above is alarming and expresses the urgency of measures for the management and conservation of biodiversity in the neotropical region. As we have a lack of information on this subject in this region, we will only address Brazil, which according to Torres-Florez et al. (2018), is the country with the largest database, with about 44% of publications on genetic conservation since 1992. Although Brazil has several initiatives on the part of the academy to

investigate the genetic basis of biodiversity and its respective management and conservation, we can observe that conservation strategies, in general, are inefficient according to the information mentioned below regarding the studies by Pires et al. (2019) and Table 20.1.

Amazon is the largest biome, covering immense social and cultural diversity surrounding ecosystem mosaics, mainly in the Tropical Rainforest (Fig. 20.1a). It currently has 43.9% of the total protected area if we consider indigenous lands (20.4%). The Cerrado is the second largest biome, occupying 24% of the territory and is

**Table 20.1** Characterization of Brazilian biomes in relation to territorial extension, percentage of protected area and biodiversity richness

| Biome | [a]Total area (thousand km$^2$) | Occupation (%) | Remaining native (%) | Total protected area (%) | Flora (ssp.) | Endangered species (ssp.) |
|---|---|---|---|---|---|---|
| Amazon | 4200 | 49 | 82 | 43.9 | 12,354 | 86 |
| Cerrado | 2000 | 24 | 55 | 8.2 | 12,070 | 645 |
| Atlantic forest | 1300 | 13 | 28 | 11 | 16,146 | 1544 |
| Caatinga | 912 | 11 | 57 | 1.3 | 3150 | 253 |
| Pantanal | 150 | 1.8 | 73 | 6.5 | 1577 | 21 |
| Pampas | 176 | 2 | 26 | 3.4 | 2215 | 120 |

Source: adapted from Pires et al. (2019)
[a]Approximated values

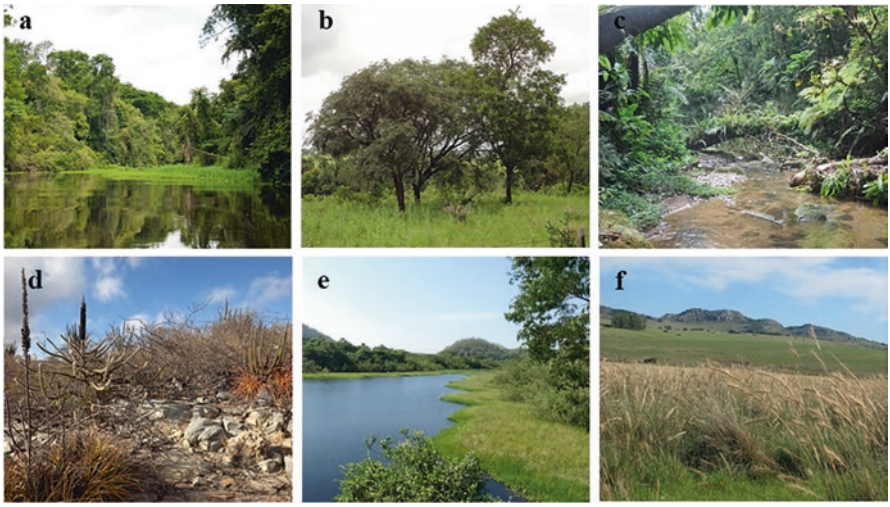


**Fig. 20.1** This data set displays the boundaries of six Brazilian continental biomes: the (**a**) Amazon. (Photo: Geraldo Majela Moraes Sálvio). (**b**) Cerrado. (Photo: Mario Luiz Teixeira de Moraes). (**c**) Atlantic Forest. (Photo: Geraldo Majela Moraes Sálvio). (**d**) Caatinga. (Photo: Roland Brack). (**e**) Pantanal. (Photo Marcos Vinicius Bohrer Monteiro Siqueira). (**f**) Pampas. (Photo: Rosa Lia Barbieri)

characterized by being the richest savanna in the world in terms of biodiversity (Fig. 20.1b). This a key role in the protection, production and maintenance of water quality in several river sources. Unfortunately, its deforestation is 2.5 times greater than in the Amazon (Strassburg et al. 2017) and its total protected area is only 8.2% considering indigenous territories (4.3%). The Atlantic Forest extends along the coastline, characterized by many vegetation formations, from semideciduous forests to restingas and mangroves (Fig. 20.1c). As it is located in the richest and most populous regions of the country, it is the most studied biome, but the second with the largest degraded area, with only 11% being protected. The Caatinga is characterized by a semi-arid climate with relatively high temperatures (annual average of 23–27 °C), low rainfall (300–1000 mm/year) and irregular rainfall distribution (Fig. 20.1d). Even occurring in a large territorial extension, there is an insignificant preservation (1.3%). The Pantanal is the sixth largest continuous wetland on Earth, consisting of an extensive plain surrounded by mountains and plateaus (Fig. 20.1e). It presents formations from forests to swamps. There is the presence of flooding that occurs by the combination of rains with the waters that overflow from its tributaries. It is the smallest national biome and has 6.5% of protected area, considering indigenous lands (1.8%). Finally, the Pampas consist of open and flat landscapes that dominate large portions. Forests can be observed along the rivers in the area bordering the Atlantic Forest (Fig. 20.1f). This occurs exclusively in the southern region of the country and has only 3.4% in a protected area.

In addition to the low coverage of protection of biomes in the in situ conservation form (see Chap. 9), Myers et al. (2000) studied the two hotspots in Brazil and found that Atlantic Rainforest has 2.7% endemic plants (% of global plants, 300,000) and 8.7 species/area ratios per 100 $km^2$ of hotspots (endemic plants). The Cerrado has 1.5% endemic plants and 1.2 species/area ratios per 100 $km^2$ of hotspots. This information reinforces the importance of these biomes and the need to establish more efficient strategies to protect these hotspots.

Regarding ex situ conservation, Brazil has satisfactory food security (Burle 2019), native tree species claim protection. Salomão et al. (2019) found that 965 accessions of 270 species, 150 genera belonging to 46 botanical families are documented in the Alelo Vegetal System (a corporate platform of systems of the Brazilian Agricultural Research Corporation (Embrapa)) used to manage data and information on Research and Development activities applied to genetic resources in their various aspects. Six accessions have only the identification of the botanical families Bignoniaceae (one species), Fabaceae (three species), Malvaceae (one species) and Solanaceae (one species). These collections are incipient compared to the magnitude of Brazilian biodiversity.

The problem with human activities is not just forest degradation (Kindermann et al. 2008; Hansen et al. 2013), but also the associated loss of biodiversity, ecosystem functions and services such as water supply, nutrient cycling and climate regulation (Thompson et al. 2013), affecting human well-being, including the economy and health (Newman et al. 2014; Liu et al. 2016).

Another question is how to proceed with the recovery of these degraded areas. The ability of forests to resist disturbance and recover is variable (Fahey et al. 2016;

O'Connor et al. 2017). Traditionally, forest restoration aims to restore the same or very close conditions as the original forest (Stanturf et al. 2014). It has used several techniques, including (i) seedling planting, (ii) agroforestry systems, (iii) assisted natural regeneration and (iv) natural regeneration. The authors emphasize that each restorative action and technique must be adapted to the local reality with a well-defined objective. Cruz et al. (2020) made a list of 405 forest recovery projects since 1950 but were not able to estimate the amount of area recovered due to lack of information and scientific production on the subject. Based on in-depth knowledge of forest restoration as a science, it will be possible to propose better actions for future projects to recover deforested and degraded areas.

In this context, for the protection of neotropical forests to become a viable policy proposal for the government, financial and market incentives must be increased by greater public awareness of the many benefits that forests provide, both locally and globally. Building this awareness through better communication of science is an essential complement to our increasingly sophisticated understanding of why rainforests are being destroyed (Seymour and Harris 2020). The best way to solve these problems is to understand the biodiversity present in forests and remnants. A series of tools can be applied to identify, use and manage the genetic variation of these tree populations. Integrated with conservation biology, conservation genetics, throughout its 40 years of existence, has enabled an arsenal of basic and applied knowledge, ranging from basic aspects of population genetics to the inference of conservationist laws.

## 20.2 Genetic Diversity Discoveries in Neotropical Trees and Proposals for Conservation and Mitigation

The conservation of genetic diversity has a profound importance for conservation efforts because genetic diversity is the basic requirement for promoting evolutionary adaptation, which is the key to the long-term survival of any species (Schemske et al. 1994). For this survival to be successful, two objectives must be met: (i) to preserve sufficient amounts of hereditary genetic variation, especially in small populations threatened with extinction and (ii) to avoid the fixation of deleterious alleles that contribute to the reduction of the capacity of adaptation and accumulation of harmful mutations (Lynch 1996).

Thus, it is possible to observe that these objectives are closely related, showing that genetic conservation is not just to preserve species statically, but as dynamic beings capable of responding to environmental changes over time. Therefore, only when an adequate sampling of genetic diversity in their natural habitat is made, species will be able to present their adaptive potential in the face of climate changes, which will persist in the long term (O'Brien 1996).

In Brazil, the urgency of protecting biomes and greater adoption of ex situ conservation strategies to prevent the mass extinction of forest species is

unquestionable. In addition, genetic erosion is another worrying factor in which these species are subject to suffer, such as the field crops discussed in Chap. 9.

Unfortunately, we do not have specific legislation on germplasm collection and conservation. Law No. 10,711 and its regulations, establishing the National System of Seeds and Seedlings, guarantee the identity, quality and multiplication of plant material for commercialization and use throughout the national territory. However, the limit number of mother trees and the minimum distance for seed collection are not specified. Sena and Gariglio (2008) suggest collecting seeds from at least 15 mother trees per species, with distances ranging from 50 to 100 meters between them. Nogueira and Medeiros (2007) recommend between 10 and 20 mother trees, with a minimum distance of 100 m. The authors emphasize the importance of collecting only 25% to 30% of seeds in each tree so as not to impact the local fauna and the perpetuation of the species. Sebbenn (2006) suggests 45 mother trees for seed collection and a minimum distance similar to the quote above. Crossa and Vencovsky (2011) emphasize the importance of the mating system at the time of sampling in natural populations. In this way, for better representation of the genetic base, it is recommended to choose 25 mother trees for outcrossing species and 50 for self-fertilization species. Thus, we assume that ex situ conservation planning is not easy. Next, we will show which parameters must be considered to carry out an adequate sampling.

In order to retain the greatest possible allele diversity in an active germplasm bank, that is, to conserve individuals outside their natural habitat *in vivo* (progeny test) and to ensure the long-term survival of these genotypes, it is necessary to estimate: (i) genetic diversity; (ii) mating system; (iii) population genetic structure and fine-scale spatial genetic structure; (iv) gene flow; (v) inbreeding depression; (vi) maintenance of genetic variation. Today it is possible to quantify all these variables, with acceptable accuracy, using genetic markers.

First it is necessary to investigate genetic diversity in natural populations from the extraction, quantification and identification of genomic DNA in plant tissues. This molecular tool has presented considerable technological advances in a short period of time. In Brazil, enzymatic markers (allozymes) were of great importance, despite having a low degree of polymorphism, being used for a short period of time, when they began to be replaced by DNA markers capable of detecting greater variability between individuals. DNA polymorphisms emerged as a result of a variation (mutation) that are generally referred to by the type of mutation that created them.

The development and use of molecular markers for the detection and exploration of these DNA polymorphisms is one of the most significant advances in the field of molecular genetics. In this way, researchers began to use this tool on a large scale. Initially, markers such as AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphism DNA) for *Baccharis concinna* (Gomes et al. 2004), *Myracrodruon urundeuva* (Reis and Grattapaglia 2004) and *Plathymenia reticulata* (Lacerda et al. 2001). The ISSR (Inter-simple sequence repeats) were used to access genetic diversity. However, microsatellites (SSR – Simple Sequence Repeats) are the most used markers, not only for studies of genetic diversity, but also to investigate mating system and gene flow, as they present in large quantities

in the genome, are codominant, multi-allele, reproducible, easy to handle and availability of high technology equipment (Turchetto et al. 2017).

Genetic diversity is important because it refers to the proportion of heterozygous individuals for a given locus, the total number of different alleles in the population, the average number of alleles per locus, the allelic richness (presence of rare alleles) among other parameters (Hartl and Clark 2007).

A brief review of the literature on genetic diversity in natural and anthropotized populations of neotropical tree species occurring in different biomes is presented in Table 20.2. The mean total number of alleles was 10.9; the allelic richness of 7.0, the expected heterozygosity of the Hardy-Weinberg Equilibrium of 0.656, the observed heterozygosity of 0.549 and the fixation index of 0.206. These results reveal evidence of genetic erosion in the species studied, as a consequence of the deforestation and the indiscriminate use of natural resources. Thus, there were higher proportions of homozygous individuals than expected, suggesting an increase in the level of kinship in the next reproductive events. These results reveal evidence of genetic erosion in the studied species, as a deforestation consequence and the indiscriminate use of natural resources.

After quantifying genetic diversity, it is essential to discover how reproductive events occur. The mating system provides the understanding at the moment that the fusion is made from gametes to zygotes and, therefore, is responsible for the genotypic structures of the progenies in a population. The most important focus of the analysis of the mating system is to describe the way in which the gametes of parents in a population come together. The way trees in a population mating system are affected by many environmental and human influences, such as population density, spatial structure, phenology and pollination vectors, deforestation and abiotic factors (Eckert et al. 2010).

Due to the relevance of the subject, Goodwillie et al. (2005) did a thorough study reclassifying the mating system in tree species, being classified according to the outcrossing rate (t) as: (i) autogamy ($t < 20\%$), (ii) outcrossed ($t > 80\%$) and (iii) mixed mating system ($20\% > t < 80\%$). It is important to emphasize that mixed

**Table 20.2** Genetic diversity and fixation index (*F*) estimated for neotropical trees

| Species | *n* | *A* | *R* | $H_o$ | $H_e$ | *F* | References |
|---|---|---|---|---|---|---|---|
| *Araucaria angustifolia* | 120 | 5.3 | 3.8 | 0.614 | 0.672 | 0.090 | Sousa et al. (2020) |
| *Bagassa guianensis* | 488 | 11.8 | 7.9 | 0.626 | 0.693 | 0.097 | Arruda et al. (2015) |
| *Casearia sylvestris* | – | – | 9.0 | – | 0.828 | 0.600 | Zucchi et al. (2017) |
| *Centrolobium tomentosum* | 79 | – | 3.4 | 0.420 | 0.515 | 0.186 | Sujii et al. (2017) |
| *Copaifera langsdorffii* | 560 | 15.6 | 15.5 | 0.741 | 0.825 | 0.102 | Tarazi et al. (2013) |
| *Dipteryx alata* | 123 | – | 4.0 | 0.435 | 0.585 | 0.260 | Tambarussi et al. (2017b) |
| *Myroxylon peruiferum* | 93 | – | 2.7 | 0.365 | 0.385 | 0.166 | Schwarcz et al. (2018) |
| *Qualea grandifora* | 45 | – | 9.7 | 0.640 | 0.750 | 0.150 | Potascheff et al. (2019) |
| Mean | – | 10.9 | 7.0 | 0.549 | 0.656 | 0.206 | – |

*n* is the sample size; *A* is average number of alleles per locus; *R* is the allelic richness; $H_o$ is observed heterozygosity and $H_e$ is expected heterozygosity

species are more common than we considered. In this context, Degen and Sebbenn (2014) made review on the mating system in neotropical tree species and found that it has a predominance of outcrossing (average outcrossing rate of 0.88) and full-sib progenies (coancestry close to 0.25). Interestingly, the authors found different outcrossing rates and coancestry values within the same species, suggesting that these parameters do not have a standard value per species as they are entirely dependent on pollinating agents and environmental conditions. This information can suggest that although the species are preferably of outcrossing, there is a certain degree of kinship, so, once again, that the devastation of our forests is directly interfering with the mating system and their respective descendants.

The mating system is closely linked to the location of genotypes in a given population. The frequency distribution of genetic types is used to calculate parameters that characterize genetic variation within or between populations (Berg and Hamrick 1997). Spatial genetic structure (SGS) in natural populations can be caused by the nonrandom spatial distribution of genotypes (Vekemans and Hardy 2004) and, it is evident that the seed and pollen dispersion vector, mainly by animals, expresses an important role in the establishment of progenies in the next generation, ensuring the survival of the species in the place over the years. Thus, if these are extinct from the area, due to anthropogenic disturbances, the SGS will be strongly altered, changing the dynamics of the spatial distribution of genotypes.

The method most often used to characterize differentiation between populations is the calculation of $F_{ST} = G_{ST}$ (Nei 1973), that is, the proportion of total genetic variation that is due to differentiation between populations (Finkeldey 1994). Another way of calculating F-statistics is using Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992; Meirmans 2012). In an AMOVA, $F_{ST}$ analogs (called $\varphi_{ST}$) are calculated as a ratio of variance components that are obtained from a matrix of squared Euclidian distances between pairwise individuals (Excoffier et al. 1992; Meirmans 2006). It is a statistical approach to partition total genetic variation within and among populations or groups at different levels of hierarchical subdivision (Allendorf and Luikart 2009).

In practical context, $F_{ST}$ is used to verify which population or species has the greatest genotype variation. Martins et al. (2015) investigated the genetic structure in populations of three species of the Atlantic Forest, *Ocotea catharinensis*, *Ocotea odorifera* and *Ocotea porosa*. There was a moderate genetic differentiation between the populations of each species. The populations of *O. catharinensis* showed greater divergence ($F_{ST} = 0.148$) and the populations of *O. odorifera* were the most similar ($F_{ST} = 0.086$). With AMOVA, it was found that most of the genetic diversity of each species was within populations (80%, 88% and 84%, respectively for *O. catharinensis, O. odorifera* and *O. porosa*). The three species have been heavily harvested because of their high timber and essential oils values. However, the authors highlight that the lower SGS found in the species may be due to their long generation times and the historical gene flow, which can guarantee many generations in the current population and the maintenance of genetic diversity.

At fine scale, the most prevalent cause of SGS probably is the formation of a local genotype structure, as a result of limited gene dispersal. Thus, under isolation

by distance within continuous populations, genetic similarity is higher among neighbors than among distant individuals (Vekemans and Hardy 2004). Within populations, it is possible to measure the fine-scale SGS based on the estimate of pairwise kinship coefficient between pairs of individuals ($F_{IJ}$). So, average pairwise $F_{IJ}$ estimates can be plotted against pairwise spatial distances, considering increasing distance intervals.

Sujii et al. (2015) investigated the genetic structure at both fine and large scale in nine populations of *Bertholletia excelsa.* The authors found at fine-scale SGS, small structuring in five populations, mainly between individuals up to 100 m apart. At large-scale, the model-based and distance-based approaches resulted in $K = 4$ clusters, dividing the Amazon basin into four groups of gene pool distribution in the west, center, north and northeast region. The AMOVA was performed considering these four groups of populations, and the analysis showed that 86.1% of the total variation was within groups and 12.06% was among groups ($p < 0.01$). The main hypothesis for the similarity between close regions and small-scale genetic structure of *B. excelsa* was the behavior of their pollinating bees, which tend to forage in more restricted areas. The fine-scale SGS was similar to that found for other tree species, with pollen and seeds dispersed by animals (Vekemans and Hardy 2004).

SGS is a consequence of the gene flow of a particular species. Gene flow is the movement of pollen and seeds between populations and its interruption increases the genetic divergence between populations, which can make them isolated (Sullivan et al. 2019). Habitat loss can disrupt this flow, affecting reproduction success and gene dispersal in human-modified landscapes (Fahrig 2003). According to the patterns of genetic variation, over space and time (Holderegger et al. 2006), a population may show speciation of some genotypes, as a consequence of natural selection, genetic drift and inbreeding (Wagar et al. 2021).

The main vector of pollen dispersion in most neotropical tree species is pollinated by animals, which can reach about 8.2 km, by bats, in *Hymenaea stigonocarpa* (Moraes et al. 2018), 3.9 km, by bees, in *Swietenia macrophylla* (Oliveira et al. 2020) and 2.8 km, by birds, in *Symphonia globulifera* (Carneiro et al. 2009). The seeds can be dispersed by the wind with about 887 m (*M. urundeuva*, Gaino et al. 2010), by animals with 8.0 km (*H. stigonocarpa*, Moraes et al. 2018), by gravity with 318 m (*Araucaria angustifolia*, Sant'Anna et al. 2013) and, in some cases, by water. This information is important to understand the dynamics of the populations, from the gene flow, and to develop strategies for the collection of seeds aiming at the establishment of a germplasm bank and future seed orchard.

Early in the development of Mendelian genetics, the researchers realized that the increase in homozygosity resulting from inbreeding (outcrossing between related individuals and/or self-fertilization) caused a loss in fitness, a phenomenon called inbreeding depression (Hedrick and Miller 1992).

In neotropical tree species the levels of inbreeding in the descendant generation are alarming. In *Cariniana legalis*, inbreeding depression of 30% and 36% was observed for the survival of seedlings, from outcrossing between relatives and self-fertilization, respectively (Tambarussi et al. 2017a). For *H. stigonocarpa*, the situation was more critical, with 71% and 52%, in the survival of seedlings, from

outcrossing between relatives and self-fertilization, respectively (Moraes et al. 2018). This has strong implications for the collection of seeds for conservation, breeding programs and environmental restoration, as the objective is to achieve maximum genetic diversity and the variance effective size in the seed samples. Therefore, collecting seed should preferably be carried out where self-fertilization and outcrossing between relatives are less frequent (Tarazi et al. 2013).

The last premise for planning seed collection is sample representativeness. One of the main challenges for genetic conservation in the long-term is the retention of sufficient genetic variation to guarantee adaptation, reproduction (expansion) and reestablishment in natural populations (Hedrick and Miller 1992). This problem would be solved if we worked with panmictic populations. However, real populations are finite, whose allelic frequencies fluctuate randomly over generations due to the sampling of gametes (Vencovsky et al. 2012). Parameters that measure genetic representativeness in a population are of critical importance to monitor and predict genetic diversity in natural and artificial populations in studies of population genetics, breeding, and conservation programs (Lindgren et al. 1996; Lindgren et al. 1997; Vencovsky and Crossa 2003; Vencovsky et al. 2012).

Retention of genetic variation is closely linked to the effective population size ($N_e$). This refers to "the number of breeding individuals in an idealized population that would have the same amount of frequency dispersion of alleles under random genetic drift or the same amount of inbreeding as the population under consideration" (Wright 1931). This is considered a fundamental measure of the representativeness of a sample of individuals because it reveals the effects of inbreeding and the variation of the allelic frequency occurring in the census population (Vencovsky et al. 2012).

Suggested as a useful tool for tree breeders to evaluate the consequences of different breeding strategies, status number is the number of unrelated and non-inbred individuals in an ideal panmictic population. Status number depends only on relatedness in the considered population and not on how gametes unite. Thus, genotypic frequencies are maintained across generations, with the same coefficient of inbreeding as in the first generation (Lindgren et al. 1996). Currently, through molecular technologies, the genetic relationship among individuals can be assessed more easily, accurately and cheaply than previously. Moreover, estimation of the inbreeding coefficient from the fixation index (Yan et al. 2017) and coancestry (Wang 2017) has become increasingly robust given the statistical tools available.

For neotropical tree species, the variance effective population size has been widely applied for the conservation of ex situ genes as it provides an estimate of the number of trees with seeds and seeds per tree that need to be collected for conservation or breeding programs (Degen and Sebbenn 2014). Some populations have high genetic variability, with about 44 matrix trees recommended for *A. angustifolia* (Costa et al. 2021) and 30 matrices for *Eugenia dysenterica* (Rodrigues et al. 2016) to retain an effective size sufficient for long-term conservation. However, other studies have already found critical scenarios, such as *Dipteryx alata* (Tambarussi et al. 2017b) and *H. stigonocarpa* (Moraes et al. 2018), which need to collect about 84 and 78 matrix trees, respectively, to provide an adequate effective size. Sousa

et al. (2015) reviewed this issue in several neotropical tree species and recommended the collection of at least 53 mother trees and a minimum distance of 110 m to avoid sampling related individuals. With this, we can infer that the best way to carry out a seed collection, aiming at the long term, is to sample at least 50 mother trees, with a distance of 100 m between them. It is also important to emphasize the sampling of the same number of seeds per mother trees, and this is not too high to obtain the lowest possible level of interference in the area.

In addition to ex situ conservation, it is necessary to focus on restoring degraded areas, in order to increase the rate of $CO_2$ mitigation and delay the effects of climate change. This activity provides improvements in the landscape structure and consequently in the quality of the ecosystem, protecting fragments from anthropic activities and using adaptive management to re-establish the successional trajectory. This, in turn, can facilitate the movement of biodiversity between fragmented landscapes by increasing the density, proximity and size of habitats suitable for species sensitive to border effects. For practical purposes, new protected areas may include not only blocks of conserved forest areas, but also degraded land to be later restored as natural habitats and/or as productive land. Less intensive means of production, such as agroforestry systems, sustainable forestry and silvopastoril systems, can increase the biological flows in the landscape and reduce the matrix hardness. Thus, biodiversity would not be "confined" to the protected area and a new habitat would be created to favor the population increase of vulnerable species (Brancalion et al. 2013).

However, the best path to solve the problems of biodiversity loss in the neotropical region is to have more public policies for better guidance in ex situ conservation, recovery of degraded areas and sustainable agriculture.

## 20.3 New Approaches Using Conservation Genomics in Neotropical Forests for Conservation Purposes

The transition of conservation genetics to conservation genomics (the study of genomic patterns or processes in any context that informs conservation efforts) leads to insights into the dynamics of selectively important variation and its interaction with environmental conditions, and into the mechanisms behind this interaction (Avise 2010; Ouborg et al. 2010; Allendorf et al. 2010). This transition has been supported by the population genomics that use genome-wide sampling to identify and to separate locus specific effects (such as selection, mutation, assortative mating and recombination) from genome-wide effects (such as drift or bottlenecks, gene flow and inbreeding) to improve our understanding of microevolution (Luikart et al. 2003). In a simplified way, population genomics allows the simultaneous study of several loci or genomic regions in many individuals from different populations for a better understanding of evolutionary processes (Alendorf and Luikart 2009).

Much of this achievement has been due to next-generation sequencing (NGS) technologies appeared on the market, which allows the assessment of sets of single nucleotide polymorphism markers (SNPs on a large scale), it was possible the burst of genome sequences from non-model organisms (Ellegren 2014). During the last years, tremendous progress has been made in terms of speed, read length, and throughput, along with a sharp reduction in per-base cost (van Dijk et al. 2014). The inexpensive production of large volumes of sequence data is the primary advantage over conventional methods (Metzker 2010). Now researchers are able to determine population structure without prior knowledge of the genome or diversity in the species.

New approaches of population genomics using NGS (Next Generation Sequence) such as RAD-Seq (Restriction-Site Associated DNA Sequencing) (Peterson et al. 2012) and GBS (Genotyping-by-Sequencing) (Elshire et al. 2011) are based on reducing genome complexity with restriction enzymes (REs). This approach is simple, quick, extremely specific, highly reproducible, and may reach important regions of the genome that are inaccessible to sequence capture approaches (Elshire et al. 2011), which offers the possibility of identifying loci with a high probability of being under selection, called outlier loci. Also these techniques used to identify outliers loci, can determine the environmental conditions they are related to in populations subjected to nutrient-poor soil conditions, with low water availability, high insolation and climatic conditions similar to those found in deforested areas. This is a promising alternative for selecting seedlings or seeds that will provide the population of the restoration with better alleles adapted to the new environmental conditions (Schwarcz et al. 2018).

However, a number of conservation genomics limitations are pointed out by Khan et al. (2016) among which (i) the most important impediment is the lack of availability of samples; (ii) the production of genomic data is easier / faster, but the data analysis technique requires a robust level of bioinformatics / computational biology; (iii) many statistical programs of population genetics need to be adapted to bigdata; (iv) the (bad) use of genetic data may result in a very restricted definition of Unit Conservation, which may prevent conservation actions, and even hinder the management of species threatened with extinction.

According to Martins et al. (2016) forest fragments are crucial for in situ species conservation and the maintenance of long-term genetic diversity in human-modified neotropical landscapes. Moreover, by establishing restoration plantations with high genetic diversity, the risk of extinctions of neotropical trees threatened by reproductive isolation in fragmented landscapes can be reduced. Zucchi et al. (2017) study four model tree species from the Brazilian Atlantic Forest, comparing two high-diversity restoration plantations, one forest fragment and one conserved remnant. The authors observed lower allelic richness in early successional species in restoration sites, suggesting that some species may be more prone to reintroduction with lower genetic diversity. This result shows important applications such as (i) populations established in restoration sites through tree plantations may have as much genetic diversity as natural populations from reference ecosystems; (ii) restoration

areas implemented with high genetic diversity may serve as sources of genetic variability to forest fragments in human-modified landscapes.

A few studies recently started to show genome results with endemic brazilian tree species. In pitanga (*Eugenia uniflora*), next-generation sequencing identified SSR sequences, both in nuclear (Sarzi et al. 2019) and chloroplast genomes (Eguiluz et al. 2017). Even though it is a non-model species, basic genomic information has been established and can be widely explored (Stefenon et al. 2019). In baru (*D. alata*), using the MiSeq Illumina platform, a nuclear genome (Antunes et al. 2020a) and chloroplast genome draft (Antunes et al. 2020b) were assembled, expanding the available genetic analysis repertoire for the species.

## 20.4 Future Implications for the Conservation of Neotropical Forests with a Genomic Approach

The advancement of molecular biology has led to great discoveries in the area of genetic conservation. In the past, the use of morphological and biochemical markers added knowledge of how phenotypes were transmitted from parents to progenies. Further on, the discovery of co-dominant markers allowed the estimation of several parameters that illustrate the contribution of alleles in a population, how they are transmitted and distributed over space and time. Currently, with the discovery of SNP markers, there is the possibility of investigating the genome of an organism further, and doing these studies multidisciplinary, not only focusing on genetics, but also on omics, such as proteomics and metabolomics. In this context, genomic data will play a crucial role in informing management and policy for species of conservation concern. Now is the time to consider carefully how to take the best advantage of these data to delineate CUs, characterize adaptive variation and then apply this information to improve conservation decision making (Funk et al. 2012).

There are few government initiatives to protect national biodiversity. In 2021 Brazil signed a law in view of the National Policy for Payment for Environmental Services (NPPES). In practice, the action gives force to the Forest + program, created by the Ministry of the Environment (MMA) in 2020. NPPES is the most recent development of a series of actions that MMA has been taking to create a market for environmental services in Brazil. The payers remunerate those who provide native forest conservation services, such as surveillance, monitoring, fighting forest fires, protection of water springs, biodiversity and nature as a whole. Other milestones are the implementation of the Forest + Amazon Pilot Project, the Forest + Carbon, which recognized the free market for forest carbon credits, and the formalization of economic conservation activities together with IBGE, allowing the issuance of invoices for environmental services (MMA 2021).

However, these initiatives are not enough, since at the beginning of this chapter there is an increase in deforestation rates and several species at risk of extinction. Even with few resources, several scientists in the country work to investigate the

loss of plant genetic resources and their consequences for society, as well as suggestions for the best way to conserve this biodiversity (Schlottfeldt et al. 2015; Souza et al. 2015; Diniz-Filho et al. 2016 and 2018; Dryflour et al. 2016; Mohebalian and Aguilar 2018; Edwards et al. 2019). Nonetheless, as already demonstrated in a previous Chap. 9, it is noted that in Brazil public institutions, such as Embrapa, State and Federal Universities, Research Institutes, among others, are most responsible for ensuring biodiversity, in terms of ex situ conservation. The Federal Government, in turn, and some ONGs are responsible for in situ conservation. One of the main obstacles is that the conservation of plant genetic resources is basically dependent on public resources. The same is true for research, where the private sector has little participation, especially in neotropical tree species. As a result, the researcher's difficulty in exercising his role in recent years is increasingly complex, due to successive investment cuts in this area.

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# Chapter 21
# Integrating Genomic and Cytogenetic Data to Study the Evolutionary History of Arapaimas and Arowanas in the Neotropics

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## 21.1 Introduction

The neotropical region has a high level of species richness, both in terrestrial and aquatic environments (Rull and Carnaval 2020). Fishes represent about half of the vertebrate diversity, making them a key taxonomic group for evolutionary studies (Nelson et al. 2016). More than 6000 fish species inhabit the neotropical region (Reis 2003; Fricke et al. 2022), an estimate that is likely low given that a considerable portion of its freshwater ichthyofauna is still unknown (Nelson et al. 2016). The Amazon region has some of the highest biodiversity levels worldwide (Antonelli et al. 2018) and the richest and most diverse fish fauna, containing representatives of all major teleost phylogenetic clades (Val 1995; Reis et al. 2016). This outstanding biodiversity may be related to both low extinction and high speciation rates associated with tropical regions when compared to the northern realms, a pattern known as the latitudinal gradient of biodiversity, which has been analyzed and

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corroborated for different environments and organisms (Hillebrand 2004). Numerous hypotheses have been proposed to explain this pattern, and recent discoveries support a scenario in which the gradient is the result of different climate conditions over time that had an impact on speciation, dispersal events, and extinction rates (Meseguer and Condamine 2019). Local events associated with the complex geological and climatic history of the Neotropics may also have promoted speciation (Albert and Reis 2011; Rull 2018). This is likely the case for inversions in the course of major rivers and marine transgressions that took place from the Paleocene to the Miocene (Hubert and Renno 2006). Moreover, the Great American Interchange, resulting from the closure of the Panamá isthmus, introduced new species and increased Amazon biodiversity (Carrillo et al. 2020; Rull and Carnaval 2020). All these processes extensively fragmented the environment providing thus "substratum" for practically endless speciation processes (Reis et al. 2016). Therefore, as a result of this complexity, the originating mechanisms of the abundant biodiversity of the neotropical region still need to be further investigated (Antonelli et al. 2010; Fine and Lohmann 2018; Ribas et al. 2022).

Better appraisal of these complex evolutionary mechanisms can be achieved by applying modern high-throughput sequencing techniques, which have enabled a large increase in the number of sampled individuals and sequenced loci in phylogenomic and phylogeographic frameworks (Ellegren 2014; Garrick et al. 2015). By analyzing the resulting genomic datasets, it is possible to prevent incomplete or unrealistic inferences associated with the stochastic features of the coalescent process when only one or a few genomic regions are considered (Edwards 2009; Beichman et al. 2018). More recently, the combination of genetic information with the fossil record has been used to better assess biogeographical history (Heath et al. 2014; Mongiardino-Koch et al. 2021) and obtain more precise estimates of lineage dispersal mode and timing (Hackel and Sanmartín 2021). Moreover, coupling these large datasets with modern analytical tools promotes a better understanding of microevolutionary processes acting at the population level (e.g., gene flow and demographic fluctuations; Oliveira et al. 2020a; Kirschner et al. 2022; Edwards et al. 2022).

Similarly, the significance of cytogenetic data in the exploration of fish biodiversity and conservation biology has been largely reported (Cioffi et al. 2018). Changes in chromosome number and/or high intrachromosomal dynamism have been associated with environmentally adapted traits, representing a strong evolutionary driver to increase biodiversity (Wellenreuther and Bernatchez 2018). For a long time, conventional chromosome banding procedures (e.g., G-banding; Q-banding, C-banding; AgNORs) have been used to assess the genetic diversity of related species, determine their diploid numbers, and reveal distinct chromosomal features, such as Bs and sex chromosomes (reviewed in Cioffi et al. 2018). Over the last decades, the advent of modern molecular cytogenetic techniques and their application in fish studies, such as comparative genomic hybridization (CGH) and chromosome painting (WCP), has allowed more fine-scaled insights into many evolutionary issues (e.g., uncovering evolutionary relationships and revealing hidden lineage diversities; Yano et al. 2021; Spangerberg et al. 2020; Symonová et al. 2015). As these procedures use a collection of genomic DNAs (gDNA) or whole microdissected chromosomes as labeling probes for fluorescence in situ hybridization (FISH), they

are particularly useful tools to highlight shared DNA sequences and detect chromosomal rearrangements (ChR) in closely related taxa. The identification of ChR events is very important since evidence regarding their putative contribution to the speciation process has steadily increased (Faria and Navarro 2010; Brown and O'Neill 2010). As further discussed in the sections below, differences in karyotype structure may favor the formation of novel species and thus increase biodiversity.

## 21.2 The Neotropical Bonytongues

The bonytongue fishes (order Osteoglossiformes) comprise around 248 species distributed in several rivers and lakes in South America, Africa, Asia, and Oceania (Hilton and Lavoué 2018). These species belong to five families: three of them endemic to the African continent (Pantodontidae, Mormyridae, and Gymnarchidae), and the other two (Notopteridae and Osteoglossidae) inhabiting Africa and Asia, and South America, Asia, and Australia, respectively. Given their ancient origin (~227 million years ago, Mya; Peterson et al. 2022), their current distribution pattern could have been associated with vicariant events that occurred after the division of Gondwana (Darlington 1957; Kumazawa and Nishida 2000). However, the fossil record and recent studies that incorporate molecular dating do not fully support this hypothesis (Wilson and Murray 2008; Inoue et al. 2010; Lavaué 2016). Based on the fossil record, common ancestors of living Osteoglossiformes were widespread throughout both Laurasia and Gondwana (Li and Wilson 1996; Newbrey and Bozek 2000). Thus, the current distribution of Osteoglossiformes may not be a completely reliable guide to their biogeographic history, as it might be a result of the extinction of clades in other regions since the Eocene (Li and Wilson 1999; Kumar et al. 2005).

Of the five extant families, only the charismatic pirarucus and arowanas (both from the Osteoglossidae family) occur in the Neotropics. Interestingly, the South American pirarucus (genus *Arapaima*) are the sister groups to the African genus *Heterotis*. Among the arowanas, the genus *Osteoglossum* from South America is sister to the *Scleropages* species present on both sides of the Wallace Line (the border that separates the zoogeographic regions of Asia and Australia; Nelson et al. 2016). This fragmented global distribution, strictly associated with freshwater habitats, together with their ancient origin, has motivated hypotheses about the relative contribution of transoceanic dispersal and vicariance associated with the breakup of Gondwana in their biogeographic history (Lavoué 2016; Hilton and Lavoué 2018).

## 21.3 Osteoglossidae Biogeography

Several studies have applied distinct approaches to obtain better insights into this intriguing continental distribution of the Osteoglossidae family. For arapaimas, evidence from the morphology of both living and fossil organisms (Bonde 2008) and

molecular assessments support long-distance marine or geodispersal (Hilton and Lavoué 2018). Indeed, the divergence time between the South American *Arapaima* and African *Heterotis* was estimated as ~85–50 Mya (Lavoué 2016; Hilton and Lavoué 2018), posterior to the separation of the two continents (~110 Mya). This scenario refutes Gondwana vicariance and supports post-fragmentation dispersal (marine or geodispersal) as the most likely hypothesis for the neotropical-afrotropical distribution of *Arapaima* and *Heterotis* (reviewed in Lavoué 2020).

For the arowanas, morphological analyses of extant and fossil species pointed out that marine dispersal cannot be discarded, while molecular analyses of sequences from a few genes suggest a possible Gondwana vicariance (Kumazawa and Nishida 2000; Wilson and Murray 2008; Lavoué 2016), and a phylogenomic assessment supports long-distance dispersal (Peterson et al. 2022). Interestingly, the recent description of a *Scleropages* fossil for the Eocene in China (Zhang and Wilson 2017) supports a divergence time between *Osteoglossum* and *Scleropages* at a period that predates the separation of the southern continents. Cioffi et al. (2019) combined cytogenetics and genomic data from more than a thousand SNPs from all Osteoglossinae (arowanas) species except for *Scleropages inscriptus*. By calibrating two nodes using fossil information, the authors recovered a dated phylogeny for this subfamily. The recovered ages supported a possible vicariant event between South American *Osteoglossum* and Australasian *Scleropages* associated with the Gondwana breakup (Fig. 21.1a, b). A more recent divergence was observed for the two *Osteoglossum* species within the Neotropics, *Osteoglossum ferreirai* and *Osteoglossum bicirrhosum* (~6 Mya; Fig. 21.1c), likely associated with the formation of the transcontinental Amazon River system (a more detailed explanation is presented in the next section).

## 21.4 Integrating Genomic and Cytogenetic Data in Neotropical *Arapaima*

The pirarucus are charismatic representatives of the Amazon (Brazil, Peru, Colombia, Ecuador, and Guyana) and Tocantins-Araguaia (Brazil) river basins (Reis 2003; Castello 2008; Fig. 21.2a). They can reach up to 200 kg of body mass and have high economic importance (Stone 2007). Several studies that assessed the genetic variation in *Arapaima* considered the genus as monotypic (Hrbek et al. 2005; Araripe et al. 2013; Torati et al. 2019; Oliveira et al. 2020a), with *Arapaima gigas* as the only species. Although we are aware that additional species have already been described (Stewart 2013a, b; Castello et al. 2013), because available literature does not still distinguish these newly described species, we adopt *A. gigas* in the following text. Two recent studies (Oliveira et al. 2020a, b) provided important evolutionary insights for *Arapaima* by investigating single nucleotide polymorphisms (SNPs) and chromosomal data (Fig. 21.2b), respectively. By integrating these two sources of evidence, it was possible to obtain a better understanding of

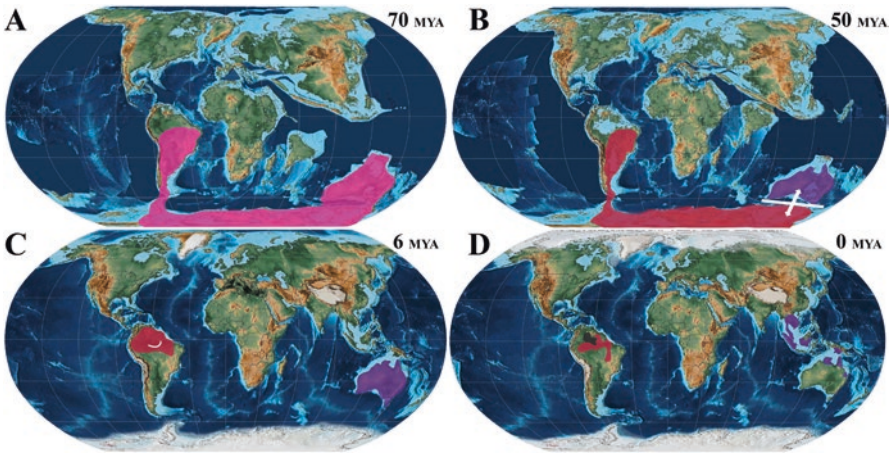


**Fig. 21.1** Schematic representation of the main biogeographic periods in the history of arowanas based on the molecular phylogeny presented by Cioffi et al. (2019). (**a**) The area in pink highlights the potential range of the ancestor of arowanas from South America to Oceania. (**b**) The last terrestrial connection between Australia and South America occurred at ~50 Mya, in congruence with the estimated divergence time between *Osteoglossum* and *Scleropages* (between 55 and 47.8 Mya). Therefore, the separation of these land masses could have been a vicariant event that mediated the divergence of *Osteoglossum* (*red*) and *Scleropages* (*purple*) genera. (**c**) At ~6 Mya the Antarctic continent was not suitable for Osteoglossidae species and the distribution of *Osteoglossum* in South America was probably restricted to the northern region. Besides, the divergence between *Osteoglossum ferreirai* and *Osteoglossum bicirrhosum* started, which coincided with the formation of the transcontinental Amazon River system. (**d**) The current distribution of *O. ferreirai* (*black*) and *O. bicirrhosum* (*red*) in South America, and *Scleropages* (*purple*) in Australia and Asia is highlighted. Maps were generated with GPlates v2.3 (Müller et al. 2018) using data from the PALEOMAP PaleoAtlas Project (Scotese 2016)

population dynamics and genetic diversity in the species (Hrbek et al. 2005; Araripe et al. 2013; Farias et al. 2015; Vitorino et al. 2017).

Oliveira et al. (2020a) used past and future species distribution models and genomic data for thousands of SNPs to estimate the genetic diversity and population structure throughout *A. gigas* distribution. The authors also compared explicit hypotheses for the demographic history of the species with a deep learning approach (sensu Flagel et al. 2019; Perez et al. 2022). The results pointed to lower genetic diversity in localities from Tocantins-Araguaia when compared to the Amazon river basin (Fig. 21.2a). Moreover, a strong population structure was detected separating both basins, with additional differentiation among the Amazon localities (Fig. 21.2c). The model comparison approach indicated that Tocantins-Araguaia localities were likely colonized by an ancestral population from the Amazon river basin at ~1 Mya ($\tau$ in Fig 21.2c), which is congruent to the river sediment estimates for the definitive separation between the two basins (Rossetti and Valeriano 2007). Interestingly, although occurring in different basins and with a marked population structure, all analyzed *Arapaima* populations possess the same diploid number (2n) equal to 56 chromosomes (28 metacentric/submetacentric + 28 subtelocentric/acrocentric) with

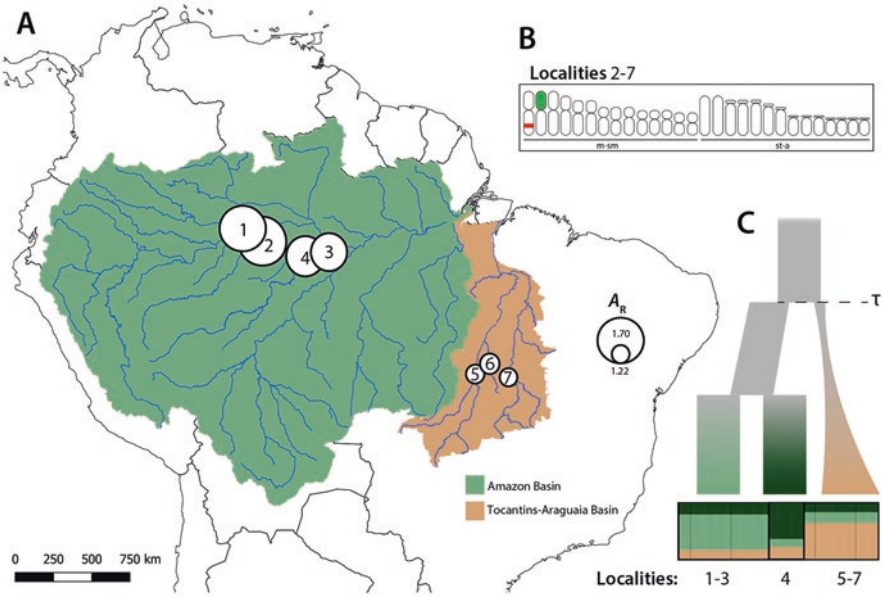


**Fig. 21.2** Map of northern South America indicating the sampling sites of *Arapaima* analyzed by Oliveira et al. (2020a, b) from the Tocantins-Araguaia (*orange*) and Amazon (*light green*) river basins. (**a**) The seven analyzed sampling sites with circle size according to allelic richness ($A_R$): 1 – Lakes of the Juruá river, Mariana Sector (AM); 2 – PantaLeão lake, Mamirauá Reserve (AM); 3 – Castanho lake (AM); 4 – Onças lake (AM); 5 – Xavantinho river (MT); 6 – Javaé river (TO); 7 – Santa Tereza river (TO). (**b**) Representative ideogram highlighting the distribution of 5S (*green* signals) and 18S (*red* signals) with a similar pattern present in all populations. (**c**) Schematic representation of the most likely demographic model for the three genetic groups (K = 3) recovered in the population structure analysis implemented in GENELAND. Each sample is represented as a vertical bar showing the proportion of their genome belonging to each of the K groups. m-sm = meta-submetacentric; st-a = subtelo-acrocentric chromosomes. AM = Amazonas; MT = Mato Grosso and TO = Tocantins states

further sharing of 18S and 5S rDNA loci, respectively, on the first and second chromosome pairs (Oliveira et al. 2020b; Fig. 21.2b). The CGH experiments using gDNA of both the Amazon and Tocantins-Araguaia populations produced intense overlapping signals, thus suggesting little genomic differentiation among them (Oliveira et al. 2020b; Fig. 21.2b), and thus remarkable chromosome stasis as found in other fish genera (Gaffaroglu et al. 2020).

These seemingly conflicting results for genomic and cytogenetic information are interesting and illustrate the importance of complementary sources of evidence that can be related to distinct stages of the speciation continuum (Henderson and Brelsford 2020).

## 21.5 Neotropical Arowanas Phylogeography

As previously mentioned in Sect. 21.3, there are two formally described species of neotropical arowanas (genus *Osteoglossum*). The black arowana, *O. ferrerai*, is an endemic and non-migratory species that occurs in the black water floodplains of the Amazon (Negro river) and Orinoco river basins in South America. In contrast, the silver arowana (*O. bicirrhosum*) is associated preferentially with clear waters from both the Amazon and Tocantins-Araguaia basins (Fig. 21.3). There is also a small region of sympatry for these two species on the lower part of the Negro river (Reis 2003). As their common names highlight, these species differ in the pattern of skin coloration, which might be associated with adaptations to the different environments they inhabit. In fact, the recovered time of ~6 Mya for the diversification among the two species (Kumazawa and Nishida 2000; Lavoué 2016; Cioffi et al. 2019) is consistent with the estimated period of formation of the Amazon river system (4.9–5.6 Mya; Albert et al. 2018). During the formation of these rivers, the skin color or visual systems of these species may have favored adaptive reproductive

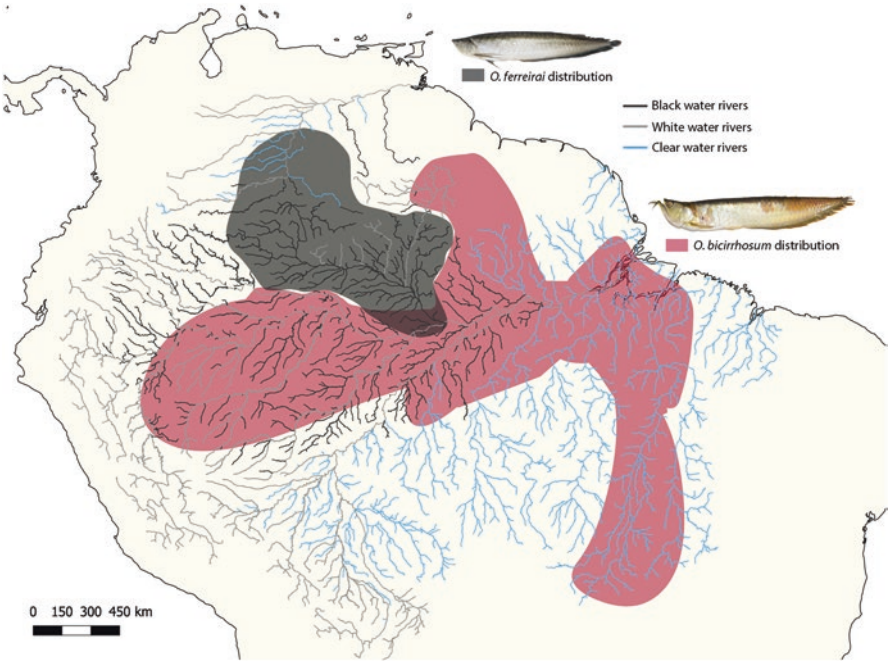


**Fig. 21.3** Map of northern South America indicating the distributional ranges of *Osteoglossum ferreirai* (*gray*) and *Osteoglossum bicirrhosum* (*red*). The distribution range was created based on a set of sampling coordinates extracted from the SpeciesLink and GBIF databases (Souza et al. 2019). River colors indicate water characteristics in the Amazon (Venticinque et al. 2016) and Orinoco (Morales-Betancourt et al. 2015) basins, with clear water rivers represented in light blue, white water rivers in light gray, and black water rivers in black

isolation due to the non-recognition of individuals bearing different characteristics. Alternatively, populations could have been differentiated as a result of allopatry, with morphological differences appearing subsequently. Wallace (1853) already postulated that dark waters may have played an important role in the diversification and formation of new fish species. The color of aquatic environments may change according to different concentrations of organic matter, sediment, and other dissolved substances (Marshall et al. 2003). South American rivers with black water, like the Negro river, present a high concentration of organic matter, an acidic pH (around 5.5), and are oligotrophic (Furch and Junk 1997). These water differences directly impact the aquatic fauna and Amazonian rivers with distinct biogeochemical compositions and colors possess different fish communities (Bogotá-Gregory et al. 2020).

A recent assessment of the demographic history of the widespread *O. bicirrhosum* (Souza et al. 2019) was performed using thousands of SNPs with a deep learning approach similar to that described above for *A. gigas* (Oliveira et al. 2020a). The most likely scenario also indicated the colonization of the Tocantins-Araguaia basin from the Amazon basin approximately 1 Mya (Souza et al. 2019). Niche modeling analyses suggest a wider range nowadays when compared to the last interglacial (~120 thousand years ago; Kya) and last glacial maximum (~21 Kya), as the suitable range for these fishes in these two past periods was recovered as restricted to some small regions, mainly in the Amazon basin.

Souza et al. (2019) also found that the genetic diversity of *O. bicirrhossum* populations in the Amazon basin was higher than that of *O. ferreirai* in the same basin. In a previous assessment, Da Silva et al. (2009) recovered a similar pattern of higher genetic diversity values for *O. bicirrhosum* than that of the black arowana when analyzing microsatellite markers. This trend can probably be related to the restricted distribution of *O. ferreirai,* as well as its current conservation status. The comparison between *O. bicirrhosum* populations from both basins evidenced that individuals from the Amazon basin have higher genetic diversity, a result similar to that of *A. gigas* (Leal and Sant-Anna 2006; Oliveira et al. 2020a). Lower genetic diversity in the Tocantins-Araguaia basin could be due to genetic drift after the founder event, coupled with flooding events that connect distinct populations and result in gene flow. Besides, the results obtained in this work suggest that the interference of climatic and demographic events that occurred in the past, together with the degradation of the current habitat, may be factors that influenced the loss of genetic diversity suffered by *O. ferreirai* (Olivares et al. 2013).

Regarding arowana cytogenetics, while the silver arowana has 2n = 56, with the karyotype formed only by subtelo/acrocentric chromosomes, the black arowana has 2n = 54, with almost all acrocentric chromosomes, except for a large pair of metacentric chromosomes (Fig. 21.4a). Furthermore, *O. bicirhossum* has four sites of 18S rDNA on the 9th and 12th chromosome pairs, while *O. ferrerai* has only two sites on the first chromosomal pair. The 5S rDNA loci observed in these species also demonstrated different patterns, with eight and four sites found, respectively, in *O. bicirhossum* and *O. ferrerai* (Cioffi et al. 2019) (Fig. 21.4a). Chromosomal painting data using this large and remarkable metacentric pair present in the black

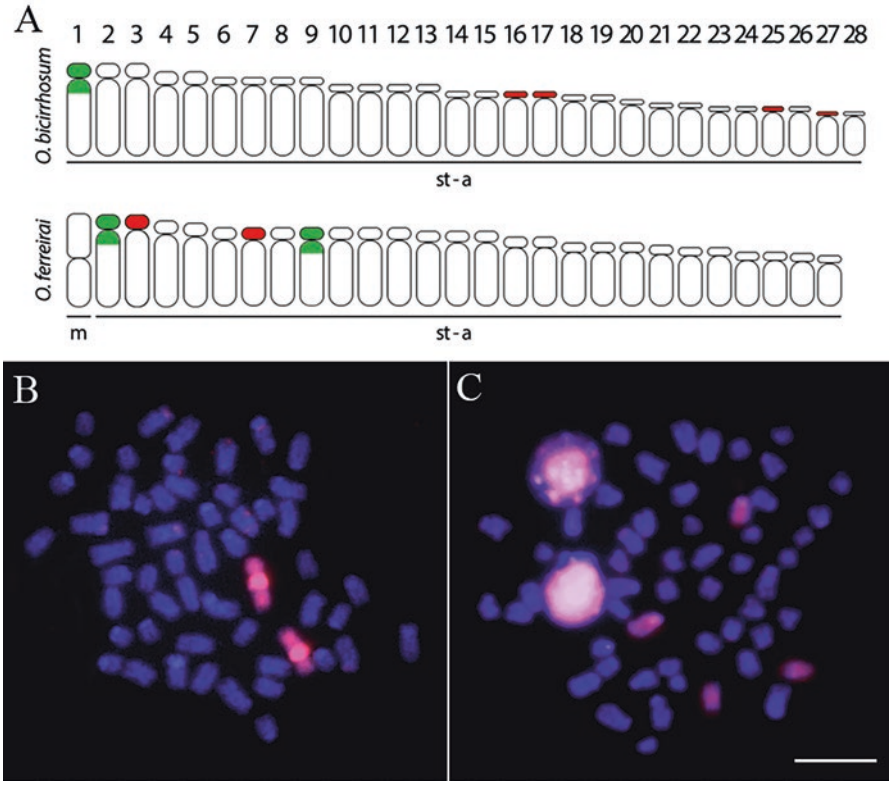


**Fig. 21.4** Cytogenetic data for the genus *Osteoglossum*. (**a**) Representative ideograms of *Osteoglossum bicirrhosum* and *Osteoglossum ferreirai* highlighting the distribution of 5S (*green* signals) and 18S (*red* signals) rDNA sites. Note that karyotypes diverge by the presence of an exclusive metacentric chromosome present in *O. ferreirai*. (**b**) and (**c**) Zoo-FISH experiments with a painting probe constructed from microdissected #1 pair of *O. ferreirai* (OFE-1) applied on the metaphase plate of the black arowana *O. ferreirai* (**b**), and silver arowana *O. bicirrhosum* (**c**) highlighting the chromosomal rearrangements involved in its origin. Hybridization patterns were retrieved from Cioffi et al. 2019. Bar = 5 μm

arowana as a probe evidenced the chromosome fusion event that separates the two karyotypes (Fig. 21.4b; Cioffi et al. 2019). The formation of new linkage groups between genes originally found on different chromosomes speeds up the accumulation of genetic incompatibilities between populations (Vieira et al. 2003).

We propose that the formation of this new linkage group through chromosomal fusions in the black arowana (pair 1) may have allowed the fixation of unique mutations that confer a local adaptive advantage to its ecological environment. This could have been achieved by, for example, trapping more locally adapted genes (Faria and Navarro 2010). If this was the case, it may have created a strong genetic barrier that could have led to speciation. Accordingly, over the last years, a considerable amount of indirect evidence has prompted the classical idea that

chromosomal rearrangements play a role in speciation, especially when they occur linked to spatial variation of selective pressures (Kirkpatrick and Barton 2006). Besides, several examples show that chromosomal rearrangements can confer adaptation and consequently lead to reproductive isolation among incipient species (Coluzzi 1982; Kirkpatrick and Barton 2006; Manoukis et al. 2008; Dobzhansky 1970; Krimbas and Powell 1992; Balanyà et al. 2003; Etges and Levitan 2004). The next steps will now include the application of Hi-C data (a high-throughput technology to capture chromatin conformation) to scaffold the genome sequences at the chromosomal level so that information on genomic content present in such new linkage groups can be found (Gao et al. 2021). This will allow us to properly identify which genes are present within such rearranged segments and their putative involvement in adaptation and speciation.

## 21.6 Concluding Remarks

In this chapter, we highlighted how the use of multipronged analysis was useful to make important advances in the biogeography, demographic history, genetic diversity, and chromosomal evolution of neotropical bonytongues. Despite some important advances that occurred regarding the biogeography of Osteoglossiformes over the last years, our future studies will incorporate genetic and genomic information with the fossil record to better assess their biogeographical history and reach more precise estimates of lineage dispersal mode and timing.

Concerning the black and silver arowanas, coloration seems to have a key role in their speciation process since it is highly related to environmental adaptation as it is important for camouflage, thermoregulation, photoresistance, and, particularly, mate-choice (Moreno Azócar et al. 2016; Duarte et al. 2017). Fishes that inhabit different depths, which implies a gradient of turbidity and luminance, may have changes to their visual system proteins (Seehausen et al. 2008; Hofmann et al. 2009). In Lake Victoria, a high correlation between water depth and Long-wavelength sensitive opsin (LWS) opsin alleles was found, and the opsin mutation rate was higher than that of neutral loci (Seehausen et al. 2008). All these characteristics, coupled with female nuptial preferences and male coloration (as both perception of color and body color are affected by water luminance), resulted in a sensory drive speciation hypothesis (Seehausen et al. 2008). Therefore, the differences in color and visual system may have favored the adaptation of *O. ferreirai* to dark waters and *O. bicirrhosum* to clear waters for reasons of camouflage or adaptations of vision to more turbid environments. However, further analysis should better assess such a hypothesis. Besides, it is evident that these species need more attention and appropriate management plans, particularly *O. ferreirai,* whose distribution is more restricted and adapted to a specific type of water. Additionally, *O. ferreirai* suffered overexploitation for decades, mainly for ornamental purposes, as it presented a higher market price (Queiroz and Camargo 2008) after the *Scleropages formosus* trade was forbidden (Yue et al. 2003).

Future studies integrating transcriptomics, molecular evolution, and Hi-C sequencing approaches will be used to better understand the molecular basis of the color phenotype and the visual system in these species, as well as to investigate the possible evolutionary implications of the adaptive divergence of this set of traits to different water environments.

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# Part VII
# Science Learning and Conservation

# Chapter 22
# Environmental Education on Practices for Biodiversity Conservation

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## 22.1 Introduction

The very beginning of environmental education is closely linked to nature conservancy, being traced to the preservation of natural areas. Historically, in some countries environmental education has been mostly carried out in protected areas with a focus on conservation. In Brazil, from the earliest days environmental education has taken a different and broader characteristic, encompassing multiple demands from movements related to the fields of environmental justice, nature conservancy and social movements. Environmental education in Brazil, following what has been observed in other countries (Sauvé 2005) usually falls into two major categories, the more traditional ones such the one called conservationist and the other group that emerged more recently, which includes one that is known as socially critical. Lima and Layrargues (2014) present three major currents when talking about environmental education in Brazil: conservationist, pragmatic and critical, although Iared et al. (2011) has discussed how the lines among those are not solid and the tendencies may be observed concurrently in the same activities when we analyze them closely.

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Environmental education for biology conservation is a topic marked by controversies involving its conceptual and political meanings (González-Gaudiano 2007). Furthermore, biodiversity is rarely being properly included at Brazilian schools or even in public policies linked to the Convention on Biological Diversity. In their book "What on earth is biodiversity?", Bensusan et al. (2006) present the many faces of this complex and polemic issue in our country. One chapter "*Biodiversity is also a matter of education*" seems ironic in the Brazilian context. We see this title as an indicator of the issues we are trying to point out, which are that biodiversity and its conservation should be a focus of environmental education, especially in programs of national parks. In that chapter, the author (John 2006) poses another kind of problem, which is the lack of awareness of our own animal species. Brazilian children learn to write using letters and names of African species, such as E is for elephant and Z is for zebra. As she says, "more than a mere curiosity, these knowledge gaps reflect a dangerous cultural trend. It shows that Brazil has still not appropriated its immense natural patrimony". It is not as if Brazilians do not know about this natural patrimony. An ongoing survey conducted every four years since 1992 by the Brazilian Environmental Ministry showed in its 2006 report that Brazilians think of forests when you ask them about biodiversity (MMA 2006). However, most Brazilians do not live near or come into contact easily with areas rich in biodiversity such as forests or savannas. For example, only 8% of the population lives in the Northern region, where the Amazon forest lies. Therefore, even though Brazilians live in a country with a rich biodiversity, most of the population may only have contact with it and biodiversity education by visiting parks, zoos, museums, aquariums and using educational materials. Yet even then biodiversity is not usually the main focus of the educational activities developed in these places. For example, when we analyze environmental education research conducted in these locations, we often find that the educational activities are centered on providing information about plants and animals, with less attention being paid to biodiversity and its conservation (Marandino and Monaco 2010). It is only recently that issues concerning biodiversity conservation have become more relevant in educational proposals of zoos and museums and other such establishments in Brazil (Marandino et al. 2010; Oliveira 2015). In this new scenario, environmental education has been increasingly included as an obligatory activity in a wide scope of research projects from ecology to genetics, showing the importance of the research and educational perspective discussed in this chapter.

## 22.2 Environmental Education in Conservation Units: Research and Projects in the Neotropics

To date, most research focusing on the relationship between environmental education and biodiversity conservation in Brazil has been developed in protected areas and their surroundings. Some examples of research that have analyzed this issue are

the studies conducted by Bizerril et al. (2011), who investigated the maned-wolf (*Chrysocyon brachyurus*) conservation at Parque Nacional da Serra da Canastra, Brazil, as well as Engels and Jacobson (2007), who evaluated the conservation of the golden lion tamarin (*Leontopithecus rosalia*) in Poço das Antas, Brazil. Other examples in Latin and Central America include Espinosa and Jacobson (2012), who studied the Andean bear (*Tremarctos ornatus*) conservation at the Reserva Ecológica Cayambe-Coca in Ecuador, Curti and Valdez (2009) who investigated the harpy eagle (*Harpia harpyja*) conservation efforts in Panamá. Additional examples were presented in Thiemann et al. (2016). In a period when an increasing percentage of the world's population is moving to urban areas, it is in protected areas that most people still experience contact with nature. This growing urbanization is also true in Brazil, a biodiversity hotspot (Mittermeier et al. 2005), where according to the World Bank, 84% of the Brazilian population is now urban.

In order to address biodiversity and biodiversity conservation in environmental education activities developed in protected areas, an initial diagnosis, such as the one carried out in the educational projects presented in this chapter, is fundamental (Thiemann 2013; Thiemann et al. 2018). The initial diagnosis allows us to explore the meanings of biodiversity and the educational possibilities of using it as a theme for environmental education in a rich landscape representative of a Brazilian ecosystem.

Even though many environmental education projects focus on children and youth and are centered in school, it has been shown that community centered environmental education is best when focused on adults. This makes sense since the adults are usually in a position to make most decisions related to biodiversity conservation in and around protected areas (Valenti et al. 2017).

### *22.2.1 The Thematic Chambers of Environmental Education*

The Thematic Chambers are instances of support to the Councils of the Conservation Units linked to the Brazilian Environmental Ministry (MMA 2014), which act in the discussion of specific subjects to support decisions and propositions of these Councils. More specifically, the Environmental Education Thematic Chamber (CTEA, acronym in Portuguese) works in the communication, development and support of educational projects and actions.

A good example of this work occurs in the Itatiaia National Park (PNI, acronym in Portuguese). The PNI is the first Conservation Unit in Brazil, founded in 1937. Located in the southeastern region, one of the most developed regions of the country, this Conservation Unit protects one of the last remnants of the Atlantic forest hotspot.

The CTEA/PNI works to mobilize, train and make tourists and the community around the park aware of the importance of the Conservation Unit. For this, CTEA/PNI supports and develops accessibility actions and projects for people with different types of physical disabilities, dissemination and conservation of fauna and flora

and socio-environmental development. This CTEA organizes seminars, courses, exhibitions, workshops, in addition to preparing and distributing educational materials aimed at students from public schools and nearby universities and colleges (Fig. 22.1). CTEA/PNI is also responsible for publicizing activities and actions with an educational focus and updating the media that publicize these actions.

### *22.2.2 The Itatiaia National Park Goes to School (PNIVE)*

The project "The Itatiaia National Park goes to School" (PNIVE) was created at the end of 2012, from the need to structure school visits in the Conservation Unit. Faced with this problem, the CTEA/PNI, in partnership with a local higher education institution, Associação Educacional Dom Bosco (AEDB), structured the project, based mainly on the National Education Policy Environmental Law (Brasil 1999).

The objectives for the project were to present the species of fauna and flora of PNI, emphasizing its endemic and threatened species; raise awareness of the importance of the Conservation Unit in the conservation of biodiversity and natural resources; and demonstrate the role of human beings in protecting nature, as they are an inseparable component of ecosystems.

To achieve the proposed objectives, the project was structured in four steps (Table 22.1). It is worth mentioning that each municipality had the autonomy to establish its programming and its work according to its viability and conditions of the participating schools.

Guided visits to PNI were carried out by accredited drivers and, sometimes, with the collaboration of environmental analysts, biologists and teachers of Environmental Education and other areas, in addition to professionals with experience in

**Fig. 22.1** Educational activity at the Itatiaia National Park Visitors Center. Child draws trees and a monkey to represent the park

**Table 22.1** Steps developed by the Project "The Itatiaia National Park goes to School – PNIVE"

| Step | Activity | Target stakeholders |
|---|---|---|
| 1 | Creation and implementation of a course on environmental initialization, focusing on PNI. | Undergraduate students |
| 2 | Planning of oriented visitations. | Municipal departments of education in surrounding municipalities |
| 3 | Visitation to the schools and to PNI. | School deans and coordinators, teachers and students from participating schools. |
| 4 | Monitoring and evaluation.<br>Presentation of the results to the CTEA/PNI and to the participating schools. | School deans and coordinators, teachers and students from participating schools. |

mountaineering and knowledge of the Conservation Unit in its geological, hydrological, climatological and biological aspects.

These visits were structured in three distinct moments:

First – The Park goes to the School through a lecture on the PNI, specific to each class, whose theme is being worked on in the curriculum of that school year. Parents and guardians, as well as teachers, are invited to sign a Free and Informed Consent Declaration, safeguarding the use of photographic material in project documentary, reports and dissemination material.

Second – The School goes to the Park, and students visit PNI;

Third – Students return to school, where work is carried out through workshops as the culmination of the visit.

The project generated important results in training students and teachers in the region on issues related to nature conservation and conservation units such as:

- certification as "Beginners in Environmental Education in Itatiaia National Park" of 70 undergraduates from the AEDB's Pedagogy, Biological Sciences, Engineering and Administration courses, as multiplier agents with public schools, exceeding the established goal of 40 multipliers in the first two years old.
- the participation of 17 Municipal schools per year, serving approximately 2000 elementary school children in the 4 years of the project.
- development of the multiplying spirit, of a new vision in Environmental Education contextualized through experiences, activities, events and didactic-pedagogical results, monitored and evaluated throughout the process.
- strengthening of interdisciplinary practice, promoting the intersection of Environmental Education and disciplines of different knowledge, especially those that constitute the foundations of education in licentiates, enriching the didactic and pedagogical practices of the future trainer.
- collective production of didactic-pedagogical material aimed at environmental awareness, dissemination and support to school and non-school visitors to the park.

In 2018, occurrences such as the problem of yellow fever and the relationship with its primate hosts in PNI, as well as difficulties in school transport by the Municipal Departments of Education, the project was temporarily suspended.

The continuity of the PNIVE was scheduled for 2020, however with the COVID-19 Pandemic it is being negotiated with schools, in the condition of remote work, providing an opportunity for written production, using the methodology of environmental interpretation with the use of videos, photos and images of PNI.

### *22.2.3 Primates of Itatiaia Project*

Itatiaia National Park is home to five native species of primates, the Black-horned Capuchino (*Sapajus nigritus*) (Fig. 22.2), the Northern Muriqui (*Brachyteles hypoxanthus*), the Brown Howler Monkey (*Alouatta guariba*), the Black-fronted Titi Monkey (*Callicebus nigrifrons*) and the Buffy-tufted-ear Marmoset (*Callithrix aurita*), as well as an exotic species, the Black-pencilled Marmoset, *Callithrix penicillata* (Aximoff et al. 2015). According to the International Union for Conservation of Nature's Red List, S. *nigritus* and *C. nigrifrons* are classified as "Near Threatened"; *C. aurita* is "Endangered"; and the most worrisome species *B. hypoxanthus* is "Critically Endangered". These species are endemic to South America, and the populations are declining mainly due to loss of forest habitat and hunting (IUCN 2022).

Although PNI is a fully protected area, the primates that inhabit it are not exempt from the impacts generated by human presence. For example, changes in the feeding activity of some primate species are very noticeable in this Conservation Unit (Fig. 22.3). Many of these animals are replacing natural food like fruits and leaves with human provisioned food like industrialized food. In addition, the monkeys can

**Fig. 22.2** Black-horned capuchino (*Sapajus nigritus*) in Itatiaia National Park

**Fig. 22.3** Black-horned capuchino (*Sapajus nigritus*) feeding on rice scattered on the ground in Itatiaia National Park

**Fig. 22.4** Black-horned capuchino (*Sapajus nigritus*) rummaging through garbage in Itatiaia National Park

feed on the garbage discarded by homes and businesses, such as hotels, located inside the Park.

In order to minimize the impacts of garbage, Park employees promote dialogue with residents and visitors, informing them about the correct ways of storing and disposing of garbage, in addition to providing adequate places for depositing this

waste. Even in the face of these actions, these animals often manage to access the stored garbage and end up feeding on the organic waste deposited (Fig. 22.4).

The Primates of Itatiaia Project was created, in 2015, to address the issue of anthropogenic food supplied to the animals. This project was developed by students, teachers and researchers from universities, in partnership with the CTEA/PNI. The species *S. nigritus* was chosen as the target species of the work, since the interaction between these primates and visitors was frequently observed. However, during the Environmental Education activities, all primate species in the park were addressed.

The main project objectives were to minimize the contact of the monkeys with the visitors of the Conservation Unit; avoid offering food to these animals; sensitize the Park's visitors, residents and visitors about the threats that anthropic food represented for the monkeys, for the human and for the ecosystem; and collect data on the ingestion of garbage by the monkeys, generating subsidies for possible actions of the management of the Park.

In order to fulfill the proposed objectives, volunteers interviewed and observed the interactions of the visitors with the *S. nigritus* target species. These actions were carried out fortnightly on Saturdays, one of the most visited days in the Park. During visiting hours (from 9 am to 5 pm), the volunteers walked about four kilometers along the main road of the Conservation Unit. The area of action was defined after a previous survey, which indicated the points of occurrence of interaction between visitors and monkeys.

At the end of the first year of the project, 72 episodes of interaction and 72 interviews were recorded. From the observations it was possible to identify the points of highest frequency of interactions. Among them were an area close to a restaurant and the Park Visitors Center. Therefore, these places were considered priorities in Environmental Education actions. Educational banners were made available in the restaurant. Banners were also available at the Visitors' Center, where lectures and workshops were held, including for people with special needs.

As a result of this study, it was observed that 32% approximately of the visitors offered food to the monkeys. The main foods offered were cookies (41%), fruits (31%) and bread (22%). Many of these foods present high values of calories, which can result in obesity, diabetes, and increased cholesterol levels (Forthman-Quick and Demment 1988).

There were also reports of food theft by monkeys in hotels and in a restaurant. Saito et al. (2010) also reported theft of food by monkeys in Brasília National Park. This behavior often causes conflicts between monkeys and humans.

The interaction distance between monkeys and humans was mostly less than one meter (78%) (Fig. 22.5). This proximity is very worrying, since in almost half of the interactions, visitors reported aggression by the monkeys (49%). The aggressions, however, did not harm anyone and no bites or scratches were reported, which are dangerous behaviors, as they allow the transmission of diseases to humans, such as herpes and rabies (Fragaszy et al. 2004). The lack of information can favor the proximity with the animals. Most respondents, 82% approximately, were unaware of the zoonotic potential of monkeys.

**Fig. 22.5** Interaction between black-horned capuchino (*Sapajus nigritus*) and a tourist in Itatiaia National Park

Based on the results obtained, several educational actions, such as lectures and workshops, were carried out in schools and at events in the surrounding areas.

## 22.3 Lived Experiences in Nature and Their Importance in Building a Relationship with Nature

Developing environmental education activities based on the theme of biodiversity in protected areas allows participants to consider biodiversity as something close and within their reach. The relationship with the place, as well as the possibility of taking action, is very important for critical environmental education. According to John (2006), only when Brazilians are exposed to elements of our biological diversity will the cultural appropriation of our natural patrimony improve. Working in PNI with groups of students from surrounding areas brings us close to what Greenwood (2013) calls *place-conscious education*. The author claims that "places provide a local focus for socioecological experience and inquiry" and likely more directly relevant this "place should be understood as a socioecological construct capable of remembering people to the natural environment that supports all life".

There is a strong relationship between being in a green area (such as a national park) and the educational possibilities and attachment to nature that may arise from it. It does not suffice to talk about nature in a classroom; people actually have to be there, to be able to feel the fresh air, hear the sounds of nature and see the beauty of it to be motivated to take care of it. People felt that being in a green area made environmental education more "real", which could increase their awareness of the need

to preserve nature and manage the resources in a more sustainable way. This corroborates the findings of Russ et al. (2015). The authors present a narrative in which the participant talks about the importance of being in the environment – "I have noticed that it's hard for students to get ideas about particular areas if you just show them a map, just talk about it or show pictures. It's hard if they are not out there to touch it and experience it. If you are not out there touching it or seeing it to make it concrete, it means nothing". The importance of experiences in nature to form a bond with the natural world/environment is cited by many authors as the origin of involvement with environmental education and the environmental movement. In the narratives collected during her research on forming an "ecological self", Carvalho (2001) notices an association between memories of experiences lived in nature, both in childhood and adult stages of life, as well as the creation of an affective bond with it. That bond is then related to future engagement with environmental concerns/issues.

Cachelin et al. (2009) reported the relevance of the experience in nature at Utah's wetlands (USA) and found that the cognitive gain and emotional involvement of the children who participated in the field trips were greater than those who learned about the ecosystem in the classroom. The same relationship is pointed out by Lindemann-Matthies (2002) when evaluating educational biodiversity programs. When reviewing the literature, the author found many examples whereby learning is significantly improved when classes take place in natural environments, as opposed to theoretical lessons inside the classroom. However, in this case the learning takes place in the city. In her own research, Lindemann-Matthies (2002) focuses on nature observation on the way to school and in children's day-to-day activities. The observation itself, as well as taxonomy-based teaching, broadens children's knowledge about plants and animals and helps them increase the number of species they know and can name, and therefore they gain perception of the biodiversity in their own neighborhood.

Direct encounters with nature are also highlighted by Sandell and Öhman (2010) as a means to develop a relationship with nature. Louv (2008) goes beyond recognizing the importance of experiences in nature. The author identifies what he calls a "nature-deficit disorder" nowadays. His research, conducted in schools and communities in the USA, focused on the relationship of children and nature. Louv (2008) found that, compared to his (and his generation's) own experience, the intimate contact with natural areas that marked his childhood is simply not there anymore. The growing urbanization and conversion of natural areas in suburbia due to urban sprawl moved even farther to what used to be nature in children's own backyards. The author talks of this deficit and questions how children's involvement with environmental issues in the future will be, since they did not have the opportunity of forging a strong/lasting bond with nature in their childhood/as children. He also claims that direct contact with nature is crucial for mental health and spiritual resilience, not only for children, but adults as well.

Rural (or non-urban) areas, such as PNI, are frequently pointed out as the ideal place for pleasant sensory contact with nature experiences. The nostalgia for virgin nature, untouched, emerges as a "contraposition to the social and environmental

violence of the urban world" (Carvalho 2001) and leads to a valuing of nature by society. Thus, areas such as PNI, surrounded by several urban areas, are able to provide the kind of encounter with nature proposed by Louv (2008) and Sandell and Öhman (2010). Easy access to these areas is very important, and as the area is nearby, people feel that this creates opportunities for learning more about animals and plants, bringing the community closer to nature without the need of traveling long distances. Proximity may influence tours to natural areas even in more economically developed countries. Wals (2010), discussing equitable access and the opportunity to participate in experiences in nature, says that "the radical Scandinavian outdoor life simply is not accessible for many children growing up in urban areas due to a number of factors including legal barriers, physical barriers, lack of transportation, over-crowded curricula or simply because of the absence of near-by outdoor areas". Brazilian legislation, both in the national Education Environmental guidelines (MMA 2012) and in the National Environmental Education Policy from 1999 (Brasil 1999) stress the importance of promoting activities in places in which students recognize themselves as part of nature. In order for this recognition to take place, such sites must retain characteristics considered "natural", such as parks and green areas. It is also necessary to adapt educational activities aimed at elderly participants, because some of them may experience a diminishing sensory capacity. Thiemann (2013) investigated the relationship between biodiversity education and aging, and proposed four suggestions for environmental education activities: (a) Memories: educational activities that focus on changes in biodiversity over a period of time may help strengthen memory, bringing back names, sensations, tastes and smells from earlier years; (b) The effects of the passage of time: here the educational activities can remind people of the passage of time for human beings and other life forms, life cycles, the seasons and the need to care for nature for future generations; (c) The senses: as some of our senses become less accurate as we age, we can work with biodiversity and the senses of sound, sight, touch, smell and taste using fruit, flowers, the texture of leaves and tree bark, bird watching and other educational activities that bring into focus both the variety of life and the changes due to aging; (d) Finally, the importance of biodiversity can be discussed, arising from many points of view, such as resilience, memories, traditional and science-based knowledge and biodiversity's role in pharmaceutical discoveries.

## 22.4 Final Considerations

The educational projects presented in this chapter, in line with other studies, highlight the importance of organizing tours and educational activities in green areas, both by the general community and students. A restorative effect of being in nature and green urban areas was reported by many authors (Hartig et al. 2003; Hipp et al. 2015; Carrus et al. 2015). Takano et al. (2002) showed that senior citizen's longevity actually increases if they have access to green areas.

We consider that lived experiences in such areas help to meet some of environmental education's most cited aims, such as creating a bond with nature, gaining knowledge about natural systems, inter-relations among all living beings and the environment and also providing opportunities for discussing issues such as urban sprawl and its impact on natural areas. These aspects are particularly relevant in the megadiverse Neotropics.

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# Chapter 23
# Phenotypic Plasticity of Plants in Formal and Non-formal Education: Genetics in Everyday Life

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## 23.1 Introduction

Plants represent more than 80% of all living biomass on our planet, while humans make up only 0.01% (Carrington 2018; Parsley 2020). However, the quantity and quality of initiatives to promote plant conservation always lag behind efforts to promote the survival of animal species. Thus, plant species that are susceptible to extinction have been receiving considerably less resources from research funding agencies (Bar-Ona et al. 2018; Silva et al. 2021).

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The insertion of plant populations in spaces dedicated to scientific studies and recreation, such as Botanical Gardens, is capable of rescuing relationships that negatively link natural resources to the right of indiscriminate use of plants (Campos et al. 2020). Therefore, we are all responsible for conserving the components of the landscape, thus ensuring that people can understand and carry out the same practices in relation to plants (Wandersee and Schussler 1999).

In this scenario, only education (formal or non-formal) ensures that the society will clearly understand the importance of plants (Wandersee and Schussler 1999; Neves et al. 2019). It is necessary to support an early, interactive, well-planned, meaningful and conscious education (both scientific and social) about plants, combined with a variety of pedagogical practices through interpersonal experiences to reduce the botanic blindness among us (Wandersee and Schussler 1999; Huey et al. 2002; Allen 2003; Salatino and Buckeridge 2016; Ferah et al. 2019; Lima 2019a, b; Silva et al. 2021).

By definition, "botanic blindness" or "plant blindness" result from a human visual information-processing system that involves the inability to see or notice plants in one's own environment. Therefore, an effort must be made for the objective of conservation or rational exploitation of plants (Wandersee and Schussler 1999; Allen 2003; Salatino and Buckeridge 2016; Colon et al. 2020).

## 23.2 Why Should We Promote the Public Appreciation for Botany?

"Plants are the basis of life on Earth as we know it and the study of plants is essential to protect our future ... their positive effect on human well-being" (Burke et al. 2022). However, why are people relatively unaffected by plants? Moreover, why are plants often ignored by us in favor of animals? (Jose et al. 2019; Margulies et al. 2019; Cowell 2020; Sundberg et al. 2002). There is a number of reasons that can answer these questions.

First, there is a misguided anthropocentric ranking of plants as inferior to animals (Wandersee and Schussler 1999). Second, most teachers invest more time and approaches to studying animals than plants (Proença and Dal-Farra 2022). Therefore, most people did not learn in school that plants are organisms with a considerable degree of importance given they comprise around 80% of all biomass on Earth, play important roles in almost all ecosystems, support humans and several animals by providing food, herbal medicines, oxygen, shelter or material to build them, and can regulate the climate (Qui and Garcia 2005; Jose et al. 2019; Burke et al. 2022, Nicotra 2022). Additionally, the general public perception regarding extinction of living organisms tends to focus on animals, and not so much in plants, however, roughly six times the number of plants is in danger of extinction (around 30,000

species), compared to that of animals (around 5000 species) yearly (Convention on International Trade in Endangered Species of Wild Fauna and Flora – Cowell 2020). People have a difficulty to perceive threats to plants the same way they do to animals as an effect evidence-supported claims of zoo chauvinism (or animal chauvinism) and general plant neglect are major causes of plant blindness (Piassa et al. 2022). Botanic studies should be improved in Environmental Education classes (Costa et al. 2019; Ferah et al. 2019). People lack a general interaction and ability to recognize the importance of plants in their day-by-day experiences (Lima et al. 2017a, b; Lima 2019a, b; Mancebo et al. 2020, 2021).

As pointed out by Hershey (2002), "there seems to be no good reason why an entertaining natural history or science TV series on plants would not be successful. Zoos frequently get tremendous publicity from the birth of animals or acquisition of new specimens such as pandas or polar bears. However, botanical gardens and arboreta rarely seem to be in the news".

The general perception regarding plants will change once these problems are properly answered. Recently, the Royal Botanical Garden of the United Kingdom together with institutions from 42 countries, including Brazil represented by the Rio de Janeiro Botanic Garden, released an estimate indicating that for every five species of plants in the world, two of them are already endangered (Lisboa 2020).

Furthermore, most people understand that it is easier to preserve several animals such as the giant panda in China, the jaguar from the Brazilian Pantanal, the golden lion tamarin from the Atlantic Forest and so forth, so that these species do not face the same extinction history as North American bison and Canadian deer (Morato et al. 2006).

Nonetheless, animal conservation requires attention to the natural features of the landscape not only as essential habitats of the animals themselves, but also for taking into account climate regulation by plants and also their use as a source of food and medicinal drugs (Qui and Garcia 2005; Lukhoba et al. 2006; Rosal 2008; Rosal et al. 2011; Malhi et al. 2020). In addition, plants provide medicines and recreation through gardening activities and in public botanical gardens (Balding and William 2016).

Thus, considering the tendency among humans not to value plants in the environment (Balding and Williams 2016), and given the impactful significance of plants, it is clearly important to be aware and understand their value for all living organisms (Malhi et al. 2020).

Therefore, promoting quality in the botany teaching-learning process is always very important. For that, a variety of dynamic didactic strategies conducive to students or of general interest as well as proactive actions through formal or non-formal education exploring plant phenetic plasticity expression can be used (Lima 2017; Lima et al. 2017a, b, 2019a, b; Sodré et al. 2018, 2019a, b; Ferah et al. 2019; Campos et al. 2020; Mancebo et al. 2020, 2021).

## 23.3 A Brief Overview of Genotypes and Phenotypes

First, it is important to recall facts from the history of genetics. Gregor Johann Mendel (1822–1884) was an Austrian botanist, Augustinian friar, and meteorologist who in 1865 published his work on crossing pea plants (Astrauskas et al. 2009). He determined, for instance, that crossings between pure lines of tall (P1) and short (P2) pea plants produced the F1 generation composed of 100% tall plants like P1. In the second generation (F2), 3/4 of the descendants were tall as P1 and 1/4 short as P2. To explain this phenomenon, Mendel coined the terms "Dominant" and "Recessive" about certain plant traits that he observed (Mendel 1866).

In 1909, Willian Bateson repeated these experiments and observed that pure tall and short strains of peas varied in their height. In tall plants, it was possible to find heights from 185 to 215 cm (1.16 times) while among short plants the sizes varied even more from 20 to 50 cm (2.5 times). Why do such phenomena occur? Organisms are the by-products of both their genetic makeup and the environment, characterizing what is named phenotypic plasticity (Doughty and Reznick 2004; Ehrenreich and Purugganan 2006; Ghalambor et al. 2007; West-Eberhard 2008; Whitman and Agrawal 2009).

Evolutionary adaptation, or simply adaptation, is the adjustment of organisms to their environment in order to improve their chances at survival in that environment (Via and Lande 1985; Sultan 1995, 2017). Thus, phenotypic plasticity is a component of evolutionary change because the genotype-environment interactions and the evolution of phenotypic plasticity represent an important key responsible for the mechanism for enabling organisms to survive in face of environmental changes (Via and Lande 1985; Doughty and Reznick 2004; Ghalambor et al. 2007; Fusco and Minelli 2010; Thompson 1991; Murren et al. 2015).

The response of the organism to environmental variations may stimulate the expression of stress proteins, disrupting physiological homeostasis, reducing acclimation capacity, increasing immune responsiveness, and changing learning responses. These responses, in turn, involve phenotypic plasticity of many characteristics such as body size, shape or color, physiological activities, and/or behavioral pattern (Lima et al. 2017a, b).

In ecological terms, we can consider phenotypic plasticity as a complex resulting from environmental interactions with gene pools that occurs in much more complicated and dynamic ways than imagined, as it can even cause changes in the structure and general organization of communities (Scheiner 1993; Scheiner 2002; Dewitt and Scheiner 2004). The continued progress in this field will help to explain the condition of plant biodiversity and will also provide important insights into how adaptation occurs at the genetic levels (Ehrenreich and Puruggana 2006).

Several studies of plant developmental pathways have revealed that genotypes are in fact differentially expressed depending upon environmental conditions (Poersch-Bortolon et al. 2016; Fenollosa et al. 2017; Steiner et al. 2017). Moreover, plants have developed several mechanisms in response to drought stress, and drought tolerance is a quantitative trait with a complex response at molecular,

metabolic and physiological levels (Poersch-Bortolon et al. 2016). However, how is it possible to demonstrate this phenomenon in plants through easy-to-perform experiments with short reactively times?

## 23.4 Plants Expressing Phenotypic Plasticity

Phenotypic plasticity has been studied most intensively in plants, which typically show dramatic effects of the environment on their growth and development. Plants can also be more readily cloned and raised in alternative environments than many other organisms. Thus, much of the current knowledge of phenotypic plasticity comes from plant studies documenting the range of phenotypes that can be produced by individual genotypes in response to contrasting conditions (Sultan 2000).

The notion that plants respond morphologically to changes in their environment has a long tradition. In a classic paper published by Josh Schmidt in 1899, and then Pedersen (1966), found that *Lathyrus maritimus* Linnaeus 1783 from the Baltic coast differs in the anatomical structure of its leaves (these being dorsiventral) from the *L. maritimus* found along the North Sea coast of Denmark (which has isolateral leaves). Some experiments showed that watering *L. maritimus* with solutions of sodium chloride induced a leaf structure typical of the North Sea coast and concluded that it is a direct effect of the sodium chloride, which is found in a higher percentage in the North Sea than in the Baltic (Pedersen 1966).

Another interesting example is presented in a paper on the modifications of *Polygonum amphibium* Linnaeus 1783 Gray, a typical aquatic plant from Europe (Massart 1902; Heizer 2008). It shows that this plant can be readily modified from its more common aquatic form into a terrestrial form or a dune form by exposing cultures from one and the same individual to the proper environmental conditions. Later studies documented the nature and extent of phenotypic responses to varied environments, including classic examples such as sun *versus* shade leaves, or responses to herbivores, among others (for review, Bradshaw 1965).

One of the most universal examples of plant plasticity occurs in the family Brassicaceae Burnett 1835. This family includes many plants of economic importance that have been extensively altered by domestication (Cabral 2016), particularly within the *Brassica* genus, such as *Brassica oleracea* Linnaeus 1753. Artificial selection based in enlarged flowering structures of *B. oleracea* created broccoli, while arrested flower development produced cauliflower from sterile flowers, among other edible plant varieties (Fig. 23.1). The genetic diversity, nutraceutical importance, and derived phytochemicals of *Brassica* still motivate the improvement of these plants in face of imminent climate changes and to meet food demands (El-Esawi 2016; Singh et al. 2022).

The evolutionary history of the genus *Brassica* involves allotetraploids and allohexaploid events, benefiting plant breeding and creation of new biotypes, specially from *B. oleracea* (Liu et al. 2014; Yin et al. 2020; Xue et al. 2020). The *Brassica* plasticity and artificial selection conducted by human societies in the past were

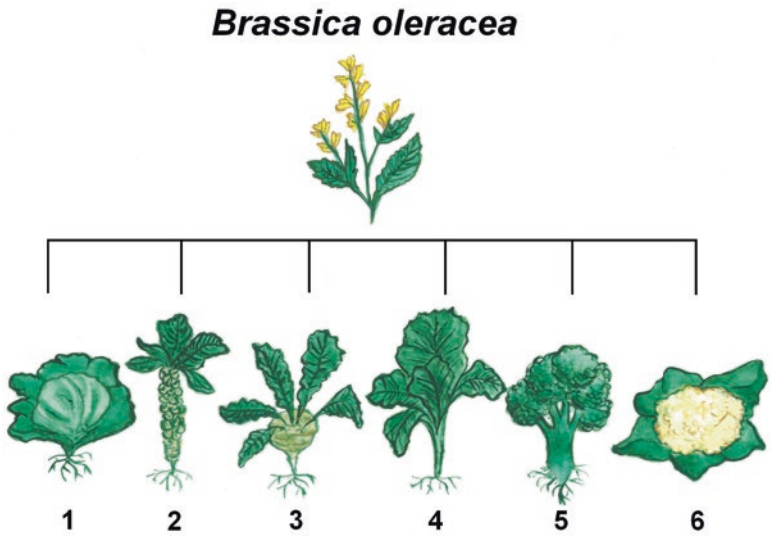


**Fig. 23.1** Illustration of wild mustard *Brassica oleracea* and its biotypes produced from the selection for: (1) terminal buds: Cabbage, (2) lateral buds: Brussels sprouts, (3) stem: Kohlrabi, (4) leaves: Kale, (5) stems and flowers: Broccoli, (5) flower cluster: Cauliflower. (Source: Art of Ms. Ana Paula da Silva Amaral Soares (2022). Modified from Stromberg (2015))

important to show the extreme morphological diversity and crop forms, with various members grown for their leaves, flowers and stems (Cabral 2016; El-Esawi 2016).

These morphological variations illustrated consequences of genome duplication and gene divergence, imparting biochemical and morphological variation to *B. oleracea*. These studies provide insights into the evolution of the *Brassica* genome and may underpin research into the many important crops of this genus (Liu et al. 2014; Yin et al. 2020; Xue et al. 2020; Saad et al. 2021), showing the economic importance of plant plasticity (Sultan 1987, 1995, 2000; Sultan and Stearns 2005; Herman and Sultan 2011; Sultan 2017).

Biologists, naturalists, botanists and others interested in plants have always been aware that organisms develop differently in many conditions, but environmental effects on phenotype were formerly regarded simply as uninformative noise obscuring the true expression of the genotype (Cabral 2016; El-Esawi 2016). In plants, for instance, individuals that have a lower resource intake level, show a lower growth in general mass size. The effects of resource availability on plant phenotypes are so profound that researchers were often frustrated in their attempts to discern genetically based adaptations from a more trivial environmental noise (Pigliucci 2001).

Due to a belief that many of the observed variations were due to this "noise", the fact that phenotypic responses to different environments may also include highly specific development adjustments that enhance function in those environments were frequently overlooked (Pigliucci 2001). This capacity for a specific and functionally appropriate environmental response is what is called adaptive plasticity, as undistinguished from the unavoidable effects of resource limitations and other suboptimal

environmental heterogeneity or stressful conditions on the phenotypic expression (Sultan 1995; Herman et al. 2014; Poersch-Bortolon et al. 2016; Fenollosa et al. 2017; Steiner et al. 2017).

## 23.5 Is Phenotypic Plasticity an Adaptive Response in Plants?

Plants cannot migrate when environmental conditions change and have to deal with different environments. Because they change their phenotype in response to environmental change, it is often assumed that phenotypic plasticity has frequently evolved as an adaptation to environmental heterogeneity (Herman et al. 2014).

Many phenotypic responses to environmental stress, however, may be the consequence of passive reductions in growth due to resource limitation (Dorn et al. 2000). Moreover, phenotypic plasticity does not necessarily evolve as an adaptation but alternatively can evolve due to genetic correlations with other traits that are under selection or due to genetic drift. So, we can assume that plants' response to novel conditions can be (but not always are) adaptive (Sultan 1987, 1995, 2000, 2017; Schmid 1992; Sultan and Stearns 2005).

A general consensus on the adaptive significance of phenotype plasticity exists only for a few plant traits, as the elongation of internodes and leaves in response to shading (Schmitt et al. 1995) and when is necessary to induce resistance against herbivores or pathogens (Agrawal 1998; Anurag, 1998). Adaptive plasticity is predicted to evolve when a species is subjected to relevant fine-scale environmental heterogeneity within the lifespan of the organism and when conditions can be predicted based on environmental cues (Herman et al. 2014; Poersch-Bortolon et al. 2016). Thus, species that are distributed across wide environmental gradients present an ideal system for examining drivers and consequences of phenotypic variation.

## 23.6 Epigenetics and Plant Phenotypic Plasticity

Several studies suggest that phenotypic plasticity can be mediated through epigenetic effects (Zhang et al. 2013; Herman et al. 2014). The most studied epigenetic effect is DNA methylation which has its variance increased in response to stressful conditions (Dowen et al. 2012) and has known effects on ecologically important phenotypes (Zhang et al. 2012, 2013; Cortijo et al. 2014; Schlichting and Wund 2014).

Because epigenetic states can be altered, epigenetic effects could provide a rapid source of phenotypic variation without any change in genetic variation, which would affect the ability of populations to persist in face of a changing environment (Sultan 1987, 1995, 2000, 2017; Sultan and Stearns 2005; Herman and Sultan 2011; Herman et al. 2014). Moreover, different epigenetic marks in the somatic line can

translate to the germline and seeds, generating a fitness patchwork in the progeny with unexplored effects on plant evolutionary dynamics (Sobral and Sampedro 2022).

A major contributor to epigenetic variability is the chromatin state, which can be altered, for instance, by ATP-dependent chromatin remodeling (Brzeski and Jarzembowski 2003). Some epigenetic roles on phenotypic plasticity of plants are now well documented. Seed dormancy, a trait that is found in many plant species and is defined as the inability of viable seeds to germinate under favorable conditions, can be overcome by environmental stimuli (Poersch-Bortolon et al. 2016). Sixty-seven epigenetic recombinant inbred lines, which are nearly isogenic but differ in their DNA methylation polymorphisms, were used to isolate *Arabidopsis* lines with quantitative differences in dormancy (Reinders et al. 2009).

A possible role for epigenetics in phenotype plasticity and evolution is well known in *Mimulus guttatus* DC. [*Erythranthe guttata* (DC.) G.L. Nesom, Phyrmaceae], plants with experimentally damaged leaves (i.e., simulated herbivory) produced offspring with higher densities of defensive leaf trichomes compared to genetically identical offspring of undamaged control plants (Agrawal 1998).

Transgenerational responses to herbivory also appear to be adaptive in the colonizing annual *Impatiens capensis* Meerb. Offspring of plants that experienced natural herbivory in the field emerged earlier, grew taller, and had significantly greater biomass than offspring of plants protected from most herbivores (Anurag, 1998; Duncan et al. 2014). These phenotypic differences have been previously attributed to natural hybridization at the subspecies level. Field studies monitoring the flower phenotypes of *I. capensis* populations, however, showed the transition in flower phenotype occurs during the lifespan of individual plants, thus ruling out the hybrid's explanation (Duncan et al. 2014).

Further research has revealed that this phenotypic transition bears signatures of an epigenetic transition (Siomos 2009). What makes this transition all the more remarkable is that it does not occur in plants grown under controlled laboratory conditions but only in the field, suggesting that an environmental factor is triggering the transition. As the transition from yellow, insect-pollinated flowers, to red, bird-pollinated flowers, leads to reproductive isolation (Duncan et al. 2014), this is a good example of how epigenetics could play a pivotal role in phenotypic plasticity, adaptation and even speciation.

## 23.7 Science Learning Activities: Phenotypic Plasticity in Boldos

### *23.7.1 What Are Boldos?*

Boldos are shrubby plants that belong to the genus *Plectranthus* L' Héritier, 1788. They are known for their richness of ornamental and medicinal species (Thoppil 1993; Nani 2011). These plants belong to the family Lamiaceae Martinov; 1820, which comprises 250 genera and more than 7000 species (Stankovic 2020).

The main area of natural distribution of this family is the Mediterranean and Middle East, up to Central Asia, and Africa (Chengyih and Hsiwen 1982). In Brazil, about 500 species of 34 genera were introduced and can be found broadly distributed across the country (Harley 2012; Harley et al. 2013).

*Plectranthus* species are well-known for their aromatic characteristics due to a complex mixture of bioactive compounds that are responsible for biological activity in both *in vitro* and *in vivo* conditions (Lukhoba et al. 2006; Rosal 2008; Rosal et al. 2011; Stankovic 2020). The secondary metabolites of these plants are very potent, with antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer effects (Lukhoba et al. 2006; Rosal 2008; Rosal et al. 2011; Stankovic 2020; Cordeiro et al. 2022).

Species of *Plectranthus* are native to tropical and subtropical regions of Africa, Australia, East Indies, Malay Archipelago, and Philippines (Lebowitz 1985). This genus, along with *Burnatastrum* Briquet 1897, *Coleus* Loureiro 1790, *Englerastrum* Briquet 1894, *Isodictyophorus* Briquet 1917 and *Neomullera* unrecorded, has already been placed in the genus *Ocimum* Linnaeus 1753. Later, *Coleus* was aggregated with *Plectranthus*, turning this grouping into an independent genus of *Ocimum* (Morton 1962a, b). The taxonomic history of *Plectranthus* has contributed to some of its species being known by many different synonyms (Lukhoba et al. 2006).

*Plectranthus* involves several species of African origin that were introduced in Brazil during the Atlantic slave trade period, up to the mid19th century (Lorenzi and Matos 2002), and mainly used as herbal medicines. *Plectranthus barbatus* Andrews 1810 is one of the most cited species in ethnobotanical surveys in Brazil (Carriconde et al. 1996; Luz 2001). The two species *P. barbatus* and *Plectranthus grandis* (Cramer 1979) Willemse 1985 are very similar species and they are usually confused, as they are used for the same medicinal purposes almost all with medicinal functions related to stomach and liver (Fernandes et al. 2021). In the other hand, *P. barbatus* and *Plectranthus neochilus* Schlecher 1896 can be differentiated by several traits as phyllotaxis, blade shape and leaf margins, coloration of glandular, trichomes, inflorescences and densification between flowers in anthesis (Fernandes et al. 2021).

Pioneering cytogenetic works in *Plectranthus* have revealed a group of species with diversified chromosome numbers from 2n = 14 to 84, with 2n = 28 most frequent (Morton 1962b). A study in four species cultivated in Brazil revealed no significant variation in the number of chromosomes (2n = 30), except for *Plectranthus amboinicus* (Lour.) Spreng. (2n = 34), although the karyotypic formula varied (Table 23.1) (Nani 2011; Nani et al. 2015).

### *23.7.2 How Did Everything Begin?*

The idea was to explore the concept of phenotypic plasticity by evaluating growth characteristics of Mirim boldo (*P. neochilus*), based on observation in two backyards located in two different regions of the state of Rio de Janeiro, Brazil. One was

**Table 23.1** Karyotypic Formula (KF), Flow Cytometry (FC) of four cultured species of *Plectranthus*. m = metacentric; sm = submetacentric chromosome

| Species | KF | FC (pg) | Culture location |
|---|---|---|---|
| *P. amboinicus* | 13 m + 4sm | 5.58 | Medicinal Plant Garden of University of Lavras (Lavras, MG, Brazil) |
| *P. grandis* | 7 m + 8sm | 5.23 | Medicinal Plant Garden of University of Santa Maria (Santa Maria, RS, Brazil) |
| *P. neochilus* | 9 m + 6sm | 5.98 | Campinas Agronomy Institute (Campinas, SP, Brazil) |
| *P. barbatus* | 8 m + 7sm | 5.35 | Medicinal Plant Garden of University of Lavras (Lavras, MG, Brazil) |
| | 9 m + 6sm | | Campinas Agronomy Institute (Campinas, SP, Brazil) |
| | 10 m + 5sm | | Medicinal Plant Garden of University of Santa Maria (Santa Maria, RS, Brazil) |

Modified from Nani et al. 2015

located in Niterói (RJ), at sea level, in the metropolitan region of the city of Rio de Janeiro, and the second in Teresópolis (RJ), in a mountain region at 871 m altitude (Lima et al. 2017a, b; Lima 2019a).

It is well known that Mirim boldo presents physiological and morphological differences when cultivated in different environmental conditions such as light intensity, altitude, and humidity (Hoffmann et al. 2009; Rosal 2008; Segal 2010; Rosal et al. 2011; Zhang et al. 2012; Lima et al. 2017a, b). These variations include differences in the reproduction (sexual or asexual), number of leaves per branch, distance between leave nodes, leaf area and external morphology of the leaves (Lima et al. 2017a, b; Lima 2019a, b; Campos et al. 2020).

### *23.7.3 The Experimental Design*

The plant species selected for experiments, observations and measurements belong to the genus *Plectranthus*. Such shrub plants were chosen because they are common in-home and medicinal gardens worldwide, regardless of their place of origin (Lukhoba et al. 2006; Rosal 2008; Rosal et al. 2011; Lima et al. 2017a, b; Campos et al. 2020; Ferah et al. 2019; Mancebo et al. 2020, 2021). Previous studies by Lukhoba et al. (2006), Rosal (2008) and Rosal et al. (2011) outlined new strategies to approach environmental education for undergraduate students (Biological Sciences, Environmental Engineering), and non-formal education for young people (university freshmen) and elderly or retired people using these plants as model.

The experiments with the Mirim boldo began by collecting apical cuttings of about 10 cm long from one adult plant and placing them to grow in the UFF greenhouse using three types of nets for covering the plants: (i) Sombrite 90, 10% sunshine, (ii) Sombrite 70, 30% sunshine, (iii) Sombrite 50, 50% sunshine, and a control without cover net (100% sunshine). The plants were irrigated once a day.

**Table 23.2** Experimental design carried out at the school and the university from October 19 to November 16, 2017

| Location | Color of the flowerpots | Flowerpots | Type of soils | Source of light | Intensity of light (%) |
|---|---|---|---|---|---|
| School | Blue | 1 | plant compost + poultry manure (3:1) | 20 W Lamp | 10 |
| | | 2 | | | 30 |
| | | 3 | | | 50 |
| | | 4 | | | 100 |
| | | 5 | earthworm humus + bovine manure (1:1) | | 10 |
| | | 6 | | | 30 |
| | | 7 | | | 50 |
| | | 8 | | | 100 |
| UFF | Black | 9 | plant compost + poultry manure (3:1) | Sun Light | 10 |
| | | 10 | | | 30 |
| | | 11 | | | 50 |
| | | 12 | | | 100 |
| | | 13 | earthworm humus + bovine manure (1:1) | | 10 |
| | | 14 | | | 30 |
| | | 15 | | | 50 |
| | | 16 | | | 100 |

Modified from Sodré et al. 2018

Two types of soil were used in the flowerpots, (i) plant compost + poultry manure (3:1) and (ii) earthworm humus + bovine manure (1:1). In parallel, a similar experiment was conducted at an elementary school (Table 23.2). In addition, both types of soil were chemically characterized (Table 23.3).

### *23.7.4 What Do We Learn from This?*

The expression of phenotypic plasticity of two species of boldos (Mirim and Brazilian) was assessed by students of formal education (schoolchildren, aged 7 to 10, and undergraduate, aged 18 to 28), and in non-formal education through workshops and expositions, involving participants in the "UFF – Cultural Trote 2017" (17 to 45), and elderly and/or retired people, participants of the Project Advanced Space of UFF (55 to 94 years old) (Table 23.4).

All activities led to important results, allowing us to discuss the importance of plants in nature, for food, medicine, holistic therapies, and healthy recreational activities, such as building, enjoying and taking healthy benefits from gardens and horticulture. The importance of plants in the maintenance of the planet earth climate was also highlighted (Nicotra 2022) (Table 23.5).

The results and observations on the participants' behavior point out that the younger audience (schoolchildren) was more active as a result of children generally expressing a greater degree of curiosity for what is new to them (Sodré et al. 2018;

**Table 23.3** Chemical composition of nutrients in the two types of soils used in the Mirim boldo experiments (1) plant compost + poultry manure, (2) earthworm humus + cattle manure) and nutritional wealth index (NWI: values in soil 1 ÷ values in soil 2)

| Elements | Symbols | Unit | Soil type 1 | Soil type 2 | NWI |
|---|---|---|---|---|---|
| Nitrogen | N | g/kg | 3.43 | 3.84 | 0.9 |
| Potassium | K | g/kg | 3.54 | 3.97 | 0.9 |
| Carbon | C | C/CHN – % | 5.65 | 11.41 | 0.5 |
| Calcium | Ca | mg/kg | 7572.0 | 4488.0 | 1.7 |
| Copper | Cu | mg/kg | 34.7 | 12.9 | 2.7 |
| Iron | Fe | mg/kg | 32,760.0 | 14,430.0 | 2.3 |
| Phosphor | P | mg/kg | 585.0 | 162.0 | 3.6 |
| Magnesium | Mg | mg/kg | 3314.0 | 2089.0 | 1.6 |
| Manganese | Mn | mg/kg | 498.0 | 133.0 | 3.7 |
| Zinc | Zn | mg/kg | 48.5 | 24.6 | 2.0 |

Modified from Sodré et al. 2018

**Table 23.4** The eight groups of people involved in our activities

| Type of activities | Target audience |
|---|---|
| (I) Formal Education | (1) Biological Science undergraduate (1st and 3st semesters)<br>(2) Agricultural and Environmental Engineering undergraduate (1st semester)<br>(3) Students of three Elementary school grades (1st and 2nd enrolled in the Science discipline, and 4th enrolled in the Environmental Education discipline) |
| (II) Non-formal Education | (1) University freshmen, and family and friends that participated of *UFF – Trote Cultural 2017* (an interactive exposition and workshops about research activities of the university)<br>(2) Older and/or retiree participants of the Project Advanced Space of UFF |

Modified from Lima 2019a

Ferah et al. 2019; Lima 2019a, b). On the other hand, the sentence "What do we learn from all this?" for instance, was said by a child (first grade) while working in the final proposed activity. This student was intrigued by the fact that part of the plants died in the mini greenhouse established inside the school. From this observation, we helped the students formulate a hypothesis that the artificial light didn't provide enough light for the complete plant development (Sodré et al. 2018; Lima 2019b).

Such hypothesis was brought in the following year, and the greenhouse experiment was conducted by the 2nd grade students using two different conditions, inside the classroom and in the schoolyard. The commitment to redo the experiments led the students to feel important for being treated as young scientists (Sodré et al. 2018; Lima 2019b).

In turn, children from the elementary school learned to measure a plant's height, check the number of leaves and leaf area comparatively, using their perception as well as intuition (Lima 2019a, b), and discussed the basic characteristics of plants

**Table 23.5** Relevant and stimulating results for building knowledge about plants

| Target group | Principal questions | Relevant findings |
|---|---|---|
| (a) Biological Science undergraduate (first semester) (Ref. 1, 2, 3, 4) | Is it possible to measure the phenotypic plasticity of plants grown in different gardens in a practical botany class using two simple methods to access the leaves and stems morphological differences? | The adopted strategy allowed the biological students to easily visualize how the variation in abiotic elements (soil, light) significantly affected plant phenotypic in a short time and at low cost. |
| (b) Biological Science undergraduate (3rd semester) (Ref. 1, 2, 4, 7) | Is it possible to discuss the concepts of phenotypic and species plasticity from a practical botany class on leaf morphology? | Environmental factors such as light intensity, altitude, humidity impact on the leaves and stalk morphological variations as well as on the number of leaves per branch, showing that phenotypic plasticity should be considered in plant identification and in approaches to the ecology and evolution of plants. |
| (c) Agricultural and Environmental Engineering undergraduate (1st semester) (Ref. 1, 2, 4, 8) | How do you show changes in the flowering pattern of plants due to climatic changes that occurred during the cultivation period?<br>How to discuss the relevance of phenotypic plasticity to produce new biotypes that serve as food? | The unexpected increase in the temperature of rainfall that occurred in June and July 2018 significantly modified the expected inflorescence pattern for the Brazilian boldo, allowing the discussion of global warming issues on plant reproduction.<br>It was possible to demonstrate and discuss the genetic improvement of plants through selective crosses that created new varieties of plants with desirable phenotypic characteristics. A photo album with several vegetables were used to reinforce or explain the importance of plants in our lives, showing that artificial selection produced new food items like different strains of tomatoes, potatoes, corns, and variants from wild mustard *Brassica oleracea,* such as cabbages and cauliflowers.<br>We used the classic example of *B. oleracea* and it mainly derivatives biotypes (broccoli, brussels sprouts, cabbage, cauliflower, collard greens, kohlrabi, kale, Savoy cabbage). |

(continued)

**Table 23.5** (continued)

| Target group | Principal questions | Relevant findings |
|---|---|---|
| (d) Students of Elementary school (the same class 1st and 2nd grade levels) (Ref. 1, 2, 4, 5, 9) | How is it possible to carry out experiments such as mini greenhouses inside the school room to show the phenotypic plasticity of plants? | It was possible to develop with children an experimental design inside the school area to observe plant plasticity in a short time. However, half of boldo cuttings did not produced seedlings and died because the roots did not grow.<br>The children committed to repeat the experiment in the following semester in order to test whether to add a substance that stimulates the production of boldo seedlings. |
| (e) Students of Elementary school (4th grade level) (Ref. 1, 2, 4, 5, 9, 10) | How can you show the importance of the environment in the expression of phenotypic plasticity of plants with experiments carried out in the school yard?<br>Is it possible to promote the construction of knowledge about phenotypic plasticity expressed by plants among children? | The activities involved two types of boldo (Mirim and Brazilian) to introduce the concept of species and teach how to measure plant growth and help to identify differences between plants.<br>Finally, the children were instructed to record their results on the plants in tables, enabling comparisons. |
| (f) University freshmen and their family and friends (Ref. 1, 2, 4, 6) | Is it possible to demonstrate the importance of phenotypic plasticity of plants from sensations (touch, odor, vision) during the UFF – Cultural Trote 2017? | The university freshmen and their companions were invited to identify differences in plants (Mirim boldo) that were grown in four experimental conditions using sight, smell and touch. In addition, photos of a variety of plants were presented showing how they were created based on artificial selection using the phenotypic plasticity of the species involving the wild mustard species. |

(continued)

**Table 23.5** (continued)

| Target group | Principal questions | Relevant findings |
|---|---|---|
| (g) Elderly and/or retirees that belonging to the "third age" University (Ref. 1, 4, 8, 11, 12) | How do you encourage senior citizens and/or retirees to build knowledge about the phenotypic plasticity of plants through ted talks, and observation of feeding items with growing plants in the garden of the university and greenhouses? | A photo album with a variety of pictures was created to demonstrate how new types of edible plants were created by human based on artificial selection and phenotypic plasticity of each species. First, elderly and/or retired people were invited to observe and classify the pots containing Mirim boldo seedlings under different growing conditions. They have the opportunity to plant some cuttings of Brazilian boldo in two types of soils in eight vases. After planting the Brazilian boldo in the pots, the elderly helps us to transfer the pots to the UFF greenhouses. In the second activity, the elderly and/or retired people were warned that phenotypic plasticity in plants can interfere with the degree of recognition of those that are beneficial to our health. We responded that preliminary results which have not yet been published showed differences in the types and concentrations of essential oils contained leaves of Mirim boldo. Finally, we promoted a visit to UFF gardens and greenhouses to observe the cultivation of Mirim boldo and Brazilian boldo, respectively. |

1 – Lima (2017); 2 – Lima et al. (2017a); 3 – Lima et al. (2017b); 4 – Lima (2019a); 5 – Lima (2019b); 6 – Sodré et al. (2019a); 7 – Sodré et al. (2019b); 8 – Campos et al. (2020); 9 – Sodré et al. (2018); 10 – Ferah et al. (2019); 11 – Mancebo et al. (2020); 12 – Mancebo et al. (2021); Stromberg (2015)

and their importance in the environmental conservation, and source of food for human and other animals (Ferah et al. 2019).

It was clear to us that children in 4th grade were very happy to compare Mirin and Brazilian boldos and understand the differences among them. By the end, they were able to relate the characteristics of the leaves and the environmental condition. Their essays they wrote revealed how they understand the importance of environmental quality as expected by the purpose of Environmental Education discipline (Ferah et al. 2019).

The drawings and essays by the children (1st, 2nd, and 4th) revealed the degree of involvement, commitment and understanding of the experimental results that were obtained (Ferah et al. 2019; Lima 2019a, b).

In most drawings there were plants, flowerpots, the two types of greenhouses (classroom and/or schoolyard), and other elements (Fig. 23.2), as detailed shown in Lima (2019b).

We also observed that children more often drew the components of the environment (grass, sky, sun, clouds, rain, and moon) more often than animals such as butterflies and flying birds (Ferah et al. 2019; Lima 2019a, b). Both the measurements of the plants and the drawings and sentences that the children made revealed that they were aware of the purpose of the study as well as its obtained results (Lima 2019a, b). With 4th grade students, we developed comparative experiments between two boldo species (Mirim and Brazilian).

Yet among the formal education students, the university students took advantage of the practical activities that mediated the phenotypic plasticity of leaves in both species and inflorescence specially for Brazilian boldo. Additionally, in theoretical-practical classes we were able to discuss and develop their knowledge about species description as well as the different concepts of species in debate in the literature (Lima et al. 2017b; Sodré et al. 2018, 2019a, b).

We adapted our strategies to discuss plant plasticity with the elderly public from the non-formal education. We took into account that most elderly people have already experienced part of these questions when using medicinal plants, such as the two selected species of boldo (Rosal 2008; Lima et al. 2017a, b). Also, this target audience could have different views on genetics and phenotypic plasticity. Fortunately, we would have a group of people who, for the most part, were already aware of many types of edible vegetables (Mancebo et al. 2020, 2021; Lima 2019a).

The workshops developed with the elderly people involved: (a) two tad talks about edible and medicinal plants, followed by (b) collection of boldo seedlings in the university gardens, (c) planting the stakes and (d) interviewing individuals. The participation of this target audience was quite diversified in terms of intensity and duration of activities. However, the comments, doubts and enthusiasm informed us that the activities were pleasant and overall positive (Mancebo et al. 2020, 2021; Lima 2019a).

Two workshops held during the UFF – Trote Cultural 2017 with different groups of freshmen university students and their families and/or friends made it possible for them to easily recognize in a playful way the phenotypic plasticity in plants and how the man took advantage of this fact to create different types of foods, as already presented in the case of mustard (Fig. 23.1), which caused a lot of curiosity among the participants (Sodré et al. 2019a).

The same enthusiasm was observed in the elderly and/or retired participants (Mancebo et al. 2020, 2021). Other biological questions, as intriguing as dog races and human origin, were raised among the elderly and retired people, demonstrating that the strategy of using plant plasticity in the core of our discussion, allowed understanding the role of genetics in everyday life.

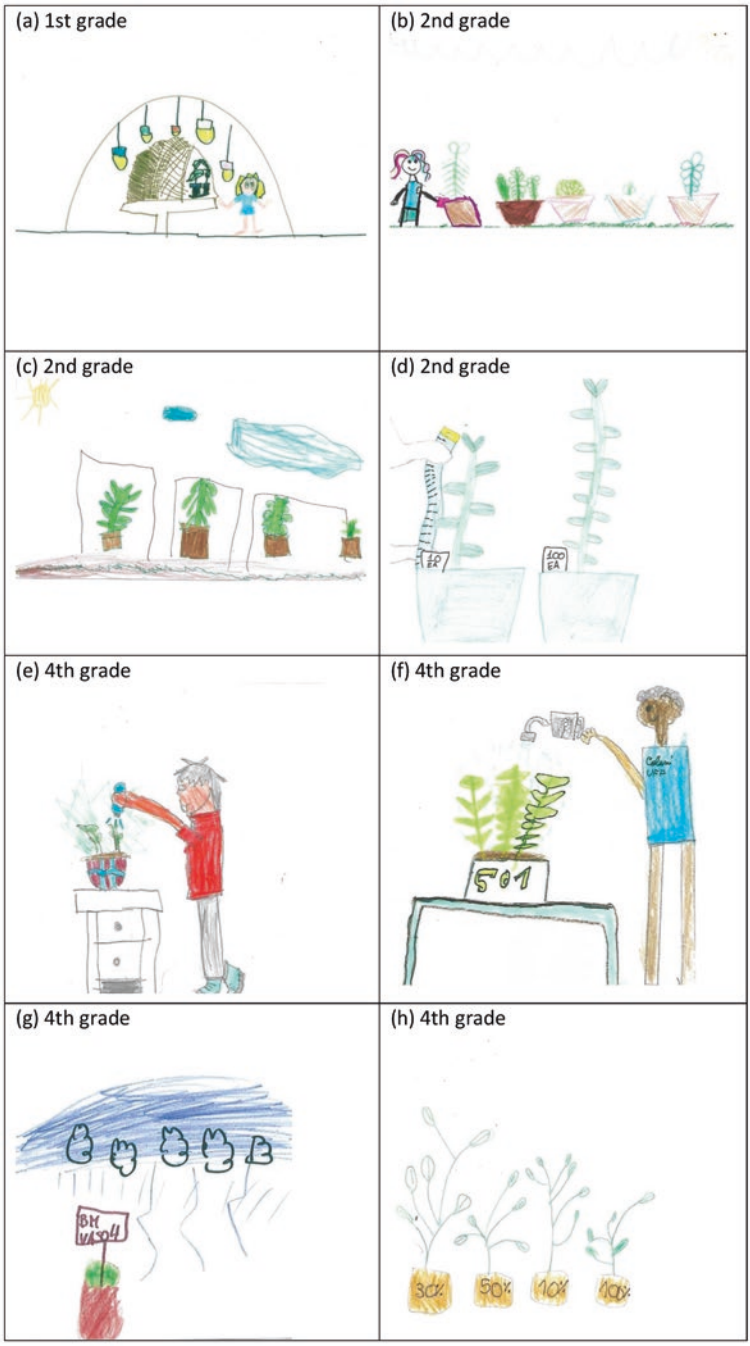


**Fig. 23.2** Illustrations of children from elementary school expressing the different experimental steps with Mirim boldo involving: (**a–c**) preparation of the experiments inside and outside the classroom, (**d**) size measures of plants, (**e–f**) water and care the plants, (**g**) watch plants being watered by the rain; (**f**) observe differential plant growth, as expected. (Source: Lima (2019a))

## 23.8 Final Considerations

The phenotypic plasticity typically observed in the genus *Plectranthus* is an important tool to exemplify genetic issues in the everyday life, and also contributes to the deconstruction of the botanic blindness ubiquitously seen among students and general people, helping to call attention to the plant conservation.

Through simple, quick and inexpensive experiments, we were able to discuss phenotype plasticity from schoolchildren to elderly people. The input given by the participants made us reflect and seek solutions to answer their questions, leading to eventual changes in the way of our initially planned strategy. All these facts were not treated as obstacles, but as new possibilities in the teaching-learning process, both for formal and non-formal education. When the basic biology of plants is treated with prominence and playfulness, it is possible to achieve greater acceptance of plants. Of note, the participants were similarly involved in several activities regardless of age, educational or professionalization degrees. In conclusion, we demonstrated that understanding plant plasticity through formal and non-formal education is a valuable strategy to reduce the phenomenon of botany blindness.

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# Correction to: Phylogeography for Neotropical Species Conservation: Lineages Through Time and Space

**Carolina B. Machado and Manolo F. Perez**

## Correction to:
## Chapter 6 in: P. M. Galetti Jr. (ed.), *Conservation Genetics in the Neotropics*, https://doi.org/10.1007/978-3-031-34854-9_6

The original version of the chapter was inadvertently published with incorrect caption and image of Fig. 6.2. This figure has now been corrected. The correct presentation is given here.

The updated original version of this chapter can be found at
https://doi.org/10.1007/978-3-031-34854-9_6



P. M. Galetti Jr. (ed.), *Conservation Genetics in the Neotropics*,
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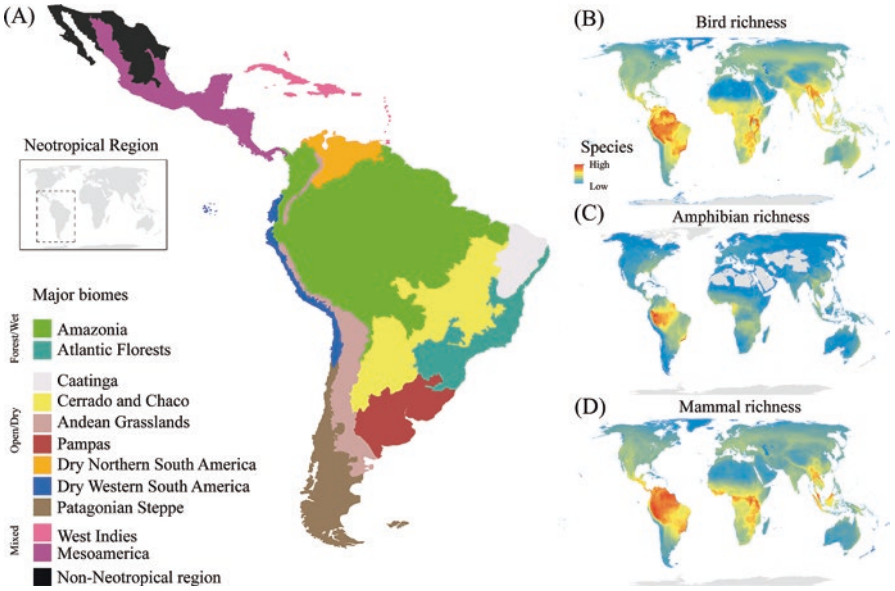


**Fig. 6.2** Map of the neotropical region (**A**) and its species richness (**B**-**D**). (**A**) Approximate natural distribution of major terrestrial neotropical biomes (adapted from Antonelli et al. (2018), Morrone (2014), and Olson et al. (2001)). Latitudinal distribution of species richness among birds (**B**), amphibians (**C**) and mammals (**D**) across the world based on Jenkins et al. (2013). Image created by Carolina Machado with the software ArcGIS v10.2 (https://www.arcgis.com). Free vector data from Löwenberg-Neto (2014) and BiodiversityMapping.org (https://biodiversitymapping.org/; Accessed September 10, 2022)

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