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Molecular phylogeny of Neotropical rock frogs reveals a long history of vicariant diversification in the Atlantic forest

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ABSTRACT

The Brazilian Atlantic coastal forest is one of the most heterogeneous morphoclimatic domains on earth and is thus an excellent region in which to examine the role that habitat heterogeneity plays in shaping diversification of lineages and species. Here we present a molecular phylogeny of the rock frogs of the genus *Thoropa* Cope, 1865, native to the Atlantic forest and extending to adjacent *campo rupestre* of Brazil. The goal of this study is to reconstruct the evolutionary history of the genus using multilocus molecular phylogenetic analyses. Our topology reveals 12 highly supported lineages among the four nominal species included in the study. Species *T. saxatilis* and *T. megatympanum* are monophyletic. *Thoropa taophora* is also monophyletic, but nested within *T. miliaris*. Populations of *T. miliaris* cluster in five geographically distinct lineages, with low support for relationships among them. Although all 12 lineages are geographically structured, some *T. miliaris* lineages have syntopic distributions with others, likely reflecting a secondary contact zone between divergent lineages. We discuss a biogeographic scenario that best explains the order of divergence and the distribution of species in Atlantic forest and adjacent areas, and outline the implications of our findings for the taxonomy of *Thoropa*.

1. Introduction

The Brazilian Atlantic forest is a highly complex and heterogeneous morphoclimatic and phytogeographic domain (Ab'Saber, 1977). It is a global biodiversity hotspot (Mittermier et al., 1998; Morellato and Haddad, 2000; Silva and Casteleti, 2003) and high habitat heterogeneity, resulting from topographic complexity and large latitudinal range, is one of the main reasons proposed for its high biological diversity (Ribeiro et al., 2009; Rodríguez et al., 2015). Therefore, the Atlantic forest is an excellent region to examine how heterogeneous habitats contribute to diversification (Rodríguez et al., 2015). Habitat heterogeneity has a potentially large effect on amphibian diversification, due to their low vagility and often specialized habitat preferences (Rodríguez et al., 2015). Indeed, the Atlantic forest is home to more than 500 frog species (Haddad et al., 2013; Toledo et al., 2014), accounting for 8.1% of the world's known anuran diversity. More than 75% of Atlantic forest anurans are endemic to the domain (Haddad et al., 2013) and many of these endemic species inhabit montane environments (Cruz and Feio, 2007; Haddad et al., 2013).

Rock frogs in the genus *Thoropa* Cope, 1865, belong to the family Cycloramphidae (*sensu* Frost, 2017) and include the following six species endemic to Brazil: *T. miliaris* (Spix, 1824), *T. petropolitana* (Wandolleck, 1907), *T. taophora* (Miranda-Ribeiro, 1923), *T. lutzi* Cochran, 1938, *T. megatympanum* Caramaschi and Sazima, 1984, and *T. saxatilis* Cocroft and Heyer, 1988. All species of the genus, except *T. megatympanum*, inhabit rocky seashores, wet rocky outcrops, and boulders and waterfalls in montane rocky streams of the Atlantic forest (Bokermann, 1965; Cocroft and Heyer, 1988; Feio et al., 2006). *Thoropa megatympanum* inhabits the Atlantic forest-Cerrado and Atlantic forest-Caatinga ecotones (Caramaschi and Sazima, 1984) in the same kinds of habitats as its congeners. *Thoropa* species have specialized breeding requirements. Males are typically territorial and exhibit parental care of

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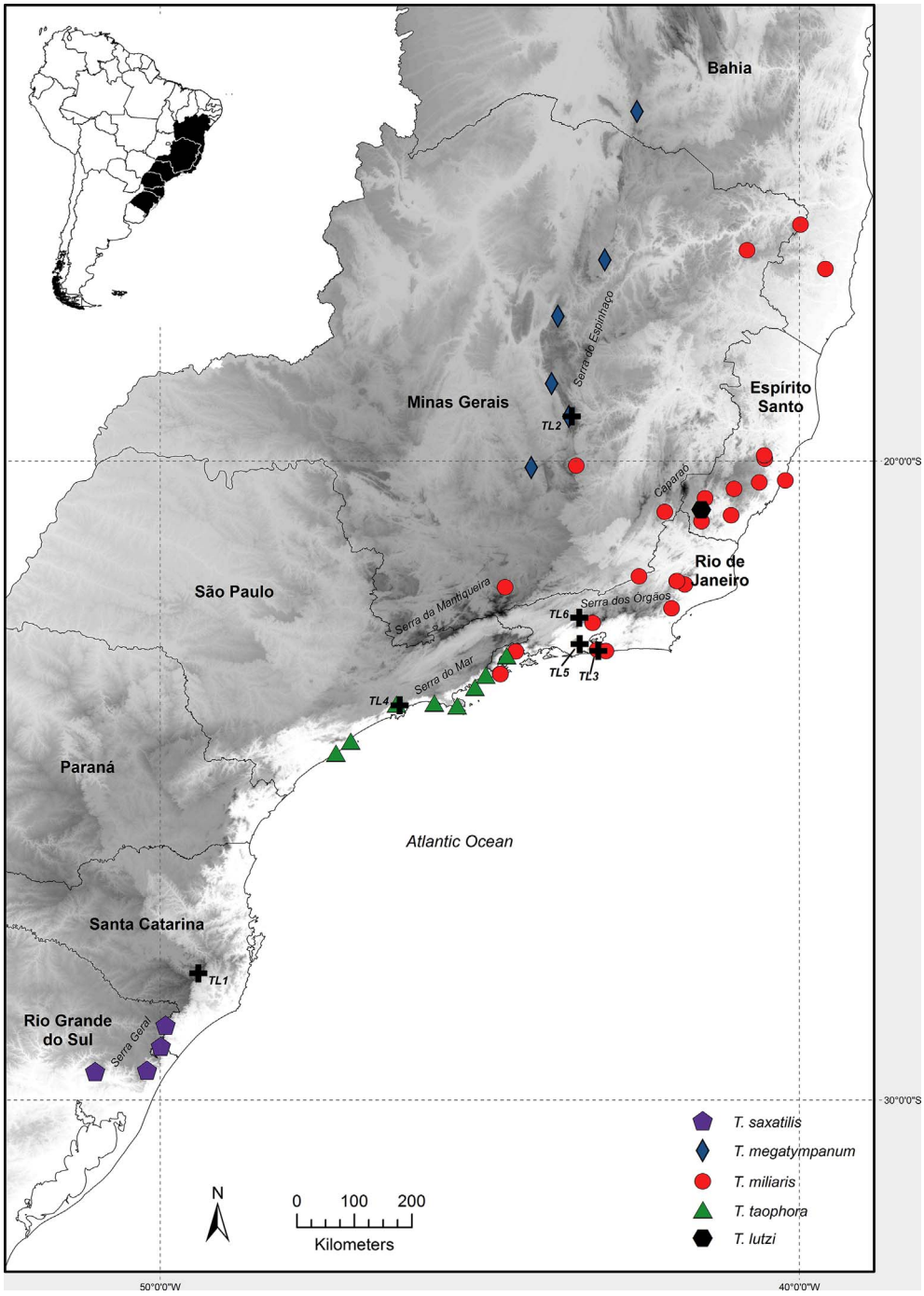

Fig. 1. Collection localities for *Thoropa* samples included in this study. Black crosses labelled *TL1-TL6* indicate type localities of *T. saxatilis*, *T. megatympanum*, *T. miliaris*, *T. taophora*, *T. lutzi* and *T. petropolitana,* respectively. Elevation is shown in gray scale ranging from white (0–50 m above the sea level) to black (3900–3950 m above sea level).

egg clutches (Giaretta and Facure, 2004; Muralidhar et al., 2014; Consolmagno et al., 2016), which are deposited on rocks in freshwater seeps or at the humid rocky margins of shallow rivulets (Barth, 1956; Bokermann, 1965; Rocha et al., 2002). Tadpoles are exotrophic and semiterrestrial, hatching and developing in the water seeps (Barth, 1956; Bokermann, 1965).

A phylogeographic study of *Thoropa miliaris* and *T. taophora* showed that populations of the southern *T. taophora* are monophyletic, and nested within the northern *T. miliaris*, and inferred a north to south expansion and differentiation within these two species (Fitzpatrick et al., 2009). The relationships of these two species to the others in the genus and the mechanisms potentially contributing to their diversification are still unknown. In this study we reconstruct a multilocus phylogeny for populations of all available species within the genus *Thoropa*.

Our specific goals are to (1) describe the spatial distribution and genetic diversity of species and cryptic lineages within the genus; (2) infer the phylogenetic relationships of *Thoropa* species using mitochondrial and nuclear markers and date divergences among species and lineages; and (3) test for monophyly of the genus *Thoropa* and each of the nominal species within the genus. We discuss our results in the context of a biogeographic scenario that best explains the order of divergence and the current distribution of species in Atlantic forest and adjacent areas.

2. Material and methods

2.1. Taxon sampling and data matrix assembly

The genus *Thoropa* is subdivided in two groups, based on morphological characters: the *T. petropolitana* group, with *T. petropolitana* and *T. lutzi*, and the *T. miliaris* group, with *T. miliaris*, *T. taophora*, *T. megatympanum*, and *T. saxatilis* (Feio, 2002). The *Thoropa petropolitana* group is characterized by small adults (up to 30 mm), males with nuptial spines only on finger II (numbers of fingers *sensu* Fabrezi and Alberch, 1996), and tadpoles with a large abdominal disc surpassing the lateral edge of the body and posterior portion of the abdomen (Feio, 2002). In contrast, species in the *Thoropa miliaris* group have medium to large (more than 35 mm) adult body sizes, males with nuptial spines on fingers II, III, and IV (numbers of fingers *sensu* Fabrezi and Alberch, 1996), and tadpoles with reduced abdominal disc (Feio, 2002). Although these characteristics have been highlighted as important differences between the two groups (Cocroft and Heyer, 1988), group monophyly and the relationships among all nominal species have not yet been established.

Species of the *Thoropa miliaris* group are common in their preferred habitats throughout their range. *Thoropa miliaris* and *T. taophora* occur in the Atlantic forest of southeastern Brazil: *T. miliaris* has a broader distribution in the states of Bahia, Espírito Santo, Minas Gerais, and Rio de Janeiro (Frost, 2017), occurring from rocky seashores to 1500 m above sea level, while *T. taophora* is restricted to eastern mountains and coastal areas in the state of São Paulo (Feio et al., 2006). *Thoropa megatympanum* occurs in the Serra do Espinhaço mountain range in the states of Minas Gerais and Bahia, where they are found in *campo rupestre* (rupestrian grasslands, *sensu* Silveira et al., 2015) at the ecotone between the Atlantic forest and Cerrado (Caramaschi and Sazima, 1984). *Thoropa saxatilis* is the species with the southernmost distribution, occurring in the Serra Geral mountain range, in the states of Santa Catarina and Rio Grande do Sul (Cocroft and Heyer, 1988).

Species of the *Thoropa petropolitana* group are far less common. *Thoropa petropolitana* was known from mountain sites (more than 700 m above the sea level) in the Serra dos Órgãos, state of Rio de Janeiro, and *T. lutzi* was known to occur in the states of Rio de Janeiro and Espírito Santo (Bokermann, 1965). *Thoropa petropolitana* has not been recorded for more than 40 years and *T. lutzi* disappeared from the state of Rio de Janeiro, but can still be found in Parque Nacional do Caparaó and in the municipalities of Alegre and Muniz Freire, state of Espírito Santo (Feio, 2002; J.L. Gasparini pers. obs.).

We sampled throughout the distributions of *Thoropa saxatilis*, *T. megatympanum*, *T. miliaris*, and *T. taophora* (Fig. 1), including topotypic specimens of *T. miliaris* (municipality of Rio de Janeiro, state of Rio de Janeiro), *T. megatympanum* (Serra do Cipó, municipality of Santana do Riacho, state of Minas Gerais), and *T. taophora* (Paranapiacaba, municipality of Santo André, state of São Paulo). We were unable to find topotypic specimens of *T. saxatilis* (road from municipality of Bom Jardim da Serra to municipality of Lauro Müller, state of Santa Catarina), and the closest locality sampled was from the municipality of Timbé do Sul (state of Santa Catarina, around 60 km in straight line to the type locality). We also included one tissue sample of a small juvenile stored directly in 100% ethanol, identified morphologically as *T. lutzi*, collected in municipality of Alegre, state of Espírito Santo (Fig. 1). We did not include topotypic specimens of *T. lutzi* (Recreio dos Bandeirantes, municipality of Rio de Janeiro, state of Rio de Janeiro) or any specimens of *T. petropolitana* (municipality of Petrópolis, state of Rio de Janeiro) because no other tissue samples are available for those two rare species.

We gathered tissue samples from herpetological collections and from our own field efforts, and supplemented those with sequences obtained from GenBank. During field trips, we collected liver, muscle tissue, or toe clips, stored in 100% ethanol at −20 °C. Voucher specimens for most of the samples are deposited in Brazilian collections (Appendix A.1). Tissue samples without voucher specimens are archived in the tissue collection of Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo state (CFBH-T) and at Cornell University (Ithaca, NY, USA).

2.2. Laboratory protocols

We assembled two multilocus matrices for analyses at two scales, one to analyze genetic diversity and phylogenetic relationships between species within the genus *Thoropa* (herein called matrix A) and the other to test the monophyly of the genus (matrix B). Matrix A included five gene fragments for 46 individuals of the *Thoropa miliaris* group (*T. megatympanum*, *T. miliaris*, *T. saxatilis*, and *T. taophora*) and four Cycloramphidae species (*Cycloramphus boraceiensis*, *C. dubius*, *C. eleutherodactylus*, and *Zachaenus parvulus*) plus *Proceratophrys boiei* as outgroup taxa. We rooted this tree with *P. boiei* (Appendix A.1). In contrast, matrix B included ten gene fragments for 13 samples from the four species of *T. miliaris* group (*T. megatympanum*, *T. miliaris*, *T. saxatilis*, and *T. taophora*), the single sample of *T. lutzi* from the state of Espírito Santo, and 33 species as outgroups (Appendix A.2). Outgroups for both matrices were chosen based on phylogenies published by Frost et al. (2006), Grant et al. (2006), Pyron and Wiens (2011), Fouquet et al. (2013), Blotto et al. (2013), Faivovich et al. (2014), and Castroviejo-Fisher et al. (2015).

We used the Qiagen DNeasy® Blood and Tissue kit (Qiagen Inc.) to extract total DNA, following the manufacturer's protocol. Extracted DNA was used directly (or diluted to 0.5–1 ng/μl in Mili-Q® water) for amplifications. For matrix A, we PCR amplified three mitochondrial and two nuclear gene fragments: the 3′ fragment of the ribosomal gene encoding *16S* rRNA amplified with primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991) (*16S*-ARBR), a fragment of the gene encoding NADH dehydrogenase subunit 2 (*ND2*), the 5′ region of the gene encoding cytochrome *c* oxidase subunit 1 (*COI*), a fragment of the intron 1 of the fibrinogen A alpha polypeptide gene (*FGA*), and a fragment of the recombination activating gene 1 (*RAG1*). For matrix B we used eight mitochondrial and three nuclear DNA fragments: *12S* rRNA (*12S*), *tRNA-Val*, *16S* rRNA (*16S*), *tRNA-Leu1*, NADH dehydrogenase the subunit 1 (*ND1*), *tRNA-Ile*, *COI*, *RAG1*, a fragment of the proopiomelanocortin gene (*POMC*) and a fragment of exon 1 of the rhodopsin gene (*RHO*) (Supplementary Material SI.1). PCR reaction conditions are in Supplementary Material SI.2. We purified positive amplicons and sequenced them in both directions using BigDye v.3.1® (Applied Biosystems) sequencing kits at Macrogen Inc. (Seoul, South Korea), Centro de Estudos de Insetos Sociais (UNESP, Rio Claro, SP, Brazil), or at the Cornell University Genomics Facility (Ithaca, NY, USA). We cleaned sequences, removed primer sequences, and built consensus sequences for each fragment and each individual using Sequencher v.4.5 (Gene Codes). We aligned sequences of each fragment separately with the software Muscle (Edgar, 2004) implemented in MEGA v.6 (Tamura et al., 2013), using default parameters (gap open penalty: −400; gap extend penalty: 0; maximum iterations: 8; clustering method: UPGMB). We translated the coding genes (*ND1*, *ND2*, *COI*, *POMC*, *RAG1*, and *RHO*) to verify the absence of frameshifts and premature stop codons.

2.3. Phylogenetic inferences and genetic diversity

For phylogenetic inferences using matrix A, we conducted four different analyses: one concatenating mitochondrial and nuclear fragments (*16S*-ARBR, *ND2*, *COI*, *FGA*, and *RAG1*), a second including only the mitochondrial fragments (*16S*-ARBR, *ND2*, and *COI*), and one for each nuclear fragment (*FGA* and *RAG1*). For phylogenetic inferences using matrix B we conducted a single analysis with all gene fragments concatenated. Concatenated matrices were built in Mesquite v.3.03 (Maddison and Maddison, 2015). We estimated the best-fit nucleotide evolution model for each gene fragment (or codon position) and the best partition strategy using Bayesian Information Criterion (BIC;

Schwarz, 1978) in PartitionFinder v.1.1.1 (Lanfear et al., 2012).

We inferred phylogenies using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) with 100 million generations, two parallel runs, and eight Markov chains for each run. The priors were unlinked for all partitions, and as follows: Dirichlet distribution prior for state frequencies and substitution rates; uniform distribution prior for gamma shape and for the proportion of invariable sites; variable partition rates; and branch lengths unconstrained. Trees were sampled each 1000 generations and the first 25% of trees were discarded as burn-in. Analyses were run at the CIPRES Science Gateway (Miller et al., 2010). To diagnose convergence of the runs, we used four criteria: the standard deviation of split frequencies (SDSF) lower than 0.01; the Potential Scale Reduction Factor (PSRF+) close to 1.0; a stable plot of the generations versus the log likelihood values; and examination of the output log in Tracer v.1.6 (Rambaut et al., 2014; Ronquist et al., 2005). We inferred a 50% majority rule consensus tree and considered posterior clade probabilities (PP) higher than 0.95 as indicative of strong nodal support (Alfaro et al., 2003; Erixon et al., 2003). We visualized and edited trees in FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

To identify significantly divergent lineages in our tree, we used geographic concordance, node support, branch lengths, and the tree topology. To calculate average genetic distances within and between species and within and between lineages for two fragments used in matrix A (*16S*-ARBR and *COI*), we used uncorrected *p*-distance with pairwise deletion and 1000 replicates of bootstrap in software MEGA v.6 (Tamura et al., 2013).

2.4. Coalescent-based species tree

We reconstructed a coalescent-based species tree using *Beast (Heled and Drummond, 2010), and estimated time to the most recent common ancestor (t_{MRCA}), in the software BEAST v.2.3 (Bouckaert et al., 2014). We prepared the input file with the BEAUti utility, using only the 35 terminal taxa from matrix A that possessed all five gene fragments sequenced (Appendix A.1).

We used the package bModelTest (Bouckaert, 2015) to find the best models of nucleotide evolution for each gene fragment, partitioned or not by codon positions, for each data set. BEAST v.2.3 runs the *Beast analysis concomitantly with nucleotide substitution model selection through the bModelTest package. Lineages were assigned as "species" in a mapping file (required by BEAUti) following the phylogenetic tree inferred using matrix A. We performed the analysis without outgroup, and including sites with missing data. The best partition scheme did not include partition by codon, and each fragment was set for an independent nucleotide evolution model. We used strict clock for *16S*-ARBR and *ND2*, and lognormal relaxed clock (Drummond et al., 2006) for *COI*, *FGA*, and *RAG1*. We also adopted the Yule speciation process and unchecked the function "fix mean substitution rates" for each gene partition. Rates of nucleotide evolution were set to "estimate", except for *ND2* fragment, to which we applied the instantaneous rate of 0.00957 base changes per lineage per million years (Crawford, 2003).

The best-fitting parameters were established after preliminary searches including different datasets (with and without outgroups and sites with missing data), best model of molecular clock rate variation, best tree model prior, and best partition schemes. Performance and accuracy were checked in Tracer v.1.6 (Rambaut et al., 2014) and an analysis was considered reliable if all ESS values were higher than 200 (Rambaut et al., 2014). Final analyses included one run of 100 million generations, sampled each 10 000 generations. The final maximum clade credibility tree was generated with TreeAnnotator v.2.3 (Bouckaert et al., 2014), discarding 10% as burn-in and using median node heights.

3. Results

3.1. Phylogenetic inferences and genetic diversity

For matrix A, including the outgroup, the mitochondrial concatenated alignment consisted of 2288 bp, the alignment of *FGA* was 535 bp long, the alignment of *RAG1* was 429 bp long, and the total concatenated alignment (mitochondrial and nuclear) consisted of 3252 bp. For matrix B, also including outgroups, the mitochondrial concatenated alignment consisted of 4330 bp and the total concatenated alignment (mitochondrial and nuclear) consisted of 5576 bp. The models of nucleotide substitution found for the fragments through BIC (Schwarz, 1978) and the best partition scheme are shown in Supplementary Material SI.3, for both matrices. The number of partitions identified by PartitionFinder v.1.1.1 was eight both for matrix A and matrix B.

3.1.1. Diversity of the Thoropa miliaris group

The Bayesian consensus tree inferred from the concatenated (mitochondrial + nuclear) dataset of matrix A showed that *Thoropa* species form a monophyletic group in relation to the outgroup species, with high PP values (Fig. 2). The other trees (mitochondrial, *FGA*, and *RAG1*) also recovered *Thoropa* as monophyletic (Supplementary Material SI.4, SI.5, and SI.6). The nuclear gene trees recovered only *T. saxatilis* as monophyletic. The species *Thoropa megatympanum*, *T. saxatilis*, and *T. taophora* were recovered as monophyletic in all concatenated phylogenetic analyses (mitochondrial + nuclear, and only mitochondrial). However, *Thoropa miliaris* was recovered as paraphyletic in relation to *T. taophora*, and populations of this species clustered in five well supported cryptic lineages (Fig. 2).

Thoropa saxatilis included two well supported and distinct lineages, both distributed in the slopes of the Serra Geral: sax-1, a northern lineage from municipality of Timbé do Sul in the state of Santa Catarina; and sax-2, a southern lineage distributed throughout the states of Santa Catarina and Rio Grande do Sul (Fig. 3), approximately 40 km distant from each other. *Thoropa megatympanum* included two well supported lineages (meg-1 and meg-2; Fig. 2) distributed in the northern and southern portions of Serra do Espinhaço, respectively (Fig. 3). The topotype (from Santana do Riacho, Minas Gerais state) belongs to the southern meg-2 lineage.

Thoropa miliaris is composed of five well-supported lineages, although paraphyletic in their relationship to *T. taophora* (Fig. 2): lineage mil-1, distributed in southern Espírito Santo state; lineage mil-2, distributed in montane regions of the states of Bahia, Minas Gerais, Espírito Santo and Rio de Janeiro; lineage mil-3, distributed in central and southern regions of Espírito Santo; lineage mil-4, located in northern Serra dos Órgãos in the state of Rio de Janeiro; and lineage mil-5 distributed primarily in the state of Rio de Janeiro, including coastal populations and the topotype sample of *T. miliaris* (Fig. 3). Finally, *Thoropa taophora* is composed of three lineages: tao-1, distributed in the northern coastal regions of the state of São Paulo; tao-2, distributed in southern coastal regions of the state of São Paulo, and including the topotype specimen of *T. taophora*; and tao-3, from Estação Ecológica Juréia-Itatins, a coastal site in southern São Paulo state.

We found five localities where lineages of *T. miliaris* were syntopic (Santa Maria Madalena, Rio de Janeiro state; Santa Teresa, Cachoeiro do Itapemirim, and Domingos Martins, Espírito Santo state; Carangola, Minas Gerais state) and one region of syntopy of lineages mil-5 of *T. miliaris* and tao-1 of *T. taophora* (Paraty, Rio de Janeiro state) (Fig. 2).

According to our analyses, the deepest phylogenetic split separates *Thoropa saxatilis* from all other *Thoropa*, with *T. megatympanum* forming the sister lineage to five lineages of *T. miliaris* plus *T. taophora* (Fig. 2). Both nuclear fragments and the concatenated analyses recovered the same results. *Thoropa miliaris* and *T. taophora* form a complex of poorly resolved populations. Lineages mil-1 and mil-2 are sister lineages (PP = 92), and combined form the sister taxon (PP = 100) to the

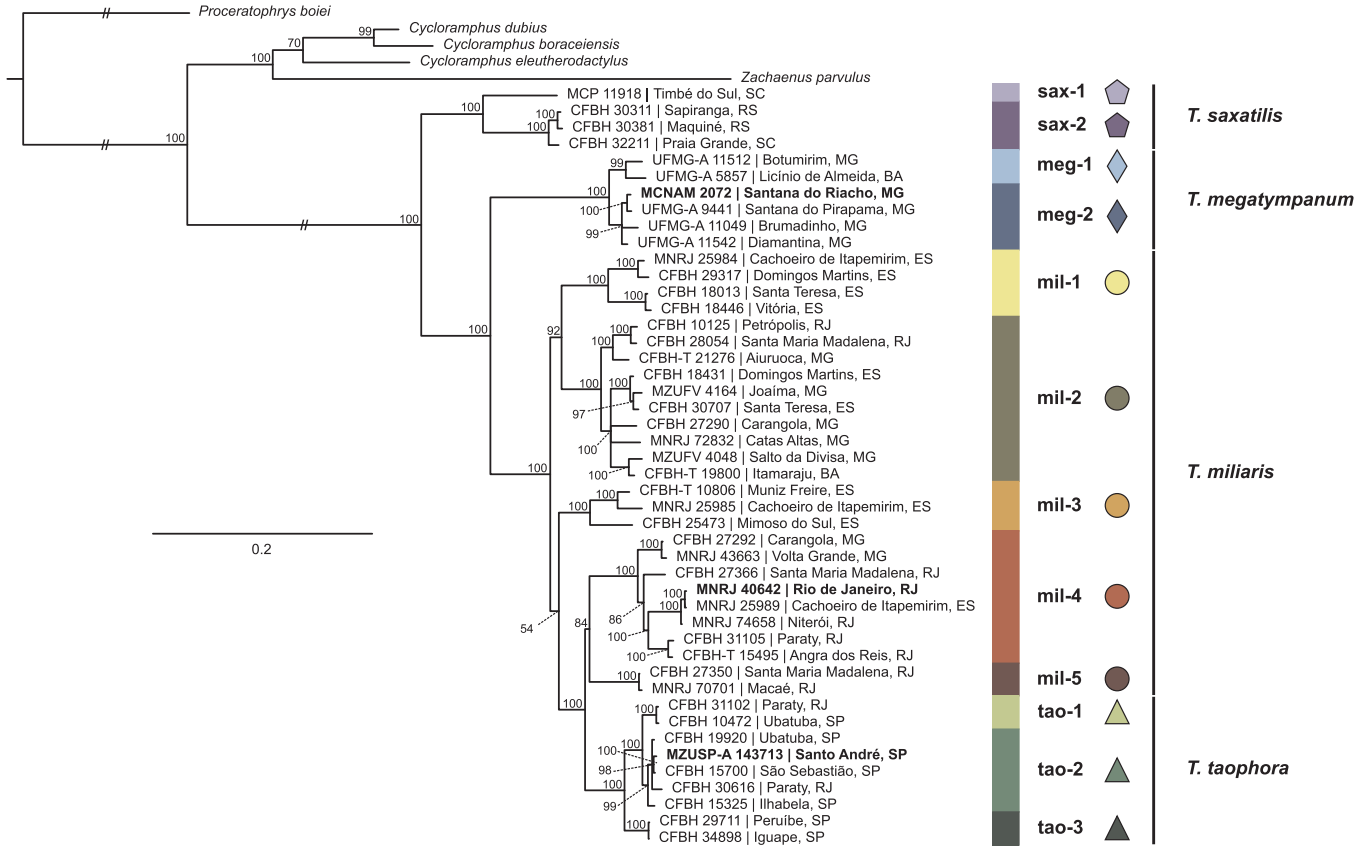

Fig. 2. Phylogeny of *Thoropa* species based on concatenated (mitochondrial + nuclear) dataset (matrix A) and Bayesian Inference (50% majority rule consensus). Numbers at each node represent posterior probabilities. Terminals in bold represent topotype specimens for *T. megatympanum*, *T. miliaris*, and *T. taophora*. The symbol // indicates branch lengths that are not to scale. Samples CFBH 30,616 and CFBH 31,105 were identified as *Thoropa miliaris* because of their collection locality (Paraty, Rio de Janeiro), but belong to *T. taophora*, thus we renamed them. The codes BA, MG, ES, RJ, SP, SC, RS refer to the states of Bahia, Minas Gerais, Espírito Santo, Rio de Janeiro, São Paulo, Santa Catarina, and Rio Grande do Sul, respectively.

remaining lineages (mil-3 + mil-4 + mil-5 + *T. taophora*). Mil-3, in turn, is the sister lineage of mil-4 + mil-5 + *T. taophora*, again with low support (PP = 69, Fig. 2). Mil-4 + mil-5 + *T. taophora* form a well-supported clade (PP = 100).

The average genetic distances found between pairwise species ranged from 4.4 to 8.1% for *16S*-ARBR and 13.5 to 17% for *COI* (Table 1). The intraspecific genetic distances ranged from 1.1 to 3.1% for *16S*-ARBR and from 3.5 to 11.4% for *COI*. The genetic distances among lineages varied from 1.1 to 9.4% for *16S*-ARBR and from 3.7 to 17.8% to *COI* (Table 1). Within lineages they varied from 0.0 to 2.0% for *16S*-ARBR and from 0.2 to 9.7% to *COI* (Table 1).

3.1.2. Monophyly of the genus Thoropa

The Bayesian consensus tree from the concatenated (mitochondrial and nuclear) analysis of matrix B showed that the genus *Thoropa* is not monophyletic, as *T. lutzi* was recovered as the sister lineage of *Cycloramphus* + *Zachaenus* with high PP support (Fig. 4). The *Thoropa miliaris* group is monophyletic, and the sister taxon of this clade is composed of *Cycloramphus* + *Zachaenus* + *T. lutzi*. Within the *T. miliaris* group, most of the relationships were recovered with PP higher than in the analyses of matrix A. The only node with low PP was the node uniting mil-5 and *T. taophora* (78%). The families Aromobatidae, Batrachylidae, Bufonidae, Centrolenidae, Ceratophryidae, Cycloramphidae, Dendrobatidae, Hylidae, Hylodidae, Leptodactylidae, Odontophrynidae and Rhinodermatidae were each recovered as monophyletic. Alsodidae is the only family not recovered as monophyletic, because *Limnomedusa macroglossa* was recovered as the sister taxon of Odontophrynidae.

3.2. Coalescent-based species tree

The *Beast species tree showed a similar topology to the matrix A phylogeny based on concatenated data, but with differences in PP (Fig. 5). The best-fit models of nucleotide evolution inferred by bModelTest are in SI.7. The topology recovered the two lineages of *Thoropa saxatilis* (sax-1 + sax-2) form the sister group to the remaining species; two lineages of *T. megatympanum* (meg-1 + meg-2) form the sister group to *T. miliaris* + *T. taophora*; mil-3 as the sister lineage to the other four lineages of *T. miliaris* + *T. taophora*; mil-1 as the sister lineage of mil-2, mil-5, mil-4, and *T. taophora*; mil-2 as the sister lineage of mil-5; mil-2 + mil-5 form the sister group to mil-4 and *T. taophora*; tao-1 as the sister lineage to tao-2; and tao-1 + tao-2 as the sister group to tao-3.

Estimates of times to the most common ancestor (t_{MRCA}) showed that divergences within *Thoropa* span a large timeframe, ranging from the Oligocene to the Pleistocene (Fig. 5). The common ancestor of the four species dates to the Oligocene. The common ancestor between the two lineages of *Thoropa megatympanum* and the common ancestor among the three lineages of *T. taophora* date to the Pliocene-Pleistocene, and the common ancestor of tao-1 and tao-2 dates to the Pleistocene.

4. Discussion

This is the broadest phylogenetic study of species in the genus *Thoropa* published to date. Additionally, the inclusion of the single available sample of *T. lutzi* raised doubts about the monophyly of the genus (see discussion below). Combined, the trees derived from mitochondrial and nuclear markers resolve relationships within *Thoropa* at different levels of divergence. The mitochondrial tree showed the

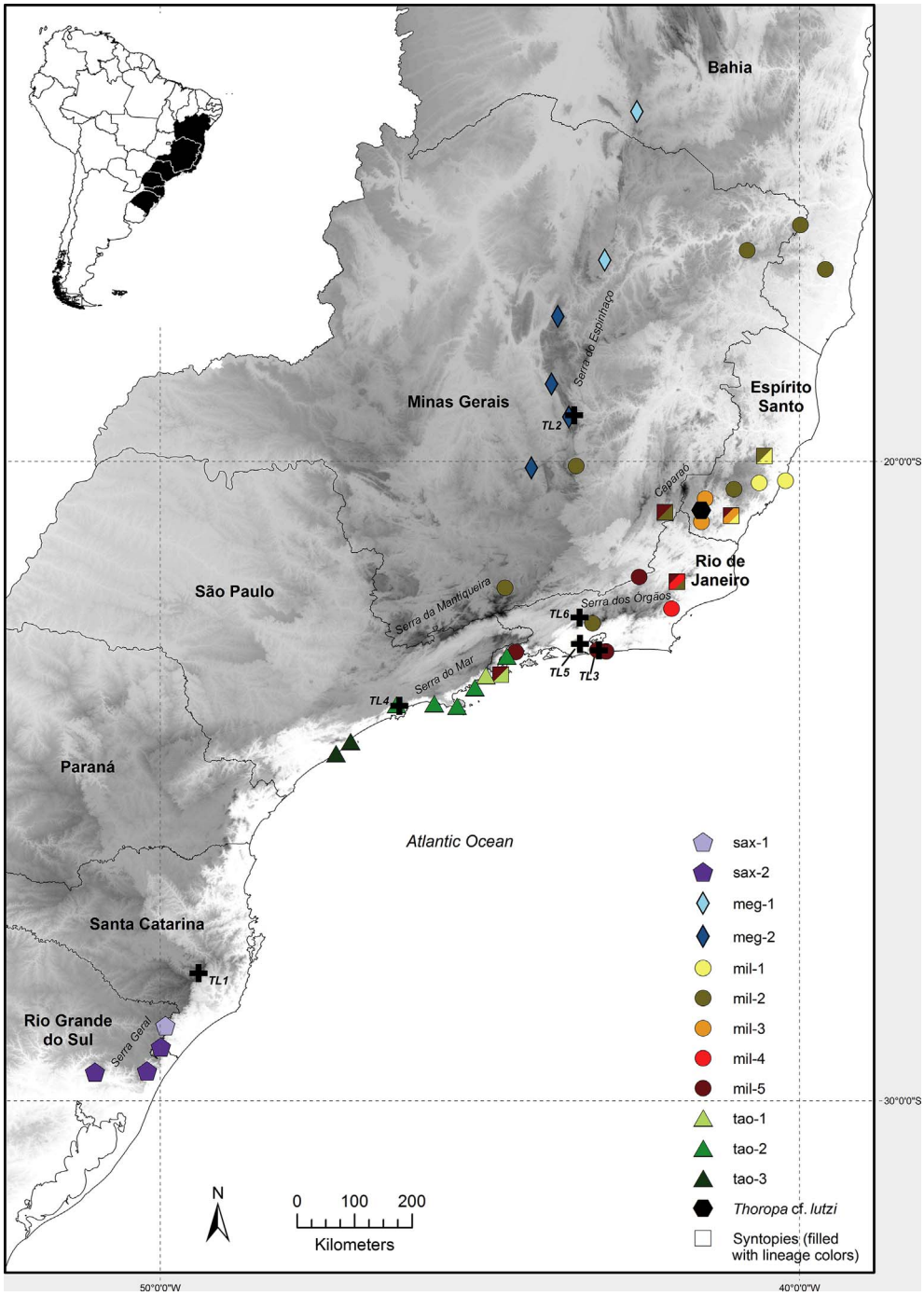

Fig. 3. Distribution of *Thoropa* samples, in different Brazilian states, identified to species (symbols) and to lineage (colors). Symbols labelled *TL1-TL6* indicate type localities of *T. saxatilis*, *T. megatympanum*, *T. miliaris*, *T. taophora*, *T. lutzi* and *T. petropolitana,* respectively. Black crosses represent type localities not sampled in this study. Localities where lineages occur in syntopy are represented by squares and multiple colors. Elevation is shown in gray scale ranging from white (0–50 m above the sea level) to black (3900–3950 m above sea level). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

monophyly of *T. saxatilis*, *T. megatympanum*, *T. taophora*, and also showed a paraphyletic *T. miliaris* with respect to *T. taophora*. The nuclear markers alone revealed well supported deeper divergences within the genus, especially the early split of *T. saxatilis* and *T. megatympanum* from other *Thoropa*.

4.1. Biogeography of diversification in Thoropa

Our data indicate that diversification of *Thoropa* began with a north-south split (between *T. saxatilis* and the other species). The distributional gap between *T. saxatilis* and the most southern locality of *T. taophora* (Peruíbe and Iguape, state of São Paulo) as well as its ancient separation from other species in the group corroborate this observation. This split was followed by a west-east division, with the separation between *T. megatympanum* and the complex *T. miliaris* + *T. taophora*. This pattern of diversification corroborates the hypothesis of allopatric speciation of *Thoropa* species from a widespread ancestor proposed by Cocroft and Heyer (1988), although the speciation events were older than they postulated. Subsequently, the diversification of *T. miliaris* + *T. taophora* extended to the coast, in a process that likely included both vicariance and dispersal. Despite the long process of diversification of lineages, the distribution of *Thoropa* species is still

Table 1
Average uncorrected *p*-distances (percentage, with standard errors in parentheses), within species (diagonal) and between species (below diagonal), and within lineages (diagonal) and between lineages (below diagonal), calculated from *16S*-ARBR and *COI* fragments. (a) Within and between species for *16S*-ARBR fragment. (b) Within and between species for *COI* fragment. (c) Within and between lineages for *16S* fragment. (d) Within and between lineages for *COI* fragment.

(a) 16S-ARBR fragment, by species

Species	N	*T. saxatilis*	*T. megatympanum*	*T. miliaris*	*T. taophora*
T. saxatilis	4	**2.0 (0.4)**			
T. megatympanum	6	8.1 (1.0)	**1.2 (0.3)**		
T. miliaris	27	6.7 (0.9)	6.4 (0.8)	**3.1 (0.5)**	
T. taophora	9	7.4 (1.0)	7.4 (0.9)	4.4 (0.7)	**1.1 (0.3)**

(b) COI fragment, by species

Species	N	*T. saxatilis*	*T. megatympanum*	*T. miliaris*	*T. taophora*
T. saxatilis	4	**6.6 (0.8)**			
T. megatympanum	6	14.6 (1.2)	**3.9 (0.5)**		
T. miliaris	24	16.3 (1.2)	16.0 (1.1)	**11.4 (0.9)**	
T. taophora	7	17.0 (1.2)	15.9 (1.3)	13.5 (1.0)	**3.5 (0.5)**

(c) 16S-ARBR fragment, by lineage

Lineage	N	sax-1	sax-2	meg-1	meg-2	mil-1	mill-2	mil-3	mil-4	mil-5	tao-1	tao-2	tao-3
sax-1	1	–											
sax-2	3	3.7 (0.9)	**0.3 (0.2)**										
meg-1	2	9.4 (1.4)	7.2 (1.0)	**1.1 (0.4)**									
meg-2	4	9.4 (1.4)	7.9 (1.1)	1.7 (0.5)	**0.7 (0.3)**								
mil-1	4	7.8 (1.2)	7.8 (1.1)	7.3 (1.0)	7.1 (1.0)	**2.0 (0.4)**							
mil-2	10	7.0 (1.2)	6.6 (1.1)	6.0 (0.9)	6.6 (1.0)	3.6 (0.6)	**1.3 (0.3)**						
mil-3	3	8.0 (1.3)	6.5 (1.1)	5.5 (0.8)	5.9 (0.9)	4.4 (0.8)	3.3 (0.6)	**1.9 (0.4)**					
mil-4	2	6.7 (1.2)	5.9 (1.0)	6.0 (1.0)	6.3 (1.0)	3.9 (0.8)	3.0 (0.6)	3.1 (0.7)	**0.2 (0.2)**				
mil-5	8	7.1 (1.1)	6.0 (0.9)	6.0 (0.9)	6.3 (0.9)	4.0 (0.7)	3.8 (0.6)	4.0 (0.7)	2.5 (0.6)	**1.6 (0.4)**			
tao-1	2	8.2 (1.2)	7.1 (1.1)	6.8 (0.9)	7.3 (1.0)	5.6 (0.9)	4.4 (0.8)	4.2 (0.8)	3.4 (0.8)	4.6 (0.8)	**0.0 (0.0)**		
tao-2	5	7.5 (1.2)	7.4 (1.1)	7.1 (1.0)	7.4 (1.0)	5.6 (0.9)	4.3 (0.7)	4.6 (0.8)	3.4 (0.7)	4.4 (0.8)	1.1 (0.4)	**0.5 (0.2)**	
tao-3	2	7.2 (1.1)	7.6 (1.1)	7.5 (1.1)	8.1 (1.1)	5.0 (0.8)	4.0 (0.7)	4.8 (0.8)	3.2 (0.7)	4.2 (0.8)	2.0 (0.6)	1.7 (0.5)	**0.0 (0.0)**

(d) COI fragment, by lineage

Lineage	N	sax-1	sax-2	meg-1	meg-2	mil-1	mil-2	mil-3	mil-4	mil-5	tao-1	tao-2	tao-3
sax-1	1	–											
sax-2	3	10.7 (1.1)	**2.5 (0.5)**										
meg-1	2	15.2 (1.5)	14.7 (1.3)	**4.4 (0.8)**									
meg-2	4	15.0 (1.5)	14.3 (1.4)	5.4 (0.7)	**1.8 (0.4)**								
mil-1	3	16.4 (1.4)	16.3 (1.4)	16.9 (1.5)	16.8 (1.5)	**5.7 (0.7)**							
mil-2	9	17.0 (1.4)	16.5 (1.3)	15.5 (1.3)	15.8 (1.4)	11.5 (1.1)	**6.8 (0.6)**						
mil-3	2	15.3 (1.4)	17.3 (1.4)	15.9 (1.2)	16.4 (1.3)	13.2 (1.0)	14.1 (1.1)	**9.7 (1.2)**					
mil-4	2	15.7 (1.5)	16.6 (1.3)	15.7 (1.3)	16.8 (1.4)	12.9 (1.2)	13.9 (1.3)	13.0 (1.2)	**0.5 (0.3)**				
mil-5	8	15.3 (1.3)	15.8 (1.3)	16.3 (1.3)	15.5 (1.3)	13.2 (1.1)	13.4 (1.1)	13.3 (1.1)	12.3 (1.1)	**5.3 (0.6)**			
tao-1	2	16.2 (1.5)	17.8 (1.3)	17.4 (1.5)	17.4 (1.5)	15.4 (1.3)	14.2 (1.2)	13.2 (1.2)	12.0 (1.3)	11.9 (1.1)	**0.2 (0.2)**		
tao-2	4	16.7 (1.5)	17.2 (1.4)	15.5 (1.4)	15.8 (1.5)	15.1 (1.3)	14.7 (1.2)	13.4 (1.1)	12.4 (1.2)	12.6 (1.1)	3.7 (0.7)	**1.0 (0.3)**	
tao-3	1	14.3 (1.4)	16.2 (1.5)	13.8 (1.3)	13.9 (1.3)	15.2 (1.5)	13.3 (1.2)	11.8 (1.2)	10.7 (1.4)	11.4 (1.1)	6.8 (0.9)	6.3 (1.0)	–

highly associated with rocky seashores or wet rocky outcrops near mountain formations, which indicates that this habitat preference is probably the ancestral state for the genus. The deep diversification times found for *Thoropa* species are similar to those found in many other South American amphibians, beginning in late Oligocene and continuing through the early Pliocene (Rull, 2008). Similar temporal patterns were found in *Adenomera* (Fouquet et al., 2014) and *Leptodactylus* (Fouquet et al., 2013).

The disjunct distribution of *Thoropa saxatilis* in relation to other species in this genus may be explained by paleogeographic changes to the landscape during the Miocene and Pliocene (Rull, 2008). *Thoropa saxatilis* and *T. megatympanum* have remained largely geographically isolated from all other species, except for a small contact zone between *T. megatympanum* and *T. miliaris* (mil-2), in Catas Altas, Minas Gerais state, and other known localities in the Espinhaço mountain range in Minas Gerais state (Feio et al., 2006). These cases of syntopy seem to represent secondary contact of *T. miliaris* and *T. megatympanum* following the west-east vicariance.

Populations of *Thoropa miliaris* and *T. taophora* show a more complex scenario of diversification. Given the largely allopatric geographic distributions of *T. miliaris* lineages and *T. taophora*, and the paraphyly of *T. miliaris*, we also consider vicariance for the split between the two forms, followed by secondary contact of lineages in some localities where the species are syntopic. This posterior contact may have occurred naturally, following past expansion and retraction hypothesized for Atlantic forest due to climatic oscillations (Carnaval and Moritz, 2008). Another possibility for syntopic distributions of *T. miliaris* lineages is anthropogenic mediated migration, but this latter hypothesis can only be ruled out after we have a better understanding of the geographic distributions of lineages. The split between *Thoropa miliaris* lineages and *T. taophora* occurred sometime between 15.5 and 4.0 million years in the late Miocene or early Pliocene (Fig. 5). However, this split was not accompanied by morphological change, as *T. miliaris* and *T. taophora* do not have any diagnostic morphological phenotypes

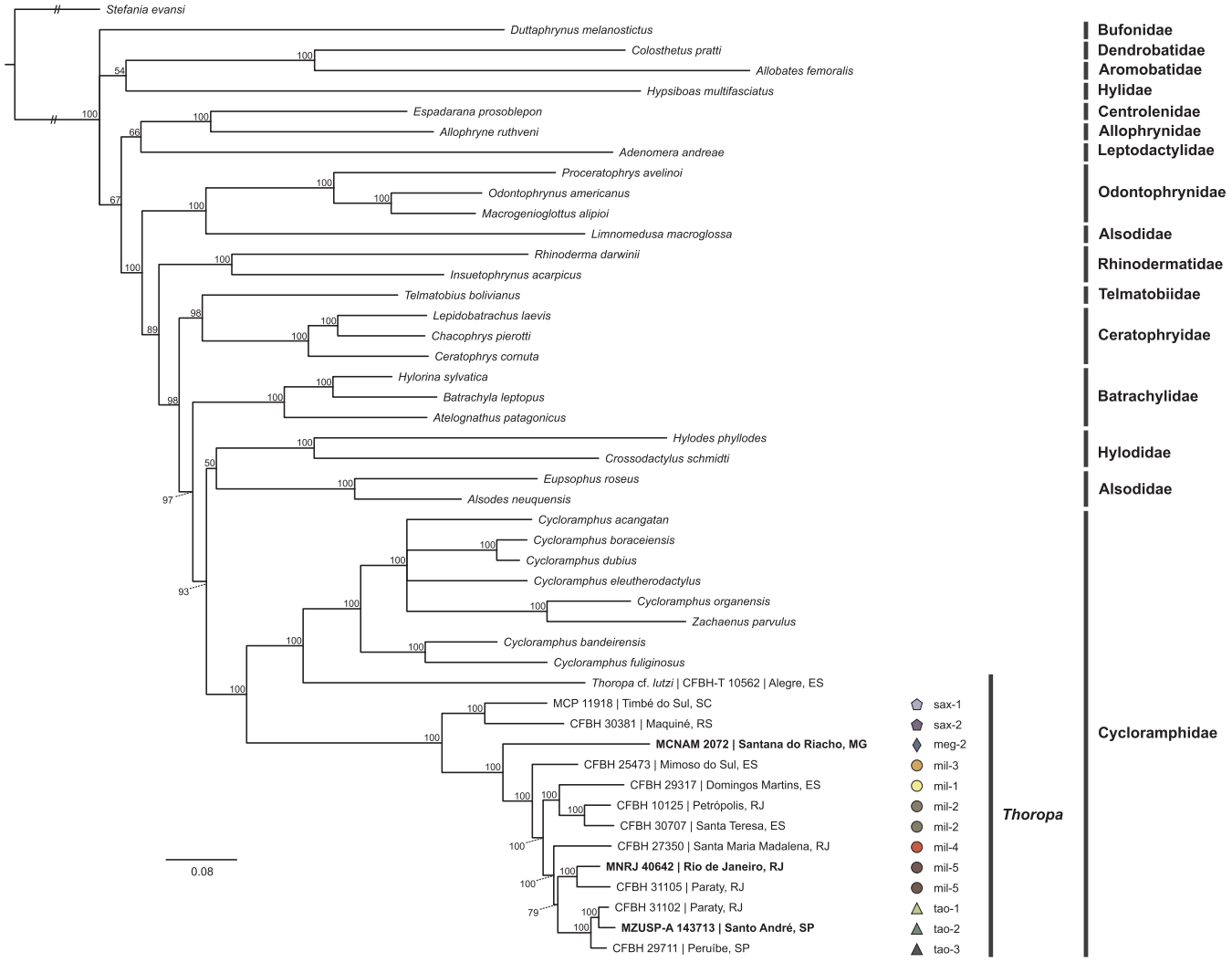

Fig. 4. Phylogeny based on concatenated (mitochondrial + nuclear) dataset (matrix B) and Bayesian Inference (50% majority rule consensus) for *Thoropa* species and outgroups. Numbers at each node represent clade posterior probabilities. The branches leading to *Stefania evansi* are not to scale. Sample CFBH 31105 was identified as *Thoropa miliaris* because of its collection locality (Paraty, Rio de Janeiro), but it has been renamed *T. taophora*. The codes MG, ES, RJ, SP, SC, RS refer to the states of Minas Gerais, Espírito Santo, Rio de Janeiro, São Paulo, Santa Catarina, and Rio Grande do Sul, respectively. Color coded lineages of Thoropa are as shown in Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Feio et al., 2006).

The more recently derived lineages of *Thoropa miliaris* + *T. taophora* complex (mil-5, tao-1, tao-2, and tao-3) include populations that reach the coast of Brazil and inhabit rocky seashores. Seashores are an odd environment for an amphibian and few species are known to tolerate high-salinity habitats (Abe and Bicudo, 1991; Gordon et al., 1961; Romspert and McClanahan, 1981). Nonetheless, the two lineages have evolved to occupy saline microhabitats and although these populations live in hostile environments, they are locally abundant (Bokermann, 1965; Brasileiro et al., 2010).

Additionally, *Thoropa saxatilis*, *T. megatympanum*, and *T. taophora* may also be experiencing ongoing diversification as our topology showed geographical structure within these species (sax-1 and sax-2; meg-1 and meg-2; tao-1, tao-2, and tao-3). The north-south divergence in *T. taophora* was identified by Fitzpatrick et al. (2009), with the possibility of a southern São Paulo refugium forming the third lineage ("Juréia" = tao-3; Carnaval et al., 2009). Our data including the four *Thoropa* species corroborate that the distribution of habitats may play an important role in genetic connectivity or discontinuity of lineages and species (Fitzpatrick et al., 2009).

4.2. On the monophyly of Thoropa

Although Cycloramphidae was recovered as a monophyletic family, its relationships with the other families remain poorly resolved, as found in previous studies (Grant et al., 2006; Pyron and Wiens, 2011; Fouquet et al., 2013; Blotto et al., 2013; and Faivovich et al., 2014). Clearly, the higher-level systematics of Athesphatanura needs further study.

The inclusion of a sample of *T. lutzi* resulted in the potential paraphyly of the genus *Thoropa* (Figs. 2 and 4), with the single *T. lutzi* sample more closely related to *Cycloramphus* plus *Zachaenus* than to other members of the genus *Thoropa*. The position of this sample in the phylogeny raises interesting questions about the phylogenetic relationships of these three genera. For example, an externally non-visible tympanum is a synapomorphy for *Cycloramphus* + *Zachaenus* (Verdade, 2005), and in our sample of *T. lutzi,* the tympanum is visible. This indicates that this feature might have two independent origins in Cycloramphidae and is thus not a synapomorphy for *Cycloramphus*, or that the Cycloramphidae ancestor had a visible tympanic membrane but it was lost in *Cycloramphus* + *Zachaenus*.

We cannot discard the possibility that our sample of *Thoropa lutzi* is not *T. lutzi sensu stricto*, because we did not analyze samples from *T. lutzi*

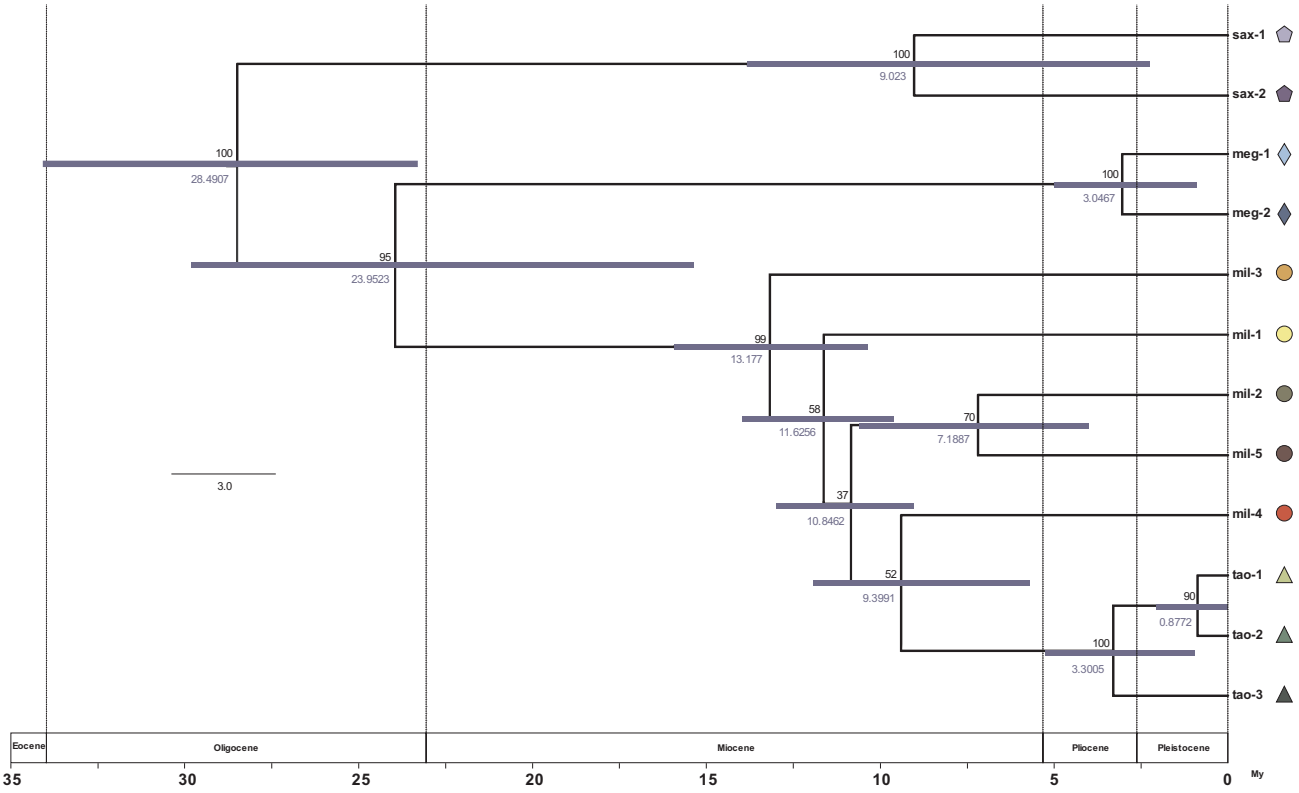

Fig. 5. Topology inferred from coalescent species tree analysis treating each lineage of *Thoropa* as distinct population, with respective t_{MRCA} for each node. Numbers above nodes indicate posterior probabilities, and numbers below nodes indicate average t_{MRCA} (in millions of years – "My"). Blue bars indicate standard deviations of t_{MRCA}.

type locality. Feio (2002) studied the morphology of a population from municipality of Muniz Freire (state of Espírito Santo), near the Alegre population we sampled, and found some morphological differences from the type series of *T. lutzi*. To test these relationships, we need more extensive sampling, with systematic analyses of molecular and morphological features, to infer the probable synapomorphies of *Thoropa*. Meanwhile, we recommend that this sample be designated as *Thoropa* cf. *lutzi*, until we can further clarify its taxonomic position.

4.3. Paraphyly and taxonomic considerations in Thoropa

Previous studies on the underestimation of diversity of frog species revealed thresholds of uncorrected *p*-distances that could be used to recognize "candidate species", based on the most commonly used barcode gene fragments for amphibians (*16S*-ARBR and *COI*) (e.g., Vences et al., 2005a; Vences et al., 2005b; Fouquet et al., 2007). For *16S*-ARBR, a threshold of 5% (Vences et al., 2005a) or 3% (Fouquet et al., 2007) of divergence have been used, while for *COI* the proposed threshold is 10% (Vences et al., 2005b). These levels of sequence divergence between lineages might correspond to different species and not merely intraspecific genetic variation.

If we analyze the four recognized species in our study, every value calculated for *16S*-ARBR is higher than 3% (threshold *sensu* Fouquet et al., 2007), and only one value (4.4%, between *Thoropa miliaris* and *T. taophora*) is slightly smaller than 5% (threshold *sensu* Vences et al., 2005a). Thus, in general, the four nominal species are well supported, although *T. miliaris* and *T. taophora* might be considered synonyms if the threshold for *16S*-ARBR is considered strictly. Our data show some evidence of deep intraspecific divergences within species. When comparing lineages within each species, they all differ genetically by less than 3% (*sensu* Fouquet et al., 2007) and less than 5% (*sensu* Vences et al., 2005a) for *16S*-ARBR, and less than 10% for *COI*, with one exception. The genetic distance of *COI* among lineages within *T. miliaris* is slightly higher at 11.4%, which could indicate that *T. miliaris* contains high internal diversity and represents a species complex.

With respect to genetic distances between species estimated from the *16S*-ARBR fragment, our values (4.4–8.1) are similar to those found in studies of *Proceratophrys* (1–11%, Dias et al., 2013), *Osteocephalus* (0.6–6.1%, Jungfer et al., 2013), *Physalaemus* (1.2–11.7%, Lourenço et al., 2015), Ceratophryidae (0.4–7.2%, Faivovich et al., 2014), and *Oreobates* (2.8–11.7%, Padial et al., 2008). For the *COI* fragment, our between-species values (13.5–17.0) are higher than the values published for species of *Alsodes* and *Eupsophus* (Blotto et al., 2013). Thus, species delimitation in *Thoropa* is well defined, even considering the *T. miliaris* + *T. taophora* complex.

Lineages of *Thoropa miliaris* and *T. taophora* may represent an ongoing diversification in this species complex, with *T. miliaris* being a "paraphyletic species" (see Hörandl and Stuessy, 2010; Schmidt-Lebuhn, 2012; Stuessy and Hörandl, 2014 for discussion). In a review of the occurrence of paraphyly and polyphyly in animal phylogenies of mitochondrial DNA, Funk and Omland (2003) found 21.3% of amphibian topologies exhibiting paraphyly or polyphyly. Similarly, 22% of Amazonia-Guianas amphibians exhibit a paraphyletic topology in phylogenies (Fouquet et al., 2007). Even with high genetic distances, the existence of syntopy between lineages of *Thoropa miliaris* and *T. taophora* makes it difficult to infer species limits. Moreover, the *T. miliaris* + *T. taophora* complex has high phenotypic variation (Feio et al., 2006), precluding unambiguous diagnosis of species lineages, because of extensive morphometric and morphological overlap found in the OTUs (Feio et al., 2006). *Thoropa miliaris* and *T. taophora* surely represent a species complex, and this lineage needs further study, with a focus on systematics and population genetics.

Combined, our analyses underscore the high genetic diversity of lineages endemic to the Atlantic forest and adjacent regions. The high degree of geographic isolation among species and lineages and the relatively old age of these divergence events indicate that high habitat heterogeneity and a dynamic history of climatic change have structured diversification in this genus. Our study shows the importance of

inferring these spatial processes to clarify taxonomy and species diversity, and the processes that might threaten species conservation in tropical biomes.

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Appendix A

Appendix A.1. Samples of *Thoropa* and outgroup species used in this study (matrix A), with accession numbers, collection localities ("simplified locality, municipality, state" or "municipality, state"), coordinates, GenBank accession numbers, and lineage membership. Samples in bold are topotype specimens. Asterisks indicate samples used in Fitzpatrick et al. (2009). Crosses indicate samples used also in matrix B, and full circles indicate samples used in coalescent species tree analysis. Samples originally recorded as *T. miliaris*, but considered as *T. taophora* because of their topology position, are shown with superscript "a". Voucher specimens are deposited in Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo (CFBH); Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro (MNRJ); Museu de Zoologia da Universidade de São Paulo, São Paulo (MZUSP); Museu de Zoologia João Moojen de Oliveira, Universidade Federal de Viçosa, Viçosa, Minas Gerais (MZUFV); Coleção Herpetológica da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais (UFMG); Museu de Ciências Naturais, Pontifícia Universidade Católica de Minas Gerais, Belo Horizonte, Minas Gerais (MCNAM); and Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul (MCP). Collection abbreviations follow Sabaj Pérez (2014).Appendix A.2. Samples of *Thoropa* and outgroup species used in this study (matrix B) sorted by family, with collection numbers and GenBank accession numbers. Samples in bold are *Thoropa* topotype specimens. Samples originally recorded as *T. miliaris*, but considered as *T. taophora* because of their topology position, are shown with superscript "a". Voucher specimens are deposited in Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo (CFBH); Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro (MNRJ); Museu de Zoologia da Universidade de São Paulo, São Paulo (MZUSP); and Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul (MCP). Collection abbreviations follow Sabaj Pérez (2014).

Species	Accession number	Collection locality	Latitude	Longitude	GenBank Accession Numbers					Clade
					16S-ARBR	*ND2*	*COI*	*FGA*	*RAG1*	
T. megatympanum ●	UFMG-A 5857	Alto da Serra Sete Quedas, Licínio de Almeida, BA	−14.524367	−42.539996	MG799589	MG799712	MG799624	MG799666	MG799754	meg-1
T. megatympanum ●	UFMG-A 11512	Serra de Botumirim, Botumirim, MG	−16.848186	−43.042217	MG799588	MG799711	MG799623	MG799665	MG799753	meg-1
T. megatympanum ●	UFMG-A 11542	Parque Nacional das Sempre Vivas, Diamantina, MG	−17.729290	−43.777300	MG799592	MG799715	MG799629	MG799670	MG799759	meg-2
T. megatympanum ●	UFMG-A 9441	Fechados, Santana do Pirapama, MG	−18.786483	−43.875649	MG799590	MG799714	MG799627	MG799668	MG799757	meg-2
T. megatympanum † ●	**MCNAM 2072**	**Parque Nacional da Serra do Cipó, Santana do Riacho, MG**	**−19.301725**	**−43.604122**	**MG799575**	**MG799713**	**MG799626**	**MG799667**	**MG799756**	**meg-2**
T. megatympanum	UFMG-A 11049	Cachoeira da Ostra, Serra do Rola Moça, Brumadinho, MG	−20.099472	−44.191089	MG799591	–	MG799628	MG799669	MG799758	meg-2
T. miliaris ●	MZUFV 4048	Fazenda Alto Cariri, Salto da Divisa, MG	−16.300000	−39.983333	MG799603	MG799727	MG799641	MG799682	MG799770	mil-2
T. miliaris	MZUFV 4164	Fazenda Ramaiana, Joaíma, MG	−16.700000	−40.816667	MG799604	–	MG799642	–	MG799771	mil-2
T. miliaris	CFBH-T 19800	Serra Grande, ao lado da Pedra do Monte do Pescoço, Itamaraju, BA	−16.993250	−39.594500	MG799605	MG799718	–	–	–	mil-2
T. miliaris * ●	CFBH 18013	Estação Biológica Santa Lúcia, Santa Teresa, ES	−19.965074	−40.540466	GQ174578	GQ174987	MG799633	MG799674	MG799763	mil-1
T. miliaris † ●	CFBH 30707	Reserva Biológica Augusto Ruschii, Nova Lombardia, Santa Teresa, ES	−19.911944	−40.549444	MG799579	MG799728	MG799643	MG799683	MG799772	mil-2
T. miliaris ●	MNRJ 72832	Cascatona, RPPN Santuário do Caraça, Catas Altas, MG	−20.070500	−43.489111	MG799602	MG799726	MG799640	MG799681	MG799769	mil-2
T. miliaris * ●	CFBH 18446	Morro da Gamela, Vitória, ES	−20.302083	−40.218333	GQ174617	GQ175017	MG799634	MG799675	MG799764	mil-1
T. miliaris ●	CFBH 18431	Pedra Azul, Domingos Martins, ES	−20.435333	−41.021500	MG799598	MG799722	MG799636	MG799677	MG799766	mil-2
T. miliaris †	CFBH 29317	Panelas, Domingos Martins, ES	−20.335556	−40.628056	MG799577	–	MG799632	MG799673	MG799762	mil-1
T. miliaris ●	CFBH-T 10806	Sítio Recanto da Mata, Muniz Freire, ES	−20.582222	−41.473611	MG799593	MG799716	MG799630	MG799671	MG799760	mil-3
T. miliaris ●	CFBH 27290	Cachoeira próxima à Pedra Dourada, Caminho da Luz, Carangola, MG	−20.796000	−42.105633	MG799599	MG799723	MG799637	MG799678	MG799767	mil-2
T. miliaris ●	CFBH 27292	Cachoeira próxima à Pedra Dourada, Caminho da Luz, Carangola, MG	−20.796000	−42.105633	MG799606	MG799729	MG799644	MG799684	MG799773	mil-5
T. miliaris	MNRJ 25984	Parque Municipal Pico do Itabira, Cachoeiro de Itapemirim, ES	−20.848906	−41.067800	MG799595	–	–	–	–	mil-1
T. miliaris	MNRJ 25985	Parque Municipal Pico do Itabira, Cachoeiro de Itapemirim, ES	−20.848906	−41.067800	MG799594	–	–	–	–	mil-3
T. miliaris ●	MNRJ 25989	Parque Municipal Pico do Itabira, Cachoeiro de Itapemirim, ES	−20.848906	−41.067800	MG799610	MG799733	MG799649	MG799688	MG799778	mil-5
T. miliaris † ●	CFBH 25473	Barra Pontões, 650 m de altitude, Mimoso do Sul, ES	−20.946197	−41.531441	MG799576	MG799717	MG799631	MG799672	MG799761	mil-3
T. miliaris ●	MNRJ 43663	Margem esquerda do Rio Paraíba do Sul, Volta Grande, MG	−21.805881	−42.508008	MG799608	MG799731	MG799647	MG799686	MG799776	mil-5
T. miliaris † ●	CFBH 27350	Parque Estadual do Desengano, Morumbeca, Santa Maria Madalena, RJ	−21.879250	−41.918617	MG799582	MG799736	MG799652	MG799691	MG799781	mil-4
T. miliaris ●	CFBH 27366	Parque Estadual do Desengano, Estrada para o Sossego do Imbé, Santa Maria Madalena, RJ	−21.930467	−41.792500	MG799607	MG799730	MG799645	MG799685	MG799774	mil-5
T. miliaris	CFBH 28054	Parque Estadual do Desengano, trilha para o pico do Desengano, Santa Maria Madalena, RJ	−21.880310	−41.916910	MG799600	MG799724	MG799638	MG799679	–	mil-2

T. miliaris ●	CFBH-T 21276	Rio Aiuruoca, Aiuruoca, MG	−21.975390	−44.603540	MG799601	MG799725	MG799639	MG799680	MG799768	mil-2
T. miliaris ●	MNRJ 70701	Parque Natural Municipal Fazenda Atalaia, Macaé, RJ	−22.304600	−41.999997	MG799612	MG799737	MG799653	MG799692	MG799782	mil-4
T. miliaris † ●	CFBH 10125	Estrada Rio de Janeiro-Petrópolis, km 86, 718m, Petrópolis, RJ	−22.533350	−43.233840	MG799578	MG799721	MG799635	MG799676	MG799765	mil-2
T. miliaris **†**	**MNRJ 40642**	**Pista Cláudio Coutinho, Praia Vermelha, Rio de Janeiro, RJ**	**−22.952931**	**−43.157719**	**MG799580**	**MG799719**	**MG799646**	**–**	**MG799775**	**mil-5**
T. miliaris ●	MNRJ 74658	Pedra de Itacoatiara, Itacoatiara, Niterói, RJ	−22.973539	−43.025100	MG799609	MG799732	MG799648	MG799687	MG799777	mil-5
T. miliaris ●	CFBH-T 15495	Paredão de pedra na Rodovia Rio-Santos (BR-101), km 487, Angra dos Reis, RJ	−22.981302	−44.436654	MG799611	MG799735	MG799651	MG799690	MG799780	mil-5
T. miliaris † ●	CFBH 31105	Costão rochoso da praia das Laranjeiras, Trindade, Paraty, RJ	−23.334416	−44.676563	MG799581	MG799734	MG799650	MG799689	MG799779	mil-5
T. saxatilis † ●	MCP 11918	Cascata da Cortina, Serra da Rocinha, Serra Velha, Timbé do Sul, SC	−28.828690	−49.915585	MG799573	MG799707	MG799619	MG799661	MG799749	sax-1
T. saxatilis ●	CFBH 32211	Cachoeira da Onça, Praia Grande, SC	−29.160000	−49.988333	MG799587	MG799710	MG799622	MG799664	MG799752	sax-2
T. saxatilis † ●	CFBH 30381	Cascata da Forqueta, Distrito de Barra do Ouro, Maquiné, RS	−29.534364	−50.203171	MG799574	MG799709	MG799621	MG799663	MG799751	sax-2
T. saxatilis ●	CFBH 30311	Reserva da Família Lima, entre a 3a e a 4a cachoeiras, Sapiranga, RS	−29.552869	−51.016900	MG799586	MG799708	MG799620	MG799662	MG799750	sax-2
T. taophora [a]	CFBH 30616	Paredão de pedra na Rodovia Rio-Santos (BR-101), próximo a Tarituba, Paraty, RJ	−23.047222	−44.577028	MG799616	–	–	–	–	tao-2
T. taophora † [a] ●	CFBH 31102	Trilha para a praia das Laranjeiras, Trindade, Paraty, RJ	−23.332247	−44.679728	MG799584	MG799740	MG799655	MG799695	MG799785	tao-1
T. taophora * ●	CFBH 10472	Ilha Redonda, Praia Almada, Ubatuba, SP	−23.351944	−44.904444	GQ174553	GQ174960	MG799656	MG799696	MG799786	tao-1
T. taophora ●	CFBH 19920	Ilha Anchieta, Ubatuba, SP	−23.541367	−45.074647	MG799615	MG799741	MG799657	MG799697	MG799787	tao-2
T. taophora **†**	**MZUSP-A 143713**	**Cachoeira da Pedra Lisa, Parque N. M. Nascentes de Paranapiacaba, Santo André, SP**	**−23.804944**	**−46.301000**	**MG799585**	**MG799720**	**MG799658**	**MG799698**	**–**	**tao-2**
T. taophora * ●	CFBH 15325	Ilha de São Sebastião, Parque Estadual de Ilhabela, Ilhabela, SP	−23.831111	−45.352778	GQ174590	GQ174990	MG799660	MG799700	MG799789	tao-2
T. taophora ●	CFBH 15700	Ilha As Ilhas, São Sebastião, SP	−23.789444	−45.710314	MG799617	MG799742	MG799659	MG799699	MG799788	tao-2
T. taophora † ●	CFBH 29711	Estação Ecológica Juréia-Itatins, Núcleo Arpoador, Peruíbe, SP	−24.382886	−47.018247	MG799583	MG799738	MG799654	MG799693	MG799783	tao-3
T. taophora	CFBH 34898	Cachoeira do Pocinho, Iguape, SP	−24.571933	−47.248519	MG799613	MG799739	–	MG799694	MG799784	tao-3
C. boraceiensis	–	–	–	–	KJ961570	–	KJ961550	MG799703	KJ961590	–
C. eleutherodactylus	–	–	–	–	KU495190	–	MG799625	MG799704	MG799755	–
C. dubius	–	–	–	–	GQ174585	MG799743	–	–	–	–
P. boiei	–	–	–	–	KU495468	EU017781	KU494675	MG799702	MG799790	–
Z. parvulus	–	–	–	–	KU495619	–	KU494826	MG799701	MG799791	–

Species	Family	GenBank Accession numbers									
		12S	*tRNA-Val*	*16S*	*tRNA-Leu*	*ND1*	*tRNA-Ile*	*COI*	*POMC*	*RAG1*	*RHO*
Allophryne ruthveni	Allophrynidae	AY843564	AY843564	AY843564	AY819458	AY819458	–	–	AY819077	EU663432	AY844538
Alsodes neuquensis	Alsodidae	AY843565	AY843565	AY843565	JX204017	JX204017	JX204017	JX203891	KP295561	AY844362	AY844539
Eupsophus roseus / E. calcaratus	Alsodidae	AY843587	AY843587	AY843587	JX204054	JX204054	JX204054	DQ502852	KC604074	–	AY844560
Limnomedusa macroglossa	Alsodidae	AY843689	AY843689	AY843689	–	–	–	KC593345	KP295579	AY844471	AY844682
Allobates femoralis	Aromobatidae	AY364543	AY364543	AY364543	HQ290951	HQ290951	HQ290951	DQ502811	HQ290831	DQ503327	DQ503215
Atelognathus patagonicus	Batrachylidae	AY843571	AY843571	AY843571	–	–	–	–	KP295562	AY844368	AY844545
Batrachyla leptopus	Batrachylidae	AY843572	AY843572	AY843572	JX204029	JX204029	JX204029	JX203910	KP295563	AY844369	AY844546
Hylorina sylvatica	Batrachylidae	JX204222	JX204222	JX204222	–	–	–	–	–	–	–
Duttaphrynus melanostictus	Bufonidae	AY458592	AY458592	AY458592	AY458592	AY458592	AY458592	AY458592	DQ158317	EU712821	DQ283967
Espadarana prosoblepon	Centrolenidae	JX564857	JX564857	JX564857	JX564857	JX564857	JX564857	FJ766591	AY819085	EU663453	AY364404
Ceratophrys cornuta	Ceratophryidae	KP295608	KP295608	KP295608	KP295542	KP295542	KP295542	KP295688	KP295567	KP295589	KP295708
Chacophrys pierottii	Ceratophryidae	KP295621	KP295621	KP295621	KP295551	KP295551	KP295551	–	KP295573	KP295597	KP295716
Lepidobatrachus laevis	Ceratophryidae	KP295631	KP295631	KP295631	KP295556	KP295556	KP295556	KP295699	KP295576	KP295599	KP295720
Cycloramphus acangatan	Cycloramphidae	HQ634162	–	FJ685683	–	–	–	–	–	FJ685103	KF214198
Cycloramphus bandeirensis	Cycloramphidae	HQ634161	–	HQ634166	–	–	–	–	–	–	–
Cycloramphus boraceiensis	Cycloramphidae	DQ283097	DQ283097	DQ283097	–	–	–	DQ502856	–	DQ503357	DQ283813
Cycloramphus dubius	Cycloramphidae	–	–	GQ174585	–	–	–	–	–	–	–
Cycloramphus eleutherodactylus	Cycloramphidae	HQ634160	–	KU495190	–	–	–	MG799625	–	MG799755	–
Cycloramphus fuliginosus	Cycloramphidae	HQ634158	–	HQ634163	–	–	–	–	–	–	–
Cycloramphus organensis	Cycloramphidae	HQ634159	–	HQ634164	–	–	–	–	–	–	–
Thoropa cf. *lutzi* (CFBH-T 10,562)	Cycloramphidae	MG799706	MG799572	MG799572	MG799572	MG799572	–	–	–	MG799748	MG799802
***Thoropa megatympanum* (MCNAM 2072)**	**Cycloramphidae**	**MG799575**	**MG799575**	**MG799575**	**MG799575**	**MG799575**	**MG799575**	**MG799626**	**–**	**MG799756**	**MG799793**
Thoropa miliaris (CFBH 29,317)	Cycloramphidae	MG799577	MG799577	MG799577	MG799577	MG799577	MG799577	MG799632	MG799745	MG799762	MG799795
Thoropa miliaris (CFBH 30,707)	Cycloramphidae	MG799579	–	MG799579	MG799579	MG799579	MG799579	MG799643	–	MG799772	MG799797
Thoropa miliaris (CFBH 25,473)	Cycloramphidae	MG799576	MG799576	MG799576	MG799576	MG799576	MG799576	MG799631	–	MG799761	MG799794
Thoropa miliaris (CFBH 27,350)	Cycloramphidae	MG799582	MG799582	MG799582	MG799582	MG799582	MG799582	MG799652	MG799747	MG799781	MG799799
Thoropa miliaris (CFBH 10,125)	Cycloramphidae	MG799705	MG799578	MG799578	MG799578	MG799578	MG799578	MG799635	MG799746	MG799765	MG799796
***Thoropa miliaris* (MNRJ 40,642)**	**Cycloramphidae**	**MG799580**	**MG799580**	**MG799580**	**–**	**–**	**–**	**MG799646**	**–**	**MG799775**	**–**
Thoropa miliaris (CFBH 31,105)	Cycloramphidae	MG799581	MG799581	MG799581	MG799581	MG799581	MG799581	MG799650	–	MG799779	MG799798
Thoropa saxatilis (MCP 11,918)	Cycloramphidae	MG799573	MG799573	MG799573	MG799573	MG799573	MG799573	MG799619	MG799744	MG799749	–
Thoropa saxatilis (CFBH 30,381)	Cycloramphidae	MG799574	MG799574	MG799574	MG799574	MG799574	MG799574	MG799621	–	MG799751	MG799792
Thoropa taophora (CFBH 31,102) [a]	Cycloramphidae	MG799584	MG799584	MG799584	MG799584	MG799584	MG799584	MG799655	–	MG799785	MG799801
***Thoropa taophora* (MZUSP-A 143,713)**	**Cycloramphidae**	**MG799585**	**MG799585**	**MG799585**	**–**	**–**	**–**	**MG799658**	**–**	**–**	**–**
Thoropa taophora (CFBH 29,711)	Cycloramphidae	MG799583	MG799583	MG799583	–	–	–	MG799654	–	MG799783	MG799800
Zachaenus parvulus	Cycloramphidae	KC593362	KC593362	KC593362	–	–	–	–	–	–	–
Colostethus pratti	Dendrobatidae	HQ290969	HQ290969	HQ290969	HQ290969	HQ290969	HQ290969	DQ502865	HQ290847	DQ503361	KP295708
Stefania evansi	Hemiphractidae	AY843767	AY843767	AY843767	AY819490	AY819490	–	–	AY819108	DQ679307	AY844755
Hypsiboas multifasciatus	Hylidae	AY843648	AY843648	AY843648	GQ366299	GQ366299	GQ366299	JN970762	GQ366036	AY844436	AY844633
Crossodactylus schmidti	Hylodidae	AY843579	AY843579	AY843579	JX204031	JX204031	JX204031	DQ502738	HQ290828	DQ503298	AY844552
Hylodes phyllodes	Hylodidae	DQ283096	DQ283096	DQ283096	–	–	–	DQ502873	–	DQ503367	DQ503253
Adenomera andreae	Leptodactylidae	KC520683	KC520683	KC520683	HQ290944	HQ290944	HQ290944	KC520689	KC604061	KC604037	KC604094
Macrogenioglottus alipioi	Odontophrynidae	KC593360	KC593360	KC593360	KC593353	KC593353	KC593353	–	–	KC593355	KC593357
Odontophrynus americanus	Odontophrynidae	AY843704	AY843704	AY843704	JX204059	JX204059	JX204059	JX203939	JX298141	FJ685706	AY844695

Proceratophrys avelinoi	Odontophrynidae	KP295643	KP295643	KP295643	–	–	–	–	–	FJ685711	KP295584	KF214204
Insuetophrynus acarpicus	Rhinodermatidae	JX204223	JX204223	JX204223	–	–	–	JX203938	–	JX204088	–	JX204152
Rhinoderma darwinii	Rhinodermatidae	DQ283324	DQ283324	DQ283324	JX564891	JX564891	JX564891	DQ502858	KP295584	–	–	AY364403
Telmatobius bolivianus/T. sibiricus/T. truebae	Telmatobiidae	JF703234	JF703234	JF703234	JF703234	JF703234	JF703234	JF703234	AY819097	AY583344	–	AY844757

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.01.017.

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