

Lost and found: Frogs in a biodiversity hotspot rediscovered with environmental DNA

Carla Martins Lopes¹  | Délio Baêta¹  | Alice Valentini²  | Mariana Lúcio Lyra¹  |
Ariadne Fares Sabbag¹  | João Luiz Gasparini³ | Tony Dejean² |
Célio Fernando Basptista Haddad¹ | Kelly Raquel Zamudio⁴ 

¹Departamento de Biodiversidade e Centro de Aquicultura, I.B., Universidade Estadual Paulista (UNESP), Rio Claro, SP, Brazil

²SPYGEN, Le Bourget-du-Lac, France

³Núcleo em Ecologia e Desenvolvimento Socioambiental de Macaé, Universidade Federal do Rio de Janeiro (UFRJ), Macaé, RJ, Brazil

⁴Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA

*Correspondence

Carla Martins Lopes, Departamento de Biodiversidade e Centro de Aquicultura, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Av. 24A, n. 1515. Rio Claro, SP, Brazil. Email: cmlopes82@hotmail.com

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Abstract

Declines and extinctions are increasing globally and challenge conservationists to keep pace with biodiversity monitoring. Organisms leave DNA traces in the environment, e.g., in soil, water, and air. These DNA traces are referred to as environmental DNA (eDNA). The analysis of eDNA is a highly sensitive method with the potential to rapidly assess local diversity and the status of threatened species. We searched for DNA traces of 30 target amphibian species of conservation concern, at different levels of threat, using an environmental DNA metabarcoding approach, together with an extensive sequence reference database to analyse water samples from six montane sites in the Atlantic Coastal Forest and adjacent Cerrado grasslands of Brazil. We successfully detected DNA traces of four declined species (*Hylodes ornatus*, *Hylodes regius*, *Crossodactylus timbuhy*, and *Vitreorana eurygnatha*); two locally disappeared (*Phasmahyla exilis* and *Phasmahyla guttata*); and one species that has not been seen since 1968 (putatively assigned to *Megaelosia bocainensis*). We confirm the presence of species undetected by traditional methods, underscoring the efficacy of eDNA metabarcoding for biodiversity monitoring at low population densities, especially in megadiverse tropical sites. Our results support the potential application of eDNA in conservation biology, to evaluate persistence and distribution of threatened species in surveyed habitats or sites, and improve accuracy of red lists, especially for species undetected over long periods.

KEYWORDS

amphibians, archival DNA, biomonitoring, endangered species, metabarcoding, population declining

1 | INTRODUCTION

A hallmark of the Anthropocene is global increases in population losses and species' extinctions. Threats to biodiversity will grow with continued human impacts on land use, climate change, and the emergence of wildlife infectious diseases (Johnson et al., 2017).

Amphibians are a particularly vulnerable group with many species already showing population declines, shrinking distributions, and presumed extinctions (Becker, Fonseca, Haddad, Batista, & Prado, 2007; Wake & Vredenburg, 2008). A challenge in assessing the conservation status of these often elusive species is the small probability of detecting individuals at low population densities.

Accurately detecting species in the environment would greatly facilitate conservation efforts.

In the 1970s, researchers reported dramatic declines and disappearances of montane stream-breeding amphibians from the Brazilian Atlantic forest. These declines were mainly documented in protected areas, where amphibian communities were well studied (Eterovick et al., 2005; Heyer, Rand, Cruz, & Peixoto, 1988; Weygoldt, 1989) (Table 1, Figure 1). The causes for these disappearances are still debated, but recent studies show that declines are most probably due to anthropogenic climate change, habitat loss, and infectious diseases (Becker et al., 2007; Carvalho, Becker, & Toledo, 2017; Eterovick et al., 2005). Most of these declined or disappeared species are listed as Least Concern or Data Deficient in IUCN and Brazilian red lists (Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio, 2016; The International Union for Conservation of Nature - IUCN, 2020; Figure 2), mainly because of lack of knowledge about their geographical distribution and populations status, hindering conservation actions. Applying various field monitoring techniques, including highly sensitive survey methods could determine whether these observations reflect true declines or disappearances, as opposed to natural population fluctuations.

Searching for DNA traces left by organisms in the environment (eDNA) can circumvent some of the challenges faced by traditional methods for surveying elusive species at low population densities (Lopes et al., 2017; Taberlet, Bonin, Zinger, & Coissac, 2018). However, to reach the full potential of eDNA for surveying threatened wildlife, adjustments of protocols depending on the taxa and ecosystem to be surveyed, and high standard laboratory and bioinformatic procedures should be systematically applied to avoid false detections or minimize the chances of missing taxa altogether (Ficetola, Taberlet, & Coissac, 2016; Goldberg et al., 2016). The completeness of the sequence reference database is fundamental for accurately identify rare species in eDNA studies, especially for organisms and geographical areas underrepresented in public databases (Zinger et al., 2020). However, one common challenge when searching for target species that have gone missing is that often good quality tissue samples are not available for accessing DNA sequences. DNA extracted from formalin preserved specimens (archival DNA) or sister species used as proxy taxa are alternatives to circumvent this problem. Advancing the development of eDNA methods in this way will increase our knowledge about the persistence and distribution of threatened species, help define conservation priorities, and improve accuracy of our red lists, especially for poorly known species and those undetected over long periods.

In this study we applied eDNA metabarcoding to survey five sites in the Brazilian Atlantic forest, a global hotspot for biodiversity (Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000), and one site in adjacent Cerrado grasslands (Figure 1). We filtered 62 water samples (Table S1) and constructed an extensive local sequence reference database to search for DNA traces of 30 target amphibian species that were considered “declining” (population densities were noticeably lower than historical levels), “locally disappeared” (not been seen or collected at our sites in the last 10 years

or more, but persist in other parts of their range), or completely “disappeared” (not seen for 10 years or more throughout their range; Table 1). These three categories increase in threat severity, and species that are completely disappeared from throughout their range for decades, are often presumed extinct.

2 | MATERIALS AND METHODS

2.1 | The target amphibian species

We chose 30 target amphibian species of conservation concern (Table 1) by consulting reports of declines in the literature, amphibian collections in Brazil (Museu Nacional - Universidade Federal do Rio de Janeiro - MNRJ, Rio de Janeiro, Rio de Janeiro; Museu de Zoologia da Universidade Estadual de Campinas, “Adão José Cardoso” - ZUEC-AMP, Campinas, São Paulo; Célio F. B. Haddad collection - CFBH, Universidade Estadual Paulista, Rio Claro, São Paulo; and Museu de Biologia Professor Mello Leitão - MBML, Santa Teresa, Espírito Santo), and the Species Link database (<http://www.splink.org.br/>). We classified target species at each locality as: “declining” ($N = 5$); “locally disappeared” ($N = 13$), or “disappeared” ($N = 14$) (Table 1).

2.2 | eDNA sampling and laboratory procedures

Environmental DNA sampling was performed from December 2015 to March 2016, which is the wet season in southeastern Brazil, and the peak of the breeding season for most amphibians in the region. We surveyed six sites, five within the Brazilian Atlantic forest (Estação Biológica de Boracéia, Parque Nacional de Itatiaia, Parque Nacional da Serra da Bocaina, Parque Nacional da Serra dos Órgãos, and mountains of Santa Teresa), and one in *campo rupestre*, a high elevation grassland in the adjacent Cerrado (Serra do Cipó) (Figure 1). We filtered 3–16 water samples from each of our six sites (Table S1), depending on the number of target species and their potential area of occurrence. At least one eDNA sample per site was collected in exact spots where declining, locally disappeared, or disappeared species were registered in the past. Other samples were collected in similar habitats in the vicinity of known historical localities. Water filtering was carried out using a Solinst 410 peristaltic pump (Solinst Canada Ltd., Georgetown, Ontario, Canada) and VigiDNA 0.45 μ M filtration capsules (SPYGEN, Le Bourget-du-Lac, France). Water was filtered directly from the source, applying a flow rate of 1.5 L/min, using new polyethylene tubes and gloves for each sample to avoid cross contamination. We filtered 2–30 L of water per sample (Table S1), from bromeliads, puddles, ponds, streams, or rivers depending on the habitat of target species. After sampling, capsules were filled with 80 ml of lysis buffer (Tris-HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1%, pH 7.5–8), mixed for 50 s, and stored at room temperature until DNA extraction. Given the sterile capsule filters, single-use materials, and previous evidence

TABLE 1 List of the 30 target amphibian species of conservation concern from six sites surveyed in Brazil using environmental DNA

Sites sampled	Species	Last registered	Range	Classification	Reference database	eDNA detection
Estação Biológica de Boracéia	<i>Crossodactylus dispar</i>	1977 (Pimenta et al., 2014)	NE	LD	3	N
	<i>Cycloramphus boraceiensis</i>	until 1979 (Heyer et al., 1990)	NE	LD	1, 2, 3	N
	<i>Cycloramphus semipalmatus</i>	until 1979 (Heyer et al., 1990)	NE	LD	2, 3	N
	<i>Hylodes asper</i>	until 1979 (Heyer et al., 1990)	NE	LD	1, 2, 3	N
	<i>Phrynomedusa vanzolinii</i>	1973 (Cruz, 1991)	NE	Dis	3	N
	<i>Thoropa taophora</i>	until 1979 (Heyer et al., 1990)	NE	LD	1, 2, 3	N
	<i>Vitreorana eurygnatha</i>	until 1979 (Heyer et al., 1990)	NE	LD	2, 3	N
Parque Nacional de Itatiaia	<i>Crossodactylus grandis</i>	1977 (NMNH)	NE	Dis	3	N
	<i>Crossodactylus werneri</i>	1978 (Pimenta et al., 2014)	NE	Dis	3	N
	<i>Holoaden bradei</i>	1978 (ZUEC-AMP)	ES	Dis	1,3	N
	<i>Hylodes glaber</i>	1978 (ZUEC-AMP)	ES	Dis	3	N
	<i>Hylodes ornatus</i>	2012 (de Sá, Canedo, Lyra, & Haddad, 2015)	ES	Dec	1, 3	Y
	<i>Hylodes regius</i>	2016 (CFBH)	ES	Dec	1, 3	Y
	<i>Paratelmatobius lutzii</i>	1978 (Pombal & Haddad, 1999)	ES	Dis	1, 3	N
	<i>Scinax obtriangulatus</i>	2009 (CFBH)	NE	LD	1, 2, 3	N
Parque Nacional da Serra da Bocaina	<i>Boana clepsydra</i>	1980 (Lyra et al., 2020)	ES	Dis	3	N
	<i>Megaelosia bocainensis</i>	1968 (Giaretta et al., 1993)	ES	Dis	3	Y
Santa Teresa	<i>Allobates capixaba</i>	1983 (MNRJ)	NE	LD	3	N
	<i>Crossodactylus gaudichaudii</i>	until 1981(Weygoldt, 1989)	NE	LD	2, 3	N
	<i>Crossodactylus timbuhy</i>	2018 (MBML)	NE	Dec	1, 3	Y
	<i>Cycloramphus fuliginosus</i>	until 1981(Weygoldt, 1989)	NE	LD	2, 3	N
	<i>Hylodes babax</i>	until 1981 (Weygoldt, 1989)	NE	LD	3	N
	<i>Phasmahyla exilis</i>	2009 (MNRJ)	ES	LD	2, 3	Y
	<i>Phrynomedusa marginata</i>	1988 (MNRJ)	NE	Dis	1, 2, 3	N
	<i>Vitreorana eurygnatha</i>	2017 (MBML)	NE	Dec	1, 2, 3	Y
Parque Nacional da Serra dos Órgãos	<i>Aplastodiscus musicus</i>	2016 (Bezerra, Passos, de Luna-Dias, Quintanilha, & de Carvalho-e-Silva, 2020)	ES	Dec	3	N
	<i>Boana claresignata</i>	1964 (Lyra et al., 2020)	NE	Dis	3	N
	<i>Cycloramphus ohausi</i>	1977 (NMNH)	ES	Dis	1, 3	N
	<i>Phasmahyla guttata</i>	1977 (NMNH)	NE	LD	2, 3	Y
	<i>Phrynomedusa vanzolinii</i>	1929 (Cruz, 1991)	NE	Dis	3	N
	<i>Thoropa petropolitana</i>	1982 (MNRJ)	ES	Dis	1, 3	N
Serra do Cipó	<i>Scinax pinima</i>	1987 (CFBH)	ES	Dis	1, 3	N

No<del author="Carla Martins Lopes" command="Delete" timestamp="1598369795372" title="Deleted by Carla Martins Lopes on 25/08/2020 12:36:35" class="reU3">te: For each target species we provide the last time species were registered at the localities we surveyed, their range of geographical distribution (NE, not endemic to the site surveyed; ES, endemic to the site surveyed), how species were classified in this study (Dec, declined; LD, locally disappeared; Dis, disappeared), the reference sequences used in our database (1, sequence of the species from the site of interest; 2, sequence of the species from another site; 3, sequence of congener/sister species), and if the species were detected (Y) or not (N) in eDNA samples. NMNH, Smithsonian's National Museum of Natural History; ZUEC-AMP, Museu de Zoologia da Universidade Estadual de Campinas, "Adão José Cardoso"; MNRJ, Museu Nacional – Universidade Federal do Rio de Janeiro; CFBH, Célio Fernando Baptista Haddad collection – Universidade Estadual Paulista "Júlio de Mesquita Filho".

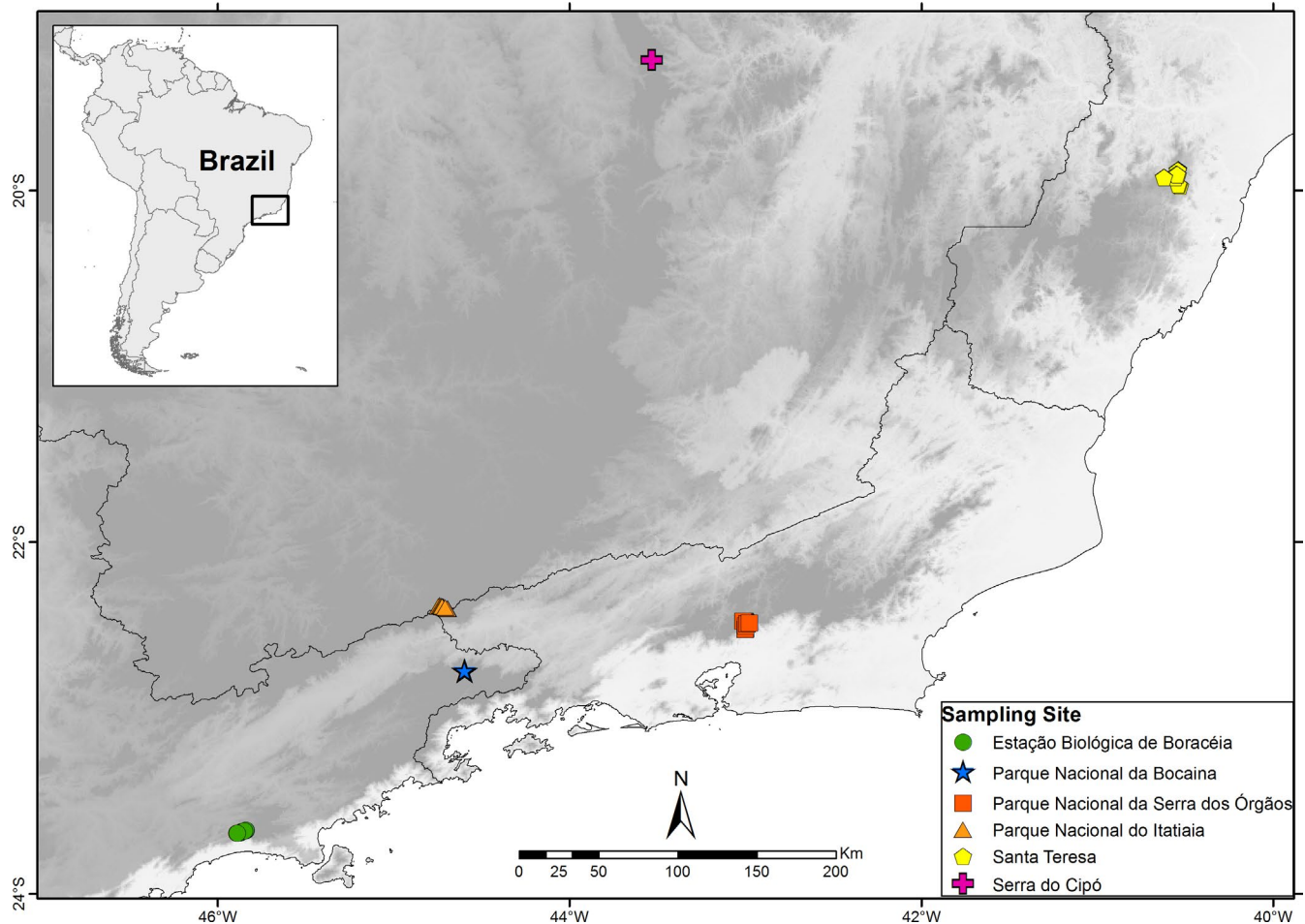


FIGURE 1 Localities surveyed for DNA traces of 30 target amphibian species of conservation concern included in this study

of no cross-contamination between samples or equipment using the same methodology (Lopes et al., 2017), we did not perform a sampling negative control, rather focused on laboratory negative controls for the more sensitive amplification-based steps.

All DNA extractions, amplifications, and purifications were performed in clean physically isolated rooms at SPYGEN (Le Bourget-du-Lac, France) equipped with positive air pressure, UV treatment, laminar flow hoods, frequent air renewal, and where personnel wear full protective clothing. For total DNA extraction we followed the protocols described in Pont et al. (2018). Three negative extraction controls were carried out in parallel to eDNA sample extractions, to monitor for possible contaminations. PCR amplifications and purification were carried out following conditions described in Lopes et al. (2017). A short fragment of the 12S rRNA mitochondrial gene was amplified from eDNA extracts, using the primers *batra_F* (5'-ACACCGCCCGTCACCCT-3') and *batra_R* (5'-GTAYACTTACCATGTTACGACTT-3') (Valentini et al., 2016). Both forward and reverse primers were 5' labelled with a unique eight-nucleotide tag for each PCR replicate, to allow sequencing assignment to appropriate samples during the sequence filtering process. The blocking primer *batra_blk* (5'-TCACCCTCTCAAGTATACTTCAAAGGCA-SPC3-3') (Valentini et al., 2016), was added to the PCR reactions to

reduce human DNA amplification. Twelve PCR replicates were run for each eDNA sample. One PCR positive and two negative controls, with 12 PCR replicates as well, were analysed in parallel with eDNA samples, to monitor for possible contamination. The positive controls were composed of DNA extracts in different known concentrations of the species *Alytes obstetricans*, *Bombina variegata*, *Bufo bufo*, *Bufo viridis*, *Calotriton asper*, *Chioglossa lusitanica* and *Epidalea calamita*, none of which are species that occur in Brazil. Paired-end sequencing was performed in five Illumina HiSeq 2500 runs (Illumina, San Diego, CA, USA), following the manufacturer's instructions at Fasteris (Geneva, Switzerland). All samples from the same site were sequenced in the same run.

2.3 | Reference database

Based on literature reports for species diversity and known species ranges, we defined a list of 161 amphibian species that could be detected in our aquatic eDNA samples and known to occur either presently or historically at each sampling site (Almeida, Gasparini, & Peloso, 2011; Folly et al., 2016; Garey, Provete, Martins, Haddad, & Rossa-Feres, 2014; Heyer, Rand, Cruz, Peixoto, & Nelson, 1990;

FIGURE 2 Thirty target amphibian species for the environmental DNA study and their conservation status. Columns indicate (i) our assigned threat level for each species; (ii) conservation status of species as listed in the IUCN Red List of Endangered Species; (iii) conservation status as listed in the Brazilian Red List; and (iv) species positively detected or not in our eDNA surveys. Photos of the seven species found using eDNA are shown



Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio, 2008, 2014; Pimenta, Cruz, & Caramaschi, 2014) (Data set S1). We constructed a reference database of the 12S rRNA mitochondrial sequences as complete as possible, to assure appropriate taxonomic assignment to sequences recovered in environmental samples. We attempted to sequence at least one individual from each of the 161 species, with priority for individuals sampled from the exact sites we surveyed eDNA. In cases when tissues from the surveyed sites were not available, we obtained tissues from the same species from nearby localities. In the few cases where tissues for target species were not available at all, we included sister species or closely related congeners as proxy taxa (Table S2).

We generated 12S rRNA sequences for 372 individuals of 138 species for the local reference database. Tissues were obtained from the Célio F. B. Haddad amphibian tissue collection (CFBHT), at Universidade Estadual Paulista, Rio Claro, Brazil; Museu Nacional - Universidade Federal do Rio de Janeiro (MNRJ), Rio de Janeiro, Brazil; and Coleção de Tecidos de Vertebrados do Departamento de Zoologia (MTR), Universidade de São Paulo, São Paulo, Brazil.

Genomic DNA was extracted from 10 mg of muscle tissue using a standard high-salt protocol (Lyra, Haddad, & de Azeredo-Espin, 2017).

We amplified the 12s rRNA mitochondrial fragment using the primers MVZ59 (5'-ATAGCACGTAAAAYGCTDAGATG-3') (Graybeal, 1997); tVal (5'-TGTAAGCGARAGGCTTTKGTAAAGCT-3') (Wiens, Fetzner, Parkinson, & Reeder, 2005); 12SA-L (5'-AAACTGGGATTAGAT ACCCACTAT-3') (Palumbi, Martin, McMillan, Stice, & Grabowski, 1991); 12SF-H (5'-CTTGGCTCGTAGTTCCTGGCG-3') (Goebel, Donnelly, & Atz, 1999); 12S-H978 (5'-CTTACRTGTTACGACT TRCCT-3') and 12SL148 (5'-ATGCAAGYMTCMGCRYCCNGTGA-3') (Walker, Lyra, & Haddad, 2018), following previously published amplification conditions (Faivovich et al., 2004; Walker et al., 2018). PCR product purification, sequencing and inspection of sequences were carried out following the protocols and methods described in Lopes et al. (2017).

One challenge was to obtain sequences for species that are not registered for more than 10 years, as tissue samples are often not available. For two target species (*Paratelmatobius lutzii* and *Thoropa petropolitana*) and the congener of *P. lutzii* (*Paratelmatobius mantiqueira*), known only from formalin preserved specimens, we obtained tissue samples from specimens housed at the amphibian collection of Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), Rio de Janeiro, Brazil and Museu de Zoologia da Universidade de

São Paulo (MZUSP), São Paulo, Brazil. For tissue samples preserved in formalin, all stages of DNA extraction and library preparation, prior to PCR amplification, were carried out in dedicated ancient and historical DNA facilities at the University of Potsdam, Germany (Fulton, 2012). Negative controls were included during DNA extraction and library preparation and screened for evidence of contamination. DNA was extracted following Dabney et al. (2013), and then converted into Illumina sequencing libraries using a protocol based on single-stranded DNA (Gansauge & Meyer, 2013). Owing to low abundance of endogenous DNA fragments in the sequencing libraries, we performed two-rounds of in-solution hybridization capture to enrich for mitochondrial DNA fragments, using DNA baits generated from long-range PCR products of *Thoropa taophora* and *Paratelmatobius cardosoi*. We sequenced the libraries on an Illumina NextSeq 500 sequencing platform, generating 75 bp paired-end reads. Quality filtering and assembly of the mitochondrial sequences of target species followed previously published protocols (Lyra et al., 2020). We assembled the 12S fragment for each species through iterative mapping using MITOBIM 1.9 (Hahn, Bachmann, & Chevreux, 2013) and used as seed 12S sequences of *T. taophora* and *P. cardosoi*. The output ".caf" files were imported in GENEIOUS R11 (<https://www.geneious.com>) to extract consensus sequences using sequences with coverage >10 only. We aligned the consensus 12S sequences with sequences available in GenBank for the specific taxonomic groups using MAFFT v.7 (Katoh & Standley, 2013) and checked the phylogenetic placement of each taxon computing neighbour joining trees using the Jukes Cantor model in GENEIOUS. We successfully retrieved the target 12S sequence for the three species.

We supplemented our local amphibian sequence reference database with sequences recovered from EMBL database, release vrt135. We downloaded from EMBL all vertebrate sequences. We extracted from our local reference database and from the EMBL database the relevant fragment of 12S sequences for metabarcoding analyses, using the programs ECOPCR 0.5.0 (Ficetola et al., 2010) and OBITOOLS 1.1.22 (Boyer et al., 2016). The final 12S metabarcoding reference database is as complete as possible, given tissues available and EMBL accessions. Of the 161 species that could potentially be detected in our aquatic eDNA samples, 144 (89.44%) have at least one sequence available in our final 12S metabarcoding reference database, either from the exact localities we surveyed and/or from other geographical locations (Data set S1). For the remaining 17 species (10.56%) we have at least one sequence available for a close congener or sister species. For our 30 target taxa, two species were known from two distinct survey sites each (*Phrynomedusa vanzolinii*, and *Vitreorana eurygnatha*), totaling 32 occurrences of 30 target species. In total, the final reference database included reference sequences for 14 target species collected at the surveyed locality, two of which were from formalin preserved individuals, six target species collected out of the survey sites, and 11 target species (12 occurrences) for which tissues were not available, we included sister species and/or close congeners (Table 1). We used the final 12S metabarcoding reference database to attribute the taxonomy to sequences retrieved in eDNA samples.

2.4 | Data processing and bioinformatic analyses

Filtering and taxonomic assignment of eDNA sequences was performed using the programs OBITOOLS, ECOPCR and R 3.3.3 (R Development Core Team, 2017), following the steps described in De Barba et al. (2014) and Lopes et al. (2017). Sequences for each sampling site were analysed independently. Briefly, we first assembled paired-end reads to construct consensus sequences, and assigned them to appropriate PCR replicates based on their molecular tags (with no mismatches allowed in the tag and two mismatches allowed for each primer). We retained the information of unique sequence read counts for each PCR replicate. We then filtered the reads and kept for analyses only sequences longer than 20 bp and with total read counts among all PCR replicates ≥ 10 . We examined the profile of sequences in the positive controls and determined a threshold for low read count sequences that were potential contaminants (not species originally included in the positive control PCR). Based on that, we kept only sequences with a frequency per PCR replicate ≥ 0.001 , both for positive controls and eDNA samples. Next, each sequence in the eDNA samples was labelled as "head" (the most common sequence within a group of sequences differing by a single indel/substitution), "internal" (sequences less frequent within the group of related sequences), or "singleton" (sequence with no other variants differing by a single indel/substitution) by PCR replicate (Shehzad et al., 2012). Sequences identified as "internal", which correspond most likely to PCR substitutions and indel errors (De Barba et al., 2014), were excluded from downstream analyses. The final 12S metabarcoding reference database was used to assign taxonomy to sequences recovered from eDNA samples. Only sequences with best identity $\geq 96\%$ with a sequence from the reference database and classified as Anura were considered for further analyses. To eliminate low-quantity PCR products more likely to produce unreliable results we retained in the data only PCR replicates ≥ 200 sequence reads count.

The eDNA sequences were identified to the finest taxonomic level possible, which was either tribe, genus, or species. Sequences identified to the genus or tribe level were double checked manually to ensure they were not a potential positive detection of one of our target species that did not get identified to species level. We calculated the proportion of sequence reads obtained for each taxon recovered in each eDNA sample based on the sum of read counts among PCR replicates, using the R software.

Recent taxonomic updates that are not yet reflected in EMBL databases have the potential to bias eDNA sequence assignments. To avoid conflicts with current taxonomy information associated to the sequences we downloaded from EMBL, we retained older taxonomy for all species currently classified in the genus *Julianus* and *Ololygon*, because they are all included as *Scinax* in current databases (Duellman, Marion, & Hedges, 2016). The sequences classified as *Hypsiboas* in EMBL were updated in our database as *Boana* to follow the current taxonomy (Frost, 2020).

To verify if our power of detection for the 30 target species varied depending on the thresholds applied during the sequence filtering

pipeline, we performed a sensitivity analysis. We reanalysed the data by combining distinct thresholds for the two parameters that could alter species assignment: sequence read counts (sequences ≥ 10 , and ≥ 100), and the level of best identity of eDNA sequences with sequences from the reference database ($\geq 92\%$, $\geq 94\%$, $\geq 96\%$, $\geq 98\%$, and $\geq 100\%$), and verified which sequences corresponding to the 30 target species were retained across these parameter combinations. All other filtering parameters are based on comparisons with our positive and negative controls, or have been validated in previous studies (De Barba et al., 2014; Lopes et al., 2017; Taberlet et al., 2018; Valentini et al., 2016) and thus, were held constant in our sensitivity analyses.

3 | RESULTS

We obtained a total of 51,569,031 consensus sequence reads across eDNA samples and controls, which were filtered for sequence quality and read frequency, compared to positive and negative controls, and assigned taxonomically (Table S3). The highest proportion of sequences filtered out from our data set were those with low read counts (< 10) among all PCR replicates. The filtering steps based on the threshold of best identity $\geq 96\%$ and sequences with a frequency per PCR replicate ≥ 0.001 also eliminated high proportions of sequences from our data. The steps that filtered out fewer sequences were those based on sequence length and cross-contaminations sources (Table S3). A list of taxa not identified as Anura and excluded from the data analyses is provided in Table S4. The final 12S metabarcoding reference database was composed of 4,731 unique amphibian sequences, for which 4,727 were identified to family, 4,689 were identified to genus, and 4,296 were identified to species level. Sensitivity analysis of best identity and number of read counts in our pipeline shows that the number of target species detected is robust to a broad range of parameter values (Table S5), and only with the most conservative parameter combination (100% best identity) do our results change for only one target species (*Phasmahyla guttata*). After the sequence filtering process, no sequence remained in extraction and PCR negative controls. All relevant sequences corresponding to the seven species mixed in PCR positive controls were recovered in the final data set.

Overall, 152 unique amphibian eDNA sequences were identified to the lowest taxonomic level possible, using a 96% similarity threshold with reference database sequences. These eDNA sequences belong to 70 amphibian taxa, of which one was identified to tribe, eight to genus, and the remaining 61 to species. In seven cases, eDNA sequences matched a reference sequence of one of our 30 target species or their closest relatives. These seven cases were considered positive detections of declining, locally disappeared, or disappeared target species (Figure 2).

At Estação Biológica de Boracéia, we detected 42 unique amphibian eDNA sequences, assigned to 22 taxa, none of which corresponded to our target species (Tables S3 and S6). At Parque

Nacional de Itatiaia, we recovered 21 eDNA sequences, assigned to 13 taxa, two of which were species with population declines (*Hylodes ornatus* and *Hylodes regius*) (Tables S3 and S7). At Parque Nacional da Serra da Bocaina, we recovered 19 eDNA sequences, assigned to 15 taxa, one matched the reference sequence of *Megaelosia jordanensis* a close relative of the disappeared *Megaelosia bocainensis* (Tables S3 and S8). At Santa Teresa, we recovered 38 eDNA sequences, assigned to 22 taxa, two matched the declined species *Crossodactylus timbuihy*, one matched the declined *Vitreorana eurygnatha*, and one matched the locally disappeared *Phasmahyla exilis* (Tables S3 and S9). At Parque Nacional da Serra dos Órgãos we detected 35 eDNA sequences, assigned to 24 taxa, one matched the locally disappeared *Phasmahyla guttata* (Tables S3 and S10). Finally, at Serra do Cipó, we detected 12 eDNA sequences, assigned to eight taxa, none of which matched our target species (Tables S3 and S11).

4 | DISCUSSION

Our results confirm that eDNA metabarcoding significantly enhances biodiversity surveys and overcomes many challenges of traditional monitoring methods (Taberlet et al., 2018). With high quality control and careful evaluation of positive detections, eDNA surveys have high potential for detecting species at low population densities (Boussarie et al., 2018). We successfully detected at least one species in each threat category, from recent population declines to those that disappeared decades ago. The target species most frequently found in our eDNA samples were those with declines, followed by locally disappeared, and then disappeared species, a pattern expected if detection is correlated with threat level and abundance in the environment.

Megaelosia bocainensis, known only from its type locality and not seen since 1968, probably persists in its natural habitat. Seven species of *Megaelosia* are described in literature, although we do not have reference sequences for *M. bocainensis*, we included proxy sequences of five described and two undescribed congeners (Table S2) and one eDNA sequence matched *M. jordanensis*. The species of *Megaelosia* have relatively small distributions, none of them occurring in syntopy, and potential sympatry between species is known only in the case of *M. bocainensis* (our target species) and *M. goeldii*, both of which occur in Serra da Bocaina (Giaretta, Bokermann, & Haddad, 1993). We included *M. goeldii* in our reference database and it was not a match with the *Megaelosia* sequence detected in the eDNA samples, therefore we have not erroneously detected a sympatric *Megaelosia* species. Confirming whether the eDNA sample is in fact from *Megaelosia bocainensis* will require sequencing the single known museum specimen or capture of a living individual. However, given all lines of evidence, we can say with certainty that a *Megaelosia* species inhabits the stream we sampled and with high probability it is the disappeared species that was described from that type locality.

As any other survey method, high standard quality control of eDNA data is important to avoid erroneous detection of absent

species (false-positives) or undetected species truly present at the site (false-negatives). Complete reference databases are crucial to avoid misdetections, especially in highly biodiverse environments (Zinger et al., 2020) where congeneric species can occur in sympatry and potentially share the same metabarcoding sequence. Our inferences of detection of target species, especially those based on proxy taxa, were interpreted with caution to avoid false positives (see Supporting Information comments on taxonomic assignments). We considered the power of the metabarcode fragment used to discriminate between species, the species complex analysed, presence of congeneric species at the site surveyed, the consistency of positive PCR replicates, and what is known about species' microhabitat distribution, before claiming rediscovery of any target species. We also considered that the DNA traces of target species detected at surveyed sites correspond to current occurrence of species in the environment, as DNA degrade in few weeks in freshwater (Dejean et al., 2011), and the probability of old DNA traces remaining preserved in sediments and resuspended and detectable after many years is extremely low.

Environmental DNA can result in imperfect detection (Cilleros et al., 2019; Tucker et al., 2016) and thus, is not a tool for confirming extinction. Besides methodological biases, such as primer bias that could result in false negatives (Taberlet et al., 2018), the disappeared species we did not rediscover may persist in nearby unsampled habitats. To maximize our power of detection, we sampled during the rainy season, when our target taxa would be reproducing. Nonetheless, sampling at other times of the year could potentially detect species with seasonal variation in habitat use. In addition, the spatial signal of eDNA varies among habitats. In streams, eDNA can be detected from a few meters to tens of kilometers from the source, depending on flow rate and water turbulence (Taberlet et al., 2018). Sampling of more discreet microhabitats, such as ponds and bromeliads, must account for occupancy and connectivity of water bodies, as exemplified by our sampling at Serra do Cipó. We filtered water samples in two ephemeral puddles and did not detect *Scinax pinima* DNA, a species last seen there in 1987. After eDNA sampling was completed, we encountered one adult of this species at a temporary puddle just 100 m from the puddles sampled for eDNA. Thus, eDNA is not foolproof, and attention to sampling design is critical to reduce false negatives. Our rediscovery of *Scinax pinima*, a species that was not detected by eDNA, underscores the importance of integrating distinct survey methods, especially if it is necessary to confirm the identity of a species or estimate population size, an application that is still debated for eDNA (Pilliod, Goldberg, Arkle, Waits, & Richardson, 2013; Piñol, Senar, & Symondson, 2019).

Our results support the potential of eDNA metabarcoding for surveying species in megadiverse tropical regions (Bálint et al., 2018; Cilleros et al., 2019; Lopes et al., 2017). Brazil has the highest diversity of amphibians in the world, with more than 1,000 species described. However, accurate distributions and population status are unknown for many. Only 40 Brazilian

amphibian species are listed at some level of threat in IUCN and Brazilian red lists, yet another 277 and 167 species are classified as Data Deficient in each list, respectively (Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio, 2016; The International Union for Conservation of Nature - IUCN, 2020). Environmental DNA metabarcoding should be considered in future efforts, together with other methods of survey to characterize species' geographical ranges, population fluctuations, and conservation status.

Our study confirms the sensitivity of eDNA sampling for detection of threatened aquatic wildlife, and serves as an incentive to apply it to other taxa of conservation concern. Rediscovery of amphibian species thought to be locally or globally disappeared gives hope that those species might persist and recover. Of course, sequences from a water filter cannot reveal species' health or potential for recovery, but they clearly signal the need for further assessment and conservation efforts.

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AUTHOR CONTRIBUTIONS

C.M.L., C.F.B.H., and K.R.Z designed research; C.F.B.H., and K.R.Z. acquired financial support; C.M.L., D.B., A.F.S., J.L.G., C.F.B.H., and K.R.Z. completed field sampling; C.M.L., M.L.L., and A.V. did the laboratory work; C.M.L., M.L.L., and A.V. analysed the data; C.M.L., and K.R.Z wrote the manuscript with contributions from coauthors.

DATA AVAILABILITY STATEMENT

DNA sequences of 12S rRNA mitochondrial gene for the local reference database were deposited in GenBank (391–569 bp) (MT771731-MT771897) and Dryad Digital Repository (50–92 bp) (doi.org/10.5061/dryad.f4qrfj6tc). NGS eDNA unfiltered data and the pipeline for filtering and assign taxonomy to eDNA sequences were deposited in Dryad (doi.org/10.5061/dryad.f4qrfj6tc).

ORCID

Carla Martins Lopes  <https://orcid.org/0000-0002-3277-1913>

Délio Baêta  <https://orcid.org/0000-0002-1427-1878>

Alice Valentini  <https://orcid.org/0000-0001-5829-5479>

Mariana Lúcio Lyra  <https://orcid.org/0000-0002-7863-4965>

Ariadne Fares Sabbag  <https://orcid.org/0000-0001-7277-0950>

Kelly Raquel Zamudio  <https://orcid.org/0000-0001-5107-6206>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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