Trabajo

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DNA barcoding in Neotropical tadpoles: evaluation of 16S rRNA gene for the identification of anuran larvae from northeastern Brazil

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ABSTRACT
The challenge in studying Neotropical tadpoles is identifying species using only their external morphology. However, the DNA barcode protocol is often implemented to help elucidate taxonomic issues. In fact, the identification of frogs through their unknown tadpoles has already been achieved accurately using this protocol. Despite the successful application of this tool, the efficiency of the 16S rRNA gene as a DNA barcode for Neotropical tadpoles has not been fully assessed. Herein we evaluate the efficacy of the 16S rRNA gene for identifying tadpoles from northeastern Brazil. Samples of 100 tadpole specimens from 12 locations were analyzed. The DNA sequences were individually submitted to a BLAST search and were then aligned with a matrix containing available sequences in the GenBank based on the anurans known to occur in the study area. The 16S rRNA fragment successfully identified the analyzed anuran species. Based on DNA barcoding, 8% of the tadpoles morphologically identified at the species level were incorrect. When an incongruence between morphological and molecular identifications was detected, the morphology of the target morphotype was reexamined, and previously neglected morphological characteristics were identified. DNA barcoding using the 16S rRNA gene facilitated the assessment of tadpole richness in northeastern Brazil. This DNA protocol can be used as a starting point for detecting high levels of genetic divergence, highlighting potential taxa that should be studied from phylogenetic and taxonomic perspectives.

Key words: Amphibia; Mitochondrial Gene; Genetic Divergence; Species Diversity.

Introduction

Anuran amphibians generally have an aquatic larval phase, representing an important trophic component of aquatic environments (Ranvestel *et al.*, 2004; Rossa-Feres *et al.*, 2004; Jordani *et al.*, 2019). During certain periods, these tadpoles are the only evidence of anuran occurrence in some environments. Tadpoles are relatively abundant where they occur, and they are also easy to collect. However, for a long time, tadpoles were neglected by naturalists and researchers, especially in megadiverse assemblages such as tropical regions (Provete *et al.*, 2012; Rossa-Feres *et al.*, 2015). Tadpoles have only recently started to be included more frequently in faunistic inventories and ecological, systematic, and taxonomic studies (Haas, 2003; Larson, 2005; Silva, 2010; Magalhães *et al.*, 2013; Dubeux *et al.*, 2020a). In fact, the tadpoles of a large portion of anuran species are unknown (Altig and McDiarmid, 1999; e.g., Provete *et al.*, 2012; Schulze *et al.*, 2015; Altig *et al.*, 2021). This is an alarming scenario, considering

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that amphibians are the most threatened vertebrate group with known declining or extinct populations (IUCN, 2022). Additionally, many species will likely become extinct without formal descriptions or identification of their tadpoles (Crawford *et al.*, 2010). In Brazil, the country with the highest worldwide anuran diversity (1,144 species; Segalla *et al.*, 2021), the tadpoles of about half of identified species are unknown (Provete *et al.*, 2012).

One significant challenge when studying tadpoles is species identification. When using only external morphology, species identification can be hampered by 1) the lack of knowledge of larval diagnostic characteristics (very similar morphologically or non-described tadpoles), 2) the absence of standardization in the nomenclature used for tadpole descriptions, or even 3) the scarcity of identification keys (Provete *et al.*, 2012; Dubeux *et al.*, 2020b). Until recently, the accurate identification of tadpoles was only possible in captivity, where tadpoles were held until they completed their metamorphosis, thereby allowing for the identification of species through their juveniles. However, this introduces the logistical challenge of reproducing environmental characteristics that mirror the natural environment where tadpoles are collected.

Currently, the integration of a molecular approach using the DNA barcode as a protocol has been promising in terms of facilitating tadpole identification (e.g., Vences *et al.*, 2005b; Grosjean *et al.*, 2015; Schulze *et al.*, 2015). Although the original DNA barcoding protocol was used for identifying the information contained in a fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene, in order to accelerate taxonomic descriptions for amphibians in general (Hebert *et al.*, 2003, 2005), the barcode procedure using the mitochondrial 16S rRNA gene has been used in studies worldwide, due to its ease of use and high amplification success rate in different laboratory conditions (Vieites *et al.*, 2009; Vences *et al.*, 2005a, b; Lyra *et al.*, 2017; Koroiva *et al.*, 2020). The information in the 16S rRNA fragment and the analysis of genetic similarity has been confirmed as an accurate method for identifying frog species, including their tadpoles. This approach has already been employed globally and has accurately identified tadpoles from Madagascar, Spain, Bolivia, and Southeast Asia (Thomas *et al.*, 2005; Vences *et al.*, 2005b, b; Grosjean *et al.*, 2015; Schulze *et al.*, 2015).

Despite the efficient application of the DNA barcode tool in refining and accelerating the process of anuran identification, few studies have investigated the efficiency of the 16S rRNA gene as a molecular identification tool in Neotropical tadpoles (e.g., Schulze *et al.*, 2015). Herein we evaluated the effectiveness of the 16S rRNA gene for the identification of specific and generic anuran taxa from northeastern Brazil, using their tadpoles. We assessed the rate of precise morphological identifications and discussed the challenges of the identification of tadpoles in megadiverse assemblages such as Brazilian ecoregions. Additionally, we included some comments on cryptic diversity in the local anurofauna based on genetic divergences and recent phylogenetic hypotheses.

Materials and methods

Sampling and DNA extraction

We used caudal musculature samples from 100 tadpoles obtained from 12 locations in the Caatinga and Atlantic Forest ecoregions in the state of Alagoas, northeastern Brazil (see Appendix I for more information about the locations). All the tadpoles were collected from 2013 to 2018 (licenses: SISBio/ICMBIO 32920, 33507; CEUA 36/2015; SISGEN A6E0CAC). Firstly, we morphologically separated the tadpoles into 51 different morphotypes. Eighteen morphotypes were only identified at the generic level and two at the family level. All tadpoles were morphologically allocated to eight families. Between one and three representatives of each morphotype were preserved in 92% alcohol, and the remaining specimens were preserved in 10% formalin. All specimens were incorporated into the Coleção Herpetológica do Museu de História Natural of Universidade Federal de Alagoas (MHN-UFAL).

The total genomic DNA was extracted from a fragment of tadpole caudal musculature from each morphotype using the Phenol-Chloroform method (Sambrook *et al.*, 1989) or by performing DNA extraction with salts (DNA Precipitation NaCl; Bruford *et al.*, 1992), and stored in 15 µl of autoclaved distilled water.

Polymerase chain reaction and sequencing

A fragment of 550 base pairs (bp) of the 16S rRNA mitochondrial gene from each sample was amplified using the forward primer 16Sar: CGC CTG TTT ATC AAA AAC AT and reverse primer 16Sbr: CCG GTC TGA ACT CAG ATC ACG T (Palumbi *et al.*, 2002) through the polymerase chain reaction

(PCR). Each PCR reaction had a final volume of 25 µl, comprised of 21.4 µl of MixMaster PCR, 0.8 µl of each primer, and 2 µl of DNA (20–100 ng/µl). The thermal cycling conditions followed an initial denaturation of 94° C (1–3 minutes), and 35 cycles of denaturation at 94° C for 45 seconds, pairing at 55° C for 45 seconds and extension at 72° C for one and half minutes. The PCR reactions were stained with syber safe, and electrophoresis in 1% agarose gel was performed to check for the presence of amplicons. The reactions were then visualized on the translumminator with ultraviolet light. The PCR products were purified with isopropanol and sequenced unidirectionally using the Sanger method after the Big Dye® terminator reaction.

Data analyses

The quality of the obtained sequences was checked and edited if necessary, using the software BioEdit Version 7.2.5 (Hall, 2011). Initially, in order to flag potential errors in tissue taxonomic labeling or DNA contamination, we performed Basic Local Alignment Search Tool (BLAST) analyzes on GenBank online platform. This analysis finds regions of local similarity between our samples and the sequences available in the repository database and calculates the statistical significance of the matches. We used the taxonomic identification of the most similar sequences in the database as a starting point to validate the identification of our samples. Each sample in our dataset was analyzed independently. The GenBank sequence with the greatest total similarity and the percentage of similarity is shown in Table 1. When an incongruence between morphological and molecular identifications was detected, the morphology of the target morphotype was reexamined.

We then calculated genetic divergence between our samples and a selection of sequences obtained from the GenBank (selection criteria are presented below) using evolutionary models. This analysis aimed to compare our samples with: (1) conspecific samples of adult specimens collected in the study area or in geographically close areas, in order to associate tadpoles with their adult counterparts; (2) conspecific topotypical samples or samples from areas that are geographically close to the type locality of the nominal taxon, to obtain a preliminary assessment of the taxonomic status of populations in the study area (considering that nominal species with highly divergent populations may present an undescribed diversity) and, (3) sequences with greater total similarity in the BLAST analysis, to validate the taxonomic identification obtained through this analysis.

Our sequences were aligned using the software MAFFT Version 7.310, implementing the iterative refinement method (automatic strategy selection) and default parameters (Katoh and Standley, 2013). They were then combined with a matrix containing sequences available in GenBank (see below for more details; Appendix II) of representatives of anuran species previously registered in the study area (following the anuran list available in Almeida *et al.*, 2016). In the matrix, up to three DNA sequences from different localities for each anuran species were included, prioritizing regions close to the study area and the type locality of the species. When sequences were not available for a certain species (last consultation in July 2020), a closely related representative was included (same genus and/or species group, following the current phylogenetic proposals [e.g., Carcerelli and Caramaschi, 1993; Lourenço *et al.*, 2015; Lyra *et al.*, 2020; specifically, *Crossodactylus dantei* (we used *C. caramaschii*), *Physalaemus caete* (we used *P. signifier*) and *Boana exastis* (we used *B. pardalis*)]. Additionally, we added the sequences with the highest total similarity in the BLAST analysis, even if they had not been previously recorded in the study area (considering the possibility of new records and/or error in the labeling of the GenBank sequences). Following alignment, the tails of sequences imported from GenBank were manually trimmed using the software AliView Version 1.27 (Larsson, 2014) in order to eliminate gene fragments that were not of interest. The FFT-NS-i alignment strategy was selected by MAFFT and the final alignment resulted in a 636 base pair matrix.

To estimate genetic divergences, distance estimates were made from the sequence matrix using the Compute Pairwise Distances function implemented with the Kimura-2-parameters evolutionary model (K2P; Kimura, 1980) using the software MEGA X (Kumar *et al.*, 2018). For a graphical visualization of the groupings, a dendrogram was generated using the Neighbor-Joining method (NJ) implemented with the K2P evolutionary model. The groupings obtained were validated using the bootstrap method (Felsenstein, 1985) with 1,000 pseudoreplicates. Bootstrap values above 97% were considered high and are indicated in the dendrogram. The sequences generated in this study were deposited in GenBank (OP022028 – OP022123; Table 1).

Table 1. Molecular identification of tadpole specimens analyzed. Locations, associated vouchers and GenBank accession numbers of our samples. Genetic divergences [GD; in percentage (%)] calculated using the Compute Pairwise Distances function implemented with the Kimura-2-parameters evolutionary model including our samples and sequences from the GenBank. Sequences with the highest similarity, and their respective percentages, in BLAST analysis.

Species genetic identification	Locality (municipality)	Voucher (MHNUFAL)	GenBank access	GD closer to study area	GD closer to type locality	BLAST (GenBank access)
Aromobatidae						
Allobates olfersioides	Teotônio Vilela	12465-1	OP022079	0	12.18	99.4 (KU495121)
Bufonidae						
Rhinella diptycha	Traipu	13874	OP022111	0	-	99.7(MH004313)
Rhinella diptycha	Arapiraca	13883	OP022116	0	-	100 (GU178784)
Rhinella diptycha	Arapiraca	13885	OP022117	0	-	99.7(MH004313)
Rhinella diptycha	Traipu	13897	OP022121	0	-	99.7 (DQ415572)
Rhinella granulosa	Junqueiro	12462	OP022077	0.51	1.02	99.6 (KP685207)
Rhinella granulosa	Arapiraca	13877	OP022112	0.51	1.02	99.8 (KP685207)
Rhinella granulosa	Batalha	13882	OP022115	0.51	1.02	100 (KP685206)
Rhinella granulosa	Batalha	13887	OP022118	0.51	1.02	99.8 (KP685205)
Rhinella hoogmoedi	Murici	12502	OP022108	1.02	1.54	99.4(KU495521)•
Hylidae						
Boana albomarginata	Limoeiro de Anadia	13901	OP022122	0	2.57	99.3 (AY549316)
Boana albomarginata	Maceió	12259	OP022070	0	2.57	99.8 (AY549316)
Boana albomarginata	Limoeiro de Anadia	12482	OP022094	0	2.57	99.8 (AY549316)
Boana albomarginata	Coruripe	12474	OP022088	0	2.57	99.8 (AY549316)
Boana albomarginata	Barra de Santo Antônio	12497	OP022104	0	2.57	99.8 (AY549316)
Boana aff. *atlantica* ◊	Maceió	12079-1	OP022051	0.51	2.57	99.8 (MK348503)
Boana aff. *atlantica* ◊	Maceió	12079-2	OP022052	0.51	2.57	100 (MK348503)
Boana faber	Limoeiro de Anadia	12478	OP022092	3.62*	3.62*	97.1 (KY002913)
Boana raniceps	Coruripe	12132	OP022055	0	0.51	100 (KU495288)
Boana semilineata	Maceió	11392-1	OP022028	0*	1.54	99.2 (MH004300)
Boana semilineata	Maceió	11392-2	OP022029	0*	1.54	99.2 (MH004300)
Boana semilineata	Maceió	12080-1	OP022053	0*	1.54	99.2 (MH004300)
Boana semilineata	Maceió	12080-3	OP022054	0*	1.54	99.2 (MH004300)
Boana semilineata	Maceió	12249-1	OP022056	0*	1.54	99.2 (MH004300)
Boana semilineata	Maceió	12249-2	OP022057	0*	1.54	99.2 (MH004300)
Corythomantis greeningi	Traipu	13889	OP022119	0	0.51	100 (MW243395)
Dendropsophus branneri	Coruripe	12470	OP022083	0.51	0.51	99.2 (MT503865)
Dendropsophus branneri	Maceió	12252-1	OP022060	0.51	0.51	99.6 (MT503865)
Dendropsophus branneri	Maceió	12252-2	OP022061	0	0	99.8 (MT503865)
Dendropsophus branneri	Maceió	12252-3	OP022062	0	0	99.8 (MT503865)
Dendropsophus sp.1 (aff. *tapacurensis)*	Barra de Santo Antônio	12491	OP022100	3.11	3.11	94.8 (MW026642)
Dendropsophus sp.2 (aff. *decipiens)*	Satuba	11866-1	OP022044	2.05	2.05	97.7 (MT503952)

Dendropsophus sp.2 (aff. *decipiens)*	Satuba	11866-2	OP022045	2.05	2.05	97.9 (MT503952)
Dendropsophus sp.2 (aff. *decipiens)*	Satuba	11866-3	OP022046	2.05	2.05	97.9 (MT503952)
Dendropsophus sp.2 (aff. *decipiens)*	Satuba	11866-4	OP022047	2.05	2.05	97.9 (MT503952)
Dendropsophus sp.2 (aff. *decipiens)*	Satuba	11866-5	OP022048	2.05	2.05	97.9 (MT503952)
Dendropsophus sp.2 (aff. *decipiens)*	Satuba	12499	OP022106	2.05	2.05	97.7 (MT503952)
Dendropsophus aff. *minutus* ◊	Limoeiro de Anadia	13879	OP022114	0	3.09	100 (MK266721)
Dendropsophus aff. *minutus* ◊	Limoeiro de Anadia	13896	OP022120	0	3.09	100 (MK266721)
Dendropsophus aff. *minutus* ◊	Limoeiro de Anadia	13906	OP022123	0	3.09	100 (MK266721)
Dendropsophus aff. *minutus* ◊	Limoeiro de Anadia	12476	OP022090	0	3.09	100 (MK266721)
Dendropsophus aff. *minutus* ◊	Limoeiro de Anadia	12477	OP022091	0	3.09	100 (MK266721)
Dendropsophus aff. *minutus* ◊	Limoeiro de Anadia	12479	OP022093	0	3.09	100 (KJ833161)
Dendropsophus soaresi	Maceió	12251-1	OP022058	0	0	100 (MT503922)
Dendropsophus soaresi	Maceió	12251-2	OP022059	0	0	100 (MT503922)
Scinax similis ▲	Maceió	12256-1	OP022068	1.02	2.06	99.4 (MH206282)
Scinax similis ▲	Maceió	12256-2	OP022069	1.02	2.06	99.4 (MH206282)
Scinax similis ▲	Maceió	12254	OP022067	0.51	1.53	99.6 (MW114955)
Scinax similis ▲	Maceió	12492	OP022101	1.54	2.59	99.4 (MH206282)
Scinax auratus	Maceió	11472-1	OP022034	5.83	5.83	95.2 (MH004316)
Scinax nebulosus	Maceió	12493	OP022102	0	5.3	100 (EU201095)
Scinax nebulosus	Maceió	11426-3	OP022031	0	5.3	100 (EU201095)
Scinax nebulosus	Maceió	11426-2	OP022030	0.51	5.28	99.7 (EU201095)
Scinax nebulosus	Maceió	11472-2	OP022035	0	5.3	99.7 (EU201095)
Scinax nebulosus	Maceió	11473-1	OP022036	0	5.3	100 (EU201095)
Scinax nebulosus	Maceió	11473-2	OP022037	0	5.3	100 (EU201095)
Scinax nebulosus	Limoeiro de Anadia	13900	OP022109	0	5.3	100 (EU201095)
Scinax x-signatus	Coruripe	12469	OP022082	0	0	100 (MW114963)
Scinax x-signatus	Junqueiro	12458	OP022074	0.51	0.51	99.7 (MW114964)
Scinax x-signatus	Maceió	12253-1	OP022063	1.02	1.02	99.0 (MW114963)
Scinax x-signatus	Maceió	12253-2	OP022064	0	0	99.0 (MW114963)
Scinax x-signatus	Maceió	12253-3	OP022065	1.02	1.02	99.0 (MW114963)
Scinax x-signatus	Maceió	12253-4	OP022066	1.02	1.02	99.0 (MW114963)
Leptodactylidae						
Leptodactylus fuscus	Junqueiro	12456	OP022073	0	-	99.6 (AY911275)
Leptodactylus fuscus	Junqueiro	12460	OP022076	0	-	99.6 (AY911275)
Leptodactylus fuscus	Igaci	12486	OP022097	0	-	99.6 (AY911275)
Leptodactylus fuscus	Teotônio Vilela	12467-2	OP022081	0	-	99.6 (AY911275)

Leptodactylus fuscus	Igaci	12487	OP022098	0	-	99.6 (AY911275)
Leptodactylus fuscus	Maceió	12490	OP022099	0	-	99.6 (AY911275)
Leptodactylus macrosternum	Arapiraca	13873	OP022110	1.02	1.02	99.8 (MH004302)
Leptodactylus macrosternum	Coruripe	12475	OP022089	0	0	100 (MH206305)
Leptodactylus aff. *mystaceus* ◊	Maceió	12500	OP022107	0	5.89	99.8 (MT117857)
Leptodactylus natalensis	Teotônio Vilela	12463	OP022078	0	1.02	99.8 (MH004304)
Leptodactylus troglodytes	Junqueiro	12459	OP022075	0	0	99.3 (KM091620)
Leptodactylus troglodytes	Coruripe	12473	OP022087	0	0	99.6 (MT117853)
Leptodactylus vastus	Maceió	11433	OP022033	0	0	100 (KU495368)
Leptodactylus vastus	Maceió	11867-1	OP022049	0.51	0.51	99.6 (KU495368)
Leptodactylus vastus	Maceió	11867-2	OP022050	0.51	0.51	99.6 (KU495368)
Physalaemus cuvieri	Coruripe	12467	OP022080	0	-	99.7 (KP146012)
Physalaemus cuvieri	Coruripe	12471	OP022084	0	-	99.5 (KP146012)
Physalaemus cuvieri	Barra de Santo Antônio	12494	OP022103	0	-	99.7 (KP146012)
Physalaemus cuvieri	Maceió	12260	OP022071	0	-	100 (KP146012)
Pseudopaludicola aff. *mystacalis* ◊	Maceió	11429	OP022032	0	3.13	100 (KJ147044)
Microhylidae						
Dermatonotus muelleri	Arapiraca	13878	OP022113	0	2.57	99.8 (MH004297)
Elachistocleis cesarii	Coruripe	12472-2	OP022086	1.02*	1.02	98.2 (KM509129)
Elachistocleis cesarii	Coruripe	12472-1	OP022085	1.02*	1.02	98.2 (KM509129)
Odontophrynidae						
Macrogenioglottus alipioi	Maceió	11865-1	OP022041	0	0.51	99.6 (FJ685684)
Macrogenioglottus alipioi	Maceió	11865-2	OP022042	0	0.51	99.6 (FJ685684)
Macrogenioglottus alipioi	Maceió	11865-3	OP022043	0	0.51	99.6 (FJ685684)
Macrogenioglottus alipioi	Maceió	12498	OP022105	0	0.51	99.2 (FJ685684)
Phyllomedusidae						
Hylomantis granulosa	Maceió	11864-1	OP022038	0	0.51	100 (GQ366224)
Hylomantis granulosa	Maceió	11864-2	OP022039	0	0.51	100 (GQ366224)
Hylomantis granulosa	Maceió	11864-3	OP022040	0	0.51	100 (GQ366224)
Pithecopus gonzagai	Junqueiro	12455	OP022072	0	0	100 (KM387490)
Pithecopus gonzagai	Igaci	12483	OP022095	0	0	100 (MW158678)
Pipidae						
Pipa carvalhoi	Igaci	12485	OP022096	0	0	100 (MT261652)

Legend of symbols: - Type location not known or not very specific; * Samples that are more than 1000 km away from the locality of interest (study area or taxon type locality); ◊ Lineage known to be distinct from that from the type locality of the taxon, which was assigned the "aff." (see Results and discussion section for more details); ▲ Species never recorded for the study area; ▪ Identified as Rhinella margaritifera (probably a misidentification).

Results and Discussion

The average length of the obtained sequences was 537 base pairs (440–594bp). The 51 analyzed morphotypes were molecularly designated to 33 taxa, representing 15 genera and eight families (Fig. 1). This represents 48% of anuran species with a registered tadpole phase in the study area (Dubeux *et al.*, 2019). In total, 32% (n = 32) of the specimens were only morphologically identified at a generic level, and only 6% (n = 6) at the family level (Fig. 2). Although incomplete, all these supra-specific identifications have been molecularly corroborated.

For the specimens that were morphologically identified at the specific level, misidentifications were made both at specific (2%, n = 2) and generic

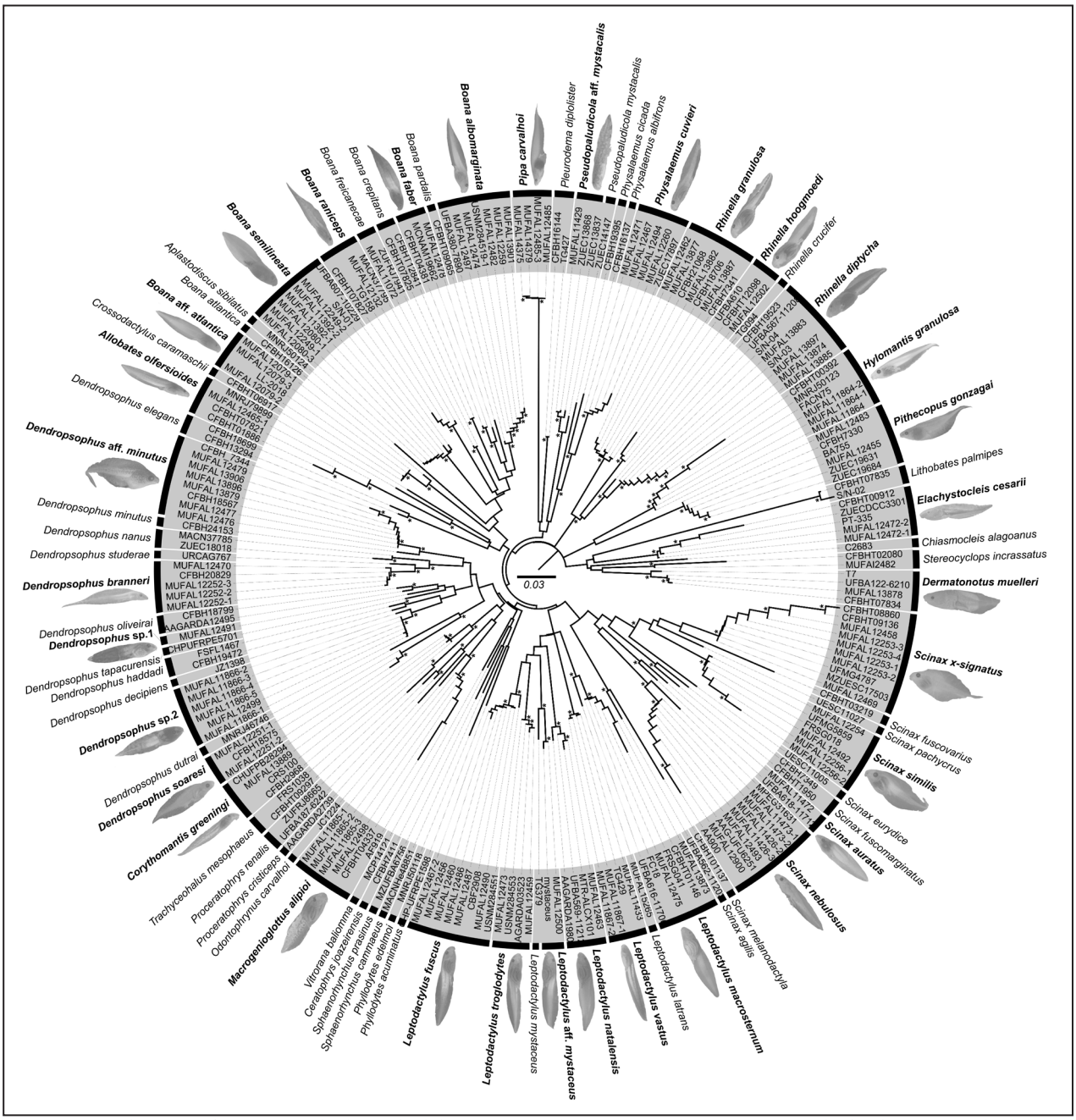

Figure 1. Dendrogram of anurans (tadpoles and adult specimens) that occur in Alagoas state, Brazil. The diagram was built based on 636-bp of DNA sequences of 16S rRNA gene, using the Neighbor-Joining method implemented with the Kimura-2-parameters evolutionary model. Bold species names were sampled in this study. The terminals with acronym MUFAL represent our sequences (except MUFAL 2482, 14375, 14379). *Bootstrap values > 97%.

(6%, n = 6) levels. Additionally, the tadpoles MHN-UFAL 11472-1 and MHN-UFAL 11472-2, both collected on the same day and in the same pond and morphologically identified as having the same morphotype [*Scinax nebulosus* (Spix, 1824)], are actually two distinct species [*S. nebulosus* and *S. auratus* (Wied, 1821)]. This misconception can be explained by the fact that both species are representatives of the *S. rostratus* clade, which are morphologically very similar, presenting the "labial arm" as a larval synapomorphy of the group (Gomes *et al.*, 2014). After revisiting the morphology of these specimens, we could easily differentiate them by the number of labial teeth present in this structure (less than 10 in *S. nebulosus*, more than 15 in *S. auratus*; Dubeux *et al.*, 2020b).

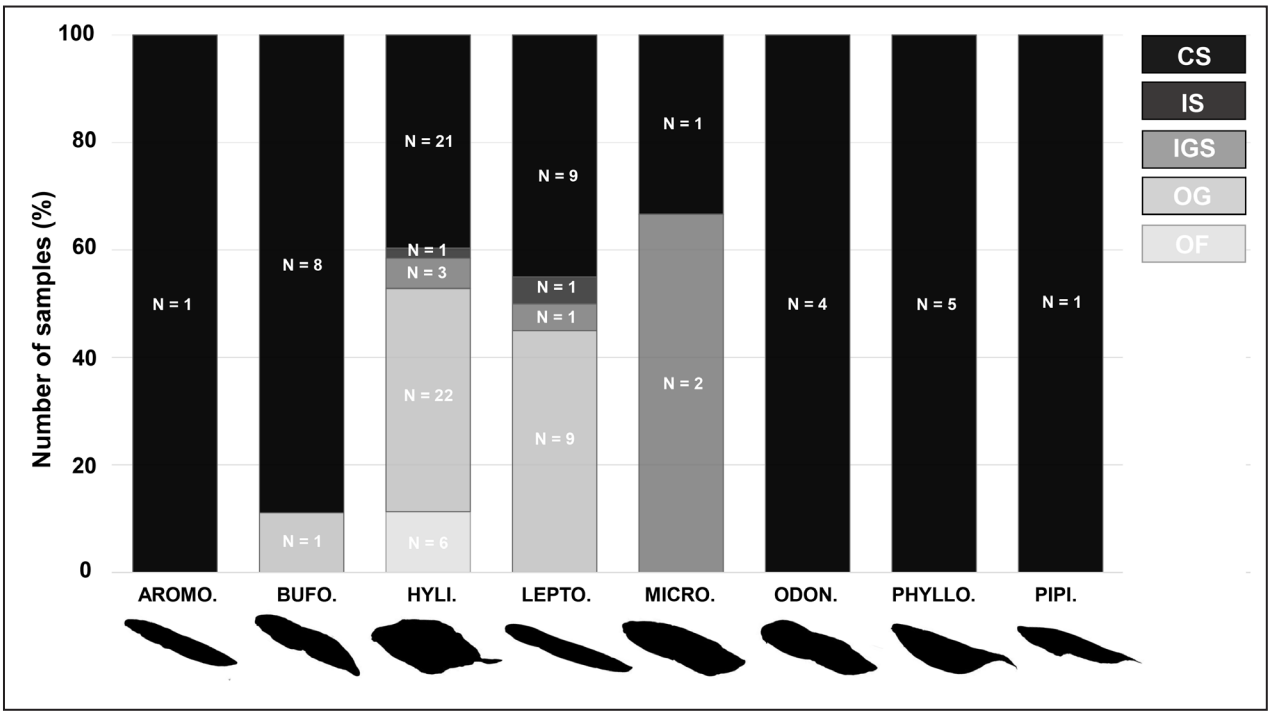

Figure 2. Percentage of morphological identification of tadpoles by family taxonomic level. CS = correct identification at specific level; IS = incorrect identification at specific level, but correct at generic level; IGS = incorrect generic and specific identifications; OG = identification only at generic level; OF = identification only at family level. Abbreviations below bars: AROMO. = Aromobatidae, BUFO. = Bufonidae, HYLI. = Hylidae, LEPTO. = Leptodactylidae, MICRO. = Microhylidae, ODON. = Odontophrynidae, PHYLLO. = Phyllomedusidae, PIPI. = Pipidae.

Additionally, some morphotypes identified as belonging to species recorded in the study area were genetically more similar to closely related taxa that have not been previously recorded in this region. Specifically, MHN-UFAL 1249, morphologically identified as *Dendropsophus oliveirai* (Bokermann, 1963), was genetically more similar to *D. tapacurensis* Oliveira, Magalhães, Teixeira, Moura, Porto, Guimarães, Giaretta & Tinôco, 2021 (GD = 3.11%), which was recently described for the state of Pernambuco (municipality of São Lourenço da Mata, ~150 km away; Oliveira *et al.*, 2021). There is no description for the tadpole of this species, which makes morphological comparisons impossible. The specimens MHN-UFAL 11866(1-5) and MHN-UFAL 12499, morphologically identified as *D. haddadi* (Bastos & Pombal, 1996), were genetically more similar to *D. decipiens* (Lutz, 1925) (GD = 2.05%). Finally, specimens MHN-UFAL 12256(1-2) and MHN-UFAL 12254, morphologically identified as *Scinax eurydice* (Bokermann, 1968), were genetically more similar to *S. similis* (Cochran, 1952) (GD = 1.53 – 2.06%), a species whose type locality is in the state of Rio de Janeiro and whose distribution is only known for the Southeast and Central-west regions of Brazil and Paraguay (Frost, 2022). The tadpoles of these two species present great morphological similarity to each other, as well as to the other representatives of the *S. ruber* clade, and no clear or unambiguous larval diagnostic characters were identified between these species (Rossa-Feres and Nomura, 2006; Dubeux *et al.*, 2020b).

Considering the intraspecific divergence between our sequences and those closest to the study area available in GenBank, the variation between conspecific pairs collected in the state of Alagoas (N = 13 species) ranged from zero (identical haplotypes) to 1.02%. Even when we added pairs of species that do not have adult sequences available for the study area, but that are available for locations up to 500km north of the state of Alagoas (covering the states of Pernambuco and Paraíba; N = 21 species), this range of variation does not change. However, when adding specimens collected up to 500km south of the state of Alagoas to the calculation (covering the states of Sergipe and Bahia; N = 29 species), the variation between conspecific pairs reached 5.83%. These high divergence values can be explained by the fact that the São Francisco River, which forms a southern boundary with the state of Alagoas, is

known to be an important geographic barrier for the dispersion of anuran amphibians (e.g., Lima *et al*., 2019; Andrade *et al*., 2020). Additionally, it delimits important biogeographic regions of the Atlantic Forest (Ribeiro *et al*., 2009) and creates zones of endemism (Asfora and Pontes, 2009; Dubeux *et al*., 2020b; França *et al*., 2020). It is expected that intraspecific genetic divergence would be greater in regions south of Alagoas compared to regions north of the state, either because they present lower gene flow or because they already represent independent evolutionary lineages.

All pairs of species had a smaller genetic divergence in relation to the area closer to the study area than to their type locality. However, some populations from the study area were significantly different from those from the type locality of the nominal taxon. Some of these populations are known to represent independent evolutionary lineages or candidates for new species, according to recent phylogenetic studies, for example *Leptodactylus* aff. *mystaceus* (GD = 3.13%; see Silva *et al*., 2020), *Dendropsophus* aff. *minutus* (GD = 3.09%; see Gehara *et al*., 2014), *Boana* aff. *atlantica* (GD = 2.57%; see Lima *et al*., 2019), and *Pseudopaludicola* aff. *mystacalis* (GD = 3.13%; see Fávero *et al*., 2011).

However, there are no recent phylogenetic studies for other species and, based on the high genetic divergence identified here, in addition to other sources of evidence available in the literature, they should be considered as potential taxa for future phylogenetic studies (see below). For example, populations of *Allobates olfersioides* (Lutz, 1925) from Alagoas showed a genetic divergence of 12.18% with samples from localities close to their type locality in the state of Rio de Janeiro. The population of Alagoas, described as *A. alagoanus* (Bokermann, 1967), was synonymized to *A. olfersioides* by Verdade and Rodrigues (2007) based on the morphological similarity of adult individuals. However, recent studies have highlighted the presence of marked differences in the larval morphology (Dubeux *et al*., 2020a, b) and acoustic repertoire (Forti *et al*., 2017) of these two populations.

Another example involves the representatives of the *Dendropsophus decipiens* species group, registered in the state of Alagoas. Although previously associated with *D. oliveirai* (hereafter referred to as *Dendropsophus* sp.1) and *D. haddadi* (hereafter referred to as *Dendropsophus* sp.2), the greater genetic similarity with *D. tapacurensis* and *D. decipiens*, respectively, raises interesting questions about the taxonomic status of these populations. Although genetically more similar (when compared to the sequences available in GenBank) and originating from locations less than 150 km away, the genetic divergence between *Dendropsophus* sp.1 and *D. tapacurensis* was 3.11%, which was higher than the divergence found between formally recognized lineages within this group (Oliveira *et al*., 2021). A similar situation was observed between *Dendropsophus* sp.2 and *D. decipiens*, where the identified divergence was 2.05%. Multiple evolutionary lineages have already been identified in this species complex, including one lineage comprising populations from the state of Alagoas (*D. decipiens* VII; Oliveira *et al*., 2021). Phylogenetic studies with adequate sampling are required in order to assess the taxonomic status of these taxa. The type localities of some species were not included in the matrix due to the lack of geographic specificity in the designation of their locations, specifically: *Rhinella diptycha* (Cope, 1862), *Leptodactylus fuscus* (Schneider, 1799), and *Physalaemus cuvieri* Fitzinger, 1826.

One of the main bottlenecks in the development of research focusing on larval life stages results as a consequence of the difficulty in identifying Neotropical anuran larvae, especially since assemblages often exceed 40 species (e.g., Brandão *et al*., 2004; Vancine *et al*., 2018) (Rossa-Feres *et al*., 2015; Dubeux *et al*., 2020b). The scarcity of this basic knowledge often becomes a barrier to the advancement of other lines of research involving neotropical tadpoles. In the northern most portion of the Atlantic Forest (north of the São Francisco River), for example, 78 anuran species have been recorded, of which 72 have a larval phase; however, 14% still lack a tadpole description [see Dubeux *et al.* (2020b) for a complete list]. Furthermore, until recently, there was no available identification key for this region (Dubeux *et al*., 2020b). In this study, following sample collection, the tadpoles were identified by different undergraduate students, all with limited experience in this area of research, and the absence of identification keys for tadpoles in the study area or nearby regions (penalties available in 2020), made accurate identification even more difficult. This lack of experience and the scarcity of accessible tools, such as taxonomic keys, may explain, in part, the high rate of incomplete (38%) and incorrect (8%) morphological identification in our data.

With the popularization and low cost of single

locus genetic analyzes, the use of molecular techniques for more accurate assessments of biodiversity has become increasingly more common (Fouquet *et al.*, 2007; Vieites *et al.*, 2009; Perl *et al.*, 2014; Grosjean *et al.*, 2015; Schulze *et al.*, 2015; Lyra *et al.*, 2017; Koroiva *et al.*, 2020; Vacher *et al.*, 2020). Several studies describing anuran larvae have used DNA barcoding to confirm the identity of species (e.g., Malkmus and Kosuch, 2000; Moravec *et al.*, 2014; Dubeux *et al.*, 2020c). However, even with the increasing number of sequenced species in recent decades, the under-sampling of species, mainly in regions such as northeastern Brazil, still persists. In the state of Alagoas, for example, 74 anuran species are currently registered (Almeida *et al.*, 2016; Roberto *et al.*, 2017; Lisboa *et al.*, 2019), of which six have no available DNA sequences (*Frostius pernambucensis*, *Crossodactylus dantei*, *Scinax cretatus*, *S. muriciensis*, *S. skuki*, and *Physalaemus caete*). This can hinder accurate assessments of local diversity, with some species remaining genetically unknown.

Despite the lack of available DNA sequences for some species, the 16S rRNA mitochondrial gene was found to be effective in associating tadpole species with their adult counterparts and aided in assessing anuran diversity in the state of Alagoas. Some advantages of using this marker in DNA barcode studies are 1) universal primers for vertebrates and 2) ease of use and high amplification success rate in different laboratory conditions (Vences *et al.*, 2005b). Additionally, for anuran amphibians, the historical use of this gene has resulted in the construction of a database with approximately 75,000 available sequences (GenBank, accessed on April 27th, 2022). Furthermore, anuran amphibian lineages are relatively old entities, and the mitochondrial gene has sufficient variation to unequivocally identify most species (Vences *et al.*, 2005b; Thomas *et al.*, 2005).

The cryptic diversity revealed through DNA barcoding has helped researchers in taxonomic revisions and estimations of local diversity (see below). In some countries anuran diversities have almost doubled when using the DNA barcode method (e.g., Fouquet *et al.*, 2007 for French Guiana and Vieites *et al.*, 2009 and Perl *et al.*, 2014 for Madagascar). In Brazil, recent large-scale studies have revealed diversities that have been neglected for decades (Lyra *et al.*, 2017; Vacher *et al.*, 2020; Koroiva *et al.*, 2020). Considering the genetic lineages documented in previous phylogenetic studies (e.g., Gehara *et al.*, 2014; Lima *et al.*, 2019; Silva *et al.*, 2020; Oliveira *et al.*, 2021) and the potential taxa for taxonomic studies based on the genetic diversity identified here, we suggest that new species should be described/revalidated in the coming years for the study area. Although the methods used here are suitable for associating tadpoles with their adult counterparts, they are not suitable for resolving phylogenetic relationships. These results should be considered as a starting point for the development of integrative studies that aim to investigate and solve taxonomic issues.

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APPENDIX I. Locations with tadpoles sampled in this study, Alagoas state, Brazil.

Ecoregion	Municipality	Coordinates	Number of samples	Number of species
Atlantic Forest	Barra de Santo Antônio	9°23'27.6"S; 35°31'30.8"W	4	3
	Coruripe	10°07'45.0"S; 36°11'09.5"W	11	8
	Junqueiro	9°55'08.0"S; 36°28'46.6"W	7	5
	Maceió	9°33'30.6"S; 35°47'57.5"W	44	16
	Murici	9°12'59.3"S; 35°51'24.2"W	1	1
	Satuba	9°34'51.4"S; 35°50'35.4"W	6	1
	Teotônio Vilela	9°54'16.6"S; 36°22'21.9"W	3	3
Caatinga	Arapiraca	9°44'32.7"S; 36°37'57.2"W	5	4
	Batalha	9°42'49.1"S; 37°06'02.4"W	2	1
	Igaci	9°32'40.0"S; 36°37'53.0"W	4	3
	Limoeiro de Anadia	9°44'16.5"S; 36°27'41.3"W	10	4
	Traipu	9°57'39.8"S; 36°57'34.6"W	3	2

APPENDIX II. Additional 16S rRNA mitochondrial gene fragment sequences of anuran species used in the study.

Species	Voucher	Locality	GenBank
Aromobatidae			
Allobates olfersioides	CFBHT01886	Alagoas: Passo de Camaragibe	KU495122
Allobates olfersioides	MNRJ79899	Rio de Janeiro: Maricá	MF624179
Bufonidae			
Rhinella crucifer	TG094	Bahia: Salvador	KU495499
Rhinella diptycha	CFBH19523	Bahia: Maracás	MW003646
Rhinella granulosa	CFBH7341	Alagoas: Passo de Camaragibe	KP685205
Rhinella granulosa	CFBH21068	Bahia: Caetité	KP685207
Rhinella hoogmoedi	UFBA610	Bahia: Ilhéus	MH538283
Centrolenidae			
Vitreorana baliomma	MCP14121	Bahia: Una	MW366909
Ceratophryidae			
Ceratophrys joazeirensis	CFBH7411	Paraíba: Araruna	KP295617
Hylidae			
Aplastodiscus sibilatus	MLL-2016	Alagoas: Murici	KU184227
Aplastodiscus sibilatus	CFBH32528	Bahia: Ibirapitanga	KU184014
Boana albomarginata	USNM284519	Pernambuco: Caruaru	KF794116
Boana albomarginata	UFBA380/7890	Bahia: Mata de São João	MH004298
Boana atlantica	MUFAL13067	Alagoas: Maceió	MK348506
Boana atlantica	CFBH16126	Bahia: Uruçuca	MK348483

Boana crepitans	CFBHT07825	Alagoas: Campo Alegre	KU495263
Boana crepitans	CFBHT12841	Bahia: Camamu	KU495262
Boana faber	CFBHT04381	Rio de Janeiro: Petrópolis	KU495265
Boana freicanecae	MUFAL11072	Alagoas: Murici	MT823773
Boana freicanecae	ZUFRJ7941	Pernambuco: Jaqueira	MT823774
Boana raniceps	CFBHT07827	Alagoas: Campo Alegre	MW197887
Boana raniceps	MACN37795	Argentina: Santa Fé	KF794140
Boana semilineata	CFBH5424	Rio de Janeiro: Duque de Caxias	AY843779
Corythomantis greeningi	CFBH2968	Alagoas: Piranhas	KF002247
Corythomantis greeningi	CRS100	Bahia: Morro do Chapéu	MW243375
Dendropsophus branneri	CFBH20829	Pernambuco: Bonito	MT503865
Dendropsophus dutrai	MNRJ46746	Sergipe: Indiaroba	MT503923
Dendropsophus elegans	CFBH13294	Sergipe: Itabaiana	MT503966
Dendropsophus elegans	CFBH18699	Bahia: Prado	KY348631
Dendropsophus haddadi	FSFL1467	Bahia: Prado	MT503942
Dendropsophus haddadi	CFBH19472	Espírito Santo: Vitória	MT503941
Dendropsophus minutus	CFBH18567	Alagoas: Campo Alegre	MK266721
Dendropsophus minutus	CFBH24153	Rio de Janeiro: Resende	MT503934
Dendropsophus nanus	ZUEC:18018	Sergipe: Aracaju	MN420278
Dendropsophus nanus	MACN37785	Argentina: Entre Rios	AY549346
Dendropsophus oliveirai	AAGARDA12495	Pernambuco: São Lourenço da Mata	MW026634
Dendropsophus oliveirai	CFBH18799	Bahia: Maracás	MT503956
Dendropsophus soaresi	CFBH18575	Alagoas: Campo Alegre	MT503922
Dendropsophus studerae	URCAG767	Alagoas: Quebrangulo	MT503894
Phyllodytes acuminatus	CHP-UFRPE1598	Pernambuco: Buíque	MN961791
Phyllodytes edelmoi	MNRJ50118	Alagoas: Maceió	MN961798
Phyllodytes gyrinaethes	CFBH44634	Alagoas: Maceió	MN961801
Scinax agilis	UFBA562/11203	Bahia: Mata de São João	MH004314
Scinax auratus	UFBA618/11712	Bahia: Conde	MH004316
Scinax eurydice	UESC11005	Bahia	OK161150
Scinax fuscomarginatus	CFBH7349	Alagoas: Passo de Camaragibe	KJ004153
Scinax fuscomarginatus	CFBHT1950	Minas Gerais: Santana do Riacho	KJ004129
Scinax fuscovarius	CFBHT03219	Minas Gerais: Araxá	KU495554
Scinax melanodactylus	CFBHT01137	Alagoas: Passo de Camaragibe	KU495535
Scinax nebulosus	AAG-UFU6251	Rio Grande do Norte: Nísia Floresta	MK503366
Scinax nebulosus	MPEG:31631	Pará: Belém	MK503362
Scinax pachycrus	UESC11027	Bahia	OK161140
Scinax x-signatus	CFBHT08860	Pernambuco: Fernando de Noronha	KU495578
Scinax x-signatus	CFBHT09136	Bahia: Caetité	KU495576
Scinax x-signatus	CFBHT09136	Bahia: Caetité	KU495576
Sphaenorhynchus cammaeus	MACNHe48851	Alagoas: Quebrangulo	MK266727
Sphaenorhynchus prasinus	MZUFBA6756	Bahia: Mata de São João	MK266752
Trachycephalus mesophaeus	CFBHT09297	Bahia: Aurelino Leal	KU495600
Trachycephalus mesophaeus	FRS1038	São Paulo: Apiaí	MH206468
Leptodactylidae			
Leptodactylus fuscus	USNM284551	Pernambuco	AY911279

Leptodactylus latrans	MUFAL15265	Alagoas: Maceió	MT495901
Leptodactylus macrosternum	CFBHT01146	Alagoas: Passo de Camaragibe	KU495341
Leptodactylus macrosternum	FC18	Bahia: Terra Nova	MT495860
Leptodactylus mystaceus	AAGARDA12501	Pernambuco: São Lourenço da Mata	MT117857
Leptodactylus mystaceus	TG379	Amazonas: São Gabriel da Cachoeira	MT117890
Leptodactylus natalensis	MTR101P28	Alagoas: Maceió	KU495347
Leptodactylus natalensis	AAGARDA1980	Rio Grande do Norte: Parnamirim	MW291330
Leptodactylus troglodytes	USNM284553	Pernambuco	KM091620
Leptodactylus vastus	TG429	Paraíba: Guarabira	KU495368
Physalaemus albifrons	CFBH16137	Ceará: Viçosa do Ceará	KP146010
Physalaemus cicada	CFBH19395	Ceará: Novas Russas	KP146064
Physalaemus cuvieri	ZUEC17897	Pernambuco: Caruaru	KP146012
Pleurodema diplolister	TG427	Paraíba: Caiçara	KU495455
Pleurodema diplolister	CFBH16144	Ceará: Viçosa do Ceara	JQ937185
Pseudopaludicola mystacalis	ZUEC:13837	Maranhão: Barreirinhas	KJ147006
Pseudopaludicola mystacalis	ZUEC:14147	Mato Grosso: Cuiabá	KJ146983
Microhylidae			
Chiasmocleis alagoanus	C2683	Alagoas: Maceió	KC180030
Dermatonotus muelleri	CFBHT07834	Alagoas: Campo Alegre	KU495218
Dermatonotus muelleri	T7	Paraguay	KC179984
Elachistocleis cesarii	ZUEC:DCC3301	Minas Gerais: Serra do Cipó	JN604511
Elachistocleis cesarii	CFBHT00912	São Paulo: Santa Fé do Sul	KU495225
Stereocyclops incrassatus	MUFAL2482	Alagoas: Marechal Deodoro	KC180046
Stereocyclops incrassatus	CFBHT02080	Espírito Santo Linhares	KU495593
Odontophrynidae			
Macrogenioglottus alipioi	CFBHT04337	Bahia: Uruçuca	KU495385
Odontophrynus carvalhoi	JC1224	Bahia: Mucugê	FJ685687
Proceratophrys cristiceps	AAGARDA2739	Ceará: Crato	KX855993
Proceratophrys renalis	ZUFRJ8665	Pernambuco: Caruaru	JN814584
Proceratophrys renalis	UFBA187/6242	Bahia: Cachoeira	MH004311
Phyllomedusidae			
Hylomantis granulosa	MNRJ50123	Alagoas: Murici	GQ366225
Hylomantis granulosa	CFBHT00392	Pernambuco: Jaqueira	KU495255
Pithecopus gonzagai	CFBH7330	Alagoas: Passo de Camaragibe	GQ366330
Pithecopus gonzagai	ZUEC19684	Pernambuco: Limoeiro	MW158582
Pipidae			
Pipa carvalhoi	MUFAL14375	Pernambuco: Buíque	MT261672
Pipa carvalhoi	MUFAL14379	Pernambuco: Buíque	MT261653
Ranidae			
Lithobates palmipes	CFBHT07835	Alagoas: Campo Alegre	KU495377
Lithobates palmipes	01	Guiana	AF467265