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Environmental DNA characterization of amphibian communities in the Brazilian Atlantic forest: Potential application for conservation of a rich and threatened fauna

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ABSTRACT

The Brazilian Atlantic forest is a highly threatened biodiversity hotspot, harboring one of the highest levels of amphibian species richness in the world. Amphibian conservation in Neotropical biomes is critical because freshwater ecosystems typically experience sharp declines in biodiversity before much is known about species that depend on those environments. Environmental DNA (eDNA) analysis is a new approach for monitoring aquatic organisms and provides valuable information on species occurrence in freshwater ecosystems. Here, we assess community diversity in Neotropical streams using eDNA metabarcoding. We compare data from a five-year traditional field survey with results from a short-term eDNA analysis in four streams of the Atlantic forest of southeastern Brazil. We recorded 19 species over 5 years using visual-acoustic methods, of which 10 species are associated with the streams during at least one life stage (egg, tadpole or post-metamorphic). We were able to detect nine of the latter species using eDNA metabarcoding from water samples collected over 4 days. Amphibian community composition as measured by both methods showed high similarity in three streams, but was not perfect, as eDNA failed to detect known species in a fourth stream. Furthermore, in one stream we detected through eDNA metabarcoding a species (*Aplastodiscus eugenioi*) found only once during the 5-year traditional survey in that stream. Also, three species (*Cycloramphus boraceiensis*, *Hylodes asper*, and *Hylodes phyllodes*) with the highest dependence on aquatic habitat showed the highest number of positive PCR detections on eDNA samples. Our results showed that eDNA metabarcoding can be a useful tool to assess community diversity in tropical streams, and although not perfect in detection, this method can potentially improve our ability to conserve Neotropical amphibians.

1. Introduction

The Brazilian Atlantic forest is home to a high diversity of amphibians, harboring over 500 species (approximately 7% of all known species) of which 88% are endemic to this biome (Haddad et al., 2013). Distributed along the coast of eastern Brazil, the Atlantic forest covers mountainous terrains with a large number of high-gradient streams (Morellato and Haddad, 2000). These freshwater environments harbor a high diversity of amphibians, including habitat specialists with most or all life cycle stages depending on lotic waters (Haddad et al., 2013). The Atlantic forest is also one of the most threatened tropical ecosystems in the world (Myers et al., 2000) with only 16% of its original vegetation cover remaining (Ribeiro et al., 2009), posing a high risk to habitat specialists endemic to this biome. Several other factors, such as invasive species, and emergent infectious diseases (Both et al., 2011; Carvalho et al., 2017; Eterovick et al., 2005) also potentially contribute to observed amphibian declines in Atlantic forest. Not surprisingly, approximately 88% of the threatened anuran species in Brazil are from the Atlantic forest (ICMBio, 2016a) and most of the documented frog declines in Brazil include species closely associated with streams (Eterovick et al., 2005). Furthermore, over one fifth of Atlantic forest amphibians that are stream specialists lack baseline data and are

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listed as Data-Deficient according to the International Union for Conservation of Nature (IUCN, 2015; ICMBio, 2016b). Thus, it is critical to describe the occurrence and distribution of stream frog species in the Atlantic forest and any method that makes this task easier and more precise will be of crucial importance for amphibian conservation.

Field techniques traditionally used to assess amphibian occurrence require a large investment in fieldwork and come with their own sources of error (Heyer et al., 1993). For instance, frog detectability through acoustic survey are highly variable among species, some species call only a few days per year making their detection less likely, and all types of traps used to sample amphibians (e.g., pitfall, funnel, pipe) provide biased results (Dodd, 2010; Petitot et al., 2014). Recently, the analysis of environmental DNA (eDNA) has emerged as a promising alternative approach for detecting aquatic species. The term eDNA refers to DNA shed by an organism in the environment, such as water, soil, or even air (Taberlet et al., 2012a), which can be then sampled for sequencing and species identification (Bohmann et al., 2014). The use of eDNA has recently gained widespread attention because it allows researchers to detect species even at low abundances (Dejean et al., 2012) and during short term field samplings (Lopes et al., 2017), making eDNA ideal for applications in conservation biology.

The number of ecological studies applying eDNA analysis to monitor species is increasing, yet few studies have sampled eDNA from aquatic environments to perform broad community surveys (but see Shaw et al., 2016; Valentini et al., 2016). Most eDNA efforts have focused on detection of a particular species using species-specific molecular markers (Thomsen and Willerslev, 2015). These efforts, while valuable, do not assess species diversity. If the goal is to survey the biodiversity of particular taxa and assess community composition, one can use DNA-based identification of a group of species at a locality with universal primers, an approach known as "DNA metabarcoding" (Taberlet et al., 2012b).

Here we report the results of a short-term eDNA metabarcoding study applied to a system of Atlantic forest streams where amphibian community composition was well known through a long-term traditional survey. By comparing our eDNA results with our traditional survey, we assess the utility of the eDNA metabarcoding for the characterization of tropical frog communities from a megadiverse region. If one or a few eDNA samples can accurately describe amphibian community composition in tropical streams, instead of long-term, time and labor-expensive traditional surveys, this method has high potential for use in conservation studies of Atlantic forest frogs.

2. Material and methods

2.1. Study site

Our study site is located within the Parque Estadual da Serra do Mar, Núcleo Picinguaba, municipality of Ubatuba, São Paulo, southeastern Brazil (see Fig. 1 in Lopes et al., 2017). The climate is tropical (Köppen, 1948) with mean annual air temperature of 26.7 °C, high and constant air humidity (monthly means 85–90%), and an annual average rainfall of 2650 mm (CIIAGRO, 2016). We collected data from four freshwater mountain streams in separate drainages. During water sampling for eDNA analyses, water temperature ranged from 20.4 to 20.9 °C and pH from 6.5 to 7.0. The structure of each stream varied as follows: *Stream 1* (23° 21′ 15.2″ S, 44° 46′ 3.2″ W) is the largest stream sampled (4–15 m wide) and runs along a flat area with many large pools and few waterfalls; *Stream 2* (23° 21′ 34.4″ S, 44° 47′ 3.2″ W) is the smallest stream sampled (1–5 m wide), it ceases to flow during the driest periods of the driest years, runs along a flat area in the first 40 m, sloping for much its length, before reaching a steep area; *Stream 3* (23° 21′ 41.2″ S, 44° 47′ 15.3″ W) has a lower inclination than the other streams sampled, with fewer waterfalls and many large pools, and its width varies from 5 to 10 m; and *Stream 4* (23° 21′ 53.7″ S, 44° 48′ 2.8″ W) is the most sloping of the four streams, with many waterfalls and few large pools, and its width varies from 5 to 10 m. We purposefully chose these four streams because they are first (Stream 2) and second (the remaining three) -order wadeable streams, but vary in habitat characteristics that potentially change amphibian community composition and abundance, and thus are excellent sites for testing the efficacy of the eDNA application to community level questions.

2.2. Traditional survey

We surveyed the four focal streams for species composition monthly from January 2007 to December 2010, and every other month in 2011, for a total of 55 monthly surveys over the course of five years. Surveys were primarily visual and complemented with call identifications. We surveyed twice per month (one diurnal and one nocturnal survey) within a 95–115 m transect along each stream with three observers. We searched for post-metamorphic individuals (froglets to adults) while walking slowly upstream for 30 to 60 min. *Phasmahyla cruzi* Carvalho-e-Silva et al., 2009 was also recorded with visual survey for tadpoles, which are easy to identify and conspicuous in stream ponds. We did not use tadpole records for other species because of their cryptic nature and difficulty in species identification.

The time gap between our traditional and eDNA surveys could be a potential problem in our study design if populations and communities of our streams vary significantly over time. However, we sampled these streams for 20 days in July 2001, every month from February 2006 to January 2011, every two months from March 2011 to December 2011, as well as during the four days we sampled for eDNA in April 2015. The average encounter rate of individuals of at least six species commonly found in these streams [*Cycloramphus boraceiensis* Heyer, 1983, *Hylodes asper* (Müller, 1924a), *H. phyllodes* Heyer and Cocroft, 1986, *Phasmahyla cruzi*, *Vitreorana uranoscopa* (Müller, 1924b), *Ololygon trapicheiroi* (Lutz and Lutz, 1954), and *Thoropa taophora* (Miranda-Ribeiro, 1923)] remained constant during this entire period (Ruggeri et al., 2015; M.M. unpublished data). Based on these observations we infer that the frog communities of these streams did not change considerably from the time of our traditional surveys to that of our eDNA survey.

2.3. Detection protocol for eDNA

Collections of eDNA samples were performed in a single four day period (22–25 April 2015; one stream each day). Air and water temperatures remained relatively constant during the sampling period. No sampling was performed during the hours following occasional rain showers. We followed the protocols described in Lopes et al. (2017) for eDNA sampling, DNA extraction, PCR amplification, DNA purification, next-generation sequencing, and bioinformatics analyses. Briefly, we filtered water at two locations per stream within the same 95–115 m transects in which traditional surveys were performed. Sampling point 1 was located at the beginning of the transect (~0 m) and sampling point 2 was located at the end of the transect (~100 m). To avoid water contamination and perturbation, sampling was first performed at sampling point 1 and proceeded upstream to sampling point 2. At each location we filtered 60 L of water directly from the water column through a disposable capsule (Envirochek HV 1 μm, Pall Corporation, Ann Arbor, MI, USA) using a peristaltic pump (1.60 L min^{-1}, model 410, Solinst, Canada). We also filtered 5 L of distilled water to serve as negative control and assess possible sources of contamination from handling procedures in the field. Capsules were filled with 150 mL of buffer solution (Tris-HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M, and *N*-lauroyl sarcosine 1%, pH 7.5–8) to prevent DNA degradation and stored at room temperature until processing in the laboratory.

DNA extraction and PCR procedures were done at SPYGEN (Le Bourget du Lac, France) following previously published extraction and amplification protocols (Lopes et al., 2017). A negative DNA extraction control was added to monitor possible laboratory contamination. Briefly, a short fragment (~52 bp) of the 12S rRNA mitochondrial gene

was amplified using the batra_F (5′-ACACCGCCCGTCACCCT-3′) and batra_R (5′-GTAYACTTACCATGTTACGACTT-3′) primers (Valentini et al., 2016). Blocking primers for human DNA (batra_blk, 5′-TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I-3′) (Valentini et al., 2016) were used to avoid undesired amplification and increase the specificity of the amplicons (Wilcox et al., 2014). We replicated each DNA extract in 12 independent PCR reactions. One negative PCR control containing ultrapure water was analyzed in parallel with the samples. Pair-end sequencing (2 × 125 bp) was carried out using an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) with HiSeq SBS Kit v4 (Illumina, San Diego, CA, USA), following the manufacturer's instructions at Fasteris facilities (Geneva, Switzerland).

2.3.1. Species reference database

To identify species whose eDNA was collected in water samples, we used the 12S rRNA mitochondrial reference database of anuran species described in Lopes et al. (2017), using the programs ecoPCR (Ficetola et al., 2010) and OBITools (Boyer et al., 2016). The reference database was composed of (i) a local database with 12S sequences for 36 anuran species, from the 44 known to occur at Núcleo Picinguaba, based on Hartmann's (2004) amphibian community list for this region and (ii) anuran species sequences from the release vrt124 of the European Molecular Biology Laboratory (EMBL) database, also corresponding to the metabarcode region. From both the EMBL sequences and the ones we generated ourselves, we identified and extracted the metabarcode sequence between the batra F and batra R primers, and imported those fragments in to the reference database. We accepted metabarcode sequences that were 20–100 base pairs in length and allowed 2 bp errors per primer. We retained 3425 sequences from 47 anuran families from the EMBL database (see Lopes et al., 2017 for more details on methodology).

2.4. eDNA data analyses

Sequences resulting from the water samples were filtered and annotated using custom pipelines in OBITools (Boyer et al., 2016) and R (v 3.1.3; R Development Core Team, 2015). We detected and eliminated sequences not properly identified by the primers (only 2 bp mismatches per primer allowed) and molecular tags (no mismatch allowed). Only sequences having a length longer than 20 bp and more than ten read counts were kept. We then assigned sequences to a corresponding taxon based on sequence similarity with the species reference database. To avoid errors from PCR and sequencing procedures, we discarded sequence records with frequency below 0.001. We excluded sequences potentially coming from cross-contamination sources with frequency below 0.003 per sample. We kept only sequences with high identity percentage (i.e. that match with > 96% to the sequences from the reference database). We considered a species present if at least one of the 12 PCR replicates showed positive detection (see Lopes et al., 2017 for more details on methodology).

Sequence conflict occurred for six species in the complete reference database due to mislabeled sequences in the EMBL database. Identical sequences for *Thoropa miliaris* (Spix, 1824) and *T. taophora* were accessioned before the recent taxonomic revision of the group (Feio et al., 2006). In this case, we assumed the query sequence to be *T. taophora* because *T. miliaris* does not occur in the study area. Similarly, taxonomic ambiguity affected species assignment to *Phasmahyla guttata* (Lutz, 1924), *P. cruzi*, *Bokermannohyla hylax* (Heyer, 1985) and *Bokermannohyla* sp. (aff. *circumdata*), taxa which lack recent taxonomic revision. In both cases, ambiguous sequences were assigned to the latter taxon, which are known to inhabit the study area.

2.5. Community-level data analyses

We quantified the constancy of each species in each stream in monthly traditional samplings, using the formula $C = P \times 100 / N$ (Silveira-Netto et al., 1976), where: P = number of months a certain species was detected; N = total number of months (all months pooled across the 5-year traditional surveys; here N = 12). We pooled the yearly data by month because the species detected in our stream varied largely in frequency, either due to detectability or species-specific densities. Some species were closely associated with the streams and easily sampled [e.g. *Cycloramphus boraceiensis*, *Hylodes asper*, *H. phyllodes*, *Ololygon trapicheiroi*, *Phasmahyla cruzi*, and *Vitreorana uranoscopa*], and other species were detected only occasionally [visually or by their calls; *Bokermannohyla* sp. (aff. *circumdata*), *Dendropsophus minutus* (Peters, 1872), *Hypsiboas albomarginatus* (Spix, 1824)]. The constancy index (C) ranges from 0 to 100%. Based on the constancy index, the species were classified as: (i) constant species, if present in 7 months or more (C > 50%), (ii) occasional species, if present in 4 to 6 months (C = 33–50%), (iii) accidental species, if present in 1 to 3 months (C = 8.3–25%), and (iv) absent species, if the species was never recorded (C = 0%; cf. Silveira-Netto et al., 1976). We also compiled species composition only for the month of April pooled across the 5 years of traditional survey, the same month that environmental DNA samplings were performed. Additionally, to allow for variation in climatic conditions in different years, and consequently, seasonal changes in community composition, we also compared eDNA results with data summed from the months of March, April, and May pooled across 5 years. We used linear regressions to assess the relationship between the constancy index for each species at each stream and the mean proportion of positive PCR replicates for each species obtained at two sampling points at each stream.

We used field observations and searched the literature to characterize the habitat of all life cycle stages for each species found in the study area. We classified egg, larval, and adult habitats as non-aquatic or ponds far (> 20 m) from streams (attributed a value = 0), ponds adjacent (< 20 m) to streams (attributed a value = 1), or along or within the watercourse of the stream (attributed a value = 2; modified from Lips et al., 2003). We summed the value attributed to each life stage habitat to calculate a riparian habitat index (HI) for each species (modified from Lips et al., 2003). The HI varies from 0 to 6 and estimates the degree of contact of a species with a riparian environment, and was used as a proxy for the expected shedding of DNA into the stream water we sampled. We tested for correlation between the HI (including only species with HI > 0, i.e., those with at least one life stage occurring adjacent or within streams) and the mean of the proportion of positive PCR replicates at the two sampling points from each stream using Spearman's rank correlation.

We quantified species composition similarity at each stream using Jaccard's index (*S*) between eDNA metabarcoding and traditional survey results obtained during: (i) all months sampled, (ii) the months of March, April, and May, and (iii) only the month of April. As we expect to detect primarily riparian species sampling aquatic eDNA, we calculated *S* taking into account only species with HI > 0. Jaccard's index is based on presence/absence data and higher values of *S* indicate higher similarity in species composition (Krebs, 1998). All analyses were performed in R (v 3.1.3; R Development Core Team, 2015).

3. Results

During five years of traditional surveys at the four focal streams we found a total of 19 amphibian species representing 11 families and 17 genera (Table 1). Ten of the 19 species found have at least one life stage occurring adjacent to or within streams (HI > 0; hereafter stream species; Table 1). The remaining nine species have no association with streams (HI = 0; hereafter non-stream species; Table 1). Among stream species, the riparian habitat index ranged from 1 to 6 (Table 1).

We detected anuran DNA at all eight eDNA sampling points. All field, DNA extraction, and PCR negative controls turned out negative after the bioinformatics filtering protocol. We detected DNA of nine of the ten stream species recorded by traditional surveys (Table 2). In

Table 1
Coded habitat of eggs, tadpoles, and adults, and riparian habitat index (HI) for all species found through traditional surveys (from 2007 to 2011). 0 = Non-aquatic habitat or ponds far (> 20 m) from streams, 1 = ponds adjacent (< 20 m) to streams, and 2 = along or within the watercourse of the stream.

Species	Egg	Tadpole	Adult	HI
Brachycephalidae				
Ischnocnema sp. (aff. *guentheri*)	0	0	0	0
Bufonidae				
Dendrophryniscus brevipollicatus Jiménez de la Espada, 1870	0	0	0	0
Rhinella ornata (Spix, 1824)	0	0	0	0
Centrolenidae				
Vitreorana uranoscopa (Müller, 1924b)	0	2	2	4
Craugastoridae				
Haddadus binotatus (Spix, 1824)	0	0	0	0
Cycloramphidae				
Cycloramphus boraceiensis Heyer, 1983	2	2	2	6
Thoropa taophora (Miranda-Ribeiro, 1923)	2	2	0	4
Hylidae				
Aplastodiscus eugenioi (Carvalho-e-Silva and Carvalho-e-Silva, 2005)	0	1	0	1
Bokermannohyla sp. (aff. *circumdata*)	0	1	0	1
Dendropsophus minutus (Peters, 1872)	0	0	0	0
Hypsiboas albomarginatus (Spix, 1824)	0	0	0	0
Ololygon perpusilla (Lutz and Lutz, 1939)	0	0	0	0
Ololygon trapicheiroi (Lutz and Lutz, 1954)	2	2	2	6
Hylodidae				
Hylodes asper (Müller, 1924a)	2	2	2	6
Hylodes phyllodes Heyer and Cocroft, 1986	2	2	2	6
Leptodactylidae				
Physalaemus atlanticus Haddad and Sazima, 2004	0	0	0	0
Microhylidae				
Chiasmocleis sp.	0	0	0	0
Odontophrynidae				
Proceratophrys belzebul Dias et al., 2013	2	2	0	4
Phyllomedusidae				
Phasmahyla cruzi Carvalho-e-Silva et al., 2009	0	2	2	4

contrast, we did not detect DNA of any non-stream species (Table 2). All species detected through eDNA metabarcoding were observed at least in one month of traditional survey. For four species, we detected DNA in those streams where they were always constant during traditional surveys (*Cycloramphus boraceiensis*, *Hylodes asper*, *H. phyllodes*, and *Phasmahyla cruzi*; Table 2). *Thoropa taophora* was also constantly found in four streams, but its DNA was only detected in streams 1 and 4 (Table 2). For species found at lower constancies, we failed to detect their DNA in some of the streams where they were observed during traditional surveys (Table 2). Yet, we were able to detect DNA of two species [*Aplastodiscus eugenioi* (Carvalho-e-Silva and Carvalho-e-Silva, 2005) in stream 3 and *Bokermannohyla* sp. (aff. *circumdata*) in stream 2], that were accidental during traditional surveys (Table 2; see Table A1 for complete constancy values for each species).

The proportion of positive PCR replicates ranged from 0.08 (1/12 PCR replicates) to 1.0 (12/12 PCR replicates). The constancy index explained approximately one third of the variation in eDNA detection (i.e., proportion of positive PCR replicates) ($r^2 = 0.3133$, $p < 0.01$) (Fig. 1a). The HI was positively correlated with eDNA detection ($\rho = 0.7604$, $p \ll 0.001$) (Fig. 1b). Three species (*Cycloramphus boraceiensis*, *Hylodes asper*, and *H. phyllodes*) with the highest constancy index and the highest HI also had a high proportion of positive detection among eDNA samples.

Although the combined richness of the four streams (10 stream species) was almost completely retrieved by eDNA metabarcoding (9 stream species), the species richness retrieved for each stream through eDNA metabarcoding was lower than that estimated based on the 5-year traditional survey (Table 2), likely because our eDNA sampling occurred in only one sampling period of four days. Similarity between species composition described by eDNA metabarcoding and by traditional surveys varied from 0.40 to 0.75 when taking into account all months sampled (Table 3). In stream 1, we detected DNA from five of seven stream species observed in traditional survey ($S = 0.71$ for all months sampled). The two species not detected in eDNA metabarcoding results were accidental species [*Bokermannohyla.* sp. (aff. *circumdata*) and *Proceratophrys belzebul* Dias et al., 2013], which were not present in stream 1 from March to May. When taking into account species composition from March to May, all species observed in traditional survey of stream 1 were detected through eDNA metabarcoding ($S = 1$). On the other hand, species composition described by eDNA metabarcoding had low similarity with traditional survey results in stream 2 for all time intervals. In stream 2, we were unable to detect DNA from *Aplastodiscus eugenioi*, *Ololygon trapicheiroi* and *Thoropa taophora*, three species commonly recorded in traditional survey. In stream 3, we detected DNA of six stream species, including an accidental species (*A. eugenioi*) that does not typically use high-gradient stream habitats (HI = 1), and was not observed in the traditional survey from March to May, but observed only once in June. On the other hand, we were unable to detect DNA from *T. taophora* and *Vitreorana uranoscopa*, two stream species (HI = 4) commonly observed in traditional survey in stream 3. In stream 4, the similarity (S) observed was not 1 at any time interval because we failed to detect DNA from an accidental species not closely associated with streams (*Bokermannohyla* sp. (aff. *circumdata*),

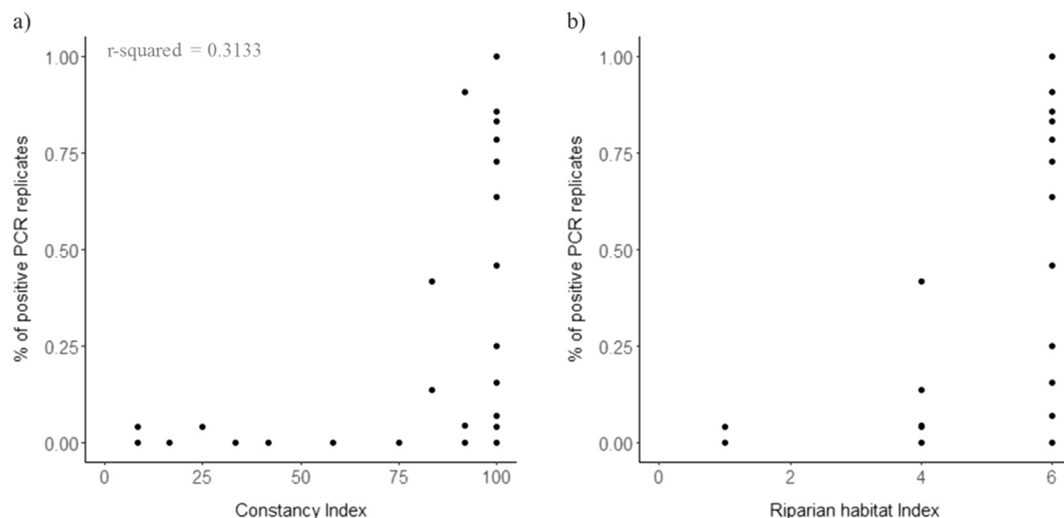
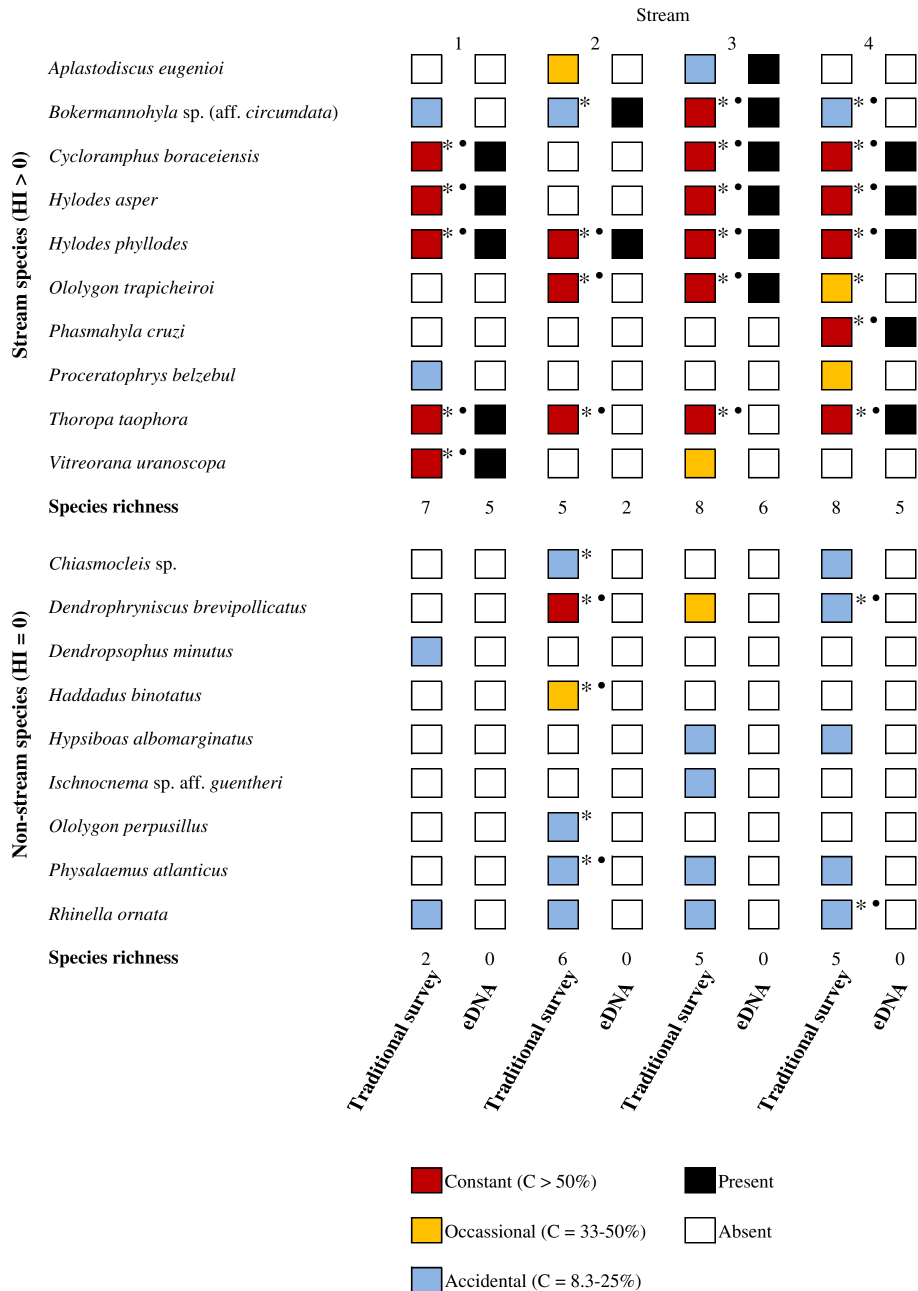


Fig. 1. Relationship between mean proportion of positive PCR replicates and: a) Constancy index calculated for all months sampled by traditional survey methods, b) Riparian habitat index (HI).

Table 2
Stream species found through traditional survey (compiling all 55 months of sampling from 2007 to 2011) and eDNA survey in April 2015 in each stream. * indicates species present from March through May pooled across 5 years of traditional survey, and • indicates species present in April pooled across 5 years of traditional survey.



	Stream 1 Traditional survey	Stream 1 eDNA	Stream 2 Traditional survey	Stream 2 eDNA	Stream 3 Traditional survey	Stream 3 eDNA	Stream 4 Traditional survey	Stream 4 eDNA
Stream species (HI > 0)								
Aplastodiscus eugenioi	Absent	Absent	Occassional	Absent	Accidental	Present	Absent	Absent
Bokermannohyla sp. (aff. *circumdata*)	Accidental	Absent	Accidental *	Present	Constant * •	Present	Accidental * •	Absent
Cycloramphus boraceiensis	Constant * •	Present	Absent	Absent	Constant * •	Present	Constant * •	Present
Hylodes asper	Constant * •	Present	Absent	Absent	Constant * •	Present	Constant * •	Present
Hylodes phyllodes	Constant * •	Present	Constant * •	Present	Constant * •	Present	Constant * •	Present
Ololygon trapicheiroi	Absent	Absent	Constant * •	Absent	Constant * •	Present	Occassional *	Absent
Phasmahyla cruzi	Absent	Absent	Absent	Absent	Absent	Absent	Constant * •	Present
Proceratophrys belzebul	Accidental	Absent	Absent	Absent	Absent	Absent	Occassional	Absent
Thoropa taophora	Constant * •	Present	Constant * •	Absent	Constant * •	Absent	Constant * •	Present
Vitreorana uranoscopa	Constant * •	Present	Absent	Absent	Occassional	Absent	Absent	Absent
Species richness	7	5	5	2	8	6	8	5
Non-stream species (HI = 0)								
Chiasmocleis sp.	Absent	Absent	Accidental *	Absent	Absent	Absent	Accidental	Absent
Dendrophryniscus brevipollicatus	Absent	Absent	Constant * •	Absent	Occassional	Absent	Accidental * •	Absent
Dendropsophus minutus	Accidental	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Haddadus binotatus	Absent	Absent	Occassional * •	Absent	Absent	Absent	Absent	Absent
Hypsiboas albomarginatus	Absent	Absent	Absent	Absent	Accidental	Absent	Accidental	Absent
Ischnocnema sp. aff. *guentheri*	Absent	Absent	Absent	Absent	Accidental	Absent	Absent	Absent
Ololygon perpusillus	Absent	Absent	Accidental *	Absent	Absent	Absent	Absent	Absent
Physalaemus atlanticus	Absent	Absent	Accidental * •	Absent	Accidental	Absent	Accidental	Absent
Rhinella ornata	Accidental	Absent	Accidental	Absent	Accidental	Absent	Accidental * •	Absent
Species richness	2	0	6	0	5	0	5	0

Constant (C > 50%); Occassional (C = 33-50%); Accidental (C = 8.3-25%); Present; Absent

Table 3
Jaccard's index (*S*) between species composition from eDNA metabarcoding and traditional-survey results, taking into account only stream species (HI > 0; see Table 1). Similarity in species composition was calculated for each stream and at three time intervals: (i) all months sampled, (ii) the months of March, April, and May, and (iii) only the month of April (same period water samples were filtered for eDNA).

Stream	All months	March + April + May	April
1	0.71	1.00	1.00
2	0.40	0.50	0.25
3	0.75	0.71	0.71
4	0.62	0.71	0.83

HI = 1), and from two occasional species (*O. trapicheiroi* and *P. belzebul*), closely associated with streams (HI ≥ 4), but which were never observed in April of any year, period when we sampled for eDNA (Table 2).

4. Discussion

Our study showed that eDNA metabarcoding is a reliable method for detecting a large proportion of the amphibian communities of stream frogs in the Atlantic forest, but it is not perfect. Remarkably, with a single four-day sampling we detected DNA of all stream species found across all four streams during our five-year traditional survey, with the exception of a single uncommon species (*Proceratophrys belzebul*). However, at a finer within-stream scale, a single eDNA sample failed to

detect species that are known to occur at those sites (Table 2). Despite these differences, our eDNA results highlighted the differential use of stream habitats by amphibians, the advantages of sampling different streams in a given region for a better assessment of species richness and community composition, and the ability of eDNA analysis to detect elusive species that are difficult to find during traditional surveys.

Overall, species composition detected by eDNA metabarcoding in each stream showed high similarities to that obtained with traditional surveys, especially when considering only constant species. With a few exceptions, species frequently recorded through traditional survey were reliably detected in eDNA metabarcoding results (Table 2). The exceptions were mainly due to the non-detection of species recorded only once or twice, and mainly in stream 2, the smallest stream that dries out in some years, reducing its use by some species or even hindering the eDNA efficacy. Estimates of species composition by the two approaches also differed because eDNA detected rare species not recovered in traditional surveys during the same time of year. The identification of rare species with eDNA metabarcoding may reflect the fact that life stages not quantified in traditional surveys (e.g., tadpoles or eggs) contributed to DNA available in the water. Alternatively, these species might be present in the region, but occurring further upstream, too far from our transect to be detected by traditional surveys, yet close enough to contribute with detectable eDNA in our downstream sampling points (Deiner and Altermatt, 2014).

Our single four-day eDNA sampling detected almost all (nine of ten) stream species found during the long-term, time and labor-expensive traditional survey. In a single eDNA sampling, we were able to detect rare and elusive species. For instance, in stream 3, we detected DNA of *Aplastodiscus eugenioi*, which was recorded only once during five years of traditional surveys at this stream. Previous studies have also compared the efficacy of eDNA analysis with traditional survey results for detecting community composition. Thomsen et al. (2012) showed that eDNA analysis recovered fish diversity from seawater samples better than or equal to nine other traditional methods. The idea that eDNA analysis is equally or more efficient than traditional surveys is becoming commonplace (Roussel et al., 2015), although this is based on just a few comparative studies where species abundance is not always reported.

We found a strong positive relationship between eDNA detection and the habitat index (HI), which estimates the degree of contact of a species at different life stages with lotic waters, confirming that specific frog life cycle characteristics are important to consider in eDNA detection. In the Brazilian Atlantic forest, frogs show a great diversity of reproductive modes and life histories, with species exploiting a diversity of humid habitats at different stages in their life cycle (Haddad and Prado, 2005). For instance, adults of *Aplastodiscus eugenioi* are arboreal, their eggs are deposited in subterranean constructed nests, and the tadpoles develop in ponds near streams or in streams (HI = 1; Hartmann et al., 2010). Adults of *Vitreorana uranoscopa* (HI = 4) and *Phasmahyla cruzi* (HI = 4) are also arboreal and their eggs are laid on leaves overhanging streams, where tadpoles drop to feed and complete development (Costa et al., 2010; Hartmann et al., 2010). Eggs and tadpoles of *Ololygon trapicheiroi*, however, can be found in isolated ponds adjacent to streams, which connect with the main stream flow during heavy rains, and calling males are mainly found on trees and shrubs along streams (HI = 6; Hartmann et al., 2010; M. Martins, pers. obs.). Postmetamorphic individuals and tadpoles of *Thoropa taophora* may be found far from streams, but always close to or on wet rocks along streams, while their eggs are semiterrestrial (HI = 4; Hartmann et al., 2010), which may decrease the chances of finding DNA in water samples in streams in which we know they occur. Unlike the species loosely associated with streams (e.g., *A. eugenioi*), *Cycloramphus boraceiensis*, *Hylodes asper*, and *H. phyllodes* use lotic waters as their primary habitat across life stages and adult individuals can be found on rocks along streams (HI = 6 for the three species). *Hylodes asper* and *H. phyllodes* lay their eggs in subaquatic chambers and tadpoles develop in streams, while eggs of *C. boraceiensis* are semiterrestrial, with tadpoles living on wet rocks along streams (Hartmann et al., 2010). Not surprisingly, the latter three species were detected in higher proportions in eDNA samples. As expected, all sampled eDNA matched amphibian species with at least one phase of its life cycle associated with streams, and we did not recover DNA from exclusively terrestrial species with direct development or from those that breed in ponds away from streams. Thus, recovery of eDNA shed in water is tightly connected with amphibian life history traits.

We found a relatively weak relationship between eDNA detection and constancy index; nonetheless, some species with the highest constancy values had higher number of positive PCR replicates. For example, common species such as *Cycloramphus boraceiensis*, *Hylodes asper*, and *H. phyllodes* that are continuous breeders (Hartmann, 2004; Ruggeri et al., 2015) were found throughout the year, had high constancy values, and also the highest proportions of eDNA positive replicates. The DNA of these species may be released in the environment constantly and in higher amounts. In the case of more seasonal species, such as *Ololygon trapicheiroi* and *Vitreorana uranoscopa* (Hartmann, 2004), tadpoles are present during many months in the water and are likely the main contributors of eDNA to water samples, but possibly in lower constancy and quantity.

Environmental DNA analyses have primarily been applied in temperate regions (Hoffmann et al., 2016), despite the fact that tropical streams host complex and often more diverse communities that can differ at very small geographic scales, as illustrated by our traditional survey results. Our study is the first effort to apply eDNA metabarcoding from aquatic samples to characterize frog communities in the Brazilian Atlantic forest, which harbors high anuran richness. Additionally, few studies to date have used eDNA from freshwater systems to detect community assemblages and to identify multiple species from one sample. Minamoto et al. (2012) were the first to report the detection of multiple vertebrate species by amplifying fish eDNA with degenerate primers in Japan. Evans et al. (2016) later measured the species richness of fish and amphibians using eDNA metabarcoding in experimental conditions and showed that some primers can accurately identify species assemblages with differing species densities. Kelly et al. (2014) and Shaw et al. (2016) accurately characterized fish communities in controlled aquatic settings and in rivers, respectively, using primers for vertebrate-specific fragments from mitochondrial genes. Finally, Valentini et al. (2016) surveyed amphibians and fishes in a wide range of aquatic ecosystems in Europe. Here, we demonstrated the value and shortcomings of eDNA detection approach for monitoring vertebrate communities in a tropical environment. Our sampling of eDNA over a single four-day period was able to characterize anuran communities and identify amphibian assemblages with differing species composition, which included elusive species and even some species not tightly associated with streams.

One challenge of working with tropical amphibians is that some species are poorly represented in public sequence databases and taxonomic errors in online databases can compromise the assignment of eDNA sequences (Hoffmann et al., 2016). Shaw et al. (2016), for instance, could not detect some fish taxa from freshwater eDNA samples using 12S or 16S rRNA mitochondrial primers due to a lack of reference data in the NCBI database. We circumvented this situation by using a local sequence reference database using specimens collected in the region of our study site (Lopes et al., 2017). This is an important stage in bioinformatics analyses, and whenever possible, future studies using eDNA should also count on a local sequence reference database to improve eDNA results, although this demands previous knowledge of species composition in the study region. General knowledge of species occurrence in the study region is also fundamental to avoid mistaken identifications. Another concern when working with amphibians from Atlantic forest is that the knowledge of species occurrence is still incomplete, with cases of taxonomic uncertainty and cryptic undescribed species. Therefore, special caution must be taken when interpreting

eDNA metabarcoding results for community studies.

5. Conclusions

Freshwater ecosystems are essential for a large percentage of the world's amphibian species (e.g., Stuart et al., 2008). Despite their value, riparian habitats are being severely disturbed and declines in freshwater biodiversity are far greater than in terrestrial ecosystems (Dudgeon et al., 2006). The conservation management required is limited by the paucity of rigorous species occurrence data or the difficulty in surveying species in nature. In light of the added sensitivity of next-generation sequencing technology, eDNA metabarcoding is becoming an important tool for addressing practical problems in vertebrate conservation, such as monitoring communities and endangered species or filling knowledge gaps of Data-deficient species. Here we investigated the possible use of eDNA analysis as an additional tool for understanding community composition within streams networks. Given the high richness and endemicity of Atlantic forest amphibians, concomitant with the high degree of endangerment (Gascon et al., 2007; Stuart et al., 2008; Verdade et al., 2012; ICMBio, 2016c), our study showed that eDNA metabarcoding can be a reliable tool to assess amphibian community diversity in streams. Thus, eDNA improves the "ecology toolbox" and will likely enhance conservation efforts of Neotropical amphibians.

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