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Thematic Review

From genes to ecosystems: a synthesis of amphibian biodiversity research in Brazil

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Abstract: Here, we summarize examples of significant advances in amphibian research supported by the São Paulo Research Foundation (FAPESP), focusing on recent discoveries in the fields of community ecology, habitat change, infection diseases, and multipurpose DNA sequencing. We demonstrated that FAPESP has been fundamental not only by directly funding research projects and scholarships, but also through its science training policy, fostering international collaborations with world-class research institutions, improving and consolidating new lines of research that often depended on a synergetic combination of different knowledge and complex tools. We emphasized that future studies will continue to focus on basic questions, such as description of new species, as well as taxonomic and systematic corrections. Furthermore, we also expect that there will be a strong integration among different disciplines using novel bioinformatics tools and modeling approaches, such as machine learning. These new approaches will be critical to further develop our understanding of foundational questions of amphibian life-history trait variation, disease transmission, community assembly, biogeography, and population forecasts under different global change scenarios such as agricultural expansion, agrochemical use, habitat loss, and climate change.

Keywords: *Anura; community ecology; habitat change; DNA sequencing; chytridiomycosis; FAPESP; financial support.*

De genes a ecossistemas: uma síntese da pesquisa em biodiversidade de anfíbios no Brasil

Resumo: No presente estudo apresentamos exemplos de avanços significativos nas pesquisas com anfíbios financiadas pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), focando em descobertas recentes nos campos de ecologia de comunidades, modificação do habitat, doenças infecciosas e o sequenciamento de DNA com múltiplos propósitos. Demonstramos que a FAPESP tem sido fundamental não somente pelo financiamento direto de projetos de pesquisa e bolsas de estudo, mas também através de sua política de formação científica, fomentando colaborações internacionais com instituições de pesquisa de excelência mundial, melhorando e consolidando novas linhas de pesquisa que frequentemente dependem da combinação sinérgica entre diferentes linhas de conhecimento e ferramentas complexas. Enfatizamos que futuros estudos continuem com foco em questões básicas, como a descrição de novas espécies, bem como correções taxonômicas e sistemáticas. Além disso, esperamos uma forte integração entre diferentes disciplinas usando novas ferramentas de bioinformática e abordagens de modelagem, como o aprendizado de máquina. Essas novas abordagens serão críticas para desenvolver ainda mais nossa compreensão a respeito de questões fundamentais sobre as características da história de vida dos anfíbios, transmissão de doenças, estrutura de comunidades, biogeografia e previsões populacionais em diferentes cenários de mudanças globais, como a expansão da agricultura, uso de agrotóxicos, perda de habitat e mudanças climáticas.

Palavras-chave: *Anura; ecologia de comunidades; modificação do habitat; sequenciamento de DNA; quitridiomicose; FAPESP; financiamento.*

Introduction

In the early 1980's, The São Paulo Research Foundation (FAPESP) began funding projects across different subfields of biodiversity research, including botany, ecology, and zoology, which gradually developed more consistently over the last four decades. The creation of the Biota/FAPESP Program in 1999 elevated Brazil to its current prominent position in biodiversity research and global leadership in science. Several lines of research involving the Class Amphibia were propelled by the Biota/FAPESP program, which not only allowed for the consolidation of basic research areas, to which the scientific community of São Paulo was already dedicated, but also facilitated wide diversification of emerging fields and consolidated more traditional disciplines already well-established in other parts of the globe. FAPESP was fundamental not only by directly funding research projects and scholarships (Figures 1 and 2), but also through its science training policy, fostering international collaborations (*i.e.*, internships and sabbaticals) with world-class research institutions, improving and consolidating new lines of research that often depended on a synergetic combination of different knowledge and complex tools. For example, several amphibian phylogenies, based mainly on DNA sequence analysis, were published by researchers who received training abroad allowing an unprecedented deepening of the understanding in evolutionary relationships and diversification in this group of vertebrates. Even the most basic areas, such as taxonomy and natural history, were leveraged by FAPESP funding. In terms of numbers, the state of knowledge of amphibian diversity in the state of São Paulo circa the late 1990's was limited (e.g., 180 described species), and was mainly based on traditional techniques using only morphology as line of evidence (Haddad 1998). The widespread use of new techniques (particularly DNA sequencing) in the 2000s allowed researchers to identify and elevate the number of species of amphibians, reaching approximately 240 species (Araújo et al. 2009a, Rossa-Feres et al. 2011); a remarkable increase of more than 30% in just over a decade. Despite the high number of new species descriptions, there is a high number of species that have disappeared or have experienced population declines (Eterovick et al. 2005, Carvalho et al. 2017). These numbers highlight that we are probably losing a significant fraction of our biodiversity even before we become aware of its existence and that it is critical to fill this knowledge gap for targeted mitigation plans. The lack of knowledge about biodiversity weakens conservation measures, facilitating the actions of predatory degradation of ecosystems and jeopardizing the survival of the species most sensitive to the anthropic changes imposed on the environment.

The use of DNA sequences is perhaps the most eloquent example of an approach that permeates across multiple fields of study in the biological sciences, allowing scientists to tackle problems from a different, and often more efficient perspective. In addition to the basic research areas, such as taxonomy, systematics, phylogeny, and natural history, the long-term support from FAPESP allowed our science to bloom in several other subfields, including biogeography, phylogeography, population genetics, community phylogenetics, trait evolution, and ecology of diseases. This has greatly contributed to a more refined understanding of important processes such as spatial distribution, evolution, and diversification of organisms, with clear applications in public policies for the conservation of populations and species. The techniques of DNA barcoding, environmental DNA, and historical DNA have revolutionized the fields of taxonomy and ecology, with relevant applications on species conservation. With multidisciplinary applications, these techniques, together with the computational tools and predictive modeling, have great power of resolution and precision, facilitating the advancement of different areas of research in biodiversity, including those focused on amphibians.

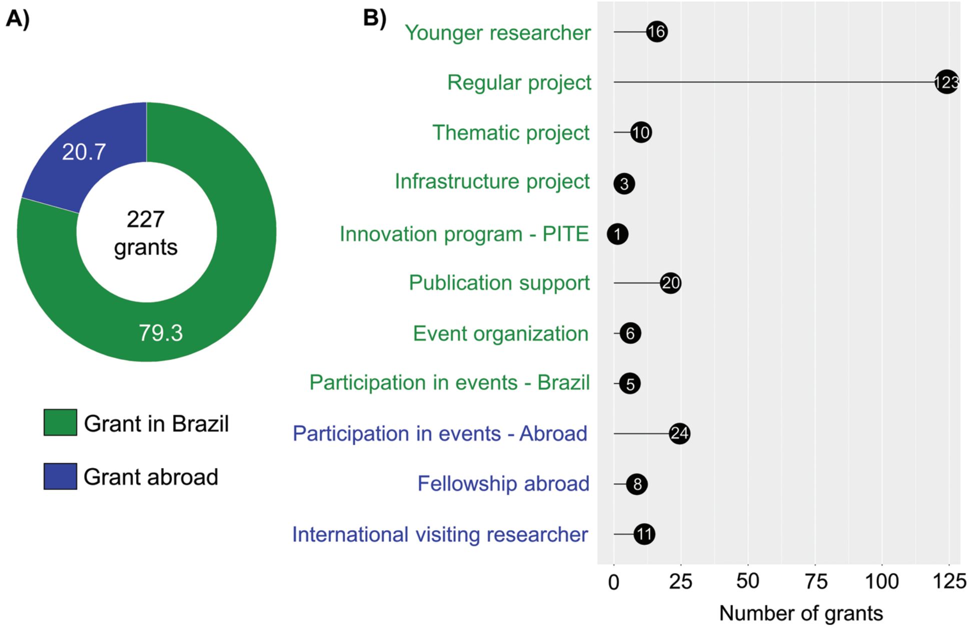

Figure 1. Proportions between the different lines of funding offered by FAPESP for projects on amphibians, since the beginning of this funding agency, 60 years ago. The data were extracted from https://bv.fapesp.br/pt/proc6291/ (from 1962 to 1991) and https://bv.fapesp.br/pt/559// (1992 to present). Selected only projects that had in the title any of these words: anur*, anfibi*, amphib*, and herpeto*.

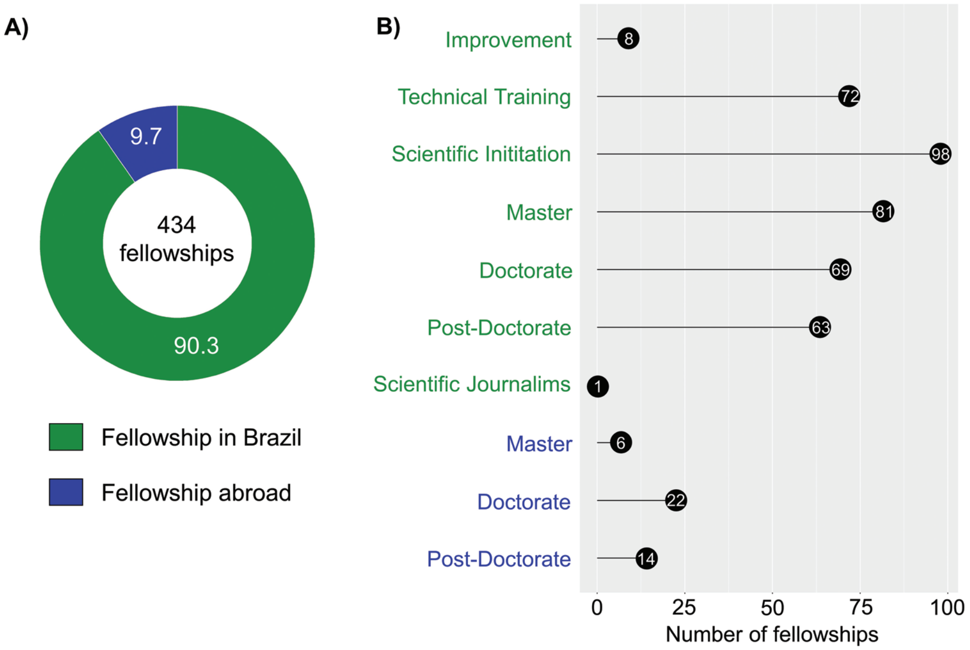

Figure 2. Proportions of scholarships awarded by FAPESP for projects on amphibians, since the beginning of this funding agency, 60 years ago. The data were extracted from https://bv.fapesp.br/pt/proc6291/ (from 1962 to 1991) and https://bv.fapesp.br/pt/559// (1992 to present). Selected only projects that had in the title any of these words: anur*, anfibi*, amphib*, and herpeto*.

Below we summarize examples of significant advances in amphibian research bolstered by FAPESP support during the last few years. Our review focuses on recent discoveries in the fields of community ecology, habitat change and disease, and multipurpose DNA sequences. These approaches are contributing to the formation of the new generation of scientists in Brazil and are generating a vast dataset for conservation policies and human wellbeing. Due to space limitations, however, we refrain from providing a revision on amphibian-centered studies focused, for example, on behavior, bioacoustics, cytogenetic, physiology, phylogeography, and toxicology. Even so, we emphasize the importance of these studies for understanding the biodiversity of Neotropical amphibians.

Community Ecology

Community ecology is defined as the study of patterns in the diversity, abundance, and composition of species in communities, and the processes underlying these patterns (Vellend 2010). Traditionally, community ecology has been separated from other disciplines such as biogeography, macroecology, and metacommunity because they have historically focused on different spatial and temporal scales (Ricklefs & Jenkins 2011). However, this separation has become indistinct as each discipline has grown and the theories that underpin the patterns of biodiversity transcend scales of space or time (e.g., Ricklefs & Jenkins 2011). Thus, the topics addressed here are representative of the intersections between evolution, biogeography, macroecology, metacommunity, and community ecology. Considering that we are evaluating only studies related to amphibians, we used the terms community (i.e., group of organisms representing multiple species living in a specified place and time) and assemblage (i.e., phylogenetically related groups within a community) interchangeably throughout the text.

Until 2007, most studies of amphibian community ecology supported by FAPESP focused on two main topics that were geographically limited in their grain (i.e., the size of the individual observational units) and extent (i.e., the overall area encompassed by a study). The first topic was related to the spatial and temporal distribution of species in breeding habitats (e.g., Haddad et al. 1988, Rossa-Feres & Jim 1996), while the second was interested in the breadth and overlap of male calling site and advertisement call (e.g., Cardoso & Haddad 1992, Rossa-Feres & Jim 2001). These studies have provided detailed information on spatiotemporal partition within assemblages and have helped other investigators by facilitating the integration of datasets on amphibian species distribution. For example, descriptive analysis using data from these foundational studies demonstrated that the pairwise similarity in amphibian species composition was better explained by corridors of vegetation (e.g., Cerrado, Semideciduous Forest, or Dense Ombrophilous Forest) than the geographic distance among these communities (e.g., Dixo & Verdade 2006, Araújo et al. 2009b). Vasconcelos et al. (2010) and da Silva et al. (2012a), gathering data on amphibian records, demonstrated that humidity-related variables are key environmental factors related to the richness of reproductive modes – reproductive modes in drier sites being a subset of reproductive modes displayed by the amphibian species in sites with higher levels of humidity. Although somewhat overlooked, surveys of species composition (e.g., checklists) and natural history information are still extremely important for studies of biogeography and community ecology in areas such as the Neotropics, where current estimates point to a very high number of undiscovered species (i.e., Linnean shortfall), and that the geographical distribution of species already described is limited (i.e., Wallacean shortfall).

Motivated by metacommunity theory, several studies have tried to understand the relative importance of niche-based (i.e., the presence and abundance of species determined by their deterministic interactions with the abiotic and biotic environment) and/or neutral-based processes (presence and abundance as a result of dispersal limitation, demographic stochasticity, and random speciation) shaping amphibian assemblages (e.g., da Silva et al. 2014, Prado et al. 2014, Provete et al. 2014, Jordani et al. 2019). However, even when addressing the same questions, amphibian communities have been defined on quite different extents and grains (Melchior et al. 2017). For example, studies have used different grain sizes or focal assemblage – ponds (Prado et al. 2014), streams (Ribeiro et al. 2018), bromeliads (Ruano-Fajardo et al. 2016), vegetation types (Thomé et al. 2021), or grid cells (Vasconcelos et al. 2019) – or spatial extents – several ponds in one national park (e.g., Provete et al. 2014) or several ponds across an entire region (Vasconcelos et al. 2010, Prado et al. 2014) – to evaluate the same processes. This difference among spatial scales is not just a theoretical matter, but it is a concrete issue indicating that the contradictory results from different studies are not comparable (Melchior et al. 2017). It has been also demonstrated that community composition-environment relationships change over time (Gomes-Mello et al. 2021). Taken together, these studies reinforce the importance of understanding that comparisons and generality about snapshot metacommunity studies performed at different spatial and temporal scales should be considered cautiously.

Studies evaluating the importance of local descriptors of breeding habitats have shown that features of habitat complexity are key factors in maintaining a high diversity of species, providing a greater variety of microhabitats, both in vertical and horizontal strata, and thus meeting diverse species-specific requirements (e.g., Prado et al. 2014, Oda et al. 2018). These results are in accordance with the habitat heterogeneity hypothesis that states that structurally complex habitats provide more niches and diverse ways to exploit environmental resources, and thus increase species diversity. Another important variable is pond hydroperiod with greatest species richness and functional diversity being found in water bodies with intermediate hydroperiod (Prado et al. 2014, Queiroz et al. 2015). Wellborn et al. (1996) suggest that in general amphibian species avoid either short-hydroperiod ponds (i.e., shallow depth) because of their high risk of desiccation, or permanent-water ponds (i.e., deep depth) because of their elevated number of predators. Considering the landscape surrounding breeding habitats, forest fragments (i.e., terrestrial habitats) are relatively less disturbed compared to agricultural and urban areas, and provide refuges and food for many species of anurans during non-reproductive periods (da Silva & Rossa-Feres 2010, Schiesari & Correa 2016, Oda et al. 2018). There is abundant empirical evidence that habitat loss and fragmentation increasing the discontinuity between aquatic breeding habitats and terrestrial habitats has negatively influenced the richness, abundance, and species composition of anurans (Becker et al. 2007, 2010a, da Silva & Rossa-Feres 2011), but not between assemblages in the interior or edge of forest fragments (Dixo & Martins 2008); see more below. Furthermore, the effects on community structure does not depend solely on isolation of terrestrial habitats, but also on the composition of the inter-habitat matrix, patch size, environmental contamination (e.g., agrochemicals), and species traits (Cushman 2006, Schiesari & Correa 2016, Dixo & Metzger 2010, Anunciação et al. 2021). Based on these results, the maintenance of semi-permanent ponds, with high complexity in vertical and horizontal strata of vegetation, and near forest fragments, could be used as an effective strategy to conserve Brazilian amphibian species dependent on aquatic breeding habitats.

The shift towards studies looking at multiple spatial and temporal scales and beyond local habitats has opened several opportunities to explore the roles of geological events (e.g., mountain uplift), past climatic oscillations (e.g., refugia during the Quaternary climate fluctuations), and present-day environmental conditions to understand the processes shaping current patterns of species distribution (Carnaval et al. 2009, Thomé et al. 2010, Vasconcelos et al. 2010, da Silva et al. 2014, Benício et al. 2021), to delimit cohesive ecoregions of similar species composition (Vasconcelos et al. 2014, 2019), to predict current and future climatically suitable areas (Haddad et al. 2008, Vasconcelos et al. 2019), and to support the delimitation of priority area-selection for conservation (e.g., Loyola et al. 2008, Vasconcelos & Prado 2018). For example, there is growing evidence that the distinct diversification rates within adjacent regions in the Atlantic Forest and the limited range expansion of species from lineages with reproductive modes dependent on high humidity have a strong role shaping local frog communities between regions (i.e., biogeographical filter), but not within them (da Silva et al. 2014, Benício et al. 2021). In the context of global environmental changes, understanding and predicting biodiversity responses is becoming imperative. Studies based on ecological niche models have predicted that some amphibian species could go extinct by 2050 and 2070 due to climate change in Atlantic Forest and Cerrado (e.g., Loyola et al. 2014). Multifaceted approaches have identified priority areas to protect key ecosystem functions and evolutionary legacy in anuran communities of different regions in Brazil (Loyola et al. 2008, Vasconcelos & Prado 2018). Therefore, these results emphasize how the integration of multiple approaches offer a more complete picture of the relative importance of evolutionary and ecological processes shaping the distribution of different facets of biodiversity.

Impacts of Habitat Change and Disease on Atlantic Forest Frogs

FAPESP has fostered a boom in field and laboratory research on anuran population declines attributed to both anthropogenic habitat change (Becker et al. 2007, Becker et al. 2010a, da Silva & Rossa-Feres 2011, da Silva et al. 2011, da Silva et al. 2012b, Rossa-Feres et al. 2018) and disease (Becker & Zamudio 2011, Schloegel et al. 2012, Rodriguez et al. 2014, Lambertini et al. 2016, Jenkinson et al. 2016, Becker et al. 2017, Carvalho et al. 2017, Greenspan et al. 2018, Ribeiro et al. 2020). In the Atlantic Forest, the historical shift in natural land cover had a significant impact on anuran biogeography, restricting amphibian groups with specialized life histories to larger remnants of continuous forests (Becker et al. 2010b). Concurrently, deforestation facilitated the expansion of a significant number of Cerrado species into the original domain of the Atlantic Forest (Haddad et al. 2013, da Silva et al. 2014). Atlantic Forest frogs also seem to be disproportionately affected by the emerging fungal disease chytridiomycosis (Carvalho et al. 2017), paradoxically in natural forest habitats (Becker & Zamudio 2011). Thus, species that cannot tolerate deforestation are threatened both by destruction of natural habitats as well as increased fungal infections in forest fragments (Belasen et al. 2022), creating a double-whammy for the most endemic and habitat-specialist amphibian species in Brazil.

Habitat disturbance has been linked to shifts in amphibian community diversity (Cushman 2006), including decline in species richness (Becker et al. 2007, da Silva & Rossa-Feres 2011, da Silva et al. 2011, da Silva et al. 2012b, Rossa-Feres et al. 2018), reduction in population sizes (Becker et al. 2010a), and decline in population genetic and immunogenetic diversity (Dixo et al. 2009, Belasen et al. 2022). For most amphibian species (i.e., aquatic-breeders), not all required habitats are present or connected in landscapes with high levels of deforestation, and these species strongly rely on high levels of spatial connectivity among multiple habitats types (Pope et al. 2000). Although habitat fragmentation does not necessarily lead to a reduction in vertebrate species diversity (Fahrig et al. 2019), reduction of spatial connectivity through forest fragmentation could significantly alter movement patterns in Atlantic forest frogs. This spatial separation among natural aquatic and terrestrial habitats (i.e., habitat split) has been shown to cause detrimental impacts on amphibian populations and communities (Becker et al. 2007).

Habitat split is a widespread phenomenon across the State of São Paulo, especially in areas of complex topography (Viana et al. 1997). In São Paulo, habitat disturbances, including livestock farming, industry, agriculture, and urbanization are often concentrated in lowlands. Forest fragments without perennial water bodies are often left on hilltops (Viana et al. 1997, Silva et al. 2007, Ribeiro et al. 2009). The process of forest fragmentation normally produces a large proportion of small upland forest fragments with limited connectivity with perennial water bodies (e.g., streams, ponds). Most amphibian species are thus forced to migrate between natural terrestrial habitats through highly disturbed environments to complete their life cycle (Becker et al. 2010a). Studies focused on Atlantic Forest frogs highlight that this process is linked to population declines at the forest fragment scale and loss of species diversity among aquatic-breeding amphibians at the landscape scale (Becker et al. 2007, Becker et al. 2010a, da Silva & Rossa-Feres 2011, da Silva et al. 2011, 2012b, Tavares & da Silva 2019). In contrast, terrestrial-breeding species (e.g., direct developers) that are usually more stationary, do not strongly respond to habitat split (Becker et al. 2007), although these species should suffer with habitat loss and fragmentation for other reasons than through breeding migrations. These findings highlight that additional conservation prioritization studies identifying regions of high habitat split that overlap with areas of high amphibian diversity and endemism should be carried out (see Becker et al. 2010b) to offer an opportunity for decision makers to implement targeted habitat restoration programs.

Batrachochytrium dendrobatidis (Bd) is the causative agent of amphibian chytridiomycosis (Voyles et al. 2009). The waterborne pathogen is extremely host generalist among amphibians, with over 700 species known to be infected in the wild (Lips et al. 2006). *Bd* has been linked to amphibian population declines and extinctions globally (Lips et al. 2006, Crawford et al. 2010, Becker & Zamudio 2011, Catenazzi et al. 2011, Lips 2016, Carvalho et al. 2017). The infective life stage of *Bd* is a free-swimming flagellated zoospore and these zoospores encyst in the keratinized skin of amphibians (Longcore et al. 1999, Berger et al. 2005). The fungus develops as a stationary zoosporangium in the host and discharges a generation of zoospores onto the environment, often leading to re-infection of the same individual or transmission to other hosts (Berger et al. 2005). Zoospores are also released into water bodies such as streams and ponds, and thus aquatic environments can serve as environmental reservoirs for *Bd*. Amphibian species with aquatic larval development thus tend to be more exposed to *Bd* than direct-developing species breeding in the forest floor.

Bd is widely distributed throughout Brazil's Atlantic Forest (Lambertini et al. 2021) and dozens of amphibian species declined during the late 1970s coincident with increased Bd prevalence (Heyer et al. 1988, Weygoldt 1989, Carvalho et al. 2017). More specifically, a recent study has linked the observed historical declines across several Atlantic Forest sites with spatiotemporal clusters of Bd in museum-preserved specimens of anurans (Carvalho et al. 2017). Clusters of Bd-positive amphibians overlapped with areas where important declines were observed, such as Reserva Biológica de Boracéia, Reserva Santa Lúcia/Santa Tereza, and Parque Nacional do Itatiaia (Heyer et al. 1988, Weygoldt 1989). The Atlantic Forest now exhibits both epizootic and enzootic Bd host-pathogen dynamics. Some of this complexity is explained by the unusual presence of multiple Bd lineages (Rodriguez et al. 2014, O'Hanlon et al. 2018), some of which may have a long history in the region (>100 years), while others appear to have arrived more recently (Rosenblum et al. 2013, Rodriguez et al. 2014, Jenkinson et al. 2016, Byrne et al. 2019). Hybridization among lineages also occurs (Jenkinson et al. 2016), which could generate Bd genotypes potentially more virulent than the parental lineages (Greenspan et al. 2018). The multiple lineages of Bd currently enzootic in the Atlantic Forest added to the history of Bd hybridization does turn a red alert for future outbreaks of conservation concern. Cleaning field gear between field campaigns and advising the general public to properly and regularly clean hiking gear could reduce the spread of Bd lineages and the likelihood of pathogen hybridization.

Habitat disturbance has clear effects on disease dynamics in Brazilian amphibians (Becker & Zamudio 2011, Liu et al. 2013, Becker et al. 2016, Becker et al. 2017). Although disturbed open-canopy habitats tend to reduce *Bd* growth and persistence by increasing temperatures beyond the pathogen's thermal optimum (Becker & Zamudio 2011), recent data indicate that tropical amphibians sampled within forest fragments have higher *Bd* prevalence than those sampled in continuous forests (Belasen et al. 2022). Reduced host immune capacity is one of several mechanisms likely increasing disease risk in amphibians from fragmented forests (Rollins-Smith et al. 2011).

Deforestation could also have less obvious impacts on disease through altering macro- and microclimate patterns. In the state of São Paulo, for instance, deforestation has been linked to prolonged droughts and higher maximum temperatures (Webb et al. 2005). In turn, shifts in rainfall patterns and temperature variability have both been linked to altered Bd dynamics in amphibians (Pounds et al. 2006, Rohr & Raffel 2010, Cohen et al. 2017, Moura-Campos et al. 2021). Recently, a study linked drought with a Bd-induced die-off in a population of pumpkin toadlets *Brachycephalus rotembergae* in an Atlantic Forest fragment in the State of São Paulo (Moura-Campos et al. 2021). Additionally, predicted increases in average temperatures were linked to higher risk of clinical chytridiomycosis and mortality in high-elevation amphibian species, according to an experimental study including three Atlantic Forest frog species from the state of São Paulo (Neely et al. 2020). This study found that Bd may lead to declines of cool-adapted montane frogs under the combined pressures of pathogen infection and warming, even at temperatures approaching the pathogen's upper thermal limit. Aside from the direct effects of habitat loss and climate change, new findings indicate that agricultural pollution could also exacerbate the risk of disease in Atlantic Forest frogs. In a recent study, agricultural runoff (swine slurry) was linked to higher Bd infection loads in the treefrog *Dendropsophus minutus* through depletion of host skin bacteria with antifungal properties that could suppress Bd growth (Preuss et al. 2020). Finally, ecosystem effects of *Bd*-related amphibian declines include changes in algal community structure, primary production, aquatic insects, and predatory dynamics (Whiles et al. 2006), and diversity declines in high-trophic levels such as snake communities (Zipkin et al. 2020), with likely shifts in energy flow between aquatic and terrestrial environments. These findings, combined, highlight the myriad of threats experienced by Atlantic Forest frogs and the importance of amphibians for ecosystem functioning, underscoring important gaps in our understanding and opportunities for future research.

DNA Barcoding and Sequence Database: A Tool for Taxonomy, Systematics, and Conservation

Amphibians are the most endangered group of vertebrates, with nearly 41% of evaluated species being threatened with extinction (IUCN 2021). Paradoxically, the number of amphibian species described has increased the most in recent years. We are therefore in a race against time to both accurately characterize and conserve amphibian diversity, and our most recent advances in conservation were only possible through the broad implementation of new molecular tools funded by FAPESP.

Molecular tools have become an indispensable part of systematic and conservation-based studies, accelerating the description of cryptic diversity, and also providing important information to studies on endemism, biological invasion, and threatened species (Sheth & Thaker 2017). DNA-based identification has emerged as a powerful tool for accelerating taxonomy by improving the discovery of new species (Hebert et al. 2003), flagging cryptic species, or identifying taxa in need of further systematic study (Vieites et al. 2009, Padial et al. 2010). The DNA barcoding approach is currently the most standard DNA-based identification tool and its functionality lies in sequencing a standardized genetic marker [a fragment of the mitochondrial encoded cytochrome c oxidase 1 (COI) gene for most animals] from many specimens, and assembling a curated database for sequencing comparison (Ratnasingham & Hebert 2007). In amphibians, amplification of the COI barcode region has proven to be difficult (Vences et al. 2005, but see Lyra et al. 2017). A fragment of the 16S rRNA gene is also commonly used as DNA barcode-like fragment, being the most common fragment on amphibians' sequences databases. Besides these two fragments, other mitochondrial sequences, such as the 12S rRNA fragment, has been used as a reference sequence for species identification, especially in cases where DNA is highly degraded (see details below on environmental DNA).

Since 2010 FAPESP has funded a large number of studies that resulted in a compilation of a public DNA barcode database for amphibians from Brazil. We currently have more than 5000 specimens barcoded (for both COI and 16S), representing around 75% of known diversity for the Atlantic Forest (M. L. Lyra unpublished data). This database has been used to support a variety of studies on the diversity, distribution, and evolution of Neotropical amphibians. For example, in our preliminary assessment of species diversity using DNA barcoding approach, we have shown that the total number of species might be underestimated by about 25% (Lyra et al. 2017). We are also using these sequences as an additional line of evidence to describe and revalidate species (e.g., de Sá et al. 2015, Gazoni et al. 2021), to flag groups of species where species diversity could be underestimated (Walker, et al. 2018, Sabbag et al 2018), or even identifying invasive species (Melo et al. 2014). In addition to COI and 16S reference sequences, we have also been adding information on the 12S gene fragment to our databases, including data from environmental DNA studies of monitoring endangered species (e.g., Lopes et al. 2021a, Lopes et al. 2021b, see below), and complete mitogenome sequences from studies of systematics and evolution (e.g., Lyra et al. 2021). Besides supporting this variety of studies, DNA barcoding is also being used as an important tool for identifying mislabeled tissues in biological collections (Lyra et al. 2017), serving as support for the selection of specimens for multi-locus phylogenies and genomic studies.

The Use of Environmental DNA to Monitor Biodiversity and its Applications for Conservation

Our ability to monitor biodiversity is reduced when dealing with elusive species, populations with low abundances, or remote locations. The environmental DNA (eDNA) rose in the last decade as a promising approach to circumvent many of the challenges faced by other methods (e.g., audio-visual encounters and traps) traditionally used for monitoring species difficult to survey (Taberlet et al. 2018). This approach has greatly improved our knowledge about species geographical distribution, population abundance, and conservation status, which is used in conservation management to reduce loss of species, protect their natural habitats, and safeguard ecosystem services (Rodriguez-Ezpeleta et al. 2021).

All organisms leave DNA traces in the environment (e.g., in soil, water, and air). This eDNA can be used to monitor species occurrence in a non-invasive (no handling or tissue sampling) and non-destructive way (Taberlet et al. 2018). Massive eDNA data have been generated in temperate zones. In tropical regions, however, the application of eDNA is still in its infancy (Lopes et al. 2017). Tropical countries are in general less developed economically, which associated with the costs of infrastructure and reagents required for molecular-based research result in constraints for eDNA data generation. Moreover, the biodiversity assessments spatially restricted to easy-to-access areas in the tropics, the high biodiversity, and the unresolved taxonomy of some taxa, in addition to limited availability of DNA sequences in public reference databases preclude scientists from taking full advantage of such approach (Zinger et al. 2020). In this sense, FAPESP has had an essential role in the development and application of eDNA-based research in Brazil, by providing financial support for research projects led by Universities in the State of São Paulo.

At Universidade Estadual Paulista (UNESP), we have been financed by FAPESP since 2014 for developing and applying eDNA metabarcoding approaches for surveying threatened anurans in the Brazilian Atlantic Forest. In our first pilot project, we evaluated the feasibility of eDNA to survey the amphibian fauna of four streams in the Brazilian Atlantic Forest and compared the results with short-term and five-year traditional visual and audio field surveys (Lopes et al. 2017, Sasso et al. 2017). The ~50bp of the 12S mitochondrial gene used for metabarcoding sequencing showed high power of taxonomic discrimination. We observed that filtering larger volumes of water in multiple sites increased our power of detection of species (egg, tadpole, or post-metamorphic) with lower population densities or with life cycles not associated with the streams. Moreover, the eDNA metabarcoding had a greater capacity of detection per sampling event than rapid field surveys, showing the potential of this approach to circumvent some of the challenges associated with traditional methods.

All information provided by our pilot project (Lopes et al. 2017, Sasso et al. 2017) was essential to guide our next studies aiming to search for DNA traces of 43 target amphibian species of conservation concern in Brazil, from species last registered in 1910 to species with historical records of severe population declines (Lopes et al. 2021a, Lopes et al. 2021b, C. M. Lopes unpublished data). We filtered water samples from streams, rivers, puddles, ponds, rocky seeps, and tank bromeliads from 13 localities distributed in the Brazilian Atlantic Forest and adjacent Cerrado grasslands. An extensive 12S mitochondrial sequence reference database was constructed to analyze the eDNA data, filling an important gap of sequences from species and geographical areas underrepresented in public databases. We successfully detected DNA traces of four declined anuran species (*Crossodactylus timbuhy*, *Hylodes ornatus*, *Hylodes regius*, and *Vitreorana eurygnatha*); two locally disappeared (*Phasmahyla exilis* and *Phasmahyla guttata*); and one species that has not been seen since 1968 (putatively assigned to *Phantasmarana bocainensis*) (Lopes et al. 2021b) (Figure 3). Our results recovered so far highlight the value of applying eDNA in conservation biology, to evaluate persistence and distribution of threatened species in their natural environment, and to improve accuracy of red lists.

We also assessed the performance of eDNA metabarcoding from leaf litter samples comparing two extraction methods, for monitoring eukaryotes, with a focus on vertebrate species, especially anurans. We recovered that total and extracellular extractions were ecologically congruent for estimating overall beta diversity and showed good potential for monitoring the biodiversity of eukaryotes, especially for active and dominant taxa in terrestrial communities, such as Fungi and invertebrates. However, our results do not support the eDNA from leaf litter as a reliable tool for monitoring vertebrate species in general (Lopes et al. 2021c).

Integrating Biological Collections and Next-Generation Sequencing: Historical DNA in Studies of Diversity, Conservation, and Evolution of Amphibians

Over the last two centuries, biological collections have provided an essential record of the planet's biodiversity, housing specimens that document dynamic changes in biodiversity (Holmes et al. 2016). In particular, the specimens from biological collections are an important source for genetic data, especially those specimens collected before the molecular era that can be used to answer many questions in taxonomy, ecology, and evolution. This is especially crucial for amphibians because the global decline in amphibian populations has resulted in the disappearance of hundreds of species since the 1980s (Stuart et al. 2004), with some species no longer found in nature, being only represented in scientific collections (ICMbio/MMA 2018).

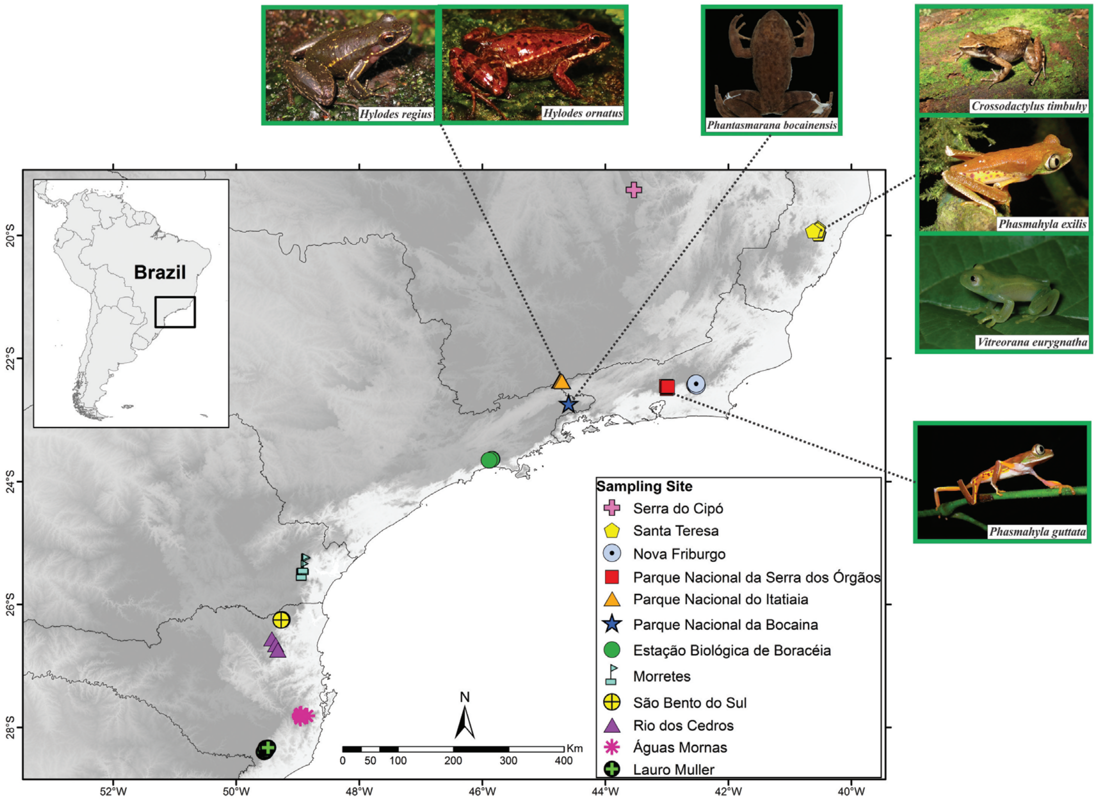

Figure 3. Localities surveyed for eDNA traces of 43 target amphibian species of conservation concern in Brazil. Photos of seven amphibian species detected through eDNA are shown.

Although DNA sequences are an extremely powerful source of evidence for biodiversity studies, the use of museum-preserved specimens in such studies has been limited because traditional methods of preserving biological specimens often result in DNA degradation, including fragmentation, base damage, and low DNA yield (Gilbert et al. 2007). With few exceptions, all type material and the millions of specimens deposited in the natural history collections around the world over the last three centuries have been historically excluded from the molecular revolution that continues to overhaul our understanding of the tree of life. These issues have been a source of frustration for amphibian systematists, creating significantly taxonomic impediments, given that even a short sequence of mitochondrial DNA from type specimens would be sufficient to solve countless phylogenetic and taxonomic problems.

Fortunately, advances in extraction protocols and next-generation sequencing methods in recent years have enabled techniques to reliably assemble mitochondrial and nuclear DNA sequences from small amounts of degraded DNA (historical DNA; hDNA) from type specimens and old or rare species from remote localities (e.g., Hykin et al. 2015, Lyra et al. 2020, Rancilhac et al. 2020, Straube et al. 2021). These molecular advances opened new perspectives for studies on evolution, ecology, taxonomy, phylogeography, and conservation.

Recently, FAPESP started funding projects, laboratory structure, and academic training focusing on the modernization, accessibility, and growth of museum collections. Within this initiative, Straube et al. (2021) recently presented a detailed protocol, including the laboratory steps and bioinformatics analysis to successfully obtain DNA from wet collection specimens. The use of this historical DNA data has already proven to be very informative to resolve outstanding questions on the systematics of some anuran groups. For example, Lyra et al. (2020), by combining morphology and historical DNA, have provided new insights into the taxonomy and phylogenetic relationships of the treefrog *Bokermannohyla claresignata* group, placing this group sister to the *Boana pulchella* group, supporting its inclusion in the genus *Boana*. These new approaches are opening the DNA archive of museum wet collections to studies on taxonomy, ecology, and evolution (e.g., Turvey et al. 2019), and are enhancing the relevance of natural history collections in the studies of tropical biodiversity (Yeates et al. 2016).

Future Perspectives

Although our understanding of Brazilian amphibians has increased over the years, there are still important knowledge gaps to be filled. The number of newly described species will continue to grow, but the predicted negative impact of environmental degradation on Brazilian amphibians, such as agricultural expansion, agrochemical use, habitat loss, and climate change, will likely intensify within the next decade. The synergistic effects of global change stressors and emerging amphibian pathogens add more complexity to the growing number of disease ecology studies. Future studies will continue to focus on basic questions, such as description of new species, as well as taxonomic and systematic corrections. However, we also expect that there will be a strong integration among different disciplines using novel bioinformatics tools and modeling approaches, such as machine learning. These new approaches will be critical to further develop our understanding of foundational questions of life-history trait variation, disease transmission, community assembly, biogeography, and population forecasts under different global change scenarios. These advances will help train the next generation of Brazilian scientists and promote a more efficient communication among scientists, the general population, and decision makers.

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Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Ethics

This study did not involve human beings and/or clinical trials that should be approved by one Institutional Committee.

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