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Power and limitations of environmental DNA metabarcoding for surveying leaf litter eukaryotic communities

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Abstract

Leaf litter habitats shelter a great variety of organisms, which play an important role in ecosystem dynamics. However, monitoring species in leaf litter is challenging, especially in highly diverse environments such as tropical forests, because individuals may easily camouflage themselves or hide in the litter layer. Identifying species based on environmental DNA (eDNA) would allow us to assess biodiversity in this microhabitat, without the need for direct observation of individuals. We applied eDNA metabarcoding to analyze large amounts of leaf litter (1 kg per sample) collected in the Brazilian Atlantic forest. We compared two DNA extraction methods, one total and one extracellular, and amplified a fragment of the mitochondrial *18S* rRNA gene common to all eukaryotes, to assess the performance of eDNA from leaf litter samples in identifying different eukaryotic taxonomic groups. We also amplified two fragments of the mitochondrial *12S* rRNA gene to specifically test the power of this approach for monitoring vertebrate species, with a focus on anurans. Most of the eukaryote sequence reads obtained were classified as Fungi, followed by Metazoa, and Viridiplantae. Most vertebrate sequences were assigned to *Homo sapiens*; only two sequences assigned to the genus *Phyllomedusa* and the species *Euparkerella brasiliensis* can be considered true detections of anurans in our eDNA samples. The detection of taxa varied depending on the DNA extraction method applied. Our results demonstrate that the analysis of eDNA from leaf litter samples has low power for monitoring vertebrate species and should be preferentially applied to describe active and abundant taxa in terrestrial communities, such as Fungi and invertebrates.

KEYWORDS
anura, beta diversity, DNA extraction, eukaryotes, sequencing, vertebrates

1 | INTRODUCTION

Leaf litter plays an important role in ecosystem dynamics, contributing to nutrient cycling and soil fertility of forests (Vitousek & Sanford, 1986). The decomposing layers of organic matter provide unique conditions to house a plethora of organisms. Besides plants, most of the biomass and species occurring in the leaf litter are involved in organic matter turnover, as detritivore species (arthropods and annelids) and microbial decomposers (bacteria and fungi) (Hättenschwiler et al., 2005). Among vertebrates, amphibians have

the highest number of species that reside in this microhabitat in tropical forests (Siqueira et al., 2009). Amphibian abundance and richness in leaf litter are correlated to several biotic and abiotic factors, including altitude, humidity, the structure and composition of the litter layer, and available food resources (Oliveira et al., 2013).

Assessing species diversity in leaf litter substrates is particularly challenging because small organisms can camouflage and hide between litter layers, making biodiversity inventories difficult, costly, and time-consuming. It is therefore not surprising that several species that specialize on leaf litter habitats are still poorly known, especially in highly diverse environments such as the Brazilian Atlantic forest. Surveys of amphibians inhabiting leaf litter are still primarily based on traditional methods of audio–visual encounters and the use of traps (Goyannes-Araújo et al., 2015; Rocha et al., 2013; Siqueira et al., 2011, 2014). These methods have some shortcomings, especially when trying to detect species with low population density or species that spend most of their time buried and appear on the surface only for brief periods of time.

The activities carried out by any organism leave DNA traces of its presence in the environment. This environmental DNA (eDNA) can be analyzed using a DNA metabarcoding approach, which describes the species diversity in the environment based on DNA barcodes and high-throughput sequencing (Taberlet et al., 2012). This approach can be standardized to overcome many of the challenges of traditional survey methods, providing access to organisms that are difficult to sample or hard to identify morphologically in the field, while also enhancing the probability of detecting new species (Taberlet et al., 2018; Taberlet, et al., 2012).

Environmental DNA has been successfully applied to describe community composition (Pansu et al., 2015; Valentini et al., 2016) and to monitor specific target species (Jerde et al., 2011; Lopes et al., 2020; Sigsgaard et al., 2015; Thomsen et al., 2012; Tréguier et al., 2014). Although these studies have explored a variety of aquatic and terrestrial environments (Bohmann et al., 2014), most effort to date for developing eDNA analysis protocols have focused on species inhabiting freshwater in temperate ecosystems (Hoffmann et al., 2016) or microbial diversity in soil samples (Bates et al., 2013; Lauber et al., 2009; Tedersoo et al., 2014). Few studies have tested the efficacy of extracting eDNA from leaf litter samples. In studies performed to date, leaf litter eDNA is obtained from a bulk sample (pool of individuals of the target taxa) or by using a small amount of leaf litter sample (0.1 g–0.5 g) (England et al., 2004; Horton et al., 2017; Yang et al., 2014). Such small amounts of sample material may not be consistent and representative enough to assess the local biodiversity in the surrounding environment. Appropriate sampling and DNA extraction design are key steps for success in any eDNA study. The amount of leaf litter sample collected, how it is processed, and which DNA extraction protocol is used are critical considerations for planning fieldwork, maximizing the DNA yield of the target organisms and reducing PCR inhibitor levels (Goldberg et al., 2016; Taberlet et al., 2018). Total DNA extraction is commonly used in eDNA studies, allowing access to free DNA molecules in the environment (extracellular DNA) and to the intracellular DNA from free spores, cells, and tissues of organisms (Taberlet et al., 2018). An alternative method is the extracellular DNA extraction (Taberlet, et al., 2012) that has been used to isolate eDNA from large soil samples (>15 g), and it is considered a fast and cheap protocol for multi-taxa analyses (Zinger et al., 2016). Thus far, no study has compared the performance of total and extracellular DNA extractions as a source of eDNA for surveying leaf litter communities.

In this study, we collected leaf litter samples in a highly diverse Neotropical site of the Brazilian Atlantic forest and extracted the DNA of the samples using one total (intra- and extracellular DNA) and one extracellular DNA extraction method. We amplified a fragment of the mitochondrial *18S* rRNA gene of eukaryotes to assess the performance of DNA extraction methods for detecting DNA traces of different taxonomic groups of eukaryotes present in the leaf litter community. We also amplified a fragment of the mitochondrial *12S* rRNA gene of vertebrates to test the feasibility of eDNA metabarcoding for monitoring leaf litter vertebrates, and more specifically, we amplified the mitochondrial *12S* rRNA gene of anurans, to test the feasibility of our protocols for monitoring anuran species. Given that plants, detritivores, and decomposer species constitute the highest number of species and greatest biomass in the leaf litter, we anticipated that the highest number of Molecular Operational Taxonomic Units (MOTUs) and sequence reads in *18S* rRNA data of eukaryotes would be attributed to taxa within these groups, regardless of extraction methods, while anuran species would be the taxa most represented in *12S* rRNA data of vertebrates.

2 | MATERIAL AND METHODS

2.1 | Study area and local biodiversity

The Reserva Ecológica de Guapiaçu (REGUA) (Figure 1) in Rio de Janeiro State, Brazil, encompasses 7,200 ha of mainly montane and submontane Brazilian Atlantic forest, ranging from 20 to 2,300 m of elevation. The climate is warm and wet, with temperature ranging from 14 to 37°C, and an average annual rainfall of 2,600 mm (Bernardo et al., 2011; Siqueira et al., 2014). The continuous well-preserved forest favors the persistence of a great variety of vertebrates, invertebrates, and plant species (http://regua.org.br/biodiversidade/), making REGUA a high priority locality for diversity conservation in the Brazilian Atlantic forest.

2.2 | Environmental DNA sampling, extraction, amplification, purification, and sequencing

We established two transects along an elevation range from 400 to 600 m in REGUA, covering areas with a high density, abundance, and diversity of leaf litter frogs (Siqueira et al., 2009, 2011, 2014). We sampled 16 plots of 2 m^2 in each transect. Plots were sampled approximately every 10 m of elevation (Figure 1 and Table S1). The leaf litter within each plot was collected in individual plastic bags
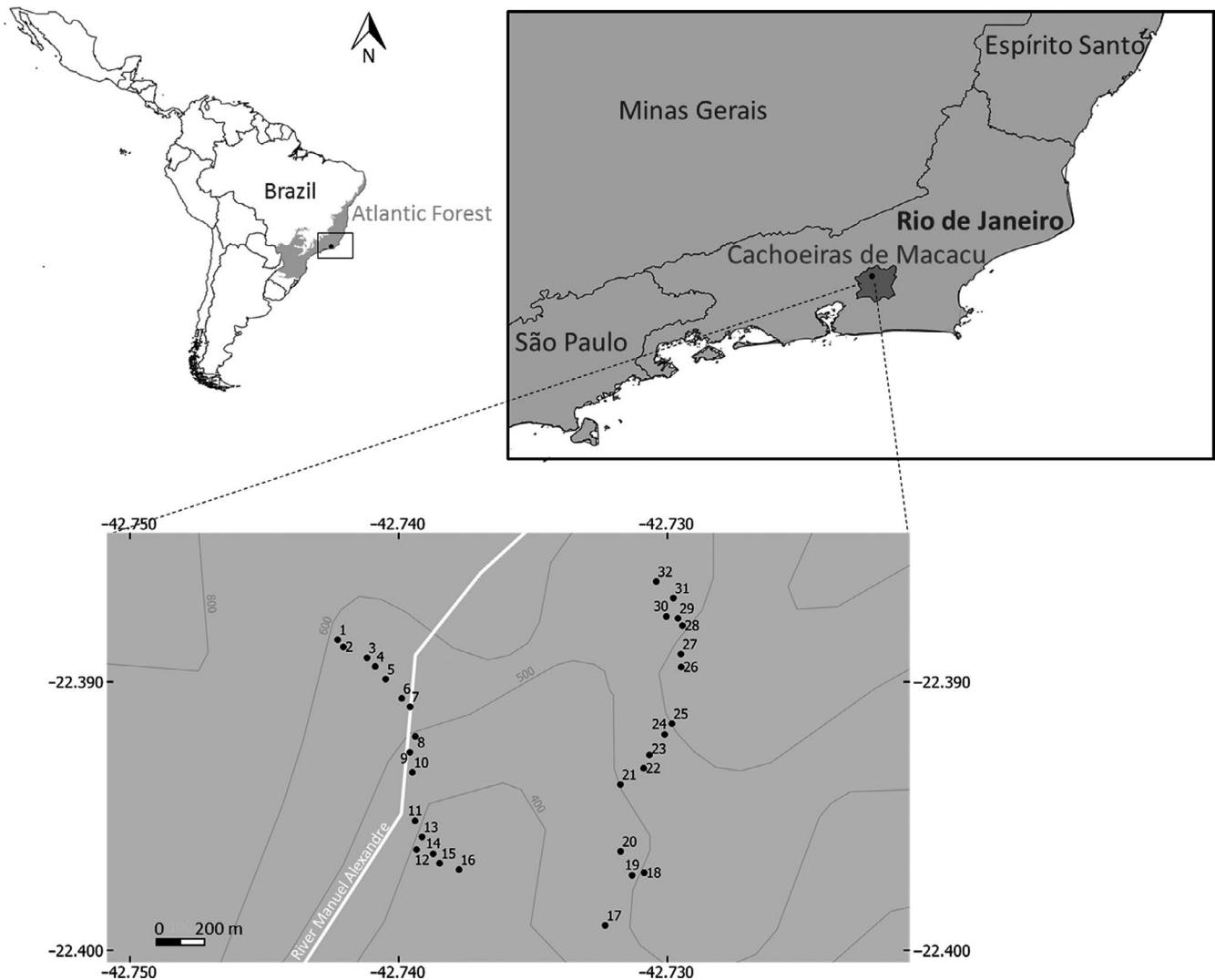
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FIGURE 1 Distribution of leaf litter plots (black dots) sampled in the Reserva Ecológica de Guapiaçu (REGUA), in Cachoeiras de Macacu, Rio de Janeiro State, Brazil

and transported to the Laboratório de Herpetologia, UNESP, Rio Claro, SP, Brazil. Samples were stored and processed in a clean and refrigerated room, within 72 hs after sampling. All sample processing was done using individual gloves, masks, and sterilized material for each sample. We compared the performance of total and extracellular DNA extraction methods by dividing the 32 samples in two subsamples of 1 kg each. For the total DNA extraction, we added a volume of 2 L of distilled water to one of the subsamples. For the extracellular method, we added a volume of 2 L of phosphate buffer (0.12 M Na_2HPO_4, pH = 8) to the other subsample. Each subsample was mixed for 10 min. The water and phosphate buffer were drained and filtered independently, using Nalgene nitrate cellulose membrane of 0.45 μm pore size (Thermo Fisher Scientific) until the membrane was clogged (20–100 ml per subsample). The membranes used for the total DNA extraction were stored in 15 ml falcon tubes filled with lysis buffer (Tris-HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1%, pH 7.5–8). The membranes used for extracellular DNA extraction

were stored dry in 15-mL falcon tubes with silica gel. All samples were stored at room temperature until DNA extraction, which was done in the Laboratoire d'Écologie Alpine, Grenoble, France, in a room dedicated to low-quantity DNA extractions. Negative sampling controls were performed applying the same procedures described above, using 2 L of distilled water and 2 L of phosphate buffer without any leaf litter sample, to certify that there was no contamination of reagents and equipment or cross-contamination among samples.

For total DNA extraction, we incubated the filters for 2 hr at 56°C to allow for cellular lysis. We transferred the 15 ml of lysis buffer to a new 50-mL falcon tube, added 33 ml of ethanol and 1.5 ml of 3 M sodium acetate, and incubated the tubes overnight at –20°C for DNA precipitation. The tubes were centrifuged at 10,000 g for 1 hr at 6°C. The supernatant was discarded, and 700 μL of lysis buffer SL2 from the NucleoSpin soil DNA extraction kit (Macherey-Nagel) was added to the precipitate. Subsequent steps of DNA extraction followed the manufacturer's instructions.

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For extracellular DNA extraction, we discarded the silica gel and added 15 ml of phosphate buffer to the dried membranes. We incubated the filters for 30 min at room temperature to recover DNA in the solution. We transferred the phosphate buffer to a new 50-mL falcon tube, added 33 ml of ethanol and 1.5 ml of 3 M sodium acetate, and incubated the tubes overnight at −20°C for DNA precipitation. The tubes were centrifuged at 10,000 g for 1 hr at 6°C. The supernatant was discarded and 700 µL of binding buffer SB from the NucleoSpin soil DNA extraction kit (Macherey-Nagel, Germany) was added to the precipitate before the tubes were vortexed for 15 s. Subsequent steps of DNA extraction followed the manufacturer's instructions.

We amplified a fragment (around 123 bp) of the v7 region of the mitochondrial *18S* rRNA gene from eukaryotes to assess the performance of the extraction methods in recovering eDNA of different taxonomic groups from leaf litter. Amplifications were performed in a final volume of 20 µL, using 2 µL of DNA extract, 1X concentrated AmpliTaq Gold® 360 Master Mix (Life Technologies), 0.2 µg/µL of bovine serum albumin (BSA, Roche Diagnostic), and 0.5 µM of the forward (5'-TTTGTCTGCTTAATTSCG-3') and reverse (5'-CACAGACCTGTTATTGC-3') primers (Guardiola et al., 2015). The PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 45°C for 30 s, 72°C for 1 min, and a final step of 72°C for 7 min.

We also amplified a fragment of the mitochondrial *12S* rRNA gene of vertebrates and anurans (around 97 and 51 bp, respectively) in a final volume of 20 µL, using 2 µL of DNA extract, 1X concentrated AmpliTaq Gold® 360 Master Mix (Life Technologies), 0.2 µg/µL of BSA (Roche Diagnostic), 0.5 µM of each the forward (5' - TTAGATACCCCACTATGC - 3') and reverse (5'- TAGAACAGGCTCCTCTAG - 3') primers for vertebrates (Riaz et al., 2011), and the batra_F (5' - ACACCGCCCGTCACCCT - 3') and batra_R (5' - GTAYACTTACCATGTTACGACTT - 3') primers for anurans (Valentini et al., 2016). For the anuran PCR, we also included 5 µM of the human blocking primer batra_blk (5' - TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I - 3') (Valentini et al., 2016). No human blocking primer was included in vertebrate PCR reactions, to avoid blocking amplification of other mammal species as well. PCR amplifications were carried out under 95°C for 10 min, followed by 45 (vertebrates) or 50 (anurans) cycles of 95°C for 30 s, 49°C (vertebrates) or 55°C (anurans) for 30 s, 72°C for 1 min, and a final step of 72°C for 7 min. One sampling (water or phosphate buffer used with no leaf litter sample), one extraction (DNA-free water used in the place of a sample), and two PCR negative controls (DNA-free water used in the place of a DNA sample) for each DNA extraction method and molecular marker were included in the experiments for monitoring contamination. Two PCR-positive controls for each DNA extraction method and molecular marker were added to monitor the detection power of the methods. The positive controls were composed of the DNA of four amphibian species obtained from the Célio F. B. Haddad collection (CFBHt) at Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo, Brazil (*Ischnocnema guentheri*—CFBHt13091, *Haddadus binotatus*—CFBHt13050, *Rhinella*

icterica—CFBHt13068, and *Adenomera marmorata*—CFBHt14596) mixed at known concentrations. We performed eight, six, and six PCR replicates for each sample to amplify the DNA of eukaryotes, vertebrates, and anurans, respectively. All primers were 5' labeled with 8 bp unique molecular tags allowing identification of sequences to the corresponding PCR replicate. The PCR products were purified using a QIAquick PCR purification kit (Qiagen GmbH,) and titrated using fluorometric quantitation (Qubit, Qiagen GmbH). One library for each marker was prepared using the Metafast protocol (https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis). The paired-end sequencing (2 × 125 bp) was carried out using the Illumina Hiseq 2,500 (Illumina Inc.) at Fasteris (http://www.fasteris.com).

2.3 | Reference database

We constructed one DNA metabarcoding reference database for each molecular marker to taxonomically assign the sequences recovered from eDNA samples. We used the sequences of the primer pairs of *18S* rRNA gene for eukaryotes, and the *12S* rRNA gene for vertebrates and anurans to extract the relevant part of the sequences from the European Molecular Biology Laboratory (EMBL) database (release 135) using the programs implemented in the OBITools 1.1.22 package (Boyer et al., 2016) and ecoPCR 0.5.0 (Ficetola et al., 2010). In addition, a local DNA reference database was constructed to improve taxonomic assignment of the eDNA anuran sequences. Anuran tissues from the species occurring in REGUA and surrounding areas (Rocha et al., 2007; Siqueira et al., 2009, 2011) were obtained from the Célio F. B. Haddad collection (CFBHt) at Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo, Brazil, and Museu Nacional— Universidade Federal do Rio de Janeiro (MNRJ), Rio de Janeiro, Brazil (Table S2). Total DNA was extracted from 10 mg of muscle tissue using a standard high-salt protocol (Lyra et al., 2017). The fragment of the mitochondrial *12S* rRNA gene was amplified using the primers 12SA-L (5'-AAACTGGGATTAGATACCCCACTAT-3'; Palumbi et al., 1991) and tVal (5'-TGTAAGCGARAGGCTTTKGTTAAGCT-3'; Wiens et al., 2005), following the protocols described by Faivovich et al. (2004). PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (Thermo Fisher Scientific) following the guidelines of the suppliers. Both DNA strands were sequenced. Sequences were visually inspected, primers were trimmed, and consensus sequences were constructed using Geneious 7.1.3 (Kearse et al., 2012). The relevant part of the sequences from the local reference database was added to the anuran sequences extracted from the EMBL database.

2.4 | Sequence filtering and annotation

Environmental DNA sequences were filtered and taxonomically annotated using the programs OBITools, ecoPCR, and R 3.3.3 (R Development Core Team, 2016), following the main steps

described by Lopes et al. (2017). We analyzed the data for each molecular marker separately, as follows: i) we constructed consensus sequences assembling paired-end reads; ii) assigned only sequences unambiguously identified by their molecular tags and maximum 2 bp errors per primer to appropriate PCR products; iii) dereplicated the reads, keeping the sequence reads count per PCR product; iv) excluded from the subsequent analyses sequences shorter than 30 bp, 20 bp, and 20 bp, for eukaryotes, vertebrates, and anurans, respectively; and v) sequences with total read counts lower than 10 among all PCR replicates for each molecular marker, to eliminate possible amplification/sequencing errors; vi) labeled each sequence as "head" (the most common sequence in a group of sequences linked by a single indel or substitution), "internal" (less frequent sequences in a group of linked sequences), or "singleton" (sequences with no variants linked to them), according to Shehzad, et al. (2012), in each PCR product, to identify possible amplification/sequencing errors; and vii) assigned the taxonomic identification to the sequences using the appropriate reference database. We filtered sequences to eliminate possible contaminations, PCR and sequencing errors from the data, by excluding from subsequent analysis: viii) sequences with frequency lower than 1% per PCR product; ix) sequences identified as "internals"; x) sequences with less than 90%, 90%, and 96% of identity with a sequence from the reference database for eukaryotes, vertebrates, and anurans, respectively. The identity thresholds applied are based on results of previous studies (Lopes et al., 2017) and the representativeness of Brazilian Atlantic forest biodiversity in the sequence reference databases; xi) sequences with maximal average read counts in negative controls; xii) sequences not identified at least to order or family taxonomic levels or as Anura, for eukaryote, vertebrates, and anuran datasets, respectively; and xiii) low-quantity PCR products (< 100 reads in total), by comparing the number of read counts of positive and negative controls and eDNA samples.

2.5 | Statistical analyses

We performed all statistical analyses using R 3.3.3. We calculated the proportion of sequence reads obtained for each taxon of interest, in each leaf litter plot, for each molecular marker and DNA extraction method based on the sum of read counts among PCR replicates. We applied the Wilcoxon signed-rank test to statistically compare differences in the number of sequence reads and MOTUs between total and extracellular DNA extractions per plot, using the whole eukaryote dataset and the Fungi, Metazoa, and Viridiplantae kingdoms, separately, under a significance of $p < .01$. We used the Spearman correlation test to identify whether the relative abundance and number of MOTUs for each phylum identified in the eukaryote dataset are correlated between the two extraction methods, under a significance of $p < .05$.

We used a site occupancy–detection model (Royle & Link, 2006) to compare probabilities of detection (p11) and site occupancy (psi)

for Fungi, Metazoa, and Viridiplantae kingdoms between total and extracellular extractions. We constructed matrices of presence/absence for each kingdom and DNA extraction, considering the 32 plots and the 8 PCR replicates performed for each sample. We applied Bayesian inference under the JAGS 4.3.0 program (Plummer, 2003) in the R package R2jags 0.6 (Su & Yajima, 2015) considering the maximum probability of false presences as 0.05, running four chains of 100,000 iterations, 50,000 as burn-in, and saving 1,000 iterations per chain.

We explored the community composition congruence between the extraction methods comparing the matrices of pairwise beta diversity estimates between plots. We used the presence/absence of MOTUs in the eukaryote dataset to calculate the beta diversity based on the Sørensen dissimilarity index. The overall beta diversity was partitioned into turnover and nestedness components to assess the dissimilarity among plots resulting from the replacement or loss/gain of MOTUs, respectively. The dissimilarity indexes were calculated using the R package betapart 1.5.1 (Baselga et al., 2018). We compared the correlation between the dissimilarity matrices of each extraction method using Mantel's test, under 999 permutation and significance of $p < .05$. We also applied Mantel's test to further explore whether a gradient in the beta diversity pattern is recovered along the altitudinal transect for both extraction methods.

3 | RESULTS

3.1 | Eukaryote data

Sequencing of the fragment of the *18S* rRNA gene from eukaryotes resulted in 11,309,111 consensus sequence reads, including the eight PCR replicates of all environmental samples, positive, and negative controls. After the filtering process, two sequences assigned to Anura were retained in positive controls. No sequence remained in sampling and PCR negative controls. Four sequences (assigned to the plant subfamily Pooideae, and the fungi families Valsaceae and Physalacriaceae and the genus *Malassezia*) were detected at low read counts in only four PCR replicates of extraction controls. We did not exclude these sequences from eDNA samples. We obtained 1,303,676 reads among eDNA samples, distributed in 598 MOTUs (Table S3). Among the MOTUs, 35.62% were identified to the species level, 21.07% to genus, 16.72% to family, 16.05% to order, and the remaining 10.54% were identified to other levels below order.

We retrieved 646,759 sequence reads, distributed in 501 MOTUs for the 32 eDNA samples obtained with the total DNA extraction method. Most of the sequence reads and MOTUs were classified as Fungi (60.68% and 247), Viridiplantae (19.26% and 50), and Metazoa (11.47% and 95), respectively. Considering the phylum level, the most represented were Ascomycota (48.35% and 149 sequence reads and MOTUs, respectively), Streptophyta (19.26% and 50), Basidiomycota (9.63% and 71), and Arthropoda (5.34% and 51) (Figure 2). Only 0.76% of the sequence reads were classified as

FIGURE 2 Proportion of sequence reads and number of Molecular Operational Taxonomic Units (MOTUs) amplified for each eukaryotic phylum for the total and extracellular DNA extraction methods. NA—Phylum classification absent for sequences in the reference database
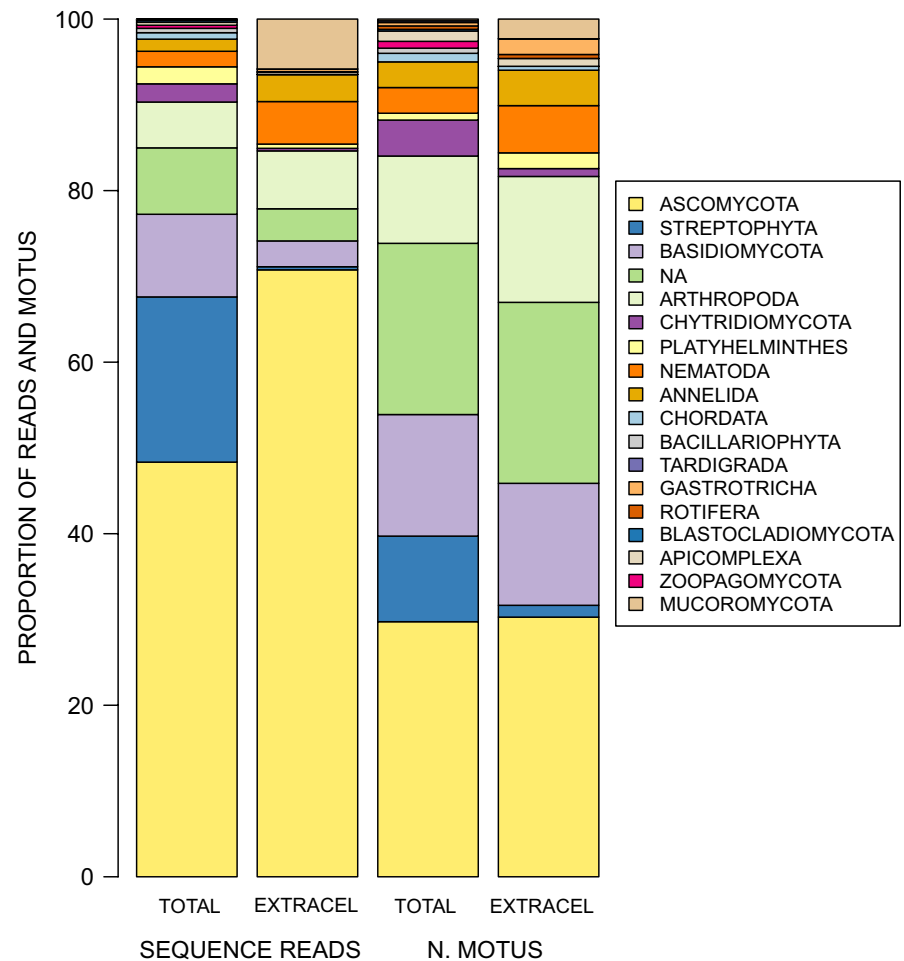
Chordata, from which only one MOTU was assigned to Anura in two PCR replicates.

We retrieved 656,917 sequence reads, distributed in 218 MOTUs for the 32 eDNA samples obtained with the extracellular DNA extraction. Most of the sequence reads and MOTUs were classified as Fungi (79.89% and 104), Metazoa (15.72% and 63), and Viridiplantae (0.36% and 3). The phyla Ascomycota (70.76% sequence reads and 66 MOTUs), Arthropoda (6.75% and 32), Mucoromycota (5.81% and 5), and Basidiomycota (3.01% and 32) correspond to most of the sequence reads and MOTUs obtained, respectively (Figure 2). No MOTU was classified as Chordata for extracellular DNA extraction samples.

The number of sequence reads was similar using both DNA extraction methods for the whole eukaryote dataset and for Fungi and Metazoa sequences. For Viridiplantae sequences, the number of sequence reads was significantly higher ($p < .01$) for the total DNA extraction, compared with extracellular extraction. The number of MOTUs was equivalent for both extractions for Metazoa. For the whole eukaryote dataset, and for Fungi and Viridiplantae sequences, the number of MOTUs was significantly higher ($p < .01$) for the total DNA extraction, compared with the extracellular extraction (Figure 3). The extraction methods showed moderate positive correlations in the relative abundances and number of MOTUs across eukaryotic phyla (Spearman's rho 0.52 and 0.72, respectively,

$p < .05$) (Figure 4). However, some phyla showed quite higher relative abundance or number of MOTUs for one of the extraction methods compared to the other (namely Chordata, Chytridiomycota, and Streptophyta for total extraction and Mucoromycota for extracellular extraction) or the phyla were only detected using the total extraction method (Bacillariophyta, Blastocladiomycota, Tardigrada, and Zoopagomycota) (Figure 2).

The estimated detection probabilities for Fungi and Metazoa were slightly higher for the extracellular DNA extraction method ($p11 = 0.939$ and 0.691, respectively) than for total extraction ($p11 = 0.885$ and 0.547). On the other hand, the highest estimated detection probability for Viridiplantae was observed with the total DNA extraction ($p11 = 0.622$), when compared to the extracellular method ($p11 = 0.205$). The estimated proportion of sites occupied (psi) for Fungi, Metazoa, and Viridiplantae for the total DNA extraction was 0.979, 0.853, and 0.756, respectively, and for the extracellular method, it was 0.979, 0.741, and 0.207, respectively (Figure 5).

We found overall congruent Sørensen, nestedness, and turnover pairwise dissimilarity estimates between plots for total and extracellular extractions ($r = 0.11$, $r = -0.07$, $r = 0.05$, $p > .05$). However, the Sørensen and turnover values were slightly more pronounced for the total DNA extraction method, while the nestedness component was higher for the extracellular extraction. Overall, the
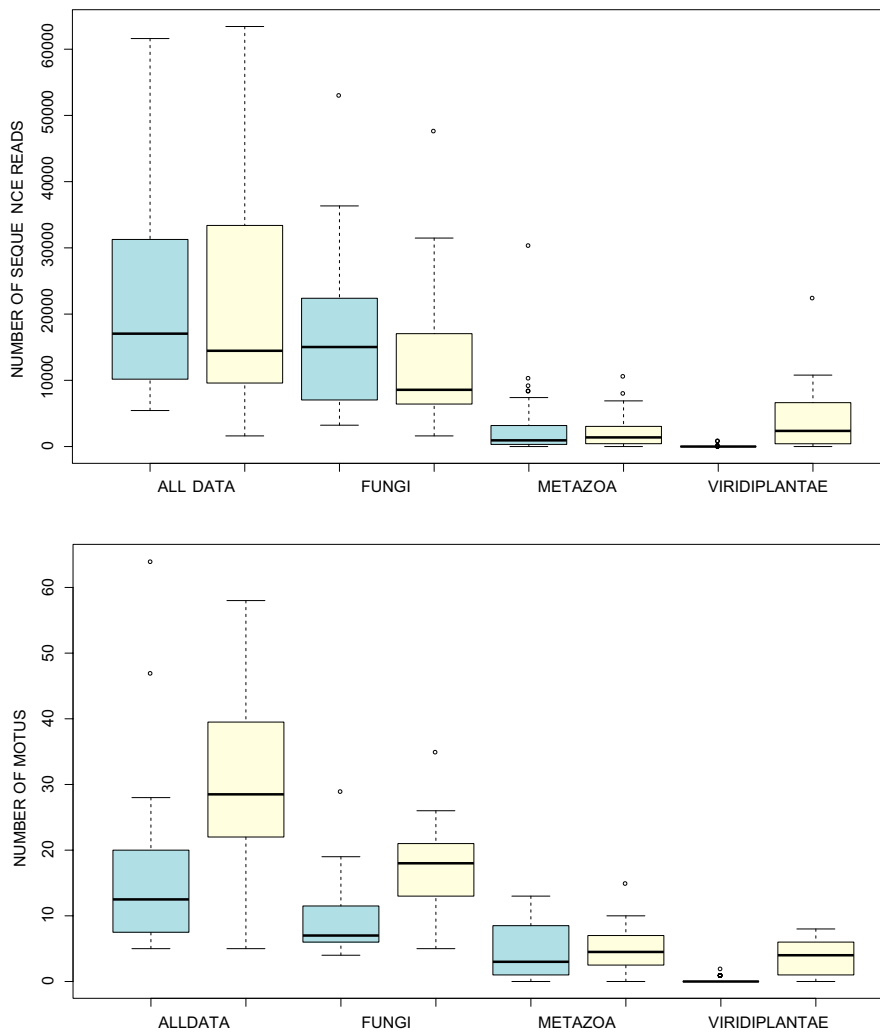
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FIGURE 3 Comparison of number of sequence reads (above) and MOTUs (below) between extracellular (light green) and total (light yellow) DNA extraction methods for the eukaryote dataset. Data were plotted considering the total data, Fungi, Metazoa, and Viridiplantae for each DNA extraction method

turnover component contributed more to the dissimilarity patterns observed for both extraction methods than the nestedness component (Table 1). A positive and significant correlation between the Sørensen dissimilarity estimates along the altitudinal transect was only observed for the total DNA extraction ($r = 0.12$, $p < .05$). All the other comparisons did not show significant values (nestedness and turnover total extraction: $r = 0.06$ and 0.06, respectively. Sørensen, nestedness, and turnover extracellular extraction: $r = 0.04$; −0.01 and 0.02, respectively; $p > .05$).
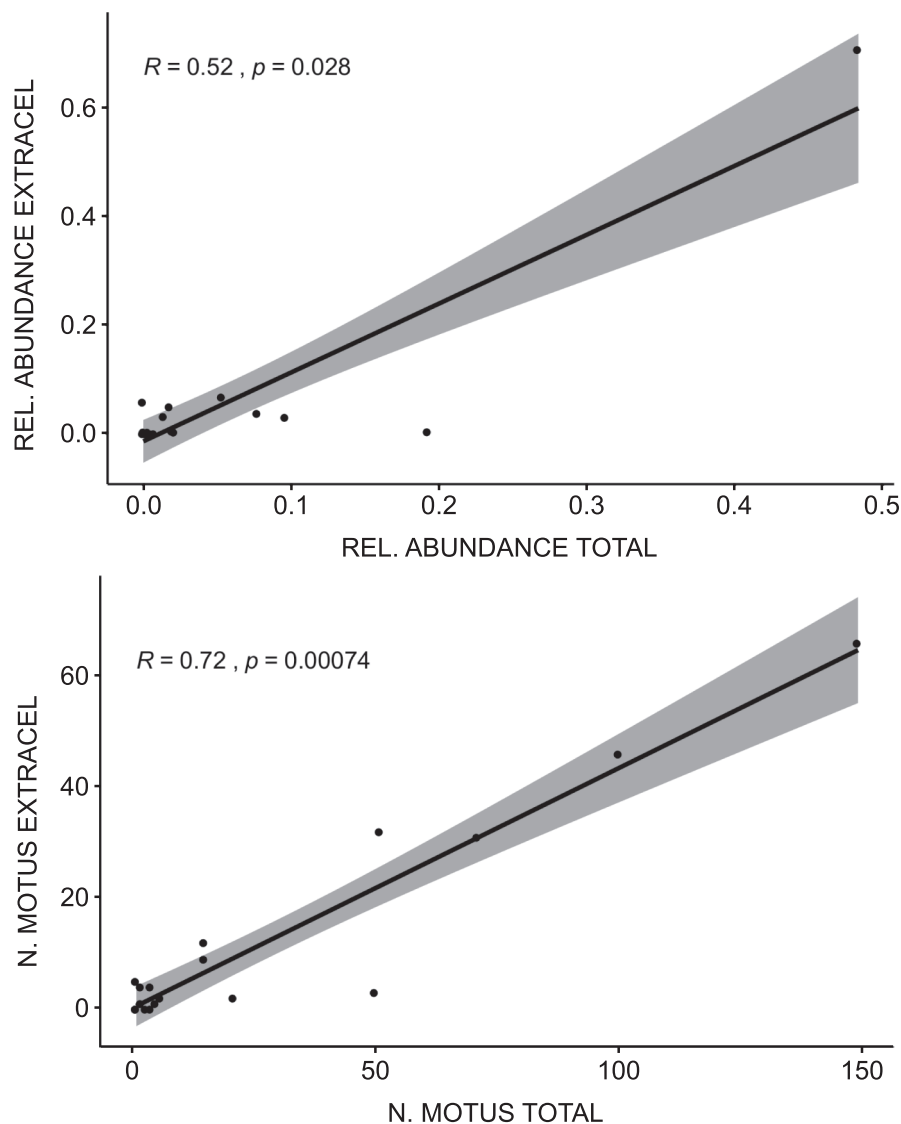
3.2 | Vertebrate data

Sequencing of the fragment of the *12S* rRNA gene from vertebrates resulted in 3,921,430 consensus sequence reads, including the six PCR replicates of all environmental samples, negative and positive controls. After the filtering process, no sequence reads remained in negative controls. We recovered in positive controls sequences corresponding to the species *Haddadus binotatus*, *Rhinella icterica*, and *Adenomera marmorata*, but we lost the sequence corresponding

to *Ischnocnema guentheri* (< 90% of identity with a sequence from the reference database). We obtained 25,945 reads from the eDNA samples, distributed in 27 MOTUs (Table S3). Among the MOTUs, 59.26% were identified to species level, 11.11% to genus, 25.93% to subfamily, and 3.70% to family.

We retrieved 19,343 sequence reads, distributed in 21 MOTUs for the 32 eDNA samples obtained with the total DNA extraction. All sequence reads were assigned to nine taxa: Hominidae, Homininae, and *Homo sapiens* (corresponding to 0.73%, 19.63%, and 50.94% of the reads, respectively), the frog species *Pseudopaludicola boliviana* (17.27%), the wild turkey *Meleagris gallopavo* (5.67%), the genera *Canis* (1.97%), *Bos* (1.13%), and *Phyllomedusa* (0.99%), and the bird subfamily Phasianinae (1.67%).

We retrieved 6,602 sequence reads, distributed in 12 MOTUs for the 32 eDNA samples obtained with the extracellular DNA extraction. All sequence reads were assigned to seven taxa: Homininae and *Homo sapiens* (corresponding to 9.36% and 75.86% of the reads, respectively), the frog species *Euparkerella brasiliensis* (4.24%), the woodcock *Scolopax rusticola* (2.23%), the shrew *Crocidura russula* (1.50%), and the genera *Canis* (1.57%) and *Bos* (5.24%).

FIGURE 4 Comparison of congruence of relative abundances (above) and number of MOTUs (below) between total and extracellular extraction methods for the eukaryotic orders. Spearman's rho (*R*), significance value (*p*), and the regression line are shown

3.3 | Anuran data

The sequencing of the fragment of the *12S* rRNA gene from Anura resulted in 2,305,405 consensus sequence reads, including the PCR replicates of all environmental samples, positive and negative controls. After the filtering process, no sequence reads remained in negative controls. The sequences corresponding to the species *Haddadus binotatus*, *Rhinella icterica*, and *Adenomera marmorata* were recovered in positive controls sequences, but we lost the sequence of *Ischnocnema guentheri* (< 96% of identity with a sequence from the reference database). No frog sequence was retained in any eDNA sample (Table S3).

4 | DISCUSSION

The analysis of eDNA from leaf litter samples is poorly explored for monitoring macroorganisms and many methodological gaps still remain. Our results demonstrate that this approach can be successfully applied to describe the biodiversity of eukaryotes, mainly of clades that are active and abundant in terrestrial communities. However, results may vary depending on the DNA extraction method applied. Both DNA extractions showed good potential for surveying Fungi and Metazoa communities in our study, despite differences in the power of detection observed for some specific phyla (total extraction favored the detection of Chordata, Chytridiomycota, Blastocladiomycota, Tardigrada, and Zoopagomycota, while extracellular extraction performed better for Mucoromycota). For Viridiplantae, the total extraction performed much better than the extracellular method in all analyses.

Total and extracellular extractions were ecologically congruent for estimating overall beta diversity. However, the total DNA extraction method was more sensitive in detecting variation in the pattern of diversity along the altitudinal transect. The replacement of MOTUs between plots, represented by the turnover component, contributed more to the pairwise dissimilarities observed, which can reflect a gradual shift in species composition along the altitudinal transect. Species distributions are known to vary along
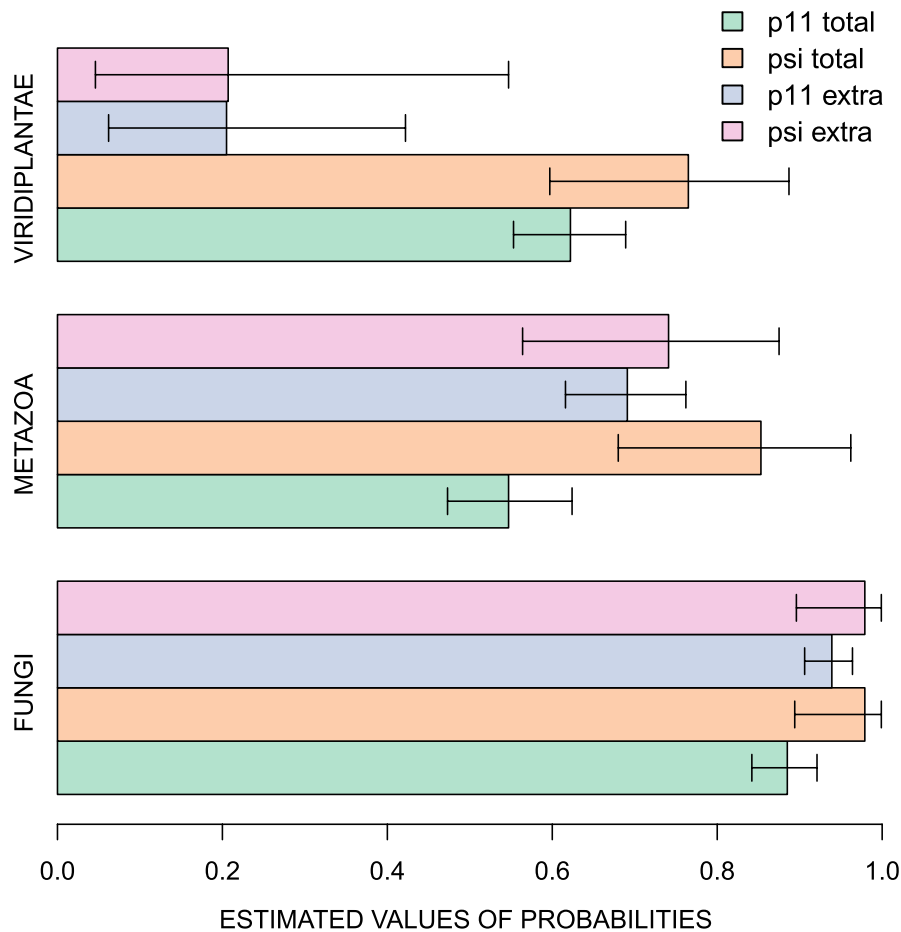
FIGURE 5 Estimated values of probability of kingdom detection (p11) and occupancy of sites (psi) for total (total) and extracellular (extra) DNA extraction methods. 95% confidence intervals are shown

TABLE 1 Variation of estimated values of Sørensen, nestedness, and turnover dissimilarity indices for the pairwise comparison between leaf litter plots for total and extracellular extraction methods, using the entire eukaryote dataset

	Total extraction			Extracellular extraction		
	Sørensen	**Nestedness**	**Turnover**	**Sørensen**	**Nestedness**	**Turnover**
Minimum	0.5789	0.0000	0.0000	0.3333	0.0000	0.0000
1st quartile	0.8197	0.0143	0.7647	0.7484	0.0318	0.6000
Median	0.8710	0.0322	0.8125	0.8286	0.0750	0.7273
Mean	0.8626	0.0693	0.7933	0.8035	0.1027	0.7008
3rd quartile	0.9080	0.0717	0.8710	0.8832	0.1368	0.8000
Maximum	1.0000	0.7297	1.0000	0.9655	0.7778	0.9643

altitudinal gradients, as demonstrated for frogs in this region (Siqueira et al., 2009, 2011, 2014). However, we do not have any data on the distribution of species other than frogs along our transects to test this assumption. Zinger et al. (2016) argue that the overall community composition of bacteria, eukaryotes, and vascular plants in soil samples were congruent between total and extracellular DNA extractions, making the extracellular method a better alternative considering costs, labor, and larger volumes of sample processed. However, soil communities may be undersampled using extracellular DNA extractions, due to the lower cell lysis compared to total DNA extractions (Zinger et al., 2016). In our study, we processed the same weight of leaf litter, using the same equipment and extraction kit for

both methods, resulting in equivalent costs. Despite some additional steps required during total DNA extraction, we concluded this was overall a good compromise to recover more reliable data for monitoring terrestrial community in our leaf litter samples.

Fungi were the kingdom most represented among the sequence reads and MOTUs recovered in the eukaryote dataset. Among Metazoa, invertebrates comprised more than 90% of sequence reads and MOTUs, represented mainly by Arthropoda, Nematoda, Annelida, and Platyhelminthes. Fungi are essential microbial decomposers in the leaf litter, which together with other litter-consuming detritivore species play a key role in the nutrient cycling in this microhabitat (Gessner et al., 2010). It is expected

that active and abundant taxa in terrestrial communities, such as Bacteria, Fungi, and invertebrates, will be highly represented in eDNA recovered from soil samples (Drummond et al., 2015; Zinger et al., 2016). However, despite leaf litter being mainly composed of plant parts (wood, leaves, and roots), Viridiplantae was the kingdom least represented in our eukaryotes data. One alternative to improve the description of plant community composition in leaf litter samples based on eDNA is to adjust protocols, for example, including a mechanical lysis step during DNA extraction that breaks plant cell walls and increases the amount of intracellular DNA recovered. Moreover, using primers specifically developed for amplification of plant DNA would increase specificity and efficiency of plant sequences recovered, avoiding amplification of undesirable taxa (Taberlet et al., 2007).

The eukaryote, vertebrate, and anuran datasets generated in our study showed that the analysis of eDNA from leaf litter is not a reliable tool for monitoring vertebrates, and more specifically, for anuran species. Only two amphibian sequences corresponding to the genus *Phyllomedusa* and the species *Euparkerella brasiliensis* can be considered true detections of the vertebrate community in our samples. Despite following strict protocols of sampling, laboratory, and bioinformatic analysis, most vertebrates we identified in our data using the fragment of the *12S* rRNA gene could be considered false positives. Some species we detected do not occur in REGUA (such as *Pseudopaludicola boliviana*), or even in Brazil (e.g., *Crocidura russula*, *Meleagris gallopavo*, and *Scolopax rusticola*). Their detection can result from contamination of our samples with exogenous DNA during handling and processing of eDNA samples or, ultimately, the incompleteness of our reference database resulted in incorrect taxonomy assignment of eDNA sequences. Incomplete and inaccurate reference databases can result in up to 30% of incorrect taxonomic assignments of sequences (Kocher et al., 2017), which is particularly tricky for Neotropical taxa that are underrepresented in public DNA reference databases (Zinger et al., 2020). Assembling a custom local reference database is an alternative to ensure the reliability of the taxa detected. The sequences of *Bos*, *Canis*, and especially *Homo sapiens* detected in our data are common contaminants of eDNA samples, even when stringent practices are adopted (Epp et al., 2012). Those are either true detections of DNA traces of these organisms in our sampling locality or could result from exogenous DNA contamination in our samples. A high amount of contaminant DNA can limit the amplification of less frequent sequences, hindering the detection of target taxa (Shehzad, et al., 2012). We applied stringent rules to remove potential PCR/sequencing errors and contaminations (false positives), which might increase exclusions of true detections (false negatives), but not at the cost of losing all true detections, especially if we consider the high number of PCR replicates used in our study (Ficetola et al., 2015). Failures in laboratory procedures are unlikely to explain the inability to recover at least part of the community of vertebrates in our eDNA samples. The primer pairs used for vertebrates and anuran amplifications have been successfully applied in previous eDNA metabarcoding studies for taxonomic assignment of species (De Barba et al., 2014; Lopes et al., 2017;

Shehzad, et al., 2012; Valentini et al., 2016). Similarly, the routine laboratory procedures, such as PCR amplification, quantification, library preparation, and eDNA sequencing, were performed in previous successful studies (Lopes et al., 2017; Taberlet et al., 2018; Valentini et al., 2016).

The stability and detectability of DNA in substrates such as soil and leaf litter might be affected by environmental conditions (pH, temperature, and UV radiation), species characteristics (population size, biomass, and behavior), and the characteristics of the molecules of DNA themselves (Taberlet et al., 2018). However, how these factors affect overall eDNA stability is still poorly understood. Moreover, DNA molecules are adsorbed to particles in the soil, which limits the capacity of DNA dispersion through the environment (Yoccoz et al., 2012). This may, therefore, require wide areas of survey to successfully detect the DNA of the target species in such substrates, especially if the species show low population densities or have limited dispersion ability. Our sampling effort was spatially distributed in a known area of frog occurrence in REGUA, totaling 64 m^2 of leaf litter. We observed several individuals of different amphibian species during the leaf litter sampling (*Adenomera marmorata*, *Euparkerella brasiliensis*, *Haddadus binotatus*, *Ischnocnema guentheri*, *Ischnocnema parva*, *Physalaemus signifier*, *Rhinella ornata*, and *Zachaenus parvulus*), both within and around plots. Surprisingly, we did not recover any amphibian sequence corresponding to these field observations in our anuran dataset. Although leaf litter shelters a wide variety of amphibian species, the release, persistence, and degradation rates of DNA in the leaf litter are unknown. Limited DNA release by amphibian species in leaf litter may limit detection probability using eDNA, as skin cells are potentially not continuously shed in leaf litter as they are periodically sloughed when frogs are in water (Taberlet et al., 2018). That, together with leaching of DNA from leaf litter into the soil layer, could contribute to the low feasibility of the anuran detection from leaf litter by means of the eDNA.

Our study was performed in a preserved Atlantic forest area, and our results have shown that detecting vertebrate DNA in leaf litter substrate is challenging, being primarily informative for a few taxa. Our sampling design and the total area sampled may have not been sufficient to detect vertebrates in general and more specifically amphibian species, mainly due to limited DNA shedding of species in the litter layer and to the low capacity of DNA dispersion in soil, which limits the signal for species detection both vertically and horizontally (Taberlet et al., 2018). Therefore, further research aiming to survey vertebrate species in terrestrial environment using eDNA should consider the representativeness of the samples collected relative to the area that will be surveyed and the sampling strategy to be applied. For example, the use of water sampling instead of soil sampling might be warranted for the search of amphibians, as water seems to be a more suitable substrate for retrieving amphibian eDNA (Lopes et al., 2017), or the potential use of DNA traps, such as sandpaper-sampling, that could detect the DNA of amphibian species, even after few contacts between the specimens and the DNA trap (Burns et al., 2020). Overall, including the use of specific primers to amplify the DNA of abundant groups such as plants, Fungi, and

other detritivore species should be of special interest for community characterization using DNA extracted from leaf litter samples.

CONFLICT OF INTEREST

The "batra" primers used to identify amphibians from environmental samples are patented. This patent only restricts commercial applications and has no impact on the use of this method by academic researchers.

AUTHOR CONTRIBUTIONS

CML, DB, and PT designed research; CFBH acquired financial support; CML, DB, TS, and AV completed field sampling; CML, TS, and AV did laboratory work; CML analyzed the data; CML wrote the manuscript with the contributions from coauthors.

DATA AVAILABILITY STATEMENT

DNA sequences of the fragment of the mitochondrial *12S* rRNA gene of anurans for the local reference database: GenBank accession numbers MW010292 - MW010337. NGS eDNA unfiltered data: Dryad Digital Repository https://doi.org/10.5061/dryad.9w0vt4bd1.

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SUPPORTING INFORMATION

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