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Multilocus phylogeny of Paratelmatobiinae (Anura: Leptodactylidae) reveals strong spatial structure and previously unknown diversity in the Atlantic Forest hotspot

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ABSTRACT

The Brazilian Atlantic Forest harbors high levels of anuran diversity and endemism, including several taxa restricted to small geographic ranges. Here, we provide a multilocus phylogeny for Paratelmatobiinae, a leptodactylid subfamily composed of small-ranged species distributed in the Brazilian Atlantic Forest and in the *campo rupestre* ecosystem. We performed Bayesian inference and maximum likelihood analyses using three mitochondrial and five nuclear markers, and a matrix comprising a broad taxonomic sampling. We then delimitated independently evolving lineages within the group. We recovered Paratelmatobiinae and each of its four genera as monophyletic and robustly supported. Five putatively new species included in our analyses were unambiguously supported in the phylogenetic trees and delimitation analyses. We also recovered other deeply divergent and geographically structured lineages within the four genera of Paratelmatobiinae. Our estimation of divergence times indicates that diversification in the subfamily began in the Eocene and continued until the Pleistocene. We discuss possible scenarios of diversification for the four genera of Paratelmatobiinae, and outline the implications of our findings for taxonomy and conservation.

1. Introduction

The Brazilian Atlantic Forest (AF) harbors one of the highest levels of endemism and biodiversity in the world (Morellato and Haddad, 2000; Silva and Casteleti, 2003; Fonseca et al., 2004). The wide latitudinal and longitudinal ranges, broad altitudinal gradients, and the environmental complexity of this forest domain are among the main factors proposed to explain its great biological diversity (Morellato and Haddad, 2000; Oliveira-Filho and Fontes, 2000; Câmara, 2003). This biodiversity hotspot also harbors exceptionally high anuran diversity. With more than 620 species, it contains nearly one-ninth of the global anuran richness and more than 50% of the megadiverse Brazilian anuran fauna (Rossa-Feres et al., 2017; Frost, 2020). More than 75% of these species are endemic to the AF and most of them are known from

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one or a few localities in montane environments (Haddad et al., 2013; Villalobos et al., 2013; Rossa-Feres et al., 2017).

These endemic taxa are susceptible to strong anthropogenic pressure. Over the last decades, the AF has suffered from alarming rates of vegetation loss (Ribeiro et al., 2009; Tabarelli et al., 2010), which represents the main threat to its species (Myers et al., 2000). In fact, 90% of the endangered Brazilian anurans occur only in the AF and are restricted to small geographic ranges (ICMBio, 2018). For instance, the first Brazilian anuran to be considered extinct, *Phrynomedusa fimbriata* (Hylidae: Phyllomedusinae), had a very small geographic distribution restricted to the AF (Baêta et al., 2016; Cruz and Pimenta, 2004; ICMBio, 2018). These factors highlight the need for comprehensive studies of small-ranged anurans endemic to the AF, as restricted geographic distributions represent a risk factor for extinctions due to stochastic events (Lande, 1999; Sodhi et al., 2008; Pimm et al., 2014). This knowledge will allow for a better understanding of evolutionary processes that have resulted in patterns of anuran endemism, which is important to improve conservation strategies.

Several non-mutually exclusive hypotheses have been proposed to explain the diversification of species in the AF. The most commonly invoked is the Pleistocene refugia hypothesis (Haffer, 1969; Hewitt, 2000; Moritz et al., 2000; Carnaval et al., 2009; Martins, 2011; Cazé et al., 2016), which rests on the idea that climatic fluctuations caused successive contractions and expansions of forests and open environments, with the persistence of forests in climatically stable areas. The hypothesis posits that isolation of forest fragments has prevented gene flow between populations of forest-adapted species, favoring diversification. A second class of hypotheses involves geographic barriers to gene flow, such as rivers systems and mountain chains (Pellegrino et al., 2005; Cabanne et al., 2007; Brunes et al., 2010; Thomé et al., 2014; Cazé et al., 2016). Additional factors that may have contributed to diversification, especially at small geographic scales, are topographic complexity of tropical mountains and climatic variation along altitudinal gradients, causing isolation among populations and subsequent speciation (Guarnizo and Cannatella, 2013; Graham et al., 2014; Rodríguez et al., 2015). Coupled with environmental complexity, life history traits play a key role in diversification by defining the vagility and the physiological tolerance of species (Wollenberg et al., 2011; Fusinatto et al., 2013; Polato et al., 2018).

Here, we focus on the diversification of one poorly known group of endemic anurans of AF, the leptodactylid subfamily Paratelmatobiinae Ohler and Dubois, 2012. This subfamily currently comprises 14 species in four genera: *Crossodactylodes* Cochran, 1938, *Paratelmatobius* Lutz and Carvalho, 1958, *Rupirana* Heyer, 1999, and *Scythrophrys* Lynch, 1971 (Frost, 2020). The four genera are distributed along a North-South gradient (Fig. 1; Fouquet et al., 2013), and all species are associated with montane environments. The monotypic genus *Rupirana* is endemic to the *campo rupestre* ecosystem (i.e., rupestrian grasslands; Silveira et al., 2016) in the northern Espinhaço Mountain Range, state of Bahia (Heyer, 1999; Juncá and Lugli, 2009). The genus *Crossodactylodes* includes five species distributed in the AF from southern Bahia to the state of Rio de Janeiro, and in one open environment of the *campo rupestre* in the state of Minas Gerais (Santos et al., 2020). The genus *Paratelmatobius* comprises seven species arranged in two groups (*P. cardosoi* group and *P. lutzii* group) distributed along the Serra da Mantiqueira and Serra do Mar mountain ranges, from southern Minas Gerais and Rio de Janeiro, through São Paulo, to the state of Paraná (Pombal and Haddad, 1999; Lourenço et al., 2008, Santos et al., 2019). *Scythrophrys* is also a monotypic genus distributed in the southern part of Serra do Mar and Serras do Leste Catarinense mountain ranges, in the states of Paraná and Santa Catarina (Lourenço et al., 2008).

Species of Paratelmatobiinae occupy a wide range of microhabitats, including shallow temporary ponds and small streams in open (*Rupirana*) or forested (*Paratelmatobius* and *Scythrophrys*) environments, and bromeliads (*Crossodactylodes*) (Heyer, 1999; Juncá and Lugli, 2009; Lourenço et al., 2008; Pombal and Haddad, 1999; Santos et al., 2020). Most species are rare and known from one or few localities (Frost, 2020); some of them have not been recorded for several years, such as *C. pintoi* (last record dating to 1909; Peixoto and Carvalho-e-Silva, 2004) and *P. lutzii* (last record dating to 1978; Pombal and Haddad, 1999). Although the subfamily occurs in a well-sampled and studied area (Oliveira et al., 2016; Moura et al., 2018), their rarity and occurrence in specific microhabitats, as well as the recent findings of new species (Barata et al., 2013; Santos et al., 2019; Teixeira et al., 2013), suggest that the number of species of Paratelmatobiinae may be underestimated.

Some life history traits of Paratelmatobiinae species such as small body size, restricted geographic range, and habitat specialization have already been linked to low population connectivity and, consequently, to elevated speciation rates (Losos, 2010; Wollenberg et al., 2011; Fusinatto et al., 2013; Condez et al., 2020). Therefore, the subfamily is a suitable model taxon to investigate diversification events across the AF mountains, at different temporal scales, as well as between the AF and the *campo rupestre*. However, phylogenetic studies focused on this subfamily have been based on limited taxonomic and/or character sampling (Fouquet et al., 2013; Lourenço et al., 2008; Santos et al., 2019, 2020; Teixeira et al., 2013), hampering inferences about the evolutionary history.

By combining fieldwork with samples from herpetological collections, we prepared a dataset with broad taxon sampling of Paratelmatobiinae, including species with unknown phylogenetic placement and lineages that may represent undescribed species. By sequencing eight markers from 11 named species and five putatively new species, we reconstructed the phylogeny of the Paratelmatobiinae to (1) test the monophyly of the subfamily and each of its genera; (2) delimit independently evolving lineages within *Crossodactylodes*, *Paratelmatobius*, *Scythrophrys*, and *Rupirana*; (3) estimate the timing of diversification events within the subfamily; and (4) identify evolutionary processes that might have driven diversification at different temporal scales. Given the strict association with montane environments, the restricted geographic ranges, and the life history traits of the species of Paratelmatobiinae, we expect to find deeply divergent and geographically structured species and lineages, consistent with ancient diversification events.

2. Material and methods

2.1. Sampling, laboratory procedures, and sequence editing

We gathered tissue samples from field efforts and seven institutional collections, complementing it with GenBank sequences (Tables S1 and S2). Our ingroup includes 60 samples of *Crossodactylodes*, 120 of *Paratelmatobius*, 45 of *Scythrophrys*, and nine of *Rupirana*. This includes 11 of the 14 nominal species within Paratelmatobiinae, covering almost the entirety of its known geographic range. Our sampling also includes five putatively new species: a lineage of *Paratelmatobius* (here referred as *Paratelmatobius* sp.), three lineages of *Crossodactylodes* (here referred as *Crossodactylodes* sp. 1, *Crossodactylodes* sp. 2, and *Crossodactylodes* sp. 3), for which morphological differences were observed in comparison with congeners, and a lineage of *Scythrophrys* previously proposed as a distinct taxonomic unit based on genetic and karyotypic differences (see Lourenço et al., 2003), here referred as *Scythrophrys* sp. Whenever the identification of a lineage was not absolutely certain, we added the qualifier *confer* (cf.) to the species names. Tissue samples were not available for (1) *C. pintoi*, known only from its type series collected in 1909 (Peixoto and Carvalho-e-Silva, 2004); (2) *P. lutzii*, last recorded in 1978 (Pombal and Haddad, 1999); and (3) *P. mantiqueira*, known only from its type series collected in 1953 (Pombal and Haddad, 1999) and a single specimen found 52 years later (Vrcibradic et al., 2010).

We obtained genomic DNA from ethanol-preserved tissues using a standard ammonium acetate extraction protocol (Maniatis et al., 1982). Then, we used Polymerase Chain Reaction (PCR) to amplify DNA

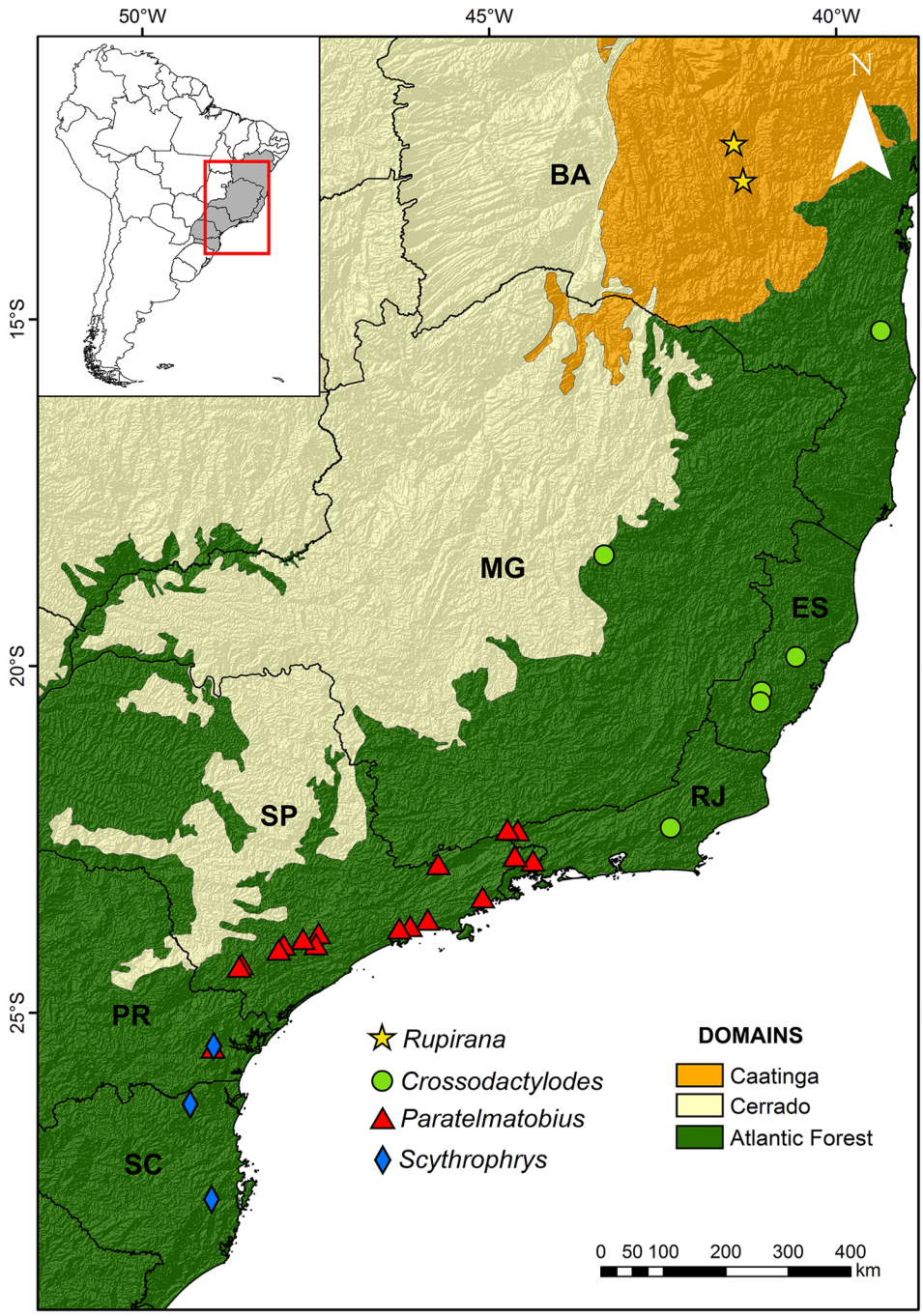

Fig. 1. Geographic distribution of the four genera of Paratelmatobiinae. Occurrence records were obtained from Juncá and Lugli (2009), Lourenço et al. (2008), and Santos et al. (2019, 2020). Brazilian states: BA (Bahia); ES (Espírito Santo); MG (Minas Gerais); PR (Paraná); RJ (Rio de Janeiro); SC (Santa Catarina); SP (São Paulo). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

sequences for three mitochondrial (mtDNA) and five nuclear (nDNA) gene fragments. The mtDNA dataset includes the non-coding heavy strand transcription unit 1 (H1: 12S and 16S rRNA genes, plus the intervening $tRNA^{valine}$ gene), a fragment of the cytochrome *c* oxidase subunit I (COI), and cytochrome *b* (cyt-*b*). The nDNA markers include fragments of the exonic genes proopiomelanocortin A (POMC), recombination-activating protein 1 (RAG-1), rhodopsin (Rhod), tensin 3 (TNS3), and tyrosinase (Tyr). PCR primers used for amplification and sequencing and reaction conditions are shown in Table S3. We purified PCR products using a mix of thermosensitive alkaline phosphatase (FastAP) and Exonuclease I, following the manufacturer's protocol (Thermo Fisher Scientific Inc.), with modifications provided by Lyra et al. (2017). The products were sequenced in both directions by Macrogen Inc. (Seoul, South Korea), with a Big Dye v.3.0 Sequencing Kit (Applied Biosystems). We checked for quality, excluded primers sequences, and assembled the chromatograms using Geneious v.7 (Biomatters Ltd.). GenBank accession numbers and voucher information are given in Table S1 (ingroup) and Table S2 (outgroup).

We performed sequence alignments with MAFFT v.7.427 (Katoh and Standley 2013) using the MAFFT online service (Katoh et al., 2017). For coding gene fragments (COI, cyt-*b*, POMC, RAG-1, Rhod, TNS3, and Tyr) we used the G-INS-i algorithm, whereas the E-INS-i algorithm was used for H1.

2.2. Phylogenetic inferences

To test the monophyly of Paratelmatobiinae we used all ingroup samples, representatives of most families of Hyloidea as outgroups, and a calyptocephalellid (*Calyptocephalella gayi*) to root the trees due to its close relationship with Hyloidea (Pyron, 2014). We performed Bayesian inference (BI) and maximum likelihood (ML) analyses concatenating mtDNA and nDNA fragments. We defined 22 *a priori* partitions, including the H1 and the three codon positions for each coding fragment, separately, to estimate the best partition scheme and substitution models in PartitionFinder v.2.1.1 (Lanfear et al., 2017) under the linked model of branch lengths, greedy search algorithm, and Corrected Akaike Information Criterion (Hurvich and Tsai, 1989). For the ML analysis, we used the resulting partition scheme, but applied the GTR + G model for all partitions due to the impossibility of applying different models in RAxML. The best partition schemes and substitution models are given in Table S4 for all analyses. The BI analysis was computed with MrBayes v.3.2.6. (Ronquist et al., 2012) using two parallel runs of 4.0×10^7 generations each, and four chains (one cold), sampling every 1000 generations. We set up the runs with unlinked character state frequencies, substitution rates of GTR model, gamma shape parameter, and proportion of invariable sites between partitions. The first 25% of generations and trees were discarded as burn-in, and the remaining trees were summarized in a majority-rule consensus tree and used to calculate Bayesian posterior probabilities (PP). We verified the minimal effective sample sizes (ESS $>$ 200) and the convergence between the runs with Tracer v.1.7 (Rambaut et al., 2018). The ML analysis was computed with RAxML v.8.2.10 (Stamatakis, 2014), with 100 independent searches for the best tree and 1000 non-parametric bootstrap replicates.

To evaluate mito-nuclear discordance, we also performed BI analyses for mtDNA and nDNA data separately. For that, we removed most of the outgroups from the previous dataset, maintaining only *Leptodactylus latrans* to root the trees due to its close relationship with Paratelmatobiinae (Faivovich et al., 2014; Santos et al., 2020; this study). We reestimated the best partition scheme and substitution models in PartitionFinder v.2.1.1 (Lanfear et al., 2017) for each dataset (Table S4). The parameters of BI analyses were the same as in the previous analyses, except for the number of generations (3.0×10^7). We performed all phylogenetic analyses using the CIPRES Science gateway portal (Miller et al., 2010).

2.3. Delimitation of independently evolving lineages

To objectively define independently evolving lineages (IELs) for subsequent species tree analysis, we followed an approach of congruence, considering the reciprocal monophyly of lineages in the phylogenetic trees, the results of the lineage delimitation analyses (Generalized Mixed Yule Coalescent model - GMYC, Pons et al., 2006; and nDNA fragments network), and the 16S rRNA genetic divergences between strongly supported clades.

For delimitation of the mtDNA IELs using the GMYC model, we first constructed an ultrametric mtDNA gene tree using the same dataset of the previous mtDNA BI analysis, yet assuming each fragment as a distinct partition, and estimating substitution models in PartitionFinder v.2.1.1 (Lanfear et al., 2017); Table S4. This ultrametric gene tree was estimated in BEAST v.2.5.2 (Bouckaert et al., 2019), using the Yule model as the tree prior. A clock was estimated independently for each fragment, assuming the log-normal uncorrelated relaxed clock, without account for absolute times in the tree. The analysis was done with two independent replicates of 5.0×10^7 MCMC generations each, sampling every 5.0×10^3 generations. The convergence between the two runs, the mixing of parameters, and the minimum effective sample size (ESS $>$ 200) were checked in Tracer v.1.7 (Rambaut et al., 2018). We combined the trees from the two runs using the LogCombiner post-processing tool (Bouckaert et al., 2019), discarding 5% burn-in. A maximum clade credibility (MCC) gene tree was then obtained through the TreeAnnotator post-processing tool (Bouckaert et al., 2019), and used in GMYC to estimate the number of IELs (Pons et al., 2006).

To run the GMYC model, we used the package *ape* v.5.3 (Paradis and Schliep, 2019) in R (R Core Team, 2017) to remove the outgroup from the MCC mtDNA tree and to keep only one random individual per locality per clade, thus avoiding the accumulation of short branches in the tree. Excessive short branches are known to result in an overestimation of candidate species in GMYC (Reid and Carstens, 2012). Specimens included in this analysis are listed in Table S1. Then we applied a maximum likelihood implementation of the GMYC using the package *splits* v.1.0.19 (Fujisawa and Barraclough, 2013) in R, assuming a single threshold among the diversification between lineages and the genealogical branching within them. Additionally, we randomly sampled 2.5×10^3 trees, using *ape* to remove the same sequences that were taken off from the MCC tree. Subsequently, we used these trees to estimate the PP of each lineage using the *bGMYC* v.1.0.2 package (Reid and Carstens, 2012) in R.

We estimated the uncorrected *p*-distances between and within the lineages delimited by the GMYC model using the 16S rRNA gene fragment limited by the primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991). We calculated the distances for each genus, as well as for each species group within *Paratelmatobius*, using Mega X (Kumar et al., 2018) and treating gaps and missing data as pairwise deletions.

Since the GMYC model tends to overestimate the number of independent units, often delimiting population structure (Talavera et al., 2013), we performed an independent lineage clustering by constructing a distance network with the nDNA fragments. For this, we first used the PHASE v.2.1.1 software (Stephens et al., 2001), with SeqPHASE input/output interconversion tool (Flot, 2010) to solve the haplotype gametic phases of each fragment, considering a threshold of 0.7 PP. From the phased sequences, we constructed a matrix of multigene distances using the algorithm *genpofad*, implemented in Pofad v.1.07 (Joly et al., 2015). We corrected multiple hits in distances using the Jukes and Cantor (1969) algorithm and inferred missing nucleotides and distances using the additive method implemented in Pofad. The analysis was performed for entire Paratelmatobiinae and for each genus separately, except for *Paratelmatobius*. In the latter, we calculated distances independently for the *P. cardosoi* and *P. lutzii* species groups, due to the high levels of genetic divergence detected between them. We visualized the resulting multigene distance matrices as phylogenetic networks in SplitsTree v.4.14.8 using the neighbor-net algorithm (Huson and Bryant, 2006).

Whenever multiple mtDNA lineages belonged to a single nDNA cluster, we treated these lineages as single terminals in the subsequent species tree analysis. This was implemented because lack of gene flow is a premise of the multispecies coalescent (Liu et al., 2015). The only exception was the mtDNA lineage D of *Paratelmatobius* cf. *segallai*. Although we recovered three lineages (B, C, and D) in the same nDNA cluster, the clade formed by these lineages was paraphyletic with respect to the lineage A in the phylogenetic analyses. In addition, deep genetic divergences distinguish lineage D from all other lineages (see Results). Because of that, we decided to keep lineage D as an independent lineage for the species tree. Although alternative methods are available for lineage validation (Rannala, 2015), we opted for the protocol described above given the relatively small number of independent gene fragments (six) available for validation procedures (Knowles and Carstens, 2007; Camargo and Sites, 2013).

2.4. Species-tree estimation and divergence times

After the definition of IELs, we built a matrix composed only by individuals with at least six gene fragments successfully sequenced, to construct a dated species-tree. This subsampling aimed to reduce the mixing problems observed in exploratory analyses of the complete dataset. Additionally, we included samples of *Odontophrynus* and *Proceratophrys* (Odontophrynidae), *Engystomops*, *Leptodactylus*,

Lithodytes, and *Pleurodema* (Leptodactylidae) as outgroups. We used phased nuclear sequences in this analysis and estimated the best substitution model for each fragment through PartitionFinder, assuming that each gene fragment had independent substitution models (Table S4). The three mtDNA genes were concatenated and considered as a single fragment.

The species tree was estimated with the StarBeast2 algorithm (Ogilvie et al., 2017) implemented in BEAST v.2.5.2 (Bouckaert et al., 2019). We used the analytical population size integration and a Yule model as prior for the species tree estimation. We assumed relaxed-clock models with uncorrelated lognormal distributions for all fragments. Due to the absence of fossil records for Paratelmatobiinae, we calibrated the analysis using two secondary calibration points from Feng et al. (2017). These authors used 20 fossil-based calibrations, testing each of them by jackknife, and a comprehensive phylogenomic dataset to estimate their divergence times. We used normally distributed priors for the divergence between *Odontophrynus* and *Proceratophrys* [mean = 32.0 million years ago (Mya), sigma = 4.1], defining a 95% range of 24.8–40.0 Mya; and for the crown age of Leptodactylidae (mean = 52.8 Mya, sigma = 2.7), defining a 95% range of 48.2–58.0 Mya. The range of divergence times of *Odontophrynus/Proceratophrys* is consistent with the fossil record of the odontophrynid *Chachaiphrynus lynchi* from the Middle Oligocene (27–29 Mya) (Nicoli, 2017). To run the calibrated analysis, we initially conducted a preliminary run with 2.5×10^9 MCMC generations, sampling every 5.0×10^4 generations. Then, we adjusted the scale-factors and size of scale operators following the report of the preliminary analysis. We ran a second analysis with the adjusted parameters, performing four independent replicates of 5.0×10^9 MCMC generations each, and sampling every 1.0×10^5 generations. We assessed stationarity, convergence, and effective sample sizes with Tracer, from what we discarded 10% of the samples for each run as burn-in. The species trees of the four runs were merged, and the MCC species tree was annotated as done for the ultrametric mtDNA gene tree.

3. Results

3.1. Phylogenetic inferences

Based on a final alignment of 6739 base pairs, we obtained similar BI and ML phylogenies which mainly differed in the relationships among some of the outgroups (particularly those distantly related to Paratelmatobiinae) and support values (Figs. 2 and S1). The monophyly of Paratelmatobiinae was robustly supported (PP = 100; ML bootstrap = 98), as well as that of each of the four genera (Figs. 2–4). In both analyses, *Rupirana* was recovered as the sister taxon of the three other genera, while *Scythrophrys* was recovered as sister to *Crossodactylodes* + *Paratelmatobius* (all relationships with PP = 100 and ML bootstrap > 95; Figs. 2 and 3). The subfamily Leptodactylinae was recovered as the sister taxon of Paratelmatobiinae in both analyses, although with moderate support in the ML inference (PP = 100; ML bootstrap = 61; Figs. 2 and S1).

Within *Scythrophrys*, the monophyly of *Scythrophrys* sp. was unambiguously supported in both analyses and this taxon was recovered as sister to the nominal species *S. sawayae* (Fig. 3). In *Crossodactylodes*, the monophyly of each of the three potentially new species (*Crossodactylodes* sp. 1, *Crossodactylodes* sp. 2, and *Crossodactylodes* sp. 3) and of *C. bokermanni*, *C. septentrionalis*, and *C. itambe* was unambiguously supported in both analyses. The monophyly of *C. izecksohni* was unambiguously supported in the BI analysis and moderately supported in the ML analysis (ML bootstrap = 84). *Crossodactylodes bokermanni* was recovered as the sister taxon of a clade comprising the remaining species. Within this clade, both analyses recovered *Crossodactylodes* sp. 3, *C. septentrionalis*, and *C. itambe* in a strongly supported clade (PP = 100; ML bootstrap = 94), separated from a clade comprising *Crossodactylodes* sp. 2, *Crossodactylodes* sp. 1, and *C. izecksohni* (PP = 100; ML bootstrap = 84; Fig. 3).

Within *Paratelmatobius*, the sampled species of the *P. lutzii* group (*P. gaigeae* and *P. poecilogaster*) were recovered as monophyletic and unambiguously supported in both analyses (Fig. 4). In the *P. cardosoi* group, *P. yepiranga* was recovered as the sister taxon of *Paratelmatobius* sp., while *P. cardosoi* + *P.* cf. *cardosoi* were recovered as sisters to *P. segallai* + *P.* cf. *segallai* (all relationships and the individual monophyly of each taxon were unambiguously supported in both analyses). Considering the ingroup, the differences between BI and ML topologies were restricted to relationships within *P. cardosoi* + *P.* cf. *cardosoi*. The lineage from the municipalities of São Paulo and Itanhaém was recovered as sister to the others in the BI analysis, whereas the lineage from the municipalities of Biritiba Mirim, Mogi das Cruzes (Biritiba Ussu), and Salesópolis was recovered as sister to the others in the ML analysis (Fig. 4).

Deeply divergent and geographically structured clades were recovered within *R. cardosoi*, *S. sawayae*, *C. bokermanni*, *C. izecksohni*, *P. poecilogaster*, *P. cardosoi* + *P.* cf. *cardosoi*, and *P. segallai* + *P.* cf. *segallai* (Figs. 3 and 4).

The topology of the BI mtDNA tree was the same as that of the multigene BI tree, varying only in clade support values (Fig. S2). On the other hand, the topology of the BI nDNA tree was distinct from that of the multigene BI tree regarding relationships within *Crossodactylodes*. While *Crossodactylodes* sp. 3 was recovered as the sister taxon of *Crossodactylodes* sp. 2 + *Crossodactylodes* sp. 1 + *C. izecksohni* in the nDNA tree (PP = 87; Fig. S3), it was retrieved as sister to *C. itambe* + *C. septentrionalis* in the multigene tree (PP = 100; Fig. 3).

3.2. Delimitation of independently evolving lineages

Thirty-one mtDNA IELs were recovered by the GMYC model (confidence interval 8–46; Fig. 5). Within *Rupirana cardosoi*, two lineages from the state of Bahia were recovered (Fig. 5), and the uncorrected *p*-distance between them varied from 2.3% to 2.5% (Appendix A). Within *Scythrophrys*, four lineages were recovered: *Scythrophrys* sp. from the Serra do Tabuleiro and vicinities, in the state of Santa Catarina; and three lineages within *S. sawayae* from localities in the states of Paraná and Santa Catarina (Fig. 5). The uncorrected *p*-distance between *Scythrophrys* sp. and the lineages of *S. sawayae* varied from 2.9% to 4.6%, while the distances among the three lineages of *S. sawayae* varied from 1.1% to 2.5% (Appendix B).

Ten lineages were recovered within *Crossodactylodes*: *C. septentrionalis* from the south of the state of Bahia; *C. itambe* and *Crossodactylodes* sp. 3 from the Espinhaço Range in the state of Minas Gerais; and *Crossodactylodes* sp. 1, *Crossodactylodes* sp. 2, two lineages within *C. izecksohni*, and three lineages within *C. bokermanni* from the state of Espírito Santo (Fig. 5). The interspecific uncorrected *p*-distance among these lineages varied from 2.3% to 7.8% (Appendix C). The distances between the two lineages of *C. izecksohni* varied from 3.4% to 4.2%, while the distances among the three lineages of *C. bokermanni* varied from 0.9% to 4.2% (Appendix C).

For *Paratelmatobius*, the GMYC model recovered 15 lineages. Within *P. lutzii* group, five lineages were recovered: *P. gaigeae* from the Serra da Bocaina, in the state of São Paulo; and four lineages within *P. poecilogaster* from localities in the states of São Paulo and Rio de Janeiro (Fig. 5). The uncorrected *p*-distance between *P. gaigeae* and the lineages of *P. poecilogaster* varied from 4.1% to 5.5%, while the distances among the four lineages of *P. poecilogaster* varied from 0.2% to 3.2% (Appendix D). Within the *P. cardosoi* group, ten lineages distributed in the states of São Paulo and Paraná were recovered: *P. yepiranga*, *Paratelmatobius* sp., four lineages within *P. cardosoi* + *P.* cf. *cardosoi*, and four lineages within *P. segallai* + *P.* cf. *segallai* (Fig. 5). The interspecific uncorrected *p*-distance among these lineages varied from 4.4% to 8.8% (Appendix E). The distances among the four lineages within *P. cardosoi* + *P.* cf. *cardosoi* varied from 1.6% to 2.4%, whereas the distances among the four lineages within *P. segallai* + *P.* cf. *segallai* varied from 1.2% to

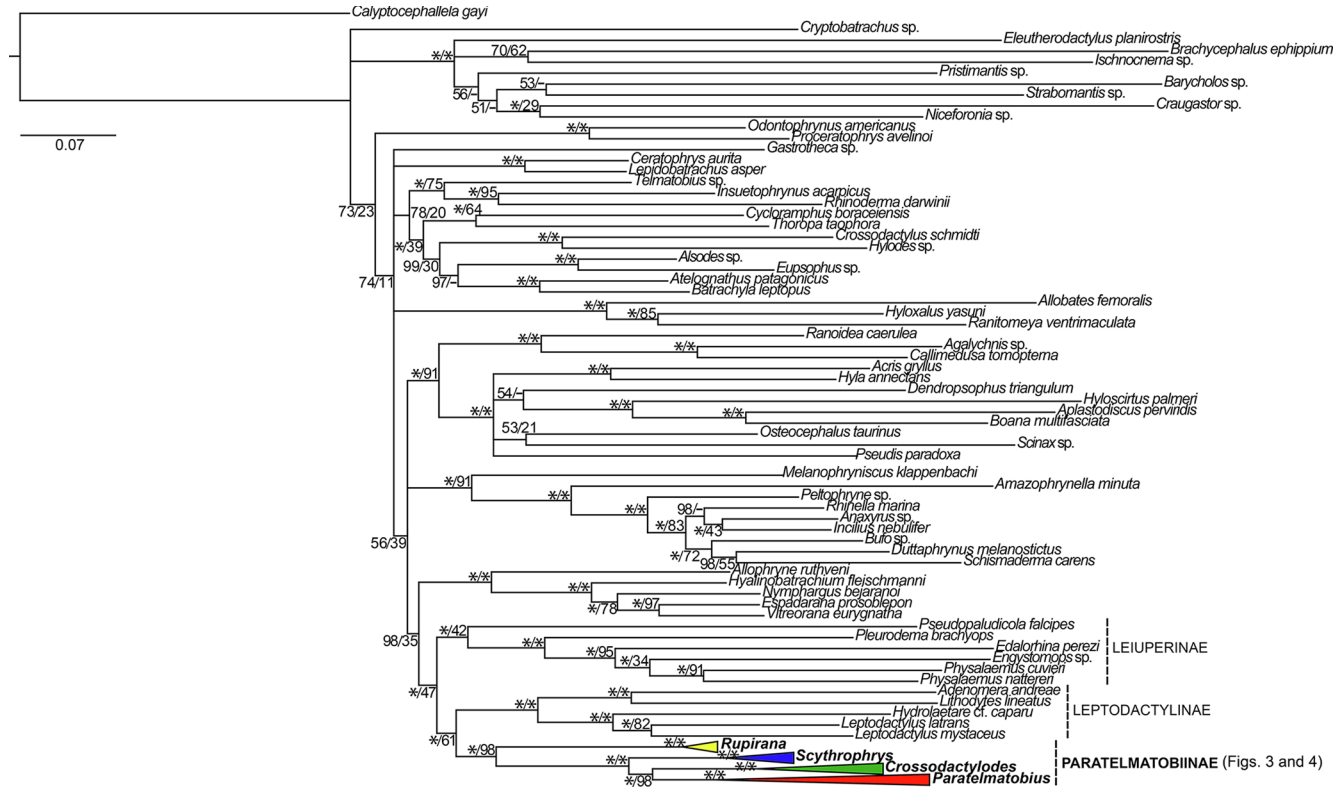

Fig. 2. The 50% majority rule consensus tree from Bayesian phylogenetic inference of concatenated mtDNA (H1, COI, cyt-*b*) and nDNA (POMC, RAG-1, Rhod, TNS3, Tyr) genes, showing the relationships of the four genera of Paratelmatobiinae with outgroups. For relationships within Paratelmatobiinae, please refer to Figs. 3 and 4. Posterior probabilities and maximum likelihood bootstraps are shown next to nodes (to the left and to the right of the bar, respectively). Asterisks (*) indicate unambiguously supported nodes and dashes (–) represent nodes not recovered in the maximum likelihood analysis. For the maximum likelihood topology, please refer to Fig. S1.

5.6% (Appendix E).

Although the structure of the nuclear neighbor-net generally agreed with the mtDNA lineages delimited by the GMYC model, several of the mtDNA lineages were placed in the same nDNA cluster (Fig. 6). This happened in *S. sawayae* (mtDNA lineages B + C), *C. bokermanni* (mtDNA lineages A + B), *C. izecksohni* (mtDNA lineages A + B), *P. poecilogaster* (mtDNA lineages A + B), *P. poecilogaster* (mtDNA lineages C + D), *P. cardosoi* (mtDNA lineages A + B + C), and *P.* cf. *segallai* (mtDNA lineages B + C + D); Fig. 6. These lineages were treated as single terminals in the subsequent species tree analysis, with the exception of the mtDNA lineage D of *P.* cf. *segallai*. This lineage, although belonging to the same nDNA cluster of lineages B + C, was recovered as sister to lineages A + B + C in the phylogenetic inferences (PP and ML bootstrap = 100; Fig. 4). In addition, high uncorrected *p*-distances distinguish lineage D from each of the other lineages (ranging 4.6–5.6%; Appendix E).

Each of the putatively new species (*Crossodactylodes* sp. 1, *Crossodactylodes* sp. 1, *Crossodactylodes* sp. 3, *Paratelmatobius* sp., and *Scythrophrys* sp.) was recovered as single nDNA clusters.

3.3. Species-tree estimation and divergence times

A total of 23 lineages of Paratelmatobiinae were included in the species tree analysis. The StarBEAST2 recovered all genera as monophyletic and unambiguously supported (Fig. 7). The relationships among the genera, as well as the relationships within each genus, were generally congruent with those of the BI and ML trees (Figs. 3 and 4). However, the relationships among *Crossodactylodes* sp. 3, *C. itambe*, and *C. septentrionalis*, as well as the relationships among the lineages within *P. segallai* + *P.* cf. *segallai,* were not robustly supported in the species tree analysis (PP < 95). The subfamily Leiuperinae was recovered as the sister taxon of Paratelmatobiinae, but with low support (PP = 53; Fig. 7).

Our estimation of divergence times (Fig. 7) suggests that the first split within Paratelmatobiinae, separating *Rupirana* from the other genera, dated back to the Middle Eocene [41.65 Mya; 35.71–48.09, 95% highest posterior density interval (HPD)]. The split between *Scythrophrys* and their sister taxa (*Crossodactylodes* and *Paratelmatobius*) was estimated to have occurred in the Upper Oligocene at 25.38 Mya (HPD 21.81–29.28), followed closely by the divergence between the latter two genera at 23.52 Mya (HPD 20.11–27.1). The split between the *P. lutzii* and *P. cardosoi* species groups dated back to the Middle Miocene at 16.57 Mya (HPD 13.85–19.27), while most of the other speciation events within the subfamily were inferred to have taken place in the Upper Miocene and the Lower Pliocene. On the other hand, divergences among what we considered intraspecific lineages appear to have taken place in the Upper Pliocene and the Lower and Middle Pleistocene (Fig. 7).

4. Discussion

This is the most comprehensive phylogenetic study of species in the subfamily Paratelmatobiinae conducted to date. The monophyly of Paratelmatobiinae and each of its genera was robustly supported, and the intrageneric relationships were generally well-resolved. Besides the 11 named species and the five potentially new species included in our dataset, we delimited seven other independently evolving lineages. We found strong support for our initial predictions of ancient diversification events and deeply divergent, geographically structured species and lineages. Our results reveal a complex scenario of diversification for Paratelmatobiinae, beginning with intergeneric divergences in the Eocene and Oligocene and continuing with most intrageneric

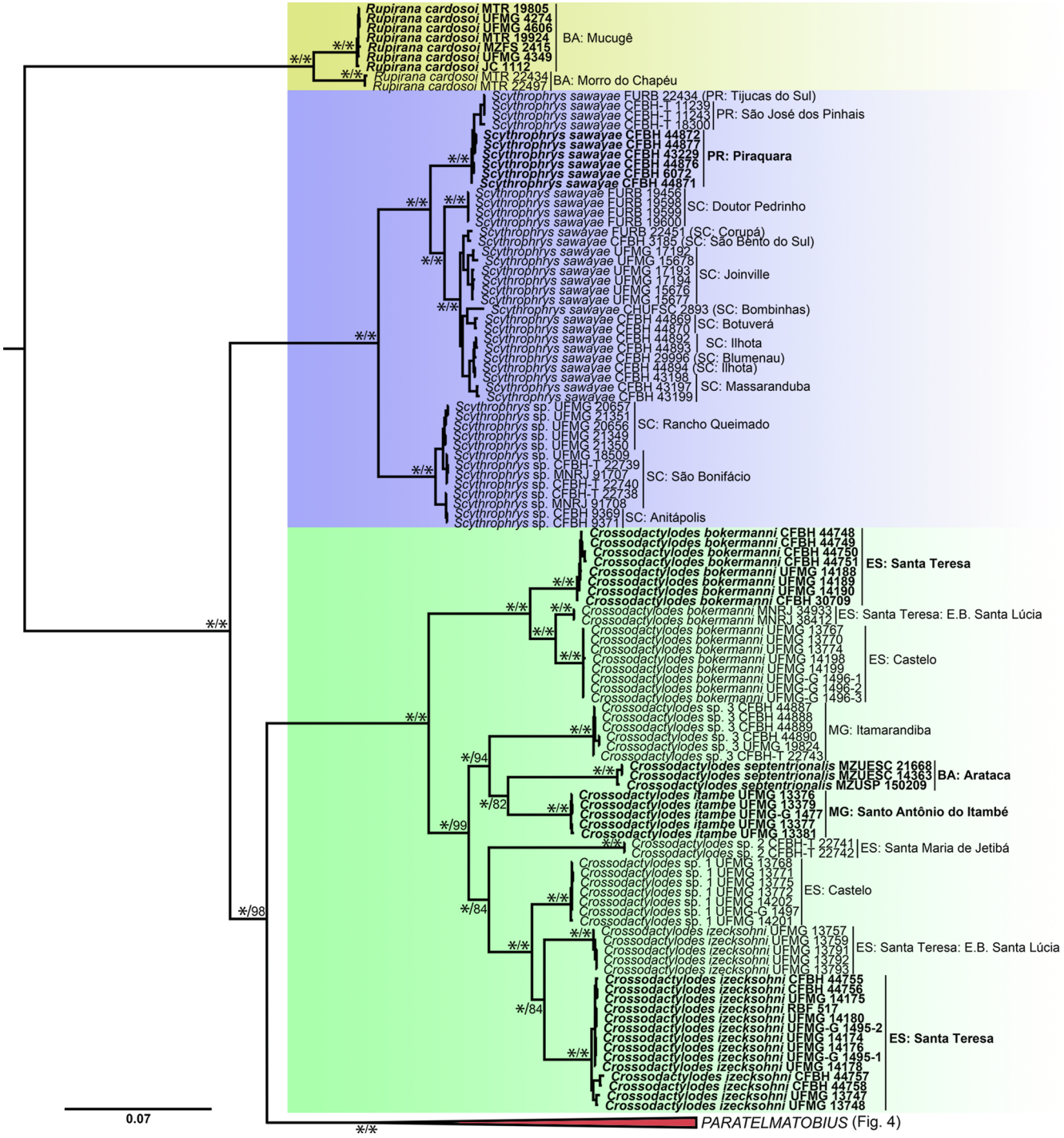

Fig. 3. The 50% majority rule consensus tree from Bayesian phylogenetic inference of concatenated mtDNA (H1, COI, and cyt-*b*) and nDNA (POMC, RAG-1, Rhod, TNS3, and Tyr) genes, showing the relationships within *Rupirana*, *Scythrophrys*, and *Crossodactylodes*. For relationships within *Paratelmatobius*, please refer to Fig. 4. Posterior probabilities and maximum likelihood bootstraps are shown next to nodes (to the left and to the right of the bar, respectively). Asterisks (*) indicate unambiguously supported nodes. Terminals in bold represent specimens from type localities.

diversification in the Miocene. Some diversification events cooccurred with past glaciations and tectonic activity in the Brazilian mountain ranges, but the heterogeneity of microhabitats in montane environments and the life history traits of the species of Paratelmatobiinae are likely important synergistic factors in diversification. Our results highlight the need of further taxonomic investigation, focusing on the unnamed lineages recovered by our analyses, as well as a reassessment of the conservation status of species, given their rarity and remarkably small geographic ranges.

4.1. Phylogenetic relationships and topological incongruences

The monophyly of Paratelmatobiinae, as well as the monophyly and relationships among the four genera, were consistent with previous studies based on smaller taxon and/or character sampling (Faivovich et al., 2014; Fouquet et al., 2013; Lourenço et al., 2008; Santos et al., 2019, 2020; Teixeira et al., 2013). We recovered the subfamily Leptodactylinae as the sister taxon of Paratelmatobiinae in both concatenated analyses, although the ML analysis shows moderate support values.

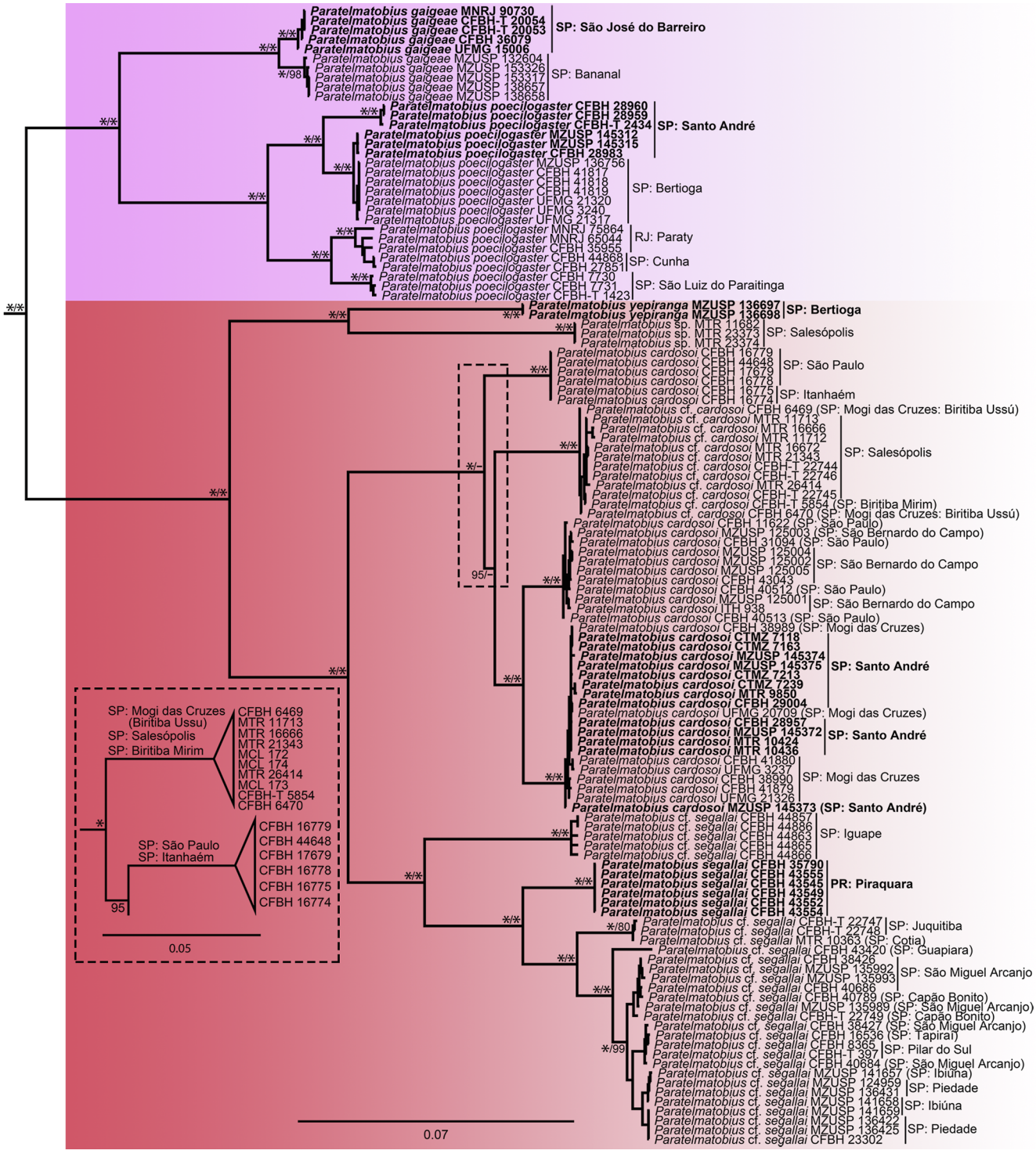

Fig. 4. The 50% majority rule consensus tree from Bayesian phylogenetic inference of concatenated mtDNA (H1, COI, and cyt-*b*) and nDNA (POMC, RAG-1, Rhod, TNS3, and Tyr) genes, showing the relationships within *Paratelmatobius*. Posterior probabilities and maximum likelihood bootstraps are shown next to nodes (to the left and to the right of the bar, respectively). Asterisks (*) indicate unambiguously supported nodes. Terminals in bold represent specimens from type localities. The dashed rectangle in the tree indicated the two nodes not recovered in the maximum likelihood analysis. For these nodes, the topology and support values achieved in the maximum likelihood analysis are shown in the dashed square.

This relationship is concordant with other phylogenetic hypotheses (Fouquet et al., 2013, complete matrix approach; Faivovich et al., 2014; Santos et al., 2020). Alternatively, the subfamily Leiuperinae was recovered as sister to Paratelmatobiinae in the species tree analysis, but with low support, in accordance with Pyron and Wiens (2011). On the other hand, Paratelmatobiinae was recovered as sister to Leiuperinae + Leptodactylinae in other studies (Fouquet et al., 2013, super matrix approach; Fouquet et al., 2014; Pyron, 2014), also with low support. Phylogenomic approaches have shown promise in resolving these deep nodes in the anuran phylogeny (Feng et al., 2017; Streicher et al., 2018). Sampling more loci and taxa may provide a better understanding of the relationships among the subfamilies of Leptodactylidae.

The relationships within *Scythrophrys*, *Crossodactylodes*, and the

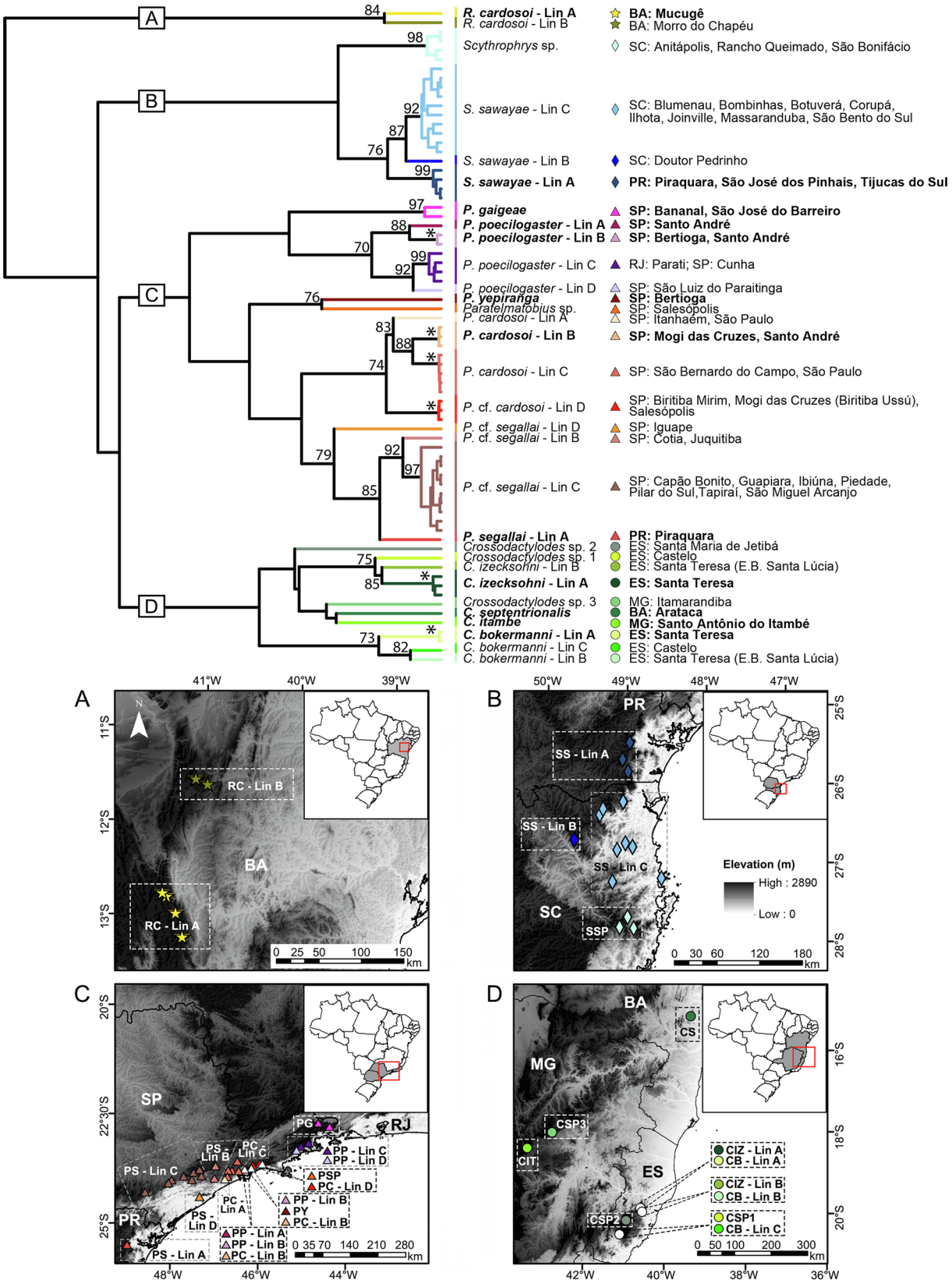

Fig. 5. The mtDNA gene tree showing the lineages delimited by the GMYC model. Symbols are the same for each genus, and distinct colors represent the delimited lineages. Terminals in bold represent lineages from type localities. Values next to the nodes represent the means of pairwise probabilities of co-specificity. Asterisks (*) indicate 100% values. The sampled localities for species and lineages are shown in the maps under the tree, for *Rupirana* (A), *Scythrophrys* (B), *Paratelmatobius* (C), and *Crossodactylodes* (D). White symbols in (C) and (D) indicate localities where two or more species/lineages occur in sympatry. Brazilian states: BA (Bahia); ES (Espírito Santo); MG (Minas Gerais); PR (Paraná); RJ (Rio de Janeiro); SC (Santa Catarina); SP (São Paulo). Other abbreviations: CB (*C. bokermanni*); CIT (*C. itambe*); CIZ (*C. izecksohni*); CS (*C. septentrionalis*); CSP1 (*Crossodactylodes* sp.1); CSP2 (*Crossodactylodes* sp.2); CSP3 (*Crossodactylodes* sp.3); Lin (Lineage); PC (*P. cardosoi*); PG (*P. gaigeae*); PP (*P. poecilogaster*); PS (*P. segallai*); PSP (*Paratelmatobius* sp.); PY (*P. yepiranga*); RC (*R. cardosoi*); SS (*S. sawayae*); SSP (*Scythrophrys* sp.). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

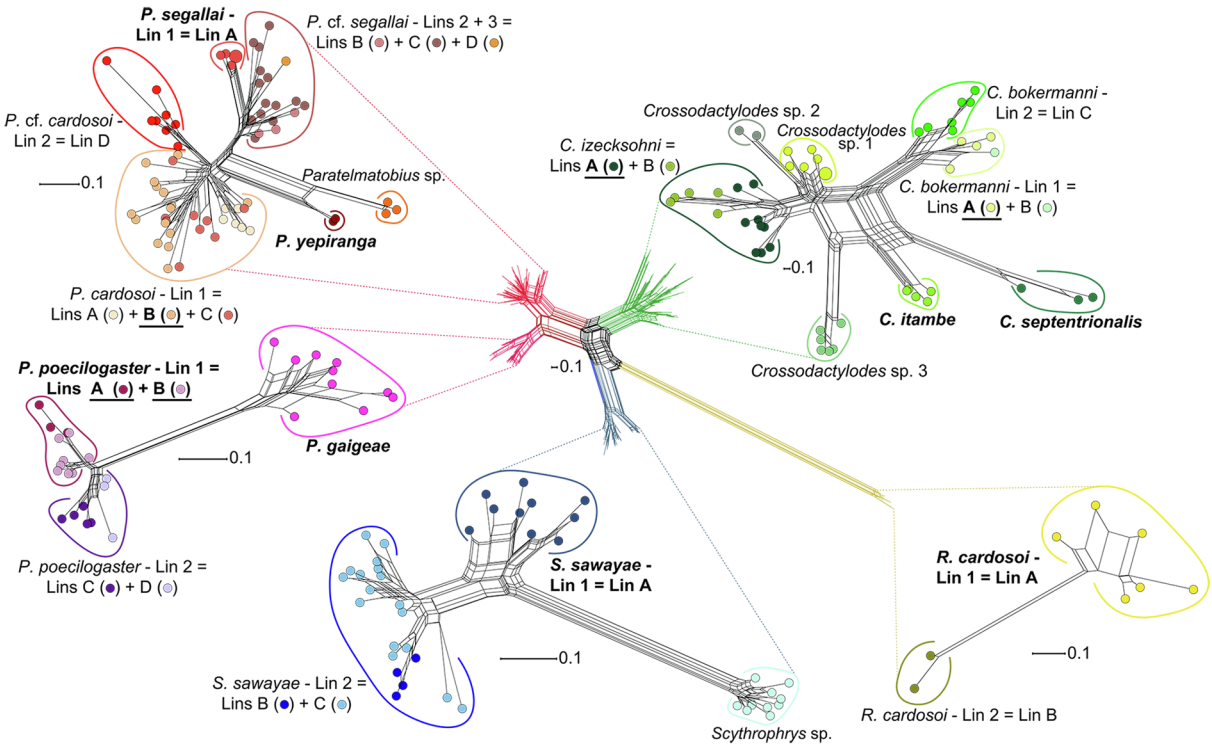

Fig. 6. Multilocus nDNA neighbor-net for the entire Paratelmatobiinae (small net in the center), for each genus, or species group (in the case of *Paratelmatobius*). Recovered clusters are delimited by ellipses. Note that some clusters are composed by more than one mtDNA lineage. Scale bars represent standardized genetic distances for each net (distances are not comparable among different nets). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

Paratelmatobius species groups (*P. cardosoi* and *P. lutzii*) are in accordance with previous hypotheses (Lourenço et al., 2008; Santos et al., 2019, 2020). However, our study is the first to provide phylogenetic placements for *Crossodactylodes* sp. 1, *Crossodactylodes* sp. 2, *Crossodactylodes* sp. 3, *Paratelmatobius* sp., and *P. yepiranga*. The phylogenetic placements of *C. pintoi*, *P. lutzii*, and *P. mantiqueira*, which were not included in our study, remain uncertain. *Crossodactylodes pintoi* was recovered as closely related to *C. itambe*, *C. septentrionalis*, and *C. izecksohni* in a total evidence analysis, but the relationships within this clade were not fully resolved (Santos et al., 2020). On the other hand, no phylogenetic hypothesis exists for *P. lutzii* and *P. mantiqueira*. Morphological data suggest that *P. mantiqueira* is possibly related to *P. cardosoi*, *P. segallai*, and *P. yepiranga*, whereas *P. lutzii* is possibly related to *P. gaigeae* and *P. poecilogaster* (Pombal and Haddad, 1999; Garcia et al., 2009; Santos et al., 2019). However, *P. lutzii* has remarkable diagnostic characters in comparison with its congeners, such as the absence of columella, foot with well-developed webs, and nuptial pads lacking spicules (Lynch, 1971; Pombal and Haddad, 1999). Therefore, the species phylogenetic placement deserves further investigation.

We found topological incongruences for some intrageneric relationships when comparing our phylogenetic inferences, mainly in the relationships with short branch lengths. The relationships within *P. cardosoi* + *P.* cf. *cardosoi* differ when comparing the multigene BI and ML analyses (Fig. 4). This may be due to differences in the evolutionary models assumed in the analyses (Suchard et al., 2003), as well as an effect of short branch lengths present in these relationships, which might be difficult to solve with strong support even using multilocus datasets (Wiens et al., 2008). We also found a variable position for *Crossodactylodes* sp. 3, when comparing the BI and ML trees (Fig. 3) with the BI nDNA tree (Fig. S3). Moreover, the relationships within *P. segallai* + *P.* cf. *segallai* are incongruent when comparing the multigene concatenated and mtDNA trees (Figs. 4 and S2) with the nDNA neighbor net (Fig. 6). In both cases, the species tree topology agrees with that of the multigene BI and ML trees, but the moderate node support values indicate the same pattern of discordant gene tree topologies (Fig. 7). Therefore, our results indicate a degree of uncertainty in the relationships of these taxa, as well as a conflicting pattern between mitochondrial and nuclear fragments. This mito-nuclear discordance may be related to specific characteristics of each genome, as the faster lineage sorting of mtDNA comparing with the nDNA (Toews and Brelsford, 2012).

4.2. Molecular dating and scenarios of diversification

The divergence times inferred with our species tree suggest an Eocene origin for the subfamily Paratelmatobiinae (~41.7 Mya) and subsequent intergeneric diversification in the Oligocene (~23.5–25.4 Mya) (Fig. 7). These estimated divergences are more recent than those found in a previous study, which estimated the split between *Rupirana* and the other Paratelmatobiinae at ~58 Mya, and that between *Crossodactylodes* and *Paratelmatobius* at ~34 Mya (Fouquet et al., 2013). These contrasting results may be due to differences in taxon and character sampling, calibration priors, and the method used for phylogenetic reconstruction (Linder et al., 2005; Pulquério and Nichols 2007; Hipsley and Müller, 2014). Fouquet et al. (2013) included a few species of Paratelmatobiinae, but a larger outgroup sampling, and calibrated the analysis using divergences between other taxa based on different secondary calibrations and one geological event. In addition, those authors used a concatenation-based method, which tends to overestimate the divergence times when compared to the coalescent method that we applied (McCormack et al., 2011). Nevertheless, both our results and those of Fouquet et al. (2013) agree on the deep intergeneric divergence times for Paratelmatobiinae.

The divergence between *Rupirana*, distributed in the *campo rupestre*, and the three remaining Paratelmatobiinae genera in the AF (35.7–48.1 Mya) is temporally coincident with the divergence time between the AF endemic frog genus *Dendrophryniscus* and its sister taxon *Amazophrynella*, endemic to Amazonia (Fouquet et al., 2012a). This

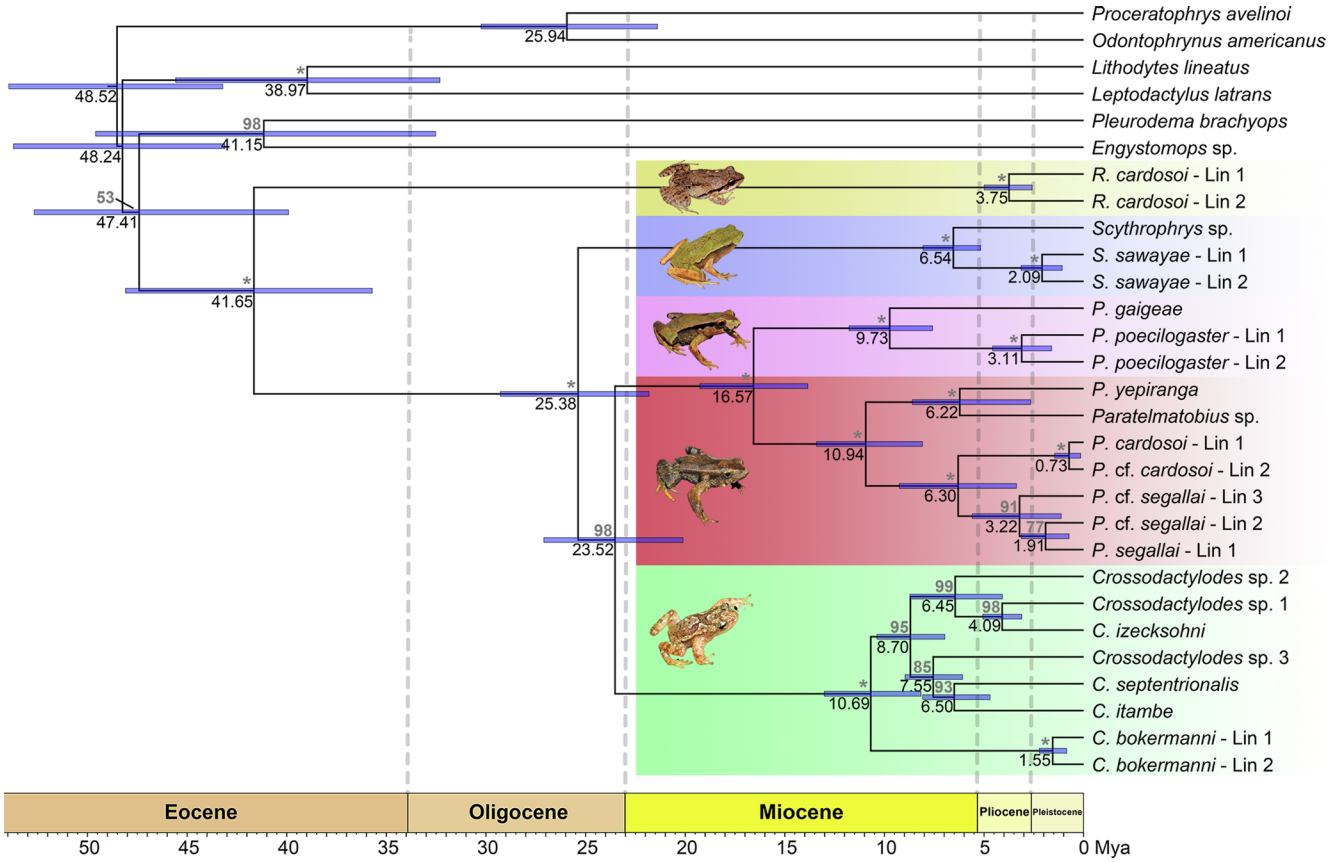

Fig. 7. Time-calibrated species tree annotated from StarBeast2 analysis. Values above branches (in gray) indicate posterior probabilities (asterisks mean 100% values). Values under branches (in black) indicate mean node ages, and the blue bars indicate standard deviation. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

timing also coincides with divergences between major clades of Brachycephaloidea and in the subfamily Phyzelaphryninae (Eleutherodactylidae), which are mainly distributed in forested habitats of either the AF or Amazonia (Fouquet et al., 2012b; Heinicke et al., 2009). The main mechanism invoked to explain these diversifications has been ancient, pre-Pleistocenic climatically-driven forest fragmentation. The Middle-Upper Eocene was characterized by the Andean uplift and remarkable climatic changes that included a shift from wet to dry conditions, as well as marine regressions (Romero, 1986; Ortiz-Jaureguizar and Cladera, 2006; Hoorn et al., 2010). Aridification is thought to have led to a partial replacement of previously widespread rainforest by open vegetation, resulting in the formation of the South American Dry Diagonal separating the AF and Amazonia (Romero, 1986; Roig-Juñent et al., 2006). Moist forest fragments embedded in the xeric Caatinga and Cerrado savannas are relicts that attest for the dynamics between the rainforest and the open vegetation in these regions (Carnaval and Bates, 2007; Thomé et al., 2016). We hypothesize that the formation of a dry corridor at that time may have isolated the *campo rupestre* endemic genus *Rupirana* from the most common ancestor of the AF Paratelmatobiinae, explaining their long-term divergence. The *campo rupestre* has been considered a climatically stable ecosystem, with several micro-refugia, favoring persistence of old lineages (Alves and Kolbek, 1994; Silveira et al., 2016). This appears to be the case of *Rupirana,* which represents a relict taxon adapted to more seasonal environments, persisting in the *campo rupestre* as its only endemic anuran genus.

Other ancient endemic elements of the *campo rupestre* are small-sized catfishes whose nearest sister taxa occur exclusively in the AF (Ochoa et al., 2017). The genera *Copionodon* and *Glaphyropoma*, endemic to the *campo rupestre*, diverged from the AF endemic subfamily Trichogeninae in the Lower Eocene (~55.9 Mya) (Ochoa et al., 2017). On the other hand, several plant genera endemic to the *campo rupestre* are inferred to have originated later, in the Miocene (Rapini et al., 2007; Antonelli et al., 2010; Silveira et al., 2016). Neither the Eocene origin nor the close affinities with the AF biota are conditions shared among all the *campo rupestre* endemic genera. Possibly, a complex scenario of multiple sources of origin drove their diversification, as proposed for species-level endemism (Bitencourt and Rapini, 2013; Silveira et al., 2016).

Following the divergence of *Rupirana*, our results support a rapid initial diversification for the three AF genera in the Oligocene and subsequent diversification within each genus, mainly in the Miocene (Fig. 7). This is consistent with divergence times recovered for several genera belonging to various anuran families (Jetz and Pyron, 2018), including other AF anurans with similar geographic range such as *Dendrophryniscus* (Fouquet et al., 2012a), *Thoropa* (Sabbag et al., 2018), and *Brachycephalus* (Condez et al., 2020). The deep divergence between *Scythrophrys* (the southernmost genus) and its sister taxa matches the pattern and timing reported for rock frogs in the genus *Thoropa* (Sabbag et al., 2018). These results suggest that at least some AF habitats in the southern portion of the AF remained relatively stable since the Oligocene, as suggested based on the Plio–Pleistocene population persistence of *Rhinella henseli* (Thomé et al., 2010). Although previous analyses suggested that contemporary climatic heterogeneity better explains lineage endemism in the southern AF relative to historical climates (Carnaval et al., 2014), our data reinforces the notion that local endemic lineages, such as *Scythrophrys, R. henseli* and *Thoropa saxatilis*, have been in place for a long time.

Past climatic changes that caused forest fragmentation and isolation may have played an important role in other diversification events. A

glaciation in the Oligocene/Miocene boundary, at ~23 Mya (Zachos et al., 2001) is coincident with divergences among the three AF genera. In turn, climatic fluctuations of the Miocene (Haffer, 1997; Zachos et al., 2001; Olsen and Whiteside, 2009; Herbert et al., 2016) match the timing of most of the intrageneric divergences, while those of the Plio-Pleistocene (Haffer, 1969; Carnaval et al., 2009) coincide with the intraspecific divergences (Fig. 7). However, these geological epochs also correspond to periods of significant tectonic activity and regressive differential erosion in the Brazilian mountain ranges (Saadi, 1993, 1995; Potter, 1997; Almeida and Carneiro, 1998; Riccomini et al., 2004; Cogné et al., 2013). These might have influenced diversification by creating barriers to dispersal (see details below). The presence of several geographically restricted lineages (Figs. 3–5) suggests that processes at smaller geographic scales related to habitat heterogeneity and life history traits might have acted as synergistic factors to shape diversification in Paratelmatobiinae (Fusinatto et al., 2013; Rodríguez et al., 2015). Species of the subfamily have a small body size and are strongly associated with humid microhabitats, characteristics that are indicative of low dispersal capacity and narrow physiological tolerances (Hillman et al., 2009; Pabijan et al., 2012). These traits, along with the differential distribution of microhabitats, may have led to reduced gene flow among populations and strong spatial structure, as proposed for other small-sized and microhabitat specialist anurans (Wollenberg et al., 2011; Fusinatto et al., 2013; Rodríguez et al., 2015).

Although similar drivers likely influenced the diversification of the Paratelmatobiinae, group-specific processes are probably important for each AF genus. Diversification within *Crossodactylodes*, for instance, may have been influenced by the fact that all species and lineages are strictly associated with bromeliads on humid mountaintops. Although these bromeliad microhabitats enable species to tolerate more seasonal conditions, even those species and lineages distributed in more inland areas (i.e., the *campo rupestre* endemics *C. itambe* and *Crossodactylodes* sp. 3; Fig. 5D) inhabit wet environments with mild temperatures due to orographic rainfall and orographic fog (Barata et al., 2013; this study). Given the patchy distribution of species and lineages of the genus on isolated mountaintops (Fig. 5D), we hypothesize that climate oscillations and changes in humidity over space and time may have influenced their evolutionary history. In this context, ancestors of *Crossodactylodes* may have had a wider distribution during the Miocene colder periods, becoming isolated on mountaintops during warmer times, as previously suggested for other montane taxa with disjunctly distributed species (Firkowski et al., 2016; Prates et al., 2020). The low vagility and habitat specificity of species may have facilitated this isolation. However, the diversity of this genus may still be underestimated, as suggested by recent discoveries of new species and populations (Barata et al., 2013; Teixeira et al., 2013; Santos et al., 2020; this study). Thus, this scenario of diversification must be interpreted with caution and remains to be tested.

Considering its geographic distribution, the genus *Paratelmatobius* comprises a relatively high number of species and lineages (Figs. 1 and 5). The spatial range of the genus strongly overlaps with the Continental Rift of Southeastern Brazil (CRSB; Riccomini et al., 2004), a large fault system originating from tectonic reactivation of Precambrian shear zones between the end of the Paleocene and the end of the Oligocene (Riccomini et al., 2004; Zalán and Oliveira, 2005; Gontijo-Pascutti et al., 2012). The CRSB has been identified as the region with the most pronounced neotectonic (Neogene–Quaternary) activity in Brazil, being associated with the evolution of river systems and the increase of topographical differences between valleys and mountain ranges (Riccomini et al., 2004; Saadi, et al., 2005; Zalán and Oliveira, 2005; Gontijo-Pascutti et al., 2012). The Neogene–Quaternary divergence times among sampled species and lineages of *Paratelmatobius* (Fig. 7), and the geographical coincidence of some genetic breaks with tectonic faults and lineaments of the CRSB, indicate that these events possibly influenced the evolutionary history of the genus.

For instance, the limit between the geographical ranges of *P. cardosoi* + *P.* cf. *cardosoi* and *P. segallai* + *P.* cf. *segallai* (Fig. 5C) is somewhat coincident with a NW-SE fault of the CRSB, which had tectonic reactivation in the Miocene (Zalán and Oliveira, 2005; Riccomini et al., 2010). This is roughly congruent in time with divergences between species of *Phyllomedusa* (Hylidae; Brunes et al., 2010) and lineages of *Proceratophrys boiei* (Odontophrynidae; Amaro et al., 2012), but the break in *Paratelmatobius* is located slightly to the north in comparison with these other taxa. The divergences among the three lineages within *P. segallai* + *P.* cf. *segallai* might also be correlated with tectonic faults and lineaments, as the Cubatão-Itariri Shear System, the Serrinha Shear Zone, and the Guapiara lineament (Riccomini and Assumpção, 1999; Saadi et al., 2002; Passarelli et al., 2007) are somewhat coincident with the limits of geographic ranges of these lineages. A striking geographical and temporal discordance occur in the diversification of some lineages in the *P. cardosoi* group. The divergence between *P. yepiranga* and *Paratelmatobius* sp. is geographically congruent with the divergence between the lineages 1 (mtDNA lineages A + B + C) and 2 (mtDNA lineage D) of *P. cardosoi* + *P.* cf. *cardosoi* (Fig. 5C), but the divergence time is much older for the former taxon pair (Fig. 7). This may be indicative of a similar barrier or process limiting connectivity between lineages across multiple time periods.

Lastly, in the genus *Scythrophrys*, *S. sawayae* (lineages 1 and 2) occurs exclusively in the south of the Serra do Mar, while *Scythrophrys* sp. occurs only in the Serra do Tabuleiro and surrounding areas, an isolated mountain range in the state of Santa Catarina (Fig. 5B). This locality harbors another endemic anuran, *Boana poaju* (Hylidae; Garcia et al., 2008) and some endemic plants (Hassemer et al., 2015; Da Silva and Külkamp, 2018), but is still poorly sampled for many taxonomic groups. The deep divergence time between *Scythrophrys* sp. and *S. sawayae* (~6.5 Mya) suggests that the Serra do Tabuleiro and Serra do Mar biota experienced independent evolution since the Upper Miocene.

4.3. Taxonomic and conservation implications

The five putative new species included in our analyses were robustly supported in the phylogenetic trees (Figs. 3 and 4) and lineage delimitation analyses (Figs. 5 and 6). In addition, we found at least seven deeply divergent and geographically structured lineages within named species in the four genera of Paratelmatobiinae (Figs. 6 and 7). These lineages are also well-supported in all analyses, suggesting that species diversity within the subfamily is highly underestimated. Karyotypic differences were previously reported for some of these independently evolving lineages. In *Paratelmatobius*, differences were reported when comparing mtDNA lineages B and D of *P. cardosoi* + *P.* cf. *cardosoi* (karyotypes not known for lineages A and C) and mtDNA lineages A and C of *P. segallai* + *P.* cf. *segallai* (karyotypes not known for lineages B and D) (Lourenço et al., 2008). For *Scythrophrys*, it was previously suggested that *Scythrophrys* sp. belongs to a distinct species based on karyotypic and mtDNA differences (Lourenço et al., 2003; Lourenço et al., 2008).

Given the identification of several potentially new species, future taxonomic investigation and the resolution of species boundaries are crucial for an accurate assessment of extinction risks in Paratelmatobiinae (Scherz et al., 2019). Factors such as rarity and reduced geographic ranges have been suggested as important indicators of extinction risk (Pimm et al., 2014; Toledo et al., 2014) and are relevant for members of the subfamily. Several species are only known from one or a few localities (i.e., *C. itambe*, *C. izecksohni*, *C. pintoi*, *C. septentrionalis*, *P. gaigeae*, *P. lutzii*, and *P. yepiranga*) and this is also true for the five putatively new species included in our analyses (*Crossodactylodes* sp. 1, *Crossodactylodes* sp. 2, *Crossodactylodes* sp. 3, *Paratelmatobius* sp., and *Scythrophrys* sp.). Moreover, none of the species are widely distributed (Fig. 5). The long periods of time that some species remain unrecorded illustrate the rarity in Paratelmatobiinae. *Crossodactylodes pintoi*, for instance, was last seen in 1909, despite several field expeditions to the type locality (Santos et al., 2020). *Paratelmatobius lutzii* was last recorded in 1978, even after multiple visits to

the type locality (Pombal and Haddad, 1999). In addition, *P. gaigeae* was only found again 73 years after its discovery (Zaher et al., 2005) and *P. mantiqueira* after 52 years (Vrcibradic et al., 2010). Beyond their tiny geographic ranges, their low abundance and use of specific microhabitats such as bromeliads, temporary ponds, or small streams in forest areas seem to be contributing to their rarity.

Red lists of threatened species are important tools to highlight conservation priorities (Miller et al., 2006; Rodrigues et al., 2006); we argue that they can be particularly relevant for Paratelmatobiinae. Within the subfamily, one species (*P. lutzii*) is classified as Critically Endangered (CR) in the Brazilian Red List (ICMBio, 2018) and another (*C. bokermanni*) as Near Threatened. In addition, it has been suggested that *C. pintoi* and *C. itambe* should be classified as CR (Barata et al., 2018; Morais et al., 2013, respectively), although they are considered as Data Deficient (DD) in the Brazilian Red List (ICMBio, 2018). Of the remaining ten species, three are listed as DD, four as Least Concern, and two were not assessed (ICMBio, 2018). Given that DD species should also be included in conservation planning (Mace et al., 2008; Trindade-Filho et al., 2012), and considering the small geographic ranges and rarity, the Paratelmatobiinae is a highly important taxon from a conservation perspective. In the light of the new information provided by this study, it will be important to reassess the conservation status of its species to safeguard their viability and survival.

CRediT authorship contribution statement

Marcus Thadeu T. Santos: Conceptualization, Formal analysis, Data curation, Writing - original draft. **Rafael F. de Magalhães:** Formal analysis, Writing - review & editing. **Mariana L. Lyra:** Data curation, Writing - review & editing. **Fabrício R. Santos:** Resources, Writing - review & editing, Funding acquisition. **Hussam Zaher:** Resources, Writing - review & editing. **Luís O.M. Giasson:** Resources, Writing - review & editing. **Paulo C.A. Garcia:** Conceptualization, Resources, Writing - review & editing. **Ana Carolina Carnaval:** Writing - review & editing, Supervision. **Célio F.B. Haddad:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Declaration of Competing Interest

We declare no conflict of interest.

Appendix A

Uncorrected *p*-distances (percentage) of mitochondrial 16S rRNA fragment (ca. 570 bp) within (highlighted in bold) and between the lineages of *Rupirana* delimited by the GMYC model. Data are shown as range (mean) when appropriate. Lin = Lineage.

Lineage	*R. cardosoi* Lin A	*R. cardosoi* Lin B
R. cardosoi Lin A	**0.0–0.2 (0.1, n = 7)**	
R. cardosoi Lin B	2.3–2.5 (2.3)	**.0 (n = 2)**

Appendix B

Uncorrected *p*-distances (percentage) of mitochondrial 16S rRNA fragment (ca. 570 bp) within (highlighted in bold) and among the lineages of *Scythrophrys* delimited by the GMYC model. Data are shown as range (mean) when appropriate. Lin = Lineage.

Lineage	*Scythrophrys* sp.	*S. sawayae* Lin A	*S. sawayae* Lin B	*S. sawayae* Lin C
Scythrophrys sp.	**0.0–0.9 (0.4, n = 13)**			
S. sawayae Lin A	3.6–4.5 (3.9)	**0.0–0.4 (0.2, n = 10)**		
S. sawayae Lin B	2.9–3.6 (3.2)	1.8–2.0 (1.9)	**0.0 (n = 4)**	
S. sawayae Lin C	3.4–4.6 (3.9)	1.6–2.5 (2.0)	1.1–1.6 (1.2)	**0.0–1.2 (0.5, n = 18)**

Appendix C

Uncorrected *p*-distances (percentage) of mitochondrial 16S rRNA fragment (ca. 570 bp) within (highlighted in bold) and among the lineages of *Crossodactylodes* delimited by the GMYC model. Data are shown as range (mean) when appropriate. Lin = Lineage.

Lineage	*C. bokermanni* Lin A	*C. bokermanni* Lin B	*C. bokermanni* Lin C	*C. itambe*	*C. izecksohni* Lin A	*C. izecksohni* Lin B	*C. septentrionalis*	*Crossodactylodes* sp.1	*Crossodactylodes* sp.2	*Crossodactylodes* sp.3
C. bokermanni Lin A	**0.0 (n = 2)**									
C. bokermanni Lin B	3.3–3.6 (3.5)	**0.0 (n = 8)**								
C. bokermanni Lin C	0.9	3.9–4.2 (4.1)	**0.0 (n = 8)**							
C. itambe	5.5–5.6 (5.5)	5.1–5.7 (5.4)	5.3–5.5 (5.3)	**0.0–0.2 (0.1, n = 5)**						
C. izecksohni Lin A	4.9–5.5 (5.2)	5.8–6.5 (6.2)	5.1–5.7 (5.4)	3.7–4.4 (3.9)	**0.0–0.4 (0.1, n = 12)**					
C. izecksohni Lin B	6.4–6.5 (6.4)	7.1–7.8 (7.4)	6.4–6.5 (6.4)	4.6–4.9 (4.7)	3.4–4.2 (3.7)	**0.0–0.2 (0.1, n = 5)**				
C. septentrionalis	5.8–6.0 (5.9)	5.7–6.1 (5.9)	5.8–6.0 (5.9)	2.8–3.2 (2.9)	4.2–4.7 (4.5)	4.8–5.1 (4.9)	**0.0–0.2 (0.1, n = 3)**			
Crossodactylodes sp.1	5.3–5.5 (5.3)	6.0–6.5 (6.2)	5.1–5.3 (5.1)	3.5–3.9 (3.6)	2.3–2.7 (2.4)	4.1–4.4 (4.1)	4.6–4.8 (4.6)	**0.0–0.2 (0.1, n = 7)**		
Crossodactylodes sp.2	5.8	6.7–7.2 (7.0)	5.4	5.4	5.6–6.2 (5.8)	6.8–7.0 (6.8)	5.4–5.6 (5.5)	5.6–5.8 (5.6)	**0.0 (n = 2)**	
Crossodactylodes sp.3	4.4–4.8 (4.6)	4.8–5.6 (5.1)	4.6–5.0 (4.7)	3.2–3.4 (3.2)	3.6–4.4 (3.9)	4.3–4.5 (4.3)	3.7–3.9 (3.8)	3.8–4.5 (4.0)	5.1–5.2 (5.1)	**0.0–0.4 (0.2, n = 6)**

Appendix D

Uncorrected *p*-distances (percentage) of mitochondrial 16S rRNA fragment (ca. 570 bp) within (highlighted in bold) and among the lineages of *Paratelmatobius lutzii* species group delimited by the GMYC model. Data are shown as range (mean). Lin = Lineage.

Lineage	*P. gaigeae*	*P. poecilogaster* Lin A	*P. poecilogaster* Lin B	*P. poecilogaster* Lin C	*P. poecilogaster* Lin D
P. gaigeae	**0.0–1.1 (0.6, n = 9)**				
P. poecilogaster Lin A	5.2–5.5 (5.4)	**0.0–0.2 (0.1, n = 3)**			
P. poecilogaster Lin B	4.3–4.8 (4.6)	0.9–1.4 (1.2)	**0.0–0.2 (0.1, n = 10)**		
P. poecilogaster Lin C	4.1–4.5 (4.4)	2.3–3.2 (2.7)	1.6–2.4 (1.9)	**0.0–1.1 (0.7, n = 5)**	
P. poecilogaster Lin D	4.1–4.7 (4.5)	2.3–3.0 (2.7)	1.6–2.2 (2.0)	0.2–1.4 (0.7)	**0.2–0.6 (0.4, n = 3)**

Appendix E

Uncorrected *p*-distances (percentage) of mitochondrial 16S rRNA fragment (ca. 570 bp) within (highlighted in bold) and among the lineages of *Paratelmatobius cardosoi* species group delimited by the GMYC model. Data are shown as range (mean) when appropriate. Lin = Lineage.

Lineage	*P. cardosoi* Lin A	*P. cardosoi* Lin B	*P. cardosoi* Lin C	*P. cardosoi* Lin D	*P. segallai* Lin A	*P. segallai* Lin B	*P. segallai* Lin C	*P. segallai* Lin D	*Paratelmatobius* sp.	*P. yepiranga*
P. cardosoi Lin A	**0.0 (n = 6)**									
P. cardosoi Lin B	2.0–2.2 (2.1)	**0.0–0.2 (0.1, n = 20)**								
P. cardosoi Lin C	1.6–1.9 (1.8)	2.1–2.4 (2.2)	**0.0 (n = 10)**							
P. cardosoi Lin D	1.6	1.8–2.0 (1.9)	1.8–2.1 (2.0)	**0.0 (n = 12)**						
P. segallai Lin A	4.8–5.0 (4.8)	5.9–6.2 (6.1)	5.4–5.6 (5.5)	5.7–5.9 (5.9)	**0.0 (n = 6)**					
P. segallai Lin B	5.4	6.4–6.8 (6.7)	5.9–6.2 (6.0)	6.3–6.4 (6.4)	1.6	**0.0 (n = 3)**				
P. segallai Lin C	4.4–5.5 (5.0)	5.1–6.4 (5.9)	5.0–6.2 (5.7)	5.0–6.0 (5.7)	1.4–2.0 (1.7)	1.2–1.8 (1.5)	**0.0–1.2 (0.3, n = 21)**			
P. segallai Lin D	5.2–5.3 (5.0)	6.6–7.0 (6.9)	5.5–5.8 (5.6)	5.9–6.1 (6.0)	4.6–4.7 (4.6)	4.6–4.8 (4.8)	5.0–5.6 (5.2)	**0.0 (n = 5)**		
Paratelmatobius sp.	7.3–7.5 (7.3)	8.2–8.6 (8.5)	8.4–8.8 (8.5)	7.7–7.8 (7.8)	7.5	7.7	7.4–8.0 (7.7)	6.8–7.0 (6.8)	**0.0 (n = 3)**	
P. yepiranga	7.3	8.5–8.8 (8.7)	7.6–8.2 (7.9)	7.9	7.0	7.2	7.2–7.9 (7.5)	5.6–5.7 (5.7)	5.5	**0.0 (n = 2)**

Appendix F. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106819.

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