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Diversity, biogeography, and reproductive evolution in the genus *Pipa* (Amphibia: Anura: Pipidae)

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ABSTRACT

The genus *Pipa* is a species-poor clade of Neotropical frogs and one of the most bizarre-looking due to many highly derived anatomical traits related to their fully aquatic lifestyle. With their African relatives, they form the Pipidae family, which has attracted much attention, especially regarding its anatomy, reproductive biology, paleontology and biogeography. However, the actual diversity and phylogenetic relationships within *Pipa* remain poorly understood, and thus so do their historical biogeography and the evolution of striking features, such as the absence of teeth and endotrophy in some species. Using short mtDNA sequences across the distribution of the genus, we identified 15 main lineages (Operational Taxonomic Units - OTUs). This more than doubles the number of the currently seven valid nominal species. Several closely related OTUs do not share nuDNA alleles, confirming species divergence. Time-calibrated phylogenies obtained from mitogenomes and from 10 nuclear loci provide highly similar topologies but strikingly distinct node ages for *Pipa*. High dN/dS ratios and the variation of substitution rates across the trees suggest a strong effect of saturation on fast evolving positions of mtDNA, producing a substantially shorter stem branch of *Pipa*. Focusing on the nuDNA topology, we inferred an early Neogene Amazonian origin of the diversification of *Pipa*, with an initial split between the Guiana-Brazilian Shields and Western Amazonia, a pattern observed in many other co-distributed groups. All the western species are edentate, suggesting a single loss in the genus. Each of these groups diversified further out of Amazonia, toward the Atlantic Forest and toward *trans*-Andean forests, respectively. These events are concomitant with paleogeographic changes and match patterns observed in other co-distributed taxonomic groups. The two Amazonian lineages have probably independently acquired endotrophic larval development.

1. Introduction

The increasing accumulation of genomic data has permitted unveiling phylogenetic relationships and divergence times with unprecedented accuracy throughout the tree of life (Delsuc et al., 2005; Burki et al., 2020). This is the case for amphibians in which phylogenomic investigations have spectacularly improved our understanding of the relationships among major lineages (e.g. Neobatrachia, Streicher

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et al., 2018), and, combined with fossil calibrations, dramatically narrowed down temporal estimates of their origins (Feng et al., 2017; Hime et al., 2021). However, many amphibian groups remain underrepresented but the reliability of future investigations critically depends on the availability/quality of priors such as node age calibration, for example from fossils, that are not evenly distributed across the tree of life or are inexistent in many groups (Donoghue and Yang, 2016).

The frogs of the family Pipidae are unique in having a fully aquatic lifestyle, and share many highly derived anatomical traits (Cannatella, 2015) and chromosomal features (Mezzasalma et al., 2015). Extant taxa of the family Pipidae include *Pipa* (7 species) in the Neotropics, and *Xenopus* (29 species, *Silurana* being considered a synonym), *Hymenochirus* (4 species), and *Pseudhymenochirus* (1 species) in Africa. The sister-taxon of Pipidae is Rhinophrynidae, with a single extant fossorial species, *Rhinophrynus dorsalis*, which occurs in Central America and southern North America. Together they form the Pipoidea clade, an ancient group of frogs whose crown age is ca. 160 Myrs (e.g., Hime et al., 2020). One pipid in particular, *Xenopus laevis*, has become a model organism and the focus of a tremendous amount of medical and fundamental laboratory research (e.g., Vleminckx, 2018). The relationships among pipids have also been the focus of many studies (e.g., Irisarri et al., 2011; Bewick et al., 2012; Hedke et al., 2013; Cannatella, 2015) including node and tip dating based upon what is probably the most abundant fossil record of all amphibian families (e.g., Trueb et al., 2005; Baez et al., 2008; Gomez, 2016). At the family level, this group has attracted much attention not only because of their bizarre anatomical features, but also because of their biogeographic history tightly linked to the break-up of western Gondwana. Phylogenomic analyses (Hedke et al., 2013; Feng et al., 2017; Irisarri et al., 2017; Hime et al., 2020), sometimes in combination with morphological data and fossils (Cannatella, 2015), largely support the monophyly of African pipids, which form the sister group of *Pipa*. Time estimates for the divergence between *Pipa* and the African pipids obtained from phylogenomic studies are highly congruent, and generally predate the final stage of the break-up of western Gondwana (ca. 105 Ma) (Feng et al., 2017; Irisarri et al., 2017; Hime et al., 2020). Nevertheless, an alternative topology, i.e., *Pipa* + Hymenochirini, has found support in a few genomic analyses (Bewick et al., 2012; Cannatella, 2015) and mitogenomic data, exclusively (Irisarri et al., 2017; Zhang et al., 2021). Even though these studies included less genomic data than the most recent studies recovering *Pipa* vs. African pipids, this alternative topology is in line with the morphological similarity between *Pipa* and Hymenochirini (Cannatella and Trueb, 1988; Gomez and Perez-Ben, 2019). Given (1) the bias inherent to mtDNA (Gissi et al., 2006; Irisarri et al., 2017; Zheng et al., 2011) and (2) the consensus provided by the most recent and most extensive phylogenomic investigations, the hypothesis of the monophyly of African pipids seems more robust, and thus either implies that the morphology of *Xenopus* is highly derived, and/or that *Pipa* and Hymenochirini underwent parallel evolution (Irisarri et al., 2011; Cannatella, 2015).

Paradoxically, despite the attention that pipids have attracted, the phylogenetic relationships within *Pipa* have been solely investigated using either morphological characters (Trueb and Cannatella, 1986; Cannatella and Trueb, 1988; Trueb and Massemin, 2001), or short mtDNA sequences with incomplete taxonomic sampling (Vacher et al., 2020; Lima et al., 2020), and consequently remain poorly understood. Multilocus phylogenetic studies focusing on pipids have either included a single or two *Pipa* terminals, and provided highly inconsistent crown ages for the genus. Among these studies, those that included the analysis of mitogenomes (Irisarri et al., 2012; Evans et al., 2019; Hemmi et al., 2020) revealed crown ages $>$ 50 Ma, which suggests ancient diversification. Conversely, Feng et al. (2017) using 88 kb of nuDNA loci found a Most Recent Common Ancestor (MRCA) for *Pipa* spp. to be only 11 Ma, implying a relatively recent diversification instead. Irissari et al. (2012) also recovered disproportionate branch lengths between phylogenetic trees inferred from mtDNA and nuDNA within *Pipa* when compared to other non-Neobatrachia anurans. Another paradox is the almost complete absence of known fossils directly related to *Pipa* (i.e., branching along the stem or nested within *Pipa*), which contrasts with an otherwise very rich Pipidae fossil record. The only exception is the ca. 10 My old fossil from Corralito (lower Urumaco formation) of a portion of the sacrum of a large *Pipa*, with posteromedial ridges on the dorsal surface of each sacral diapophyse, suggesting close relationships with *Pipa pipa* (Delfino and Sanchez, 2018). However, given its poor state of preservation, the phylogenetic position of this fossil remains unclear.

These knowledge gaps hamper a clear understanding of the biogeographic history as well as the evolution of reproductive modes and of the morphology of the genus. With four species occurring in Amazonia (*Pipa pipa* and *P. snethlageae* extend further), one in the Atlantic Forest (*P. carvalhoi*) and two with a *trans*-Andean distribution (*P. parva* and *P. myersi*), one can reasonably assume that the genus originated in Amazonia. However, it remains conjectural to formulate any biogeographic hypothesis for *Pipa* in the absence of a robust time calibrated phylogeny, and since both ancient, i.e., 40–15 Ma (Fouquet et al., 2012a, 2012b; 2013, 2014; Réjaud et al., 2020) and recent, i.e., $<$ 5 Ma (Fouquet et al., 2014) dispersals have been documented in anurans and in other vertebrates in the region (e.g. Ledo and Colli, 2017; Dal Vechio et al., 2018; Prates et al., 2018). It is also noteworthy that some characters are strikingly variable among *Pipa* spp., notably habitat preferences and body size. *Pipa pipa* and *P. snethlageae* are large-bodied species (qualified as "macropipa") and are distributed throughout Amazonia and even further into the Orinoco (Acosta-Galvis et al., 2016) and the Cerrado (Vaz Silva and Andrade, 2009; Dantas et al., 2019), occupying many different types of lotic and lentic aquatic environment, such as seasonally flooded forests. Their occurrence in large lentic systems should theoretically allow for long-distance dispersal along these aquatic corridors. Species such as the Amazonian *P. aspera* and *P. arrabali* are small-bodied ("micropipa") and occupy various types of small water bodies. They show comparatively high small-scale mobility across the terrestrial matrix and are therefore able to colonize temporary ponds not connected to any rivers. *Pipa parva* also occupies temporary water bodies in the coastal deserts and semi-deserts of the Maracaibo basin and lowlands of the northeastern Caribbean region of Colombia (Galvis et al., 2011; Blanco-Torres et al., 2013). Considering these traits, we expect dispersal ability of the macropipa to be high (e.g., Fonte et al., 2021 reported *P. pipa* in floating meadows of the Amazon main course) and their genetic structures to be relatively homogeneous over long distances vs. weaker dispersal abilities and more profound genetic structures in micropipa. Members of the genus also show striking differences in several morphological and life-history traits. Teeth, for example, are present only in *P. carvalhoi*, *P. aspera* (Trueb and Massemin, 2001) and *P. arrabali* (Trueb and Cannatella, 1986), and although all *Pipa* species incubate their eggs in a specialized skin layer growing on the female's back after going through complex breeding behaviors (Rabb and Rabb, 1960; Weygoldt, 1976), only the Amazonian species have a completely endotrophic larval development, while *P. carvalhoi*, *P. parva* and *P. myersi* have tadpoles that hatch from the female's back and subsequently complete their development as free living aquatic larvae (Greven, 2011). The evolution of edentulism (Paluh et al., 2021) and of reproductive mode (Vagi et al., 2019, Furness and Capellini, 2019) has recently attracted renewed attention thanks to the availability of extensive phylogenetic sampling and the development of new methods (Revell, 2012). However, as for the biogeographical history, the monophyly of edentate and of endotrophic species has never been tested using molecular data. Therefore the evolution of these characters remains ambiguous since either one of these characteristics necessarily implies parallel acquisition or loss.

In order to (1) delimit candidate species (2) evaluate phylogenetic relationships and divergence times within *Pipa* and (3) reconstruct the biogeographic history, the evolution of reproductive modes and teeth loss/gain, we gathered mitogenomic and 10 nuDNA loci for all *Pipa* species and major Pipoidea lineages. Since discrepancies in divergence

times have been previously recovered among *Pipa* spp., we furthermore investigated the origin of this bias by comparing the substitution rate across mt and nuDNA and tested for variation in the dN/dS ratio across the genes and branches.

2. Materials and methods

2.1. Species delimitation

Our first objective was to delimit major mtDNA lineages within *Pipa* since Vacher et al. (2020), Motta et al. (2018) and Lima et al. (2020) suggested that unrecognized species exist within *P. arrabali*, *P. aspera*, *P. pipa* and *P. carvalhoi*. Our sampling included 16S rDNA sequences from 115 specimens of *Pipa* (Table S1) covering the entire distribution of the genus. These samples were obtained through fieldwork and loans, and completed with available sequences from GenBank (Table S1). Newly acquired sequences were obtained from Sanger sequencing (details of primers are available in Table S5). DNA sequences were aligned on the MAFFT online server under the E-INS-i option with default parameters (Katoh et al., 2019) leading to a matrix of 595 base pairs (bp).

We applied three DNA-based single-locus species delimitation approaches using this matrix: (a) a distance-based method, the Automated Barcode Gap Discovery (ABGD; Puillandre et al., 2012), (b) a multi-rate coalescent-based method, the multi-rate Poisson Tree Processes model approach (mPTP; Kapli et al., 2019) and (c) a single-threshold coalescent-based method, the Generalized Mixed Yule Coalescent approach (single threshold GMYC; Pons et al., 2006; Monaghan et al., 2009). The ABGD delimitation was performed using the online web server (available at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with a prior of intraspecific divergences (K80) between 0.001 and 0.1 (P = 0.001–0.1), a proxy for minimum relative gap width of 1 (X = 1), and a number of steps equals to 30 (n = 30). For the mPTP delimitation, we first reconstructed a ML tree with RAxML v.8.2.4 (Stamatakis, 2014) using a CAT + Γ model, which was estimated to be a suitable model via PartitionFinder V2.1.1 (Lanfear et al., 2017). The mPTP delimitation was undertaken on the tree rooted on *Rhinophrynus*, with 5 million Markov chain Monte Carlo (MCMC) iterations, sampling every 10,000th iteration and discarding initial 10% iterations as burn-in. For the GMYC delimitation, we reconstructed a time-calibrated phylogeny using BEAST 2.5.2 (Bouckaert et al., 2014). We used a Birth-Death population model to account for extinction processes and incomplete sampling. We used a single partition with a GTR + I + Γ substitution model, with an uncorrelated relaxed lognormal clock model of rate variation among branches (Drummond et al., 2006). We used the estimated age of the MRCA of Pipidae of Hime et al. (2020) as a calibration point, assuming normal prior distributions of 116.0 Ma (SD = 9 Ma). For the MCMC parameters, we used four independent chains of 100 million iterations, recording every 10,000th iteration. We combined the log and tree files of the four independent runs, discarding the first 30% iterations as burn-in, using LogCombiner 2.5 (Bouckaert et al., 2014) and checked the convergence of our parameters, confirmed by all ESS being above 200. We then extracted the maximum clade credibility tree (from 28,004 trees) using Tree annotator 2.5 (Bouckaert et al., 2014). We performed a GMYC delimitation on the ultrametric tree using the GMYC function of the {splits} R package (Ezard et al., 2009), with a threshold interval between 0 and 10 Ma and by using the single threshold method. Operational Taxonomic Units (OTUs) were defined using a majority-rule consensus from the results of the three methods, i.e., a lineage is considered as being an OTU if supported by at least two of the three methods.

2.2. nuDNA differentiation

We gathered sequences of four nuDNA loci (*RAG1*, *POMC*, *BDNF*, *NCX1*) for a subset of individuals in order to evaluate the degree of congruence of differentiation with the mtDNA-based delimitation (Table S2). Two OTUs (*P.* sp. "Negro", *P.* sp. "Nordeste") were not represented by any nuDNA sequences, one (*P. parva*) by only two nuDNA loci. Six OTUs were represented in all four datasets, but by a single terminal. Conversely, sequences from several individuals were obtained for six OTUs (only five OTUs for *POMC*). The sequences of *NCX1* were incomplete in 3' for five specimens or in 5' for five others; therefore, we considered both subsets independently (*NCX1*a & b). We reconstructed Median-Joining networks (Bandelt et al., 1999) from PopArt 1.7 (Leigh and Bryant, 2015). We considered the absence of nuDNA allele sharing among specimens assigned to closely related OTUs as indicative of their differentiation.

2.3. Molecular dating

We selected one representative of each delimited OTU for estimation of phylogenetic relationships and divergence times, except for two OTUs that were represented only by a limited amount of mtDNA and were discarded. We obtained whole mitogenomic data for representatives of 11 OTUs via shotgun sequencing (Table S3; methodological details are available in Supplementary material S4). We completed the mtDNA matrix for the remaining two *Pipa* terminals for 12S, 16S, *COI* and *CYTB* using data available in GenBank (Table S4). We further complemented this mitogenomic dataset with 10 nuDNA loci (Table S4) via Sanger sequencing and sequences available in GenBank (details of primers are available in Table S5). During the matrix building, we noticed two sequences in GenBank from Irisarri et al. (2011) that appear to be swapped (AY341762 = *Pipa parva,* AY341763 = *Hymenochirus*), which were thus relabeled. We also retrieved from GenBank mitogenomes and homologous nuDNA sequences for five outgroups representing each African Pipidae genus and *Rhinophrynus dorsalis*, the sister group of Pipidae, including annotations that were transferred to the new mitogenomes. DNA sequences were realigned on the MAFFT online server under the E-INS-i option for 12S and 16S and considering the reading frame option for each CDS with default parameters (Katoh et al., 2019). The control region and tRNA were discarded as well as flanking regions that were not available for most terminals.

The final alignment consisted of 21,762 bp, comprising 14,115 bp for mtDNA (12S-16S: 2784; 11,331 for mtDNA exons) and 7,647 bp for concatenated nuDNA (*RAG1*: 1374, *NCX1*: 1278, *POMC*: 558, *BDNF*: 693, *CXCR4*: 630, *TYR*: 675, *SLC8A3*: 1092, *H3a*: 288, *RAG2*: 744, *RHO*: 315). Two OTUs (*P. parva* and *P. carvalhoi*) had only partial mitogenomic data (12-16S, *COI* and *CYTB* and 16S and *COI* respectively) and 6 and 3 nuDNA loci available, respectively. All the other terminals had complete mitogenomes and at least 4 nuDNA loci (Table S3). Preliminary Maximum Likelihood analyses of each nuDNA locus using RAxML (see above for method) suggested overall topological congruence with mtDNA except the position of *P.* sp. "Guyana" (see results).

We selected the best-fit partition scheme and model of evolution for each partition using PartitionFinder V2.1.1 (Lanfear et al., 2017), according to the Bayesian Information Criterion (BIC) using the greedy scheme and linked branch length. We predefined 14 blocks, one for rRNA genes (12S and 16S), one for each codon position of concatenated mtDNA CDS regions, and one for each nuDNA CDS region. This analysis produced a best partition scheme of seven partitions (1: 12-16S; 2: mtDNApos1, 3: mtDNApos2, 4: mtDNApos3, 5: *POMC* + *RAG2* + *CXCR4* + *RAG1* + *TYR*, 6: *NCX1*, 7: *RHO* + *BDNF* + *SLC8A3* + *H3a*).

We reconstructed a time-calibrated gene tree in BEAST 2.5.2 using a Birth-Death tree prior to account for extinction processes. We parameterized unlinked substitution models and unlinked clock models according to the models suggested by the PartitionFinder analysis. Trees were linked. Divergence-time estimation was implemented using an uncorrelated relaxed lognormal clock model of the distribution of rates among branches for each partition (Drummond et al., 2006). We relied on secondary calibrations based on Hime et al. (2020), an extensive nuclear genomic dataset (220 loci 291 kb) of all major frog lineages and the last land bridge between South America and Africa ca. 105 Ma

(Torsvik et al., 2008). We enforced the monophyly of Pipidae since this clade has been strongly supported in all molecular phylogenetic (e.g., Irisarri et al., 2011, 2017; Cannatella, 2015; Feng et al., 2017; Hime et al., 2020) and paleontological analyses (e.g., Gomez, 2016). We also enforced the monophyly of African Pipidae (*Xenopus*, *Hymenochirus* and *Pseudhymenochirus*), thus favoring the topology in which *Pipa* is the sister group of other Pipidae, following the results from the analyses of molecular data of Irissari et al. (2011, 2017); Feng et al. (2017); Hime et al. (2020) and of Cannatella (2015). This last work used a combination of molecular, morphological and fossil data providing higher support for this topology and dates compatible with phylogenomic studies (Irissari et al., 2011, 2017; Feng et al., 2017; Hime et al., 2020). We acknowledge that the interrelationships among the three main Pipidae lineages remain contentious, but the scope of our study being the relationships and the timing of divergence within *Pipa*, we believe these priors to be reasonable as they should have no influence on the crown age of *Pipa* or any divergence times within the genus. Specifically, we assumed a uniform prior distribution for three nodes (1) the MRCA of Pipidae (between 129.1 the lower HPD from Hime et al., 2020 and 105.0 Ma the western Gondwana final break-up), (2) the crown age of African Pipidae (between 114.4 Ma and 85.9 Ma HPDs from Hime et al., 2020) and (3) the crown age of Pipoidae (between 172.6 and 150.0 Ma HPDs from Hime et al., 2020) which corresponds to the root of the tree.

We independently analyzed the four mtDNA partitions and the three nuDNA partitions because preliminary analyses suggested highly incongruent posterior distributions of node ages (regardless of the inclusion of 3rd codon position, the use of either Yule or Birth-Death, linkage of the clocks among mtDNA partitions and among nuDNA partitions i.e., two clock vs seven). The concatenated analyses produced intermediary ages for the nodes among *Pipa* spp. that were thus considered incorrect. We set two independent Markov chain Monte Carlo (MCMC) runs of 200 million iterations each, recording every 10,000th iteration and using the first 10% of iterations as burn-in. We combined the log and the tree files and the resulting posterior samples of trees of the two independent runs using LogCombiner 2.5 (Bouckaert et al., 2014) and checked convergence of model parameters via time-series plots. Chain mixing was considered adequate when parameters achieved an effective sample size above 500 (obtained for all parameters). We extracted a maximum clade credibility tree (based on the 36,002 resulting trees) using Tree annotator 2.5 (Bouckaert et al., 2014).

2.4. Biogeographic analyses

We used the time-calibrated phylogeny obtained from nuDNA to infer ancestral areas and biogeographic events via the BioGeoBEARS package in R (Matzke, 2013). We compared three models: (i) a likelihood version of the Dispersal-Vicariance model (DIVALIKE; Ronquist, 1997) (ii) a likelihood version of the BayArea (BBM) model (Landis et al., 2013), and (iii) the Dispersal-Extinction Cladogenesis model (DEC; Ree and Smith, 2008). We also compared versions of these models allowing jump-dispersal as described by the J parameter (Matzke, 2013; Ree and Sanmartín, 2018; Klaus and Matzke, 2020). Models were compared using the Akaike Information Criterion (AIC).

To identify spatial processes of diversification, we considered five main geographic areas where known species currently occur: Guiana Shield (GS), Western Amazonia (WA), Brazilian Shield (BS), Atlantic Forest (AF) and a *trans*-Andean region (TA). The three Amazonian regions correspond to major geological features of Amazonia (Hoorn et al., 2010) and to the large biogeographic regions known as Wallace's districts (Wallace, 1854), roughly delimited by modern riverine barriers: the Madeira River, the Caquetá/Japurá – Solimões, and the lower course of the Amazon River. This spatial partitioning into three areas allows us to investigate the putative connectivity across Neotropical regions and dispersal routes across Amazonia (Réjaud et al., 2020; Fouquet et al., 2021a, 2021b). Even though we included only two populations of *Pipa snethlageae* (French Guiana and central Amazonia), mtDNA sequences are identical despite geographical distance. This large-sized species is associated with large swamps and in French Guiana, it reaches a northwestern distributional limit much like several other species associated with this habitat, such as *Leptodactylus intermedius*, *Hydrolaetare schmidti*, *Typhlonectes compressicauda*, *Dracaena guianensis*, *Melanosuchus niger*, among others (Lescure and Marty, 2000; Vacher et al., 2020; Gazoni et al., 2021) it is therefore likely that the range of this species extends throughout the Amazon basin and we thus considered its range to be panamazonian.

2.5. Teeth evolution and mode of reproduction

Several works have investigated the evolution of morphological and reproductive features within *Pipa* (Trueb and Cannatella, 1986, Canatella and Trueb, 1988, Trueb and Massemin, 2001), although not in the light of a robust molecular phylogeny and without including *P. arrabali* (see results, correspondence with the nominal *P. pipa* remained also ambiguous) and the candidate species identified herein (see results). Using a LEICA MZ75 stereomicroscope, we verified the presence (*P.* sp. "South"); or absence of teeth (*P. pipa*; *P.* sp. "WGU", *P.* sp. "WAM", *P.* sp. Negro", *P.* sp. "Central") in specimens corresponding to nine OTUs (see results) in addition to the species already examined by Trueb and Cannatella (1986) and Trueb and Massemin (2001).

Data on reproductive modes were available for all the outgroups and most candidate species (*P. aspera*: Trueb and Massemin, 2001), *P.* sp. "Guyana" (RE, pers. obs.), *P. arrabali* (Garda et al., 2006), *P. carvalhoi* (Fernandez et al., 2011), *P. myersi* (Trueb, 1984), *P. pipa* (Linnaeus, 1758); P. sp. "WGU" (RE, pers. obs.), *P. snethlageae* (Massemin et al., 2007), *P. parva* (Sokol, 1977). However, no data could be found for *P.* sp. "South", *P.* sp. "WAM", *P.* sp. "Central", *P.* sp. "Negro", *P.* sp. "ES", *P.* sp. "Nordeste" for which we assumed they display the same breeding behavior as their close relatives.

We used a parsimony character mapping approach to infer the ancestral states of teeth presence/absence and exotrophic/andotrophic tadpole development in Mesquite 3.70 (Maddison and Maddison, 2007).

2.6. Substitution rates estimates

We observed important discrepancies between the temporal estimates in the *Pipa* genus obtained from mtDNA vs. nuDNA, while the topologies are almost identical (see results). This discrepancy may be resolved by invoking two hypotheses: (1) nuDNA temporal estimates are correct and the mtDNA substitution rate is underestimated i.e., in fact greater in *Pipa* relative to the rest of the tree, or (2) mtDNA temporal estimates are correct and the nuDNA substitution rate is overestimated. In the absence of fossils, it remains virtually impossible to tease these two hypotheses apart. However, given that (1) the biogeographic histories of co-distributed taxa of frogs (e.g. Fouquet et al., 2014; Réjaud et al., 2020) are mostly circumscribed to the Neogene, display many similarities (see results), and that previous works have regularly evidenced overestimation of ancient divergence when using mtDNA (Zheng et al., 2011; Zhang et al., 2013; Hime et al., 2020), we estimated that the first hypothesis is the most likely.

In order to investigate the origin of this bias in mtDNA substitution rate estimation and to quantify it, we performed a second phylogenetic analysis combining mtDNA and nuDNA, identical to our first analysis, but constraining the crown age of *Pipa* to the estimate obtained with nuDNA only (using the nuDNA 95% HPD interval as a uniform calibration prior). This additional analysis allowed us to estimate the average mtDNA rate within *Pipa*, conditional on a Neogene diversification of this genus. It also allowed us to compare this rate with the rates found in other branches in the same tree, and with other studies on Neobatrachian taxa, such as *Allobates* (Réjaud et al., 2020) and *Boana* gr. *albopunctata* (Fouquet et al., 2021b), for which mitogenomic data were analyzed within the scope of a similar framework (i.e., using the same partitioning and secondary time calibration from phylogenomic studies

of Feng et al., 2017 and Hime et al., 2020). BEAST tree files were obtained from the authors. For each partition and phylogenetic analysis, we calculated the average rate applying to a specific subtree (e.g., in *Pipa*, in African pipids, etc.) as the average of the branch-specific rates (we used a relaxed molecular clock with one rate per branch) across all the branches of the subtree, weighted by the lengths of the branches.

2.7. Detecting changes in dN/dS

Selection may modulate gene sequence evolution within Pipidae and may at least partly explain changes in evolutionary rates across branches. Simulation studies have shown that analyses of selection coefficients are rather robust to sequence divergence (Yang, 2006) (as is the case in the present study), having been successfully used in various

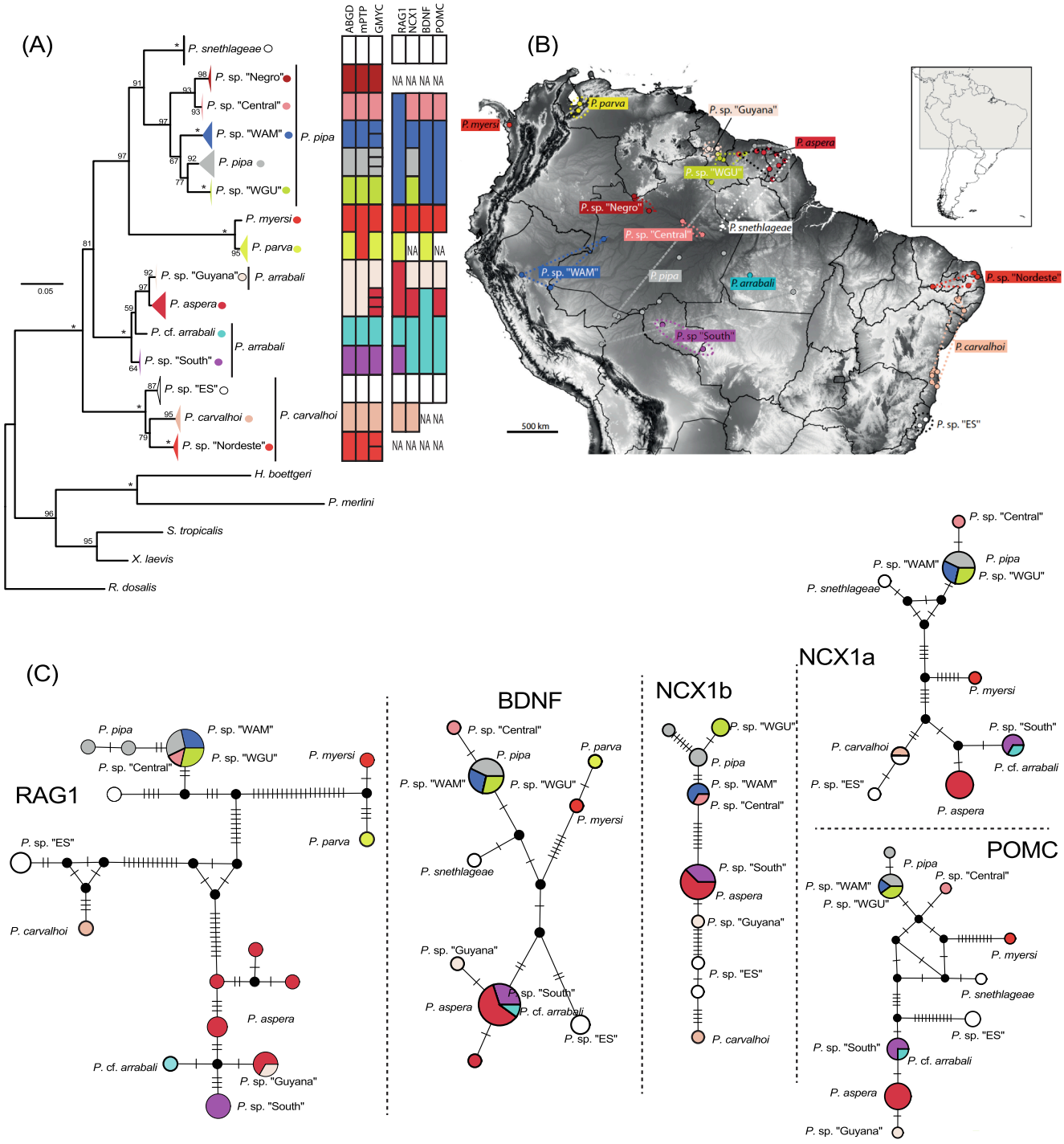

Fig. 1. (A) Maximum Likelihood phylogenetic tree obtained from the analysis of 115 sequences of 16S (595 bp) of *Pipa*. Bootstraps > 50 % are indicated on the left side of the nodes and depicted with * when > 99%. For sake of clarity, terminal branches are collapsed according to the results of the DNA-based species delimitation (ABGD, mPTP, GMYC) (complete tree given in Figure S6). Congruence across DNA-based species delimitation methods and with nuDNA neworks (absence of allele sharing) is illustrated using colored columns on the right. Absence of available data for a given lineage is indicated with "NA". (B) Maps of northern South America showing the distribution of the sampled material color-coded as in the ML tree according to the species delimitation. (C) Median Joining networks based on four loci (NCX1 being divided in two, because parts of the individual sequences mostly overlapped either on 3' or the 5' ends) with corresponding colour code.

studies with highly divergent species (e.g., Buschiazzo et al., 2012). Therefore, we tested alternative models with different assumptions about ratios of non-synonymous/synonymous substitution rates (ω). The software PAML v.3.15 (Yang, 1997) was used to estimate the likelihood and the ω values of different models derived from the topologies and sequence information from single-gene alignments with all codon positions, as well as the mt and nuclear nucleotide data sets. Branch lengths were first optimized for each data set assuming a single ω for the whole tree, and they were fixed when all other parameters were estimated under alternative models. The null model had a single ω value for all branches, and it was compared against four alternatives, which allowed a second ω value on (i) the stem branch of *Pipa*, (ii) all *Pipa* branches. All models were compared using the AIC.

3. Results

3.1. mtDNA-based species delimitation

The phylogenetic trees obtained from the ML and the Bayesian analyses of the 16S locus strongly supported *Pipa* as monophyletic as well as the existence of four major clades within the genus. Two of these clades (*Pipa aspera/arrabali* and *Pipa pipa/snethlageae*) are mainly Amazonian (Fig. 1A), one occurs in the Atlantic Forest (*Pipa carvalhoi*) and one in *trans*-Andean regions (*Pipa parva/myersi*). Several deeply diverging lineages are also supported within *Pipa pipa*, *Pipa carvalhoi* and *Pipa arrabali*, suggesting that the current recognition of only seven nominal taxa may be a significant underestimation of the actual species diversity within the genus (Fig. 1A, S6). *Pipa arrabali* is recovered paraphyletic with respect to *Pipa aspera*.

Of the three species-delimitation methods, mPTP was the most conservative, delimiting 13 OTUs. With 14 delimited OTUs, ABGD produced a very similar partitioning. We kept the 12–17th partitions ($P = 0.0092$) based on two criteria: (i) they correspond to a plateau for group number, and (ii) it is close to the 1% arbitrary threshold of intraspecific divergence recognized in other vertebrate delimitation studies with the 16S locus (Puillandre et al., 2012). By contrast, GMYC delimited 21 OTUs (Fig. 1A). Mean interspecific p distances among these OTUs reaches a minimum value of 1.6% between *P. aspera* and *P.* sp. "Guyana" and between *P. parva* and *P. myersi* and is below 3% in two more instances, between *P. arrabali* and *P.* sp. "South", and between *P.* sp. "Negro" and *P.* sp. "Central" (Table S7). The consensus of the results obtained through the three methods led to the delimitation of 14 DNA-based OTUs (Fig. 1A).

3.2. nuDNA differentiation

The relationships obtained using the four nuDNA loci are overall largely congruent with the mtDNA-based delimitation. For example, *Pipa* sp. "Central" is consistently recovered as sharing no alleles with the other three OTUs recognized as *P. pipa*. These three OTUs delimited within *Pipa pipa* are also recovered as sharing no alleles in *NCX1*, but this case is more ambiguous since allele sharing is observed on the other loci. The groups delimited within *P. carvalhoi* are also differentiated on *RAG1* and *NCX1*. The case of *P. aspera* and the populations from Guyana (assigned to *P. arrabali*) is noteworthy since these species are recovered as a single OTU based on mtDNA. However, they do not share any alleles on any nuDNA loci except *RAG1*. Considering their distinct morphology (Trueb and Massemin, 2001) and their divergence on nuDNA loci, we considered them distinct, leading to 15 OTUs.

3.3. Molecular dating

We assigned the best-fit models suggested by the PartitionFinder analysis to each of the seven partitions. The two combined BEAST analyses of both the mtDNA and the nuDNA data led to all parameters having ESS > 200. The resulting phylogenetic relationships are all highly supported in both the mtDNA and the nuDNA trees (posterior probability pp > 0.99) and completely congruent between the mtDNA and the nuDNA, except the position of *Pipa* sp. ''Guyana'', which is supported as the sister species of *P. aspera* with mtDNA and as the sister species of the clade formed by *P. arrabali* and *P.* sp. ''South'' according to nuDNA. *Pipa* is structured in two major clades, one centered in the eastern part of its range (*P. carvalhoi* - Atlantic Forest; *P. aspera*, *P. arrabali* - Eastern Amazonia), and the other clade in the western part (*P. myersi*, *P. parva* - *trans*-Andes and *P. pipa*, *P. snethlageae* - Amazonia; Fig. 2).

However, major differences are observed in the timing of divergence between the two trees. The crown age of *Pipa* is found to date back to ca. 85 Ma (76–94, 95% HPD) according to mtDNA (Fig. 2A), but to ca. 18 Ma (14–22, 95% HPD), i.e., 4.7 times younger according to nuDNA (Fig. 2B). Since these dates are largely incompatible, either the rates of the mtDNA, the nuDNA, or both are erroneously estimated. In the absence of fossils branching along the *Pipa* stem, this question will inevitably remain contentious. Nevertheless, the mtDNA rates are notoriously more subject to underestimation by BEAST, notably for ancient periods (Molak and Ho, 2015), as could be the case for *Pipa*. Therefore, we considered an additional analysis based on the crown age obtained from the analysis of nuDNA (see below) and favoured the relationships and temporal estimates of the nuDNA analysis hereafter.

3.4. Substitution rates estimates

The posterior distributions of the rates of each mtDNA partition analyzed alone and with the three old priors (Cretaceous) is relatively stable across the tree, i.e., among African pipids and for the stem and the crown of *Pipa* (Fig. 3). The only notable exception is the high rate of the stem of *Pipa* for pos2 using Cretaceous calibrations compared to the ones of other branches, while this rate is similar in other partitions. Moreover, when adding the Neogene prior on the crown age of *Pipa* (based on the results with nuDNA), the rates were found to be, for all partitions, about four times higher in crown *Pipa*, and half as high in the stem, as compared to the analysis without the Neogene prior. The rate in African pipids was similar in both analyses. The rates estimated in *Allobates* and *Boana* are comparable (although slightly higher) to the rates we estimated in crown *Pipa* with the Neogene prior, except for pos 3 (Fig. 3). The rates of the nuDNA partitions were similar across branches (Fig. S9). The tree resulting from the analysis of the concatenated mt + nuDNA with the same Neogene prior is available in Fig. S10.

3.5. Changes in dN/dS

We compared a model with ω constant across the whole tree (single-ω model) with a "three-ω model" assuming independent ω values in (1) the stem branch of *Pipa*, (2) the branches within the crown of *Pipa* and (3) in other branches of the tree. The ω value estimated with the single-ω model was well below 1 for all different mt and nuclear genes (0.012–0.194) (Table 1), indicating extreme purifying selection to maintain gene function (Castellana et al., 2011).

With the three-ω model, for all individual mt genes (except *ATP8* but consists of a short 159 bp locus), ω were also all very low but always higher (ca. X2) in the stem branch of *Pipa* than within crown *Pipa*, or in the other Pipoidea branches (Table 1). The ω estimate for stem *Pipa* was also about twice higher than the overall rate estimated with the single-ω model (Table 1). When all mitochondrial genes were considered jointly, the three-ω model was favored overwhelmingly ($\Delta AIC = 124$, Table 1). For nuclear genes (*H3A* having been excluded given this locus was short − 288 bp - and only represented by six terminals including a single *Pipa*), the three-ω model never significantly outperformed ($\Delta AIC < 2$) the single-ω model, and the single-ω model was best for four genes (*BDNF*, *RAG2*, *RHO* and *SLC8A3*), see Table 1.

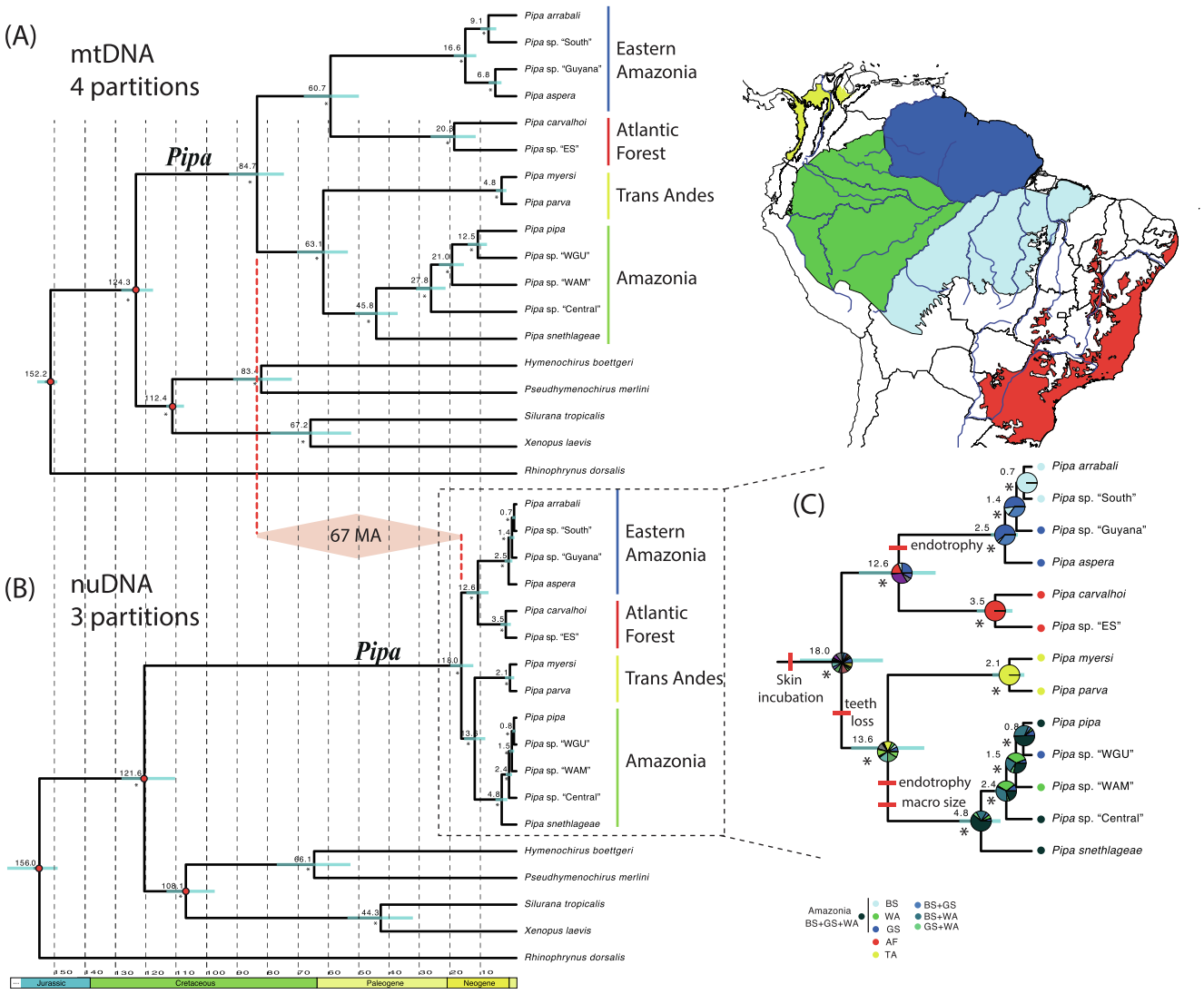

Fig. 2. Maximum clade credibility chronogram inferred in BEAST 2 based on (A) mitogenomic and (B) nuDNA, and (C) ancestral areas for *Pipa*, based on the favored nuDNA topology, inferred in BioGeoBEARS under the DEC model (results of the DEC + J model are available in Table S8). The temporal discrepancy across mt and nuDNA found for the Pipa crown-age is highlighted by a red diamond and red dotted lines. We hypothesized that it emerges from the effect of saturation on mtDNA (see discussion). Nodes with maximum posterior probability are indicated with an asterisk under the branches. Calibrated nodes are indicated with a red circle. Node bars indicate the 95 % highest posterior distribution of node dates. Colored circles on the tips of the tree indicate the geographical distribution of sampled OTUs. Pie charts on nodes show the proportion of most likely ancestral areas. Colors of node pie charts correspond to the geographic areas shown in the map. Changes in teeth, body size and larval development states are indicated with red bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Biogeography, reproductive mode evolution and teeth loss

Pipa diversified throughout the Neogene with two concomitant splits ca. 13 Ma between Amazonia and the Atlantic Forest and between Amazonia and *trans*-Andes. Subsequent diversification within *Pipa* is circumscribed to the Pliocene and Pleistocene i.e., <5 Ma. Inference of ancestral areas using BioGeoBEARS equally favored the DEC and DEC + J models (Table S8) and provided very ambiguous results for the early diversification events within *Pipa*. Nevertheless, when the three Amazonian areas are combined, an Amazonian origin is supported for the genus and for the two major clades. Concerning *P. pipa* and *P. snethlageae*, the combined probabilities suggest a western Amazonian origin of their common ancestor and recent (Middle Pleistocene) dispersals of two lineages to the Guiana Shield. In the opposite direction, but concomitantly, a dispersal from the Guiana Shield toward the Brazilian Shield is suggested for *P. arrabali*.

The most parsimonious scenario suggests that endotrophy has probably evolved independently in the two Amazonian clades (the "macropipa" and the *P. arrabali*/*P. aspera* clades). Maxillary teeth have been lost once during the early Miocene in the western clade formed by "macropipa" and the *trans*-Andean clade.

4. Discussion

Our results shed a new light on species diversity, phylogenetic relationships, divergence time, biogeography and the evolution of edentulism and of reproductive mode in the genus *Pipa*. Our comparative results on mt and nuDNA also strikingly exemplify how molecular dating (even though based on full mitogenomes) could lead to spurious time estimates along long branches (ca. 100 Myrs) when no other constraints can be applied to rate these estimates.

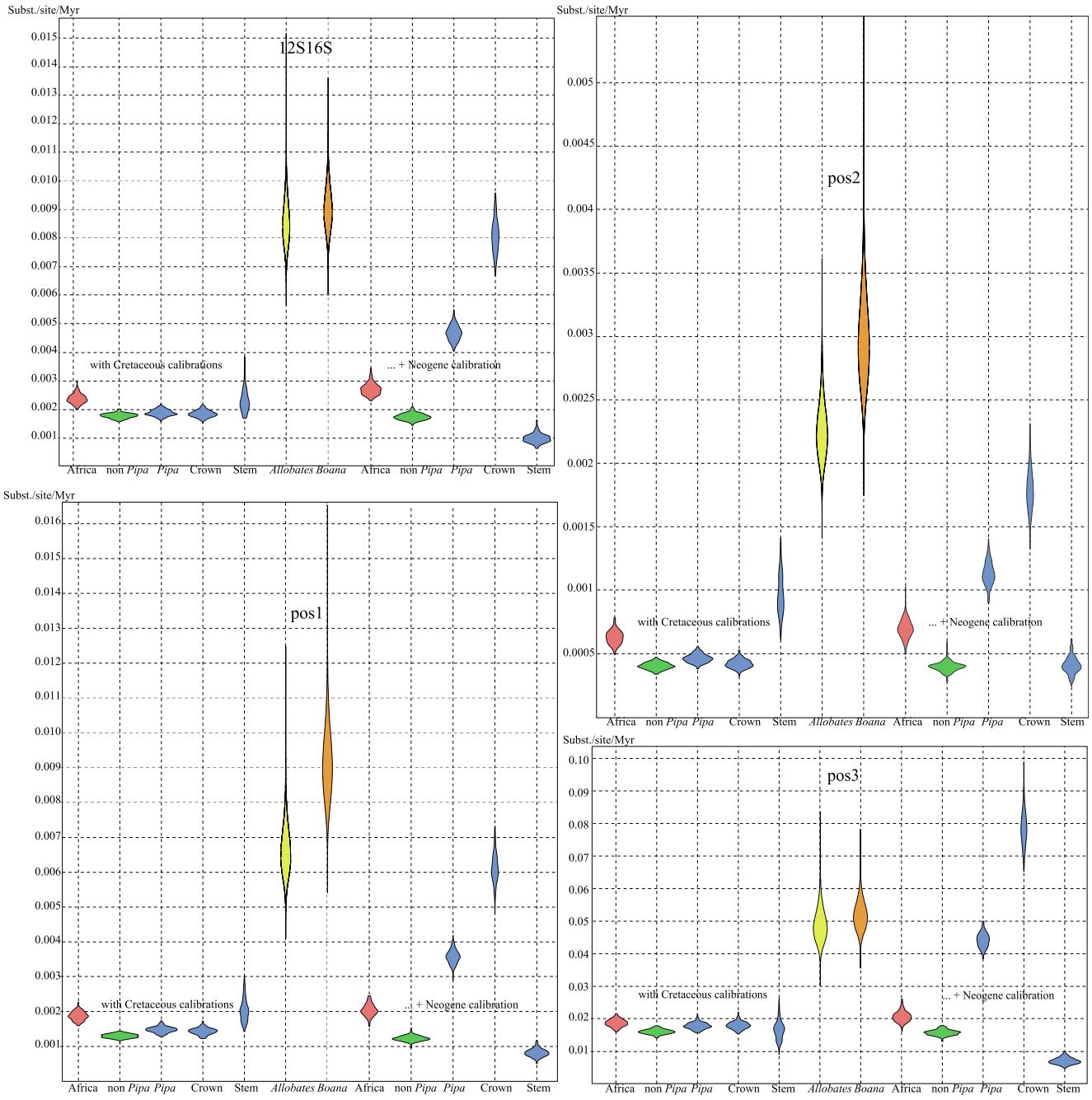

Fig. 3. Comparison among posterior distributions, depicted as mustache plots, of the rates across the four mtDNA partitions (ribosomal genes, three codon positions for combined protein-coding genes) either when mtDNA is analyzed with calibrations dating back to the Cretaceous or when a Neogene calibration for the *Pipa* crown age is added (from nuDNA results). "*Pipa*" includes both "crown" and "stem". Comparative rates for two groups of Neobatrachia (*Allobates* and *Boana* are also depicted for comparative purposes). The rates found for non-*Pipa* branches are similar regardless of the calibration used and are also similar to the ones found within *Pipa* (crown) using a Cretaceous calibration. However, when using a Neogene calibration, the rates found within *Pipa* (crown) are about four-times higher and closer to the ones found in Neobatrachia.

4.1. Species diversity

Almost all studies that have explored the question of how many species exist within particular amphibian clades in the Neotropics have uncovered high numbers of candidate species. Most often these cases correspond to populations that were previously considered to belong to species with wide distributions (e.g., Fouquet et al., 2014; 2021a, 2021b; Gehara et al., 2014; Carvalho et al., 2021; Vacher et al., 2020). This situation is also exemplified in our results for *Pipa*, with a possible 2.1 times increase in species diversity (15 instead of seven) since eight OTUs possibly correspond to unnamed species (or taxa requiring revalidation such as *Pipa laevis* Cuvier, 1831 from the Rio Negro), in addition to the seven species already described. This figure is not surprising and even lower than what has been found in many other co-distributed clades. Among these nine candidate species, most are supported as distinct by nuDNA; however, the situation remains ambiguous between some closely related pairs such as *P. arrabali* vs. *P.* sp "South"; *P. pipa* vs. *P.* sp. "WGU" and *P.* sp. "WAM"; *P. carvalhoi* vs *P.* sp. "Nordeste" and *P.* sp. "Central" vs *P.* sp. "Negro", i.e., could represent false positives, either because allele sharing suggests conspecificity or simply because of the absence of data. The cases of *P. carvalhoi*, *P.* sp. "ES" and *P.* sp. "Nordeste" were already documented by Lima et al. (2020), who

Table 1
Tests for constancy in the ratio ω of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site among branches in the tree. Values of ω are presented for the tree as a whole (null), branches outside *Pipa* (non-*Pipa*), branches within *Pipa* (Pipa crown) and the most recent common ancestral branch of *Pipa* (*Pipa* stem). Results are from the CodeML analyses for each locus and mitochondrial and nuclear loci combined. The highest ω values are in red and values in bold indicate models significantly outperforming the others according to the AIC. Values of ω well below 1, as recovered here, indicate strong purifying selection for conserving the amino acid sequence of the encoded protein.

Genes	Single-ω model (null)	Three-ω model			Delta AIC null	Delta AIC three-ω model
		non Pipa	Pipa crown	Pipa stem		
atp6	0.07458	0.06117	0.08291	0.13577	1.55	0.00
atp8	**0.16524**	0.16417	0.17029	0.12765	0.00	**3.79**
cob	0.05246	**0.05421**	**0.04754**	**0.16684**	**6.74**	0.00
cox1	**0.01312**	0.0131	0.0125	0.0272	0.00	**2.25**
cox2	0.03999	0.04029	0.03716	0.08989	0.00	0.81
cox3	0.03549	0.0322	0.03547	0.11395	0.78	0.00
nad1	0.07213	**0.083716**	**0.061665**	**0.168248**	**6.39**	0.00
nad2	0.1235	**0.14276**	**0.10034**	**0.2643**	**20.46**	0.00
nad3	0.1159	**0.14233**	**0.0899**	**0.21379**	**3.84**	0.00
nad4	0.09141	**0.08673**	**0.08854**	**0.21757**	**9.24**	0.00
nad4L	0.09169	**0.11485**	**0.06896**	**0.12563**	**2.43**	0.00
nad5	0.09683	**0.10923**	**0.08168**	**0.20561**	**23.26**	0.00
nad6	0.13259	0.15081	0.11618	0.15133	0.00	0.42
all mt prot-cod genes	0.08258	**0.09028**	**0.07151**	**0.19111**	**123.86**	0.00
bdnf	**0.0518**	0.05395	0.01992	0.06853	0.00	**2.63**
cxcr4	0.05564	0.04489	0.10037	0.07888	0.85	0.00
pomc	0.13991	0.15623	0.07654	0.10665	0.00	0.12
ncx1	0.04037	0.03954	0.06772	0.0263	0.00	1.35
rag1	0.08992	0.08365	0.12903	0.07478	0.52	0.00
rag2	**0.1939**	0.20566	0.13296	0.16237	0.00	**2.46**
rho	**0.09248**	0.0968	0.12761	0.04355	0.00	**2.50**
slc8a3	**0.03126**	0.02816	0.03978	0.04252	0.00	**3.11**
tyr	0.15588	0.12673	0.2416	0.18597	0.33	0.00
all nuclear genes	0.09206	0.08986	0.11794	0.08305	1.66	0.00

found little morphological, but clear chromosomal differences. Interestingly, interspecific genetic distances on 16S among closely related OTUs are lower than 3% in four instances (Table S7), notably between *P. aspera* and *P.* sp. "Guyana" (1.6%) and between *P. parva* and *P. myersi* (1.6%). This 3% threshold in 16S rDNA distances has frequently been suggested to be indicative of candidate species in anurans (Fouquet et al., 2007; Vieites et al., 2009). Nonetheless, these two pairs are phenotypically distinct (Trueb, 1984; Trueb and Cannatella, 1986), and congruent nuDNA divergences are recovered, thus confirming their status as distinct species. *Pipa aspera* and *P.* sp. "Guyana" illustrate a case of a false negative since mtDNA-based species delimitation failed to distinguish them. Here, as in similar cases, additional data are needed, notably an extended spatial sampling, as well as morphological and bioacoustics data that need to be integrated in genetic data sets to provide truly integrative, multi-evidence based deductions (Padial et al., 2010).

Our results highlight that geographic ranges previously assigned to *Pipa pipa* (possibly limited to Eastern Amazonia), *P. arrabali* (possibly limited to the northern part of the Madeira-Tapajos interfluvium) and *P. carvalhoi* (possibly limited to the Brazilian states of Bahia, Pernambuco and Alagoas) may be much more restricted. It is also likely that additional species remain unnoticed as a result of sampling gaps. The species *P. arrabali*, for example, has previously been recorded from southeastern Amazonia (e.g., Garda et al., 2006 from Serra do Cachimbo, Para; da Silva et al., 2020 in Tocantins; Pinheiro et al., 2012 from Carajas, Para), and two species of "macropipa" are reported from the Orinoco basin (Acosta-Galvis et al., 2016), but samples from these populations were not included in this study. The extent of these basic knowledge gaps for such a charismatic group of frogs is particularly striking and exemplifies the logistical challenges associated with undertaking fieldwork in remote Amazonian regions. Nevertheless, even with 15 newly identified candidate species, *Pipa* remains a relatively poorly diversified genus as compared to most co-distributed genera of terrestrial frogs (e.g., Réjaud et al., 2020) that also diversified throughout the Neogene.

4.2. Estimating mtDNA substitution rate in Pipa

Due to their small size, lack of recombination, and rapid evolution rate, mitogenomes have been commonly used for analyzing phylogenetic relationships and divergence time (Igawa et al., 2008; Zhang et al., 2021). However, mitogenomes are densely packed with functional information/genes; thus, the protein-coding genes primarily evolve under highly conservative selection. This extreme purifying selection is obvious in the very low dN/dS we observed (Table 1). It also implies that synonymous substitutions are rapidly saturated over time. Here we interpret higher dN/dS on the stem of *Pipa* for mtDNA primarily as the effect of synonymous substitution saturation on mtDNA along the stem branch of *Pipa* rather than being consistent with a relaxation of the purifying selection (e.g. Irissari et al., 2012). This bias may particularly affect dN/dS estimation on very long branches, dN/dS increasing with saturation (Yang, 2006; Cannarozzi and Schneider, 2012). This means that saturation leads to underestimate dS for ancient time periods because saturation is reached faster on sites that have higher substitution rates (e.g., pos1 and pos3) and thus the relative amount of dN (e.g., pos2) is artificially overestimated. Fig. 3 clearly depicts this phenomenon, by showing the rate of the stem of *Pipa* of pos2 using Cretaceous calibrations as being the highest. Over long time periods, sequences experience full substitution saturation, and the similarity between the sequences will depend entirely on the similarity in nucleotide frequencies (Steel et al., 1993). Consequently, the BEAST analysis cannot correctly estimate mtDNA substitutions rates on the long stem of *Pipa* and overestimates the divergence time. A possible reason why BEAST overestimates divergence time rather than underestimating it might rely in the algorithms correcting for saturation. This may also have been the case in the reanalysis of Neobatrachia (Irissarri et al., 2012) and relaxed purifying selection may in fact not be the main process involved in rate estimates heterogeneity. Therefore, ancient divergence times (50–100 Ma) may simply be too old to be estimated using mitogenomes along very long stems, particularly in the absence of fossil constraints from the Tertiary, which would allow substitution rates to be bounded.

4.3. Phylogeographic patterns in Amazonian Pipa

Within the *Pipa pipa/snethlageae* clade, the biogeographic analysis suggests a western Amazonian origin that is driven by early diverging lineages in western Amazonia and in the Negro River. This western origin is expected given that these species are tightly linked to large bodies of water that are currently widespread in the seasonal floodplains of the region but absent from the Guiana and the Brazilian Shields. Moreover, the historic presence of large lacustrine systems (Acre and Pebas systems; see Hoorn et al., 2010) in western Amazonia and the increasing eastward expansion of the flooded ecosystems since late Pleistocene (Aleixo and Rossetti, 2007, Bicudo et al., 2019) also support that hypothesis. However, the existence of five OTUs recovered within what has been so far considered *P. pipa* is more surprising since it suggests limited connectivity across the main Amazonian tributaries, whereas one would expect these large aquatic frogs to disperse efficiently along the vast hydrological network of the Amazon basin. In fact, there is an apparent paradox in *P. pipa* because it does display a shallow genetic structure throughout eastern Amazonia (Purus, Madeira, Tapajós, Xingú), but also including the easternmost part of the Guiana Shield (from Amapá, Brazil, to a western limit in the Suriname River in Suriname), while distinct lineages occupy western Amazonia (*P.* sp. "WAM") and the Negro River (*P.* sp. "Negro" and *P.* sp. "Central"). The rivers of the Guiana Shield are not directly connected to the Amazon basin (although recent connection through the Rio Branco existed; de Souza et al., 2020), but these connection exist between the rivers of western Amazonia and the Negro River. This pattern, along with the existence of two diverging lineages along the Negro River, is particularly noteworthy since spatially similar genetic structures are observed in fish (Piranhas: Hubert et al., 2007), and in the highly aquatic chelid turtle *Chelus fimbriata* (Vargas-Ramirez et al., 2020), to which *Pipa pipa* may actually be ecologically closer than to other frogs, but also in birds associated with flooded forests (Thom et al., 2020). Therefore, historical and/or ecological processes have likely driven the observed phylogenetic pattern. Avulsion between the Japura and the course of the lower Negro River during Pleistocene (Ruokolainen et al., 2018) may further explain this pattern: i.e., with ancient barriers that no longer exist, but also major paleo-mega-wetlands that were scattered in Amazonia during late Neogene (Albert et al., 2018). Currently the strong ecological differences between the main types of floodplains (white waters in western Amazonia vs. black waters of the Negro River, Cooke et al., 2014; Beheregaray et al., 2015; Oliveira et al., 2019) may also play a role in maintaining ecological isolation of the Rio Negro populations from the rest of the Amazon basin. Nevertheless, the nuDNA supports these differences only for the Negro River OTUs. More spatial sampling and nuclear genomic data are needed to investigate the phylogeography within *Pipa pipa* at a finer scale.

In contrast, the genetic structure in the *P. aspera/arrabali* clade was expected to be more pronounced than within *Pipa pipa* since these species are associated with smaller water bodies in terra firme forest of the Brazilian and the Guiana Shields. However, even if a marked genetic structure exists, divergences remain relatively low among these lineages and even across the Amazon River. Nevertheless, the distribution of the different OTUs is more similar to the one found in terrestrial anurans i.e., distribution breaks matching major Amazonian tributaries and bisecting the Guiana Shield (Vacher et al., 2020). Interestingly, we recovered a mtDNA/nuDNA topological incongruence within the *Pipa aspera/arrabali* clade with the Brazilian Shield OTUs being nested within Guiana Shield ones according to nuDNA vs. a sister-taxon relationship between the Brazilian Shield and Guiana Shield populations according to mtDNA.

These topological differences suggest either (1) introgression with mtDNA capture among GS lineages after the dispersal of *P. arrabali* from the GS or (2) introgression between BS and Guyana populations after dispersal from the BS, or (3) different coalescent gene histories. The origin of the group in the Guiana Shield was supported by the biogeographic analysis and the lower diversity among Brazilian Shield populations on the different nuDNA loci supports the first hypothesis. However, both topologies imply *trans*-amazon dispersals, nuDNA suggesting relatively a recent event (<1.4 Ma) when the transcontinental Amazon River was already established, which is surprising given the ecology of the species.

4.4. Biogeography

The geographic center of diversification of most Neotropical groups remains difficult to infer not only because of putative extinctions but also because subsequent dispersals and intense landscape dynamics reshuffled the spatial distribution of organisms throughout the Cenozoic (e.g., Antonelli et al., 2018). Consequently, our ability to investigate the spatial origins of focal groups is usually restricted to more recent periods (Smith et al., 2014; Silva et al., 2019; Cracraft et al., 2020). The case of *Pipa* is no different, with little signal for the early ancestral range of the main groups. Nevertheless, (1) two of the main *Pipa* lineages occupy Amazonia and they possibly originated in the eastern (*P. aspera/P. arrabali*) and the western portions (*P. pipa/P. snethlageae*) of that region, and (2) their respective sister groups occupy the Atlantic Forest (*P. carvalhoi*) on the east and the *trans*-Andean region (*P. myersi/P. parva*) on the west, which all speaks in favor of an Amazonian origin (14–22 Ma) and subsequent dispersal/vicariance some 12–13 Ma towards neighboring regions. Such a scenario seems particularly plausible since several co-occurring amphibians have similar trajectories with an Amazonian origin dating back to the early Neogene and also displaying an east–west pattern in Amazonia as well as concomitant dispersal/vicariance with Atlantic Forest and *trans*-Andean regions (see below).

The main split in *Pipa* separates an eastern Amazonian and the Atlantic Forest clade from a panamazonian (with a likely western Amazonian origin see above) and a *trans*-Andean clade. This East-West split dates back to the early Neogene (14–22 Ma) and coincides with the formation/existence of the Pebas system, a large freshwater system initially connected to the Caribbean Sea, that may have covered a surface of up to 13% of the current extent of Amazonia (800,000 km^2; Albert et al., 2018). The Pebas system supposedly formed during the early Miocene (23 Mya) and occupied most of the Western Amazonian lowlands until around 10–9 Ma, when this system was progressively drained eastward into the Atlantic Ocean and transitioned into the modern Amazon watershed (Albert et al., 2018; Hoorn et al., 2017). Recent advances in Amazonian biogeography of amphibians (Rojas et al., 2018; Réjaud et al. 2020; Carvalho et al., 2021; Fouquet et al. 2021a, 2021b) and other groups of terrestrial vertebrates recovered this east–west pattern within Amazonia. Interestingly, the edentate species of *Pipa* correspond to this western clade, which suggests a single loss, and is therefore much more parsimonious than what was previously suggested by Trueb and Cannatella (1986). The origin of edentulism in this group of *Pipa* remains highly speculative but may be linked to feeding habits in an ancestral habitat in a large lacustrine system. How these frogs feed has been investigated by Fernandez et al. (2017) and Cundall et al. (2017) but only in *P. pipa*. A comparison between edentate and dentate species may provide some insights about the evolution of feeding habits.

The Andes represent a formidable barrier to dispersal between Amazonia and the *trans*-Andean region. The Andean orogeny has been an ongoing process over the last 40 Ma punctuated by several intensive phases notably 12 Ma (Mora et al., 2010; Hoorn et al., 2010). The 12–13 Ma dispersal vicariance between Amazonia and the *trans*-Andean region in *Pipa* coincides with this middle Miocene phase of orogeny. Moreover, similar *trans*-Andean events occurred in other forestrial groups such as *Allobates* (Réjaud et al., 2020), *Engystomops* (Funk et al., 2012) and several lineages of Dendrobatidae (Santos et al., 2009), *Dendropsophus* (Pirani et al., 2020) and *Pristimantis* (Mendoza et al., 2015). Conversely, in amphibian taxa with higher dispersal abilities, notably having frequently adapted to altitude, such as glassfrogs or toads, some species have been isolated by the Andes (Bessa-Silva et al., 2020; Castroviejo-Fisher et al., 2014), while other species even display a cross-Andean distribution (e.g., *Boana boans*, Caminer and Ron, 2020; *Cochranella resplendens*; Molina-Zuluaga et al., 2017). Given that *Pipa* spp. are exclusively found in lowlands, we hypothesize that the Andes acted as a barrier to dispersal 12 Ma onward since the intense orogeny phase.

Similarly, the savannas of the Cerrado that today separates Amazonia and the Atlantic Forest represent a barrier for most forest amphibians. Middle Miocene divergence appears relatively concomitant with other Neotropical frog and lizard complexes occurring in both regions (*Adenomera*: Fouquet et al., 2014; *Adelophryne*: Fouquet et al., 2012a; *Dendropsophus*: Pirani et al., 2020; *Leposoma*: Pellegrino et al., 2011), as well as birds (Batalha-Filho et al., 2013). The occurrence of successive dispersal routes between Amazonia and the Atlantic Forest has been largely recognized since this pattern has been recovered for frogs and other vertebrates (Ledo and Colli, 2017). However, both timing and approximate location of these connections remain largely debated. Nevertheless, there is little doubt that recurrent phases of forest extension have allowed recurrent connectivity and exchanges of fauna between these forests notably during the Miocene. The Middle Miocene Climatic Optimum (MMCO) (17–15 Ma) was a warm and wet period followed by periods of stronger seasonality associated with open vegetation expansion (Steinthorsdottir et al., 2021). Such environmental changes may have isolated forest-adapted species. Batalha-Filho et al. (2013) hypothesized a southern pathway between these biomes during the middle Miocene (Por, 1992; Costa, 2003). Widespread vegetation opening is documented after the MMCO (Flower and Kennett, 1994) with drastic climatic changes linked to major uplift of the Andes (Hoorn et al., 2010) and sea-current modifications (Herold et al., 2009).

The *Pipa* species with endotrophic development form two distinct Amazonian clades. Furness and Capellini (2019) demonstrated that complex parental investment such as brooding and "viviparity" are very unlikely to undergo reversals. Therefore, we can reasonably assume that skin incubation has predated two independent acquisitions of endotrophy: one in macropipa and one in the eastern Amazonian *P. aspera/arrabali* clade. Nevertheless, some reversals from endotrophy to exotrophy have been suggested in *Anomalogossus* (Fouquet et al., 2019), in Hemiphractidae (Castroviejo-Fisher et al., 2015) and in *Adenomera* (Fouquet et al., 2014), and since the reproductive modes of many species remain undocumented, the occurrence of endotrophy-exotrophy shifts may have been more common than currently considered. Given that there are only two pairs of lineages with both modes in *Pipa*, reversals from endotrophy to exotrophy in the Atlantic Forest and *trans*-Andes clades remain plausible. The question of sequential acquisition of endotrophic larval development in anurans deserves further investigation notably concerning its historical and environmental determinants as well as genomic research aiming at identifying genic function involved.

5. Conclusion

This work revealed that (1) the genus *Pipa* may contain two-times more species than previously assumed, (2) that the diversification of the genus probably started during the early Neogene in Amazonia and that it dispersed to the Atlantic Forest and *trans*-Andean region secondarily and (3) that endotrophic development was most likely acquired twice, while teeth were lost only once. Much still remains to be investigated on each of these aspects, notably improving the sampling in Amazonia and combining morphology and bioacoustic with molecular data to describe the actual diversity in the genus. Our understanding of the biogeography and the morphological evolution of *Pipa* is hampered by large data gaps that only fossils from Amazonia (Antoine et al., 2021)

and systematic microtomography could help to fill. We hope that this work will foster and stimulate studies in this direction.

CRediT authorship contribution statement

Antoine Fouquet: Conceptualization, Resources, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Josselin Cornuault:** Formal analysis, Writing – original draft. **Miguel T. Rodrigues:** Resources, Writing – review & editing. **Fernanda P. Werneck:** Resources, Writing – review & editing. **Tomas Hrbek:** Resources, Writing – review & editing. **Andrés R. Acosta-Galvis:** Resources, Writing – review & editing. **David Massemin:** Resources, Writing – review & editing. **Philippe J.R. Kok:** Resources, Writing – review & editing. **Raffael Ernst:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107442.

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