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

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29 **Abstract.** The genus *Pipa* is a species-poor clade of Neotropical frogs and one of the most  
30 bizarre-looking due to many highly derived anatomical traits related to their fully aquatic life-  
31 style. With their related African lineages, they form the Pipidae family, which has attracted  
32 much attention, especially regarding its anatomy, reproductive biology, paleontology and  
33 biogeography. However, the actual diversity and relationships within *Pipa* remain poorly  
34 understood, and thus, so do their historical biogeography and the evolution of striking  
35 features, such as the absence of teeth and endotrophy in some species. Moreover,  
36 phylogenomic results display substantial temporal incongruences suggesting substitution rate  
37 heterogeneity across pipids. Using short mtDNA sequences across the distribution of the  
38 genus we identified 15 OTUs, a ~~two~~-twofold increase compared to the seven nominal species  
39 currently considered valid. Some of the closely related OTUs do not share nuDNA alleles,  
40 confirming species divergence. Time-calibrated phylogenies obtained from mitogenomes and  
41 from 10 nuclear loci provide highly similar topologies but strikingly distinct node ages for  
42 *Pipa*. We identified high dN/dS ratios along the mtDNA stem branch of *Pipa*, probably  
43 because an acceleration of the substitution rate in *Pipa* increases the effect of saturation on  
44 fast evolving positions. Focusing on the nuDNA topology, we hypothesize an early Neogene  
45 amazonian origin of the diversification of *Pipa*, with an initial split between the Guiana-  
46 Brazilian Shields and Western Amazonia, a pattern found in many other codistributed groups.  
47 All the western species are edentate, suggesting a single loss in the genus. Each of these  
48 groups diversified further out of Amazonia, toward the Atlantic forest and toward Trans-  
49 Andean forest, respectively, and these events are concomitant with palaeogeographic changes  
50 and recovered in codistributed groups. The two amazonian lineages have probably  
51 independently acquired endotrophic larval development.

52 **Keywords.** Amazonia; Atlantic Forest; Endotrophy; Neogene; Species delimitation

53

## 54 **1. Introduction**

55 The increasing accumulation of genomic data has permitted unveiling phylogenetic  
56 relationships and divergence times with unprecedented accuracy throughout the tree of life  
57 (Delsuc et al., 2005; Burki et al., 2020). This is the case for amphibians in which  
58 phylogenomic investigations have spectacularly improved our understanding of the  
59 relationships among major lineages (e.g. Neobatrachia, Streicher et al., 2018), and, combined  
60 with fossil calibrations, dramatically narrowed down temporal estimates of their origins (Feng  
61 et al., 2017; Hime et al., 2020). These progresses also notably led to the perception that  
62 mtDNA, which has been used for decades to decipher evolutionary patterns, sometimes  
63 underwent rapid and important shifts in substitution rates (Irisarri et al., 2012; 2017),  
64 sometimes leading to long-branch attraction artefacts (Gissi et al., 2006; Irisarri et al., 2017).  
65 The impact of the variations in substitution rate across loci and along the branches on  
66 phylogenetic reconstructions has been recognized for a long time and methods such as relaxed  
67 clocks implemented in BEAST (Drummond and Rambaut, 2007; Drummond et al., 2012;  
68 Bouckaert et al., 2014) are specifically designed to take into account these variations in order  
69 to provide more reliable time estimates. However, the reliability of these methods still largely  
70 depends on the availability/quality of priors such as node age calibration, for example from  
71 fossils, that are not evenly distributed along the tree of life and are simply absent in many  
72 groups (Donoghue and Yang, 2016).

73 The frogs of the family Pipidae are the only ones with a fully aquatic lifestyle, and  
74 harbor many highly derived anatomical traits (Cannatella, 2015) and chromosomal features  
75 (Mezzasalma et al., 2015). Extant taxa of the family Pipidae include *Pipa* (7 species) in the  
76 Neotropics, and *Xenopus* (29 species, *Silurana* being considered a synonym), *Hymenochirus*  
77 (4 species), and *Pseudhymenochirus* (1 species) in Africa. The sister-taxon of Pipidae is  
78 Rhinophrynidae, with a single extant fossorial species, *Rhinophrynus dorsalis*, which occurs

79 in central and southern North America. They form altogether the Pipoidea clade, an ancient  
80 group of frogs whose crown age is c.a. 160 Ma old (e.g., Hime et al., 2020). One pipid in  
81 particular, *Xenopus laevis*, has become a model organism and the focus of a tremendous  
82 amount of medical and fundamental lab research (e.g., Vleminckx, 2018). The relationships  
83 among pipids have also been the focus of many studies (e.g., Irisarri et al., 2011; Bewick et  
84 al., 2012; Hedke et al., 2013; Cannatella et al., 2015) including node and tip dating based  
85 upon what is probably the most abundant fossil record of all amphibian families (e.g., Baez  
86 and Pugener, 2003; Trueb et al., 2005; Gomez, 2016). At the family level, this group has  
87 attracted much attention not only because of their bizarre anatomical features, but also  
88 because of their biogeographic history tightly linked to the break-up of western Gondwana.  
89 Phylogenomic analyses (Hedke et al., 2013; Feng et al., 2017; Irisarri et al., 2017; Hime et al.,  
90 2020), either alone or in combination with morphological data and fossils (Cannatella, 2015),  
91 largely support the monophyly of African pipids which form the sister group of *Pipa*. Time  
92 estimates for the divergence between *Pipa* and the African pipids obtained from  
93 phylogenomic studies are highly congruent, and generally predate the final stage of the break-  
94 up of western Gondwana (c.a. 105 Ma) (Feng et al., 2017; Irisarri et al., 2017; Hime et al.,  
95 2020). Nevertheless, an alternative topology, i.e., *Pipa* + Hymenochirini, has found support in  
96 a few genomic analyses (Bewick et al., 2012; Cannatella, 2015) and mitogenomic data alone  
97 (Irisarri et al., 2017; Zhang et al., 2021). Even though these studies included less genomic  
98 data than the most recent studies recovering *Pipa* vs. African pipids, this alternative topology  
99 is in line with the morphological similarity between *Pipa* and Hymenochirini (Cannatella and  
100 Trueb, 1988; Gomez and Perez-Ben, 2019). Given (1) the bias inherent to mtDNA and (2) the  
101 consensus provided by the most recent and most extensive phylogenomic investigations, the  
102 hypothesis of the monophyly of african pipids seems more robust, and thus either implies that


103 the morphology of *Xenopus* is highly derived, and/or that *Pipa* and Hymenochirini underwent  
104 convergent evolution (Irisarri et al., 2011; Cannatella, 2015).

105 Paradoxically, despite the attention that pipids have attracted, the phylogenetic  
106 relationships within *Pipa* have been solely investigated using morphological characters  
107 (Trueb and Cannatella, 1986; Cannatella and Trueb, 1988; Trueb and Massemin, 2001), or  
108 based on short mtDNA sequences with incomplete taxonomic sampling (Vacher et al., 2020;  
109 Lima et al., 2020), and consequently remain poorly understood. Multilocus phylogenetic  
110 studies focusing on pipids have either included a single *Pipa* terminal or two, and provided  
111 highly inconsistent crown ages for the genus. Among these studies, the ones that included the  
112 analysis of mitogenomes (Irissari et al., 2012; Evans et al., 2019; Hemmi et al., 2020) found  
113 crown ages > 50 Ma, which suggests ancient diversification. Conversely, Feng et al. (2017)  
114 using 88 kb of nuDNA loci found a Most Recent Common Ancestor (MRCA) for *Pipa* spp. to  
115 be only 11 Ma, implying a relatively recent diversification instead. As a corollary, Irisarri et  
116 al. (2012) recovered disproportionate branch lengths between mtDNA and nuDNA within  
117 *Pipa* as compared to other non-Neobatrachia anurans. They also identified such imbalance in  
118 Neobatrachia and demonstrated that this was partly due to an acceleration of the substitution  
119 rate of the mtDNA, possibly resulting from a relaxed purifying selection on mtDNA.  
120 Therefore, incongruences among time estimates within *Pipa* could be due to a similar  
121 variation of mtDNA substitution rates. However, another paradox is the almost complete  
122 absence of known fossils directly related to *Pipa* (i.e., branching along the stem or nested  
123 within *Pipa*), which contrasts with an otherwise very rich Pipidae fossil record. The only  
124 exception is the c.a. 10 My old fossil from Corralito (lower Urumaco formation) of a portion  
125 of the sacrum of a large *Pipa*, with posteromedial ridges on the dorsal surface of each sacral  
126 diapophyses, suggesting close relationships with *Pipa pipa* (Delfino and Sanchez, 2018).

127 However, given its poor state of preservation, the phylogenetic position of this fossil remains  
128 unclear.

129         These knowledge gaps hamper an understanding of the biogeographic history as well  
130 as the evolution of reproductive modes and of the morphology of the genus. With four species  
131 occurring in Amazonia (*Pipa pipa* and *P. snethlageae* extend further), one in the Atlantic  
132 Forest (*P. carvalhoi*) and two with a ~~Trans~~-Andean distribution (*P. parva* and *P. myersi*), one  
133 can reasonably assume that the genus originated in Amazonia. However, it remains  
134 conjectural to formulate any biogeographic hypothesis for *Pipa* in the absence of a robust  
135 time calibrated phylogeny, and since both ancient, i.e., 40–15 Ma (Fouquet et al., 2012a,b;  
136 2013, 2014; Réjaud et al., 2020) and recent, i.e., < 5 Ma (Fouquet et al., 2014) dispersals  
137 events have been documented in anurans and in other vertebrates in the region (e.g. Ledo and  
138 Colli, 2017; Dal Vechio et al., 2018; Prates et al., 2018). It is also noteworthy that some  
139 characters are strikingly variable among *Pipa* spp., notably habitat and body size. *Pipa pipa*  
140 and *P. snethlageae* are large-bodied species (qualified as “macropipa”) and are distributed  
141 throughout Amazonia and even further into the Orinoco (Acosta-Galvis et al., 2016) and the  
142 Cerrado (Vaz Silva and Andrade, 2009; Dantas et al., 2019), occupying many different types  
143 of lotic and lentic aquatic environment, such as seasonally flooded forests. The other species  
144 like the Amazonian *P. aspera* and *P. arrabali* are small-bodied (“micropipa”), occupy various  
145 types of small water bodies, are occasionally terrestrial being able to colonize temporary  
146 ponds not connected to any rivers. *Pipa parva* also occupies temporary water bodies in the  
147 coastal deserts and semi-deserts of the Maracaibo basin and lowlands of the northeastern  
148 Caribbean region of Colombia (Galvis et al., 2011; Blanco et al., 2013). Considering these  
149 traits, we expect dispersal ability of the macropipa (e.g., da Fonte et al. 2021 reported *P. pipa*  
150 in floating meadows of the Amazon main course) to be high and their genetic structures to be  
151 relatively homogeneous over long distances vs. weaker dispersal abilities and more profound

152 genetic structures in micropipa. Moreover, teeth are ~~only~~ present in *P. carvalhoi*, *P. aspera*  
153 (Trueb and Massemin, 2001) and in *P. arrabali* (Trueb and Cannatella, 1986), and although  
154 all the species of *Pipa* incubate their eggs in a specialized skin layer growing on the female's  
155 back after complex breeding behaviours (Rabb and Rabb, 1960; Weygoldt, 1976), only the  
156 amazonian species have a completely endotrophic larval development while the tadpoles of *P.*  
157 *carvalhoi*, *P. parva* and *P. myersi* hatch from the skin of the female and develop freely in the  
158 water instead (Greven, 2011). The evolution of edentulism (Paluh et al., 2021) and of  
159 reproductive mode (Vagi et al., 2019, Furness and Capellini, 2019) has recently attracted new  
160 attention thanks to the availability of extensive phylogenetic sampling and the development of  
161 new methods (Revell, 2012). However, like their biogeographical history, the monophyly of  
162 the edentate and of the endotrophic species has never been tested using molecular data, and  
163 the evolution of these characters remains ambiguous since one or the other of these  
164 characteristics necessarily imply ~~convergent~~ acquisition or loss.

165 In order to (1) delimit candidate species (2) evaluate phylogenetic relationships and  
166 divergence times within *Pipa* and (3) investigate biogeographic history, the evolution of  
167 reproductive mode and teeth loss/gain, we gathered mitogenomic and 10 nuDNA loci for all  
168 *Pipa* species and major Pipoidea lineages. Since temporal discrepancies have been previously  
169 recovered among pipids, we furthermore compared the substitution rate ross mt and  
170 nuDNA and tested for variation in the dN/dS ratio across the genes and branches.

171

## 172 2. Materials and Methods

### 173 2.1. Species delimitation

174 Our first objective was to delimit major mtDNA lineages within *Pipa* since Vacher et al.  
175 (2020), Motta et al. (2018) and Lima et al. (2020) suggested that unrecognized species exist

176 within *P. arrabali*, *P. aspera*, *P. pipa* and *P. carvalhoi*. Our sampling included 16S rDNA  
177 sequences from 115 specimens of *Pipa* (Supplementary table S1) covering the entire  
178 distribution of the genus. These samples were obtained through fieldwork and loans, and  
179 completed with available sequences from GenBank (Supplementary table S1). Newly  
180 acquired sequences were obtained from Sanger sequencing (details of primers are available in  
181 Supplementary table S5). DNA sequences were aligned on the MAFFT online server under  
182 the E-INS-i option with default parameters (Kato et al., 2017) leading to a matrix of 595  
183 base pairs (bp).

184 We applied three DNA-based single-locus species delimitation approaches using this  
185 matrix: (a) a distance-based method, the Automated Barcode Gap Discovery (ABGD;  
186 Puillandre et al., 2012), (b) a multi-rate coalescent-based method, the multi-rate Poisson Tree  
187 Processes model approach (mPTP; Kapli et al., 2017) and (c) a single-threshold coalescent-  
188 based method, the Generalized Mixed Yule Coalescent approach (single threshold GMYC;  
189 Pons et al., 2006; Monaghan et al., 2009). The ABGD delimitation was performed using the  
190 online web server (available at <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>) with a  
191 prior of intraspecific divergences (K80) between 0.001 and 0.1 ( $P = 0.001-0.1$ ), a proxy for  
192 minimum relative gap width of 1 ( $X = 1$ ), and a number of steps equals to 30 ( $n = 30$ ). For the  
193 mPTP delimitation, we first reconstructed a ML tree with RAxML v.8.2.4 (Stamatakis, 2014)  
194 using a CAT+ $\Gamma$  model which was estimated to be a suitable model via PartitionFinder V2.1.1  
195 (Lanfear et al., 2017). The mPTP delimitation was undertaken on the tree rooted on  
196 *Rhinophrynus*, with 5 million Markov chain Monte Carlo (MCMC) iterations, sampling every  
197 10,000th iteration and discarding initial 10% iterations as burn-in. For the GMYC  
198 delimitation, we reconstructed a time-calibrated phylogeny using BEAST 2.5.2 (Bouckaert et  
199 al., 2014). We used a birth and death population model to account for extinction processes  
200 and incomplete sampling. We used a single partition with a GTR+I+ $\Gamma$  substitution model,



201 with an uncorrelated relaxed lognormal clock model of rate variation among branches  
202 (Drummond et al., 2006). We used the estimated age of the MRCA of Pipidae of Hime et al.  
203 (2020) as a calibration point, assuming normal prior distributions of 116.0 Ma (SD = 9 Ma).  
204 For the MCMC parameters, we used four independent chains of 100 million iterations,  
205 recording every 10,000th iteration. We combined the log and tree files of the four independent  
206 runs, discarding the first 30% iterations as burn-in, using LogCombiner 2.5 (Bouckaert et al.,  
207 2014) and checked the convergence of our parameters, confirmed by all ESS being above  
208 200. We then extracted the maximum clade credibility tree (from 28,004 trees) using Tree  
209 annotator 2.5 (Bouckaert et al., 2014). We performed a GMYC delimitation on the ultrametric  
210 tree using the GMYC function of the {splits} R package (Ezard et al., 2009), with a threshold  
211 interval between 0 and 10 Ma and by using the single threshold method. Operational  
212 Taxonomic Units (OTUs) were defined using a majority-rule consensus from the results of  
213 the three methods, i.e., a lineage is considered as being an OTU if supported by at least two of  
214 the three methods.

215


## 216 2.2. *nuDNA differentiation*

217 We gathered sequences of four nuDNA loci (RAC1, POMC, BDNF, NCX1) for a subset of  
218 individuals in order to evaluate the degree of congruence of differentiation with the mtDNA-  
219 based delimitation (Supplementary table S2). Two OTUs (*P. sp.* “Negro”, *P. sp.* “Nordeste”)  
220 were not represented by any nuDNA sequences, one (*P. parva*) by only two nuDNA loci. Six  
221 OTUs were represented in all four datasets, but by a single terminal. Conversely, sequences  
222 from several individuals were obtained for six OTUs (only five OTUs for POMC). The  
223 sequences of NCX1 were incomplete in 3' for five specimens or in 5' for five others,  
224 therefore, we considered both subsets independently (NCX1a & b). We reconstructed  
225 Median-Joining networks (Bandelt et al., 1999) from PopArt 1.7 (Leigh and Bryant, 2015).

226 We considered the absence of nuDNA allele sharing among specimens assigned to closely  
227 related OTUs as indicative of congruent differentiation.

228

### 229 2.3. Molecular dating

230 We selected one representative of each delimited OTU, with the exception of two OTUs that  
231 were ~~only~~ represented by a limited amount of mtDNA and were discarded, for estimation of  
232 phylogenetic relationships and divergence times. We obtained whole mitogenomic data for  
233 representatives of 11 OTUs via shotgun sequencing (Supplementary table S3, GenBank  
234 accession numbers will be added upon manuscript eptance; methodological details are  
235 available in Supplementary material S4). We completed the mtDNA matrix for the remaining  
236 two *Pipa* terminals for 12S, 16S, COI and Cytb using data available in GenBank (Table S4).  
237 We further complemented this mitogenomic dataset with 10 nuDNA loci (Supplementary  
238 table S4) via Sanger sequencing and sequences available in GenBank (details of primers are  
239 available in Supplementary table S5). During the matrix building, we noticed two sequences  
240 in GenBank from Irisarri et al. (2011) that appear to be swapped (AY341762=*Pipa parva*,  
241 AY341763=*Hymenochirus*), which were thus relabeled. We also retrieved from GenBank  
242 mitogenomes and homologous nuDNA sequences for five outgroups representing each  
243 African Pipidae genus and *Rhinophrynus dorsalis*, the sister group of Pipidae, including  
244 annotations that were transferred to the new mitogenomes. DNA sequences were realigned on  
245 the MAFFT online server under the E-INS-i option for 12S and 16S and considering the  
246 reading frame option for each CDS with default parameters (Kato et al., 2017). The control  
247 region and tRNA were discarded as well as flanking regions that were not available for most  
248 terminals.

249 The final alignment consisted of 21,762 bp, comprising 14,115 bp for mtDNA (12S-16S:  
250 2784; 11331 for mtDNA exons) and 7,647 bp for concatenated nuDNA (RAG1: 1374, NCX1:  
251 1278, POMC: 558, BDNF: 693, CXCR4: 630, TYR: 675, SLC8A3: 1092, H3a: 288, RAG2:  
252 744, RHO: 315). Two OTUs (*P. parva* and *P. carvalhoi*) had only partial mitogenomic data  
253 (12-16S, COI and Cytb and 16S and COI respectively) and 6 and 3 nuDNA loci available,  
254 respectively. All the other terminals had complete mitogenomes and at least 4 nuDNA loci  
255 (Supplementary table S3). Preliminary Maximum Likelihood analyses of each nuDNA loci  
256 using RAxML (see above for method) suggested overall topological congruence with mtDNA  
257 except the position of *P. sp.* “Guyana” (see results).

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

265 We reconstructed a time-calibrated gene tree in BEAST 2.5.2 using a birth-death tree  
266 prior to account for extinction processes. We parameterized unlinked substitution models and  
267 unlinked clock models according to the models suggested by the PartitionFinder analysis.  
268 Trees were linked. Divergence time estimation was implemented using an uncorrelated  
269 relaxed lognormal clock model of the distribution of rates among branches for each partition  
270 (Drummond et al., 2006). We relied on secondary calibrations based on Hime et al. (2020), an  
271 extensive nuclear genomic dataset (220 loci 291 kb) of all major frog lineages and the last  
272 land bridge between South America and Africa ca. 105 Ma (Torsvik et al., 2008). We  
273 enforced the monophyly of Pipidae since this clade has been strongly supported in all

274 phylogenetic (e.g. Irisarri et al., 2011, 2017; Cannatella, 2015; Feng et al., 2017; Hime et al.,  
275 2020) and paleontological analyses (e.g. Gomez, 2016). We also enforced the monophyly of  
276 African Pipidae (*Xenopus*, *Hymenochirus* and *Pseudhymenochirus*), thus favoring the  
277 topology in which *Pipa* is the sister group of other Pipidae following the results from the  
278 analyses of molecular data of Irissari et al. (2011, 2017); Feng et al. (2017); Hime et al.  
279 (2020) and of Cannatella (2015). This last work used a combination of molecular,  
280 morphological and fossils resulting in higher support for this topology and dates compatible  
281 with phylogenomic studies (Irisarri et al., 2011, 2017; Feng et al., 2017; Hime et al., 2020).  
282 We acknowledge that the interrelationships among the three main Pipidae lineages remain  
283 contentious, but the scope of our study being the relationships and the timing of divergence  
284 within *Pipa* we believe these priors to be reasonable and should have no influence on the  
285 crown age of *Pipa* or any divergence times within the genus. Specifically, we assumed a  
286 uniform prior distribution for three nodes (1) the MRCA of Pipidae (between 129.1 the lower  
287 HPD from Hime et al., 2020 and 105.0 Ma the western Gondwana final break-up), (2) the  
288 crown age of African Pipidae (between 114.4 Ma and 85.9 Ma HPDs from Hime et al., 2020)  
289 and (3) the crown age of Pipoidae (between 172.6 and 150.0 Ma HPDs from Hime et al.,  
290 2020) which corresponds to the root of the tree.

291 We analyzed independently the four mtDNA partitions and the three nuDNA  
292 partitions because preliminary analyses suggested highly incongruent posterior distribution of  
293 node ages (regardless of the inclusion of 3<sup>rd</sup> codon position, the use of either Yule or Birth-  
294 Death, linkage of the clocks among mtDNA partitions and among nuDNA partitions i.e., two  
295 clock vs seven). The concatenated analyses resulted in intermediary age for the nodes among  
296 *Pipa* spp. that were thus considered incorrect. We set two independent Markov chain Monte  
297 Carlo (MCMC) runs of 200 million iterations each, recording every 10,000th iteration and  
298 using the first 10% of iterations as burn-in. We combined the log and the tree files and the

299 resulting posterior samples of trees of the two independent runs using LogCombiner 2.5  
300 (Bouckaert et al., 2014) and checked convergence of model parameters via time-series plots.  
301 Chain mixing was considered adequate when parameters achieved an effective sample size  
302 above 500 (obtained for all parameters). We extracted a maximum clade credibility tree  
303 (based on the 36,002 resulting trees) using Tree annotator 2.5 (Bouckaert et al., 2014).

304

#### 305 *2.4. Biogeographic analyses*

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

314 To identify spatial processes of diversification, we considered five main geographic  
315 areas where known species currently occur: Guiana Shield (GS), Western Amazonia (WA),  
316 Brazilian Shield (BS), Atlantic Forest (AF) and a Trans-Andean region (TA). The three  
317 Amazonian regions correspond to major geological features of Amazonia (Hoorn et al., 2010)  
318 and to the large biogeographic regions known as Wallace's districts (Wallace, 1854), roughly  
319 delimited by modern riverine barriers: the Madeira River, the Caquetá/Japurá – Solimões, and  
320 the lower course of the Amazon River. This spatial partitioning into three areas allows us to  
321 investigate the putative connectivity across Neotropical regions and dispersal routes across  
322 Amazonia (Réjaud et al., 2020; Fouquet et al., 2021). Even though we ~~only~~ included two

323 populations of *Pipa snethlageae* (French Guiana and central Amazonia), mtDNA sequences  
324 are identical despite geographical distance. This large-sized species is associated with large  
325 swamps and in French Guiana, it reaches a northwestern distributional limit much like several  
326 other species associated with this habitat, such as *Leptodactylus intermedius*, *Hydrolaetare*  
327 *schmidti*, *Typhlonectes compressicauda*, *Dracaena guianensis*, *Paleosuchus niger*, among  
328 others (Lescure and Marty, 2000; Vacher et al., 2020; Gazoni et al., 2021) it is therefore likely  
329 that the range of this species extends throughout the Amazon basin and we thus considered its  
330 range to be panamazonian.

331

### 332 2.5. Teeth evolution and mode of reproduction

333 Several works have investigated the evolution of morphological and reproductive features  
334 within *Pipa* (Trueb and Cannatella, 1986, Cannatella and Trueb, 1988, Trueb and Massemin,  
335 2001), although not in the light of a robust molecular phylogeny and without including *P.*  
336 *arrabali* (see results, correspondence with the nominal *P. pipa* remained ~~also~~-ambiguous) and  
337 the candidate species identified herein (see results). We verified the presence (*P.* sp. “ES”, *P.*  
338 sp. “Nordeste”, *P.* sp. “South” and *P. arrabali*); or absence of teeth (*P. pipa*; *P.* sp. “WGU”,  
339 *P.* sp. “WAM”, *P.* sp. Negro”, *P.* sp. “Central”) in specimens corresponding to nine OTUs  
340 (see results) in addition to the ones already examined by Trueb and Cannatella (1986) and  
341 Trueb and Massemin (2001), under stereomicroscope LEICA MZ75.

342 Data on reproductive mode was available for all the outgroups and most candidate  
343 species (*P. aspera*: Trueb and Massemin, 2001), *P.* sp. “Guyana” (RE, pers. obs.), *P. arrabali*  
344 (Garda et al., 2006), *P. carvalhoi* (Fernandez et al., 2011), *P. myersi* (Trueb, 1984), *P. pipa*  
345 (Linnaeus, 1758); *P.* sp. “WGU” (RE, pers. obs.), *P. snethlageae* (Massemin et al., 2007), *P.*  
346 *parva* (Sokol, 1977). However, no data could be found for *P.* sp. “South”, *P.* sp. “WAM”, *P.*

347 sp. “Central”, *P.* sp. “Negro”, *P.* sp. “ES”, *P.* sp. “Nordeste” for which we assumed they  
348 display the same breeding behaviour than their close relatives

349

## 350 2.6. Rates shifts

351 We observed important discrepancies between the temporal estimates in the *Pipa* genus  
352 obtained from mtDNA vs. nuDNA, while the topologies are almost identical (see results).  
353 This discrepancy may be resolved by invoking two hypotheses: (1) nuDNA temporal  
354 estimates are correct and the mtDNA substitution rate is underestimated i.e. in fact greater in  
355 *Pipa* relative to the rest of the tree, or (2) mtDNA temporal estimates are correct and the  
356 nuDNA substitution rate is overestimated. In the absence of fossils it remains virtually  
357 impossible to tease these two hypotheses apart. However, given that the biogeographic history  
358 of codistributed frog groups is mostly circumscribed to the Neogene and that previous works  
359 have identified large variations in mtDNA rates across frog lineages (Irissari et al., 2012), we  
360 estimated that the first hypothesis is the most likely.

361 Consequently, we ~~carried out~~ a second phylogenetic analysis combining mtDNA and  
362 nuDNA, identical to our first analysis, but constraining the crown age of *Pipa* to the estimate  
363 obtained with nuDNA only (using the nuDNA 95% HPD interval as a uniform calibration  
364 prior). This additional analysis allowed us to estimate the average mtDNA rate within *Pipa*,  
365 conditional on a Neogene diversification of this genus, and to compare this rate with the rates  
366 found in other branches in the same tree, and with other studies (e.g., in two Neobatrachia  
367 groups *Allobates* (Réjaud et al., 2020) and *Boana* gr. *albopunctata* (Fouquet et al., 2021b) for  
368 which mitogenomic data were analysed in similar ways than for *Pipa* (i.e., using the same  
369 partitioning and secondary time calibration from phylogenomic studies of Feng et al., 2017  
370 and Hime et al., 2020). BEAST tree files were obtained from the authors. For each partition

371 and phylogenetic analysis, we calculated the average rate applying to a specific subtree (e.g.,  
372 in *Pipa*, in African pipids, etc.) as the average of the branch-specific rates (we used a relaxed  
373 molecular clock with one rate per branch) across all the branches of the subtree, weighted by  
374 the lengths of the branches.

375

## 376 *2.7. Detecting changes in dN/dS*

377 Simulation studies have shown that analyses of selection coefficients are rather robust to  
378 sequence divergence (Yang, 2006) (as is the case in the present study), having been  
379 successfully used in various studies with highly divergent species (e.g., Buschiazzo et al.,  
380 2012). In order to understand whether acceleration of evolutionary rates in *Pipa* is due to  
381 changes in selective pressure, we tested alternative models with different assumptions about  
382 ratios of non-synonymous/synonymous substitution rates ( $\omega$ ). The software PAML v.3.15  
383 (Yang, 1997) was used to estimate the likelihood and the  $\omega$  values of different models derived  
384 from the topologies and sequence information from single-gene alignments with all codon  
385 positions, as well as the mt and nuclear nucleotide data sets. Branch lengths were first  
386 optimized for each data set assuming a single  $\omega$  for the whole tree, and they were fixed when  
387 all other parameters were estimated under alternative models. The null model had a single  $\omega$   
388 value for all branches, and it was compared against four alternatives, which allowed a second  
389  $\omega$  value on (i) the stem branch of *Pipa*, (ii) all *Pipa* branches. All models were compared  
390 using the AIC.

391

## 392 **3. Results**

### 393 *3.1. mtDNA-based species delimitation*



394 The phylogenetic trees obtained from the ML and the Bayesian analyses of the 16S locus  
395 strongly supported *Pipa* as monophyletic as well as the existence of four major clades within  
396 the genus. Two of these clades (*Pipa aspera/arrabali* and *Pipa pipa/snethlageae*) are mainly  
397 Amazonian (Figure 1A), one occurs in the Atlantic Forest (*Pipa carvalhoi*) and one in Trans-  
398 Andean regions (*Pipa parva/myersi*). Several deeply diverging lineages are also supported  
399 within *Pipa pipa*, *Pipa carvalhoi* and *Pipa arrabali*, indicating that the seven currently  
400 recognized species represent a vast underestimation of the actual species diversity within the  
401 genus (Figure 1A, S6). *Pipa arrabali* in particular is recovered paraphyletic with respect to  
402 *Pipa aspera*.

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

414

### 415 3.2. nuDNA differentiation

416 The relationships obtained using the four nuDNA loci are overall largely congruent with the  
417 mtDNA-based delimitation. For example, *Pipa* sp. “Central” is consistently recovered as

418 sharing no alleles with the other three OTUs related to *P. pipa*. These three OTUs delimited  
419 within *Pipa pipa* are also recovered as sharing no alleles in NCX1, but this case is more  
420 ambiguous since allele sharing is observed on the other loci. The groups delimited within *P.*  
421 *carvalhoi* are also differentiated on RAG1 and NCX1. The case of *P. aspera* and the  
422 populations from Guyana (assigned to *P. arrabali*) is noteworthy since these species are  
423 recovered as a single OTU based on mtDNA. However, they do not share any alleles on any  
424 nuDNA loci except RAG1. Considering their distinct morphology and their divergence on  
425 nuDNA loci we considered them as distinct, leading to 15 OTUs.

426

### 427 3.3. Molecular dating

428 We assigned the best-fit models suggested by the PartitionFinder analysis to each of the seven  
429 partitions. The two combined BEAST analyses of both the mtDNA and the nuDNA data led  
430 to all parameters having ESS > 2000. The resulting phylogenetic relationships are all highly  
431 supported in both the mtDNA and the nuDNA trees (posterior probability pp > 0.99) and  
432 completely congruent between the mtDNA and the nuDNA, except the position of *Pipa* sp.  
433 "Guyana", which is supported as the sister species of *P. aspera* with mtDNA and as the sister  
434 species of the clade formed by *P. arrabali* and *P. sp.* "South" according to nuDNA. *Pipa* is  
435 structured in two major clades, one centered in the eastern part of its range (*P. carvalhoi* -  
436 Atlantic Forest; *P. aspera*, *P. arrabali* - Eastern Amazonia), and the other clade in the western  
437 part (*P. myersi*, *P. parva* - Trans-Andes and *P. pipa*, *P. snethlageae* - Amazonia; Figure 2).

438 However, major differences are observed in the timing of divergence between the two  
439 trees. The crown age of *Pipa* is found to date back to ca. 85 Ma (76–94, 95% HPD) according  
440 to mtDNA (Figure 2A), but to ca. 18 Ma (14–22, 95% HPD), i.e., 4.7 times younger  
441 according to nuDNA (Figure 2B). Since these dates are largely incompatible, either the rates

442 of the mtDNA, the nuDNA, or both are erroneously estimated. In the absence of fossils  
443 branching along the *Pipa* stem, this question will remain contentious. Nevertheless, the  
444 mtDNA rates are notoriously more subject to underestimation by BEAST, notably for ancient  
445 periods (Molak and Ho, 2015), as it could be the case for *Pipa*. Therefore, we considered an  
446 additional analysis based on the crown age obtained from the analysis of nuDNA (see below)  
447 and favoured the relationships and temporal estimates of the nuDNA analysis hereafter.

448

#### 449 3.4. Rates shifts

450 The posterior distributions of the rates of each mtDNA partition analysed alone and with the  
451 three old priors (Cretaceous) is relatively stable across the tree i.e., among African pipids and  
452 for the stem and the crown for *Pipa* (Figure 3). The only notable exception is the high rate of  
453 the stem of *Pipa* for pos2 using Cretaceous calibrations compared to the ones of other  
454 branches, while this rate is similar in other partitions. Moreover, when adding the Neogene  
455 prior on the crown age of *Pipa* (based on the results with nuDNA), the rates were found to be,  
456 for all partitions, about four times higher in crown *Pipa*, and half as high in the stem, as  
457 compared to the analysis without the Neogene prior. The rate in African pipids was similar in  
458 both analyses. The rates estimated in *Allobates* and *Boana* are comparable (although slightly  
459 higher) to the rates we estimated in crown *Pipa* with the Neogene prior, except for pos 3  
460 (Figure 3). The rates of the nuDNA partitions were similar across branches (Supplementary  
461 figure S9).

462

#### 463 3.5. Changes in dN/dS


464 We compared a model with  $\omega$  constant across the whole tree (single- $\omega$  model) with a “three- $\omega$   
465 model” assuming independent  $\omega$  values in (1) the stem branch of *Pipa*, (2) the branches  
466 within the crown of *Pipa* and (3) in other branches of the tree. The  $\omega$  value estimated with the  
467 single- $\omega$  model was well below 1 for all different mt and nuclear genes (0.012–0.194) (Table  
468 1), indicating the action of purifying selection to maintain gene function (Castallena et al.  
469 2011).

470 With the three- $\omega$  model, for all individual mt genes (except *atp8* but consists of a short  
471 159 bp locus),  $\omega$  was always estimated to be higher (ca. X2) in the stem branch of *Pipa* than  
472 within crown *Pipa*, or in the other Pipoidea branches (Table 1). The  $\omega$  estimate for stem *Pipa*  
473 was also about twice higher than the overall rate estimated with the single- $\omega$  model (Table 1).  
474 The three- $\omega$  model significantly outperformed ( $\Delta\text{AIC} < 2$ ) the single- $\omega$  model for seven genes  
475 (*cob*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*), and the single- $\omega$  model significantly  
476 outperformed the three- $\omega$  model for two genes (*atp8* and *cox1*), as shown in Table 1. When all  
477 mitochondrial genes were considered jointly, the three- $\omega$  model was favoured  
478 overwhelmingly ( $\Delta\text{AIC} = 124$ , Table 1). For nuclear genes (*h3a* having been excluded given  
479 this locus was short - 288 bp - and only represented by six terminals including a single *Pipa*),  
480 the three- $\omega$  model never significantly outperformed ( $\Delta\text{AIC} < 2$ ) the single- $\omega$  model, and the  
481 single- $\omega$  model was best for four genes (*bdnf*, *rag2*, *rho* and *slc8a3*), see Table 1.

482 Overall, our results suggest a relaxation of the purifying selection acting on mtDNA in  
483 *Pipa* which could be responsible, at least in part, for the general acceleration of the mtDNA  
484 substitution rates in *Pipa*. It is important to note that results derived from the comparison of  
485 different selection regimes should be taken with caution, because the analyzed sequences are  
486 highly divergent and silent substitutions might be saturated, thus compromising the correct  
487 estimation of  $\omega$  values (Yang 2006).

488

### 489 3.6. Biogeography, reproductive mode evolution and teeth loss

490 *Pipa* diversified throughout the Neogene with two concomitant splits ca. 13 Ma between  
491 Amazonia and the Atlantic Forest and between Amazonia and Trans-Andes. Subsequent  
492 diversification within *Pipa* is circumscribed to the Pliocene and Pleistocene i.e., <5 Ma.  
493 Inference of ancestral areas using BioGeoBEARS equally favored the DEC and DEC+J  
494 models (Table S8) and provided very ambiguous results for the early diversification events  
495 within *Pipa*. Nevertheless, when the three amazonian areas are ~~considered~~ combined an  
496 amazonian origin is supported for the genus and for the two major clades. Concerning the   
497 clade (*P. pipa*/*P. snethlageae*) the combined probabilities suggest ~~rather~~ a western Amazonian  
498 origin and a recent (Middle Pleistocene) dispersal to the Guiana Shield. In the opposite  
499 direction but concomitantly, a dispersal from the Guiana Shield toward the Brazilian Shield is  
500 suggested for the clade (*P. aspera*/*P. arrabali*).

501 Endotrophy has either evolved independently in the two Amazonian clades (the  
502 “macropipa” and the *P. arrabali*/*P. aspera* clades) or has been acquired ancestrally and  
503 independently lost in the Atlantic Forest and the Trans-Andean clades. Maxillary teeth  
504 have been lost once during the early Miocene in the western clade formed by “macropipa” and  
505 the Trans-Andean clade

506

## 507 4. Discussion

508 Our results shed a new light on species diversity, phylogenetic relationships, divergence time,  
509 biogeography and the evolution of edentulism and of reproductive mode in the genus *Pipa*.  
510 Our comparative results on mt and nuDNA also strikingly exemplify how molecular dating

511 based on single locus (even though full mitogenomes) could lead to spurious time estimates  
512 when substitution rates vary drastically along long branches and no fossils are available along  
513 these long branches.

514

#### 515 4.1. Species diversity

516 Almost all of the studies that have explored the question of how many species exist within  
517 particular amphibian clades in the Neotropics have uncovered high numbers of candidate  
518 species. Most often these cases correspond to populations that were previously considered to  
519 belong to species with wide distributions (e.g., Fouquet et al., 2014; 2021a,b; Gehara et al.,  
520 2014; Carvalho et al., 2021; Vacher et al., 2020). This situation is also exemplified in our  
521 results for *Pipa*, with a possible 2.1 times increase in species diversity (15 instead of seven)  
522 since eight OTUs possibly correspond to unnamed species (or taxa requiring revalidation such  
523 as *Pipa laevis* Cuvier, 1831 from the Rio Negro), in addition to the seven species already  
524 described. This figure is not surprising and even lower than what has been found in many  
525 other codistributed clades. Among these nine candidate species, most are supported as distinct  
526 by nuDNA, however the situation remains ambiguous between some closely related pairs  
527 such as *P. arrabali* vs. *P. sp.* “South”; *P. pipa* vs. *P. sp.* “WGU” and *P. sp.* “WAM”; *P.*  
528 *carvalhoi* vs *P. sp.* “Nordeste” and *P. sp.* “Central” vs *P. sp.* “Negro”, i.e., could represent  
529 false positives, either because allele sharing suggests conspecificity or simply because of the  
530 absence of data. The cases of *P. carvalhoi*, *P. sp.* “ES” and *P. sp.* “Nordeste” were already  
531 documented by Lima et al. (2020) who found little morphological, but clear chromosomal  
532 differences. Interestingly, interspecific genetic distances on 16S among closely related OTUs  
533 are lower than 3% in four instances (Table S7) notably between *P. aspera* and *P. sp.*  
534 “Guyana” (1.6%) and between *P. parva* and *P. myersi* (1.6%). This 3% threshold in 16S

535 rDNA distances has frequently been suggested to be indicative of candidate species in  
536 anurans (Fouquet et al., 2007; Vieites et al., 2009). Nonetheless, these two pairs are  
537 phenotypically distinct (Trueb, 1984; Trueb and Cannatella, 1986), and congruent nuDNA  
538 divergences are recovered, thus confirming their status as distinct species. *Pipa aspera* and *P.*  
539 sp. “Guyana” illustrate a case of a false negative since mtDNA-based species delimitation  
540 actually failed to distinguish them. ~~The use of single genetic marker distance criteria may~~  
541 ~~inherently result in a proportion of false negatives and false positives using short mtDNA~~  
542 ~~locus and should only be used as a preliminary approximation of species boundaries and with~~  
543 ~~the necessary precaution in species delimitation.~~ Here more data are needed, notably spatial  
544 sampling, morphology and bioacoustics, and we once more advocate the pertinence of  
545 integrative approaches that combine both genetic and phenotypic data (Padial et al., 2010).

546 Our results also highlight that the recognized species’ geographic ranges of *Pipa pipa*  
547 (possibly limited to Eastern Amazonia), *P. arrabali* (possibly limited to the northern part of  
548 the Madeira-Tapajos interfluvium) and *P. carvalhoi* (possibly limited to the Brazilian states of  
549 Bahia, Pernambuco and Alagoas) may be much more restricted than currently admitted.  
550 Moreover, it is likely that additional species remain unsampled, notably in southeastern  
551 Amazonia where records of *P. arrabali* not included in this study have been documented  
552 (e.g., Garda et al., 2006 from Serra do Cachimbo, Para; da Silva et al., 2020 in Tocantins;  
553 Pinheiro et al., 2012 from Carajas, Para), and in the Orinoco basin where two species of  
554 “macropipa” not included here may occur (Acosta-Galvis et al., 2016). The extent of these  
555 basic knowledge gaps for such a charismatic group of frogs is particularly striking and  
556 exemplifies the logistical challenges associated with undertaking fieldwork in remote  
557 Amazonian regions. Nevertheless, even considering 15 candidate species, *Pipa* remains a  
558 relatively poorly diversified clade as compared to most codistributed clades of terrestrial frogs  
559 (e.g., Réjaud et al., 2020) that also diversified throughout the Neogene.

560

561 4.2. Acceleration of mtDNA substitution rate in *Pipa*

562 Due to their small size, lack of recombination, rapid evolution rate, conserved gene content  
563 and genomic organization, and maternal inheritance, mitogenomes have been commonly used  
564 for analyzing phylogenetic relationships and divergence time (Igawa et al., 2008; Zhang et al.,  
565 2021). Although mitogenomes are usually thought to be under neutral or nearly neutral  
566 selection, evidence has accumulated for positive selection associated with environmental  
567 adaptations acting on mitochondrial genes (Carapelli et al., 2019; Shen et al., 2010). When  
568 shifts in selective forces occur in groups with long branches, along which no other constraints  
569 are available, this could lead to important bias in estimating these rates and divergence times.

570 Here we hypothesize that an acceleration of the substitution rate of mtDNA in *Pipa*  
571 relative to other pipoids largely explains incongruences across temporal estimates. This  
572 scenario is probable since (1) fixation of  $\omega$  values is detected on all mtDNA genes, not only  
573 on a few genes which could have indicated positive selection on particular mt loci; (2)  
574 biogeography of codistributed group such as *Allobates*, *Amazophrynella*, *Adenomera*,  
575 *Pristimantis*, *Chiasmocleis* for instance display many topological and temporal similarities  
576 with *Pipa* when considering the nuDNA analysis, implying a mtDNA rate ca. four times  
577 higher than in other pipids, which still remains lower than in Neobatrachia; (3) Irisarri et al.  
578 (2012) already observed this pattern when comparing mt and nuDNA molecular evolution in  
579 anurans.

580 Irisarri et al. (2012) hypothesised that relaxed purifying selection on mtDNA may at  
581 least partly explain the acceleration of mtDNA rates observed in Neobatrachia and discussed  
582 a number of possible reasons for such changes, notably in life history traits. In *Pipa*, the  
583 acquisition of skin incubation of the eggs and/or endotrophic development in the two



584 amazonian clades could be related to that acceleration of substitution rates, although the  
585 precise causality remains totally speculative. Nevertheless, relaxed purifying selection on  
586 mtDNA has been hypothesized in other groups, such as in salamanders, concomitantly with  
587 the acquisition of direct development (Takehashi and Kurabayashi, 2021). However, a  
588 somewhat more trivial but not mutually exclusive, hypothesis may explain the observed  
589 variation in dN/dS ratio: the synonymous substitution saturation effect (i.e., several  
590 substitutions occurring sequentially at the same site on the same branch of the phylogenetic  
591 tree). This bias may particularly affect dN/dS estimation on very long branches, since dN/dS  
592 increases with saturation (Cannarozzi and Schneider, 2012). In other words, saturation leads  
593 to underestimate dS for ancient time periods because saturation is reached faster on sites that  
594 have higher substitution rates (e.g., pos1 and pos3) and thus the relative amount of dN (e.g.,  
595 pos2) is artificially overestimated. This phenomenon is actually observable on Figure 3, in  
596 which the rate of the stem of *Pipa* of pos2 using Cretaceous calibrations is the highest.  
597 Consequently, the analysis via BEAST cannot correctly estimate mtDNA substitutions rates  
598 on the long stem of *Pipa* and overestimates the divergence time. This may also have been the  
599 case in Neobatrachia (Irissarri et al., 2012) and relaxed purifying selection may in fact not be  
600 the main process explaining the acceleration of mtDNA rates. Ancient divergence times (50–  
601 100 Ma) may simply be too old to be estimated using mitogenomes of lineages with rapid  
602 substitution rates such as *Pipa*, in the absence of fossil constraints from the Tertiary allowing  
603 substitution rates to be bounded.

604

#### 605 4.3. Phylogeographic patterns in Amazonian *Pipa*

606 Within the *Pipa pipa/snethlageae* clade, the biogeographic analysis suggests a western  
607 Amazonian origin that is driven by ~~the fact that~~ early diverging lineages ~~occur~~ in western

608 Amazonia and in the Negro River. This western origin is expected given ~~the ecology of~~ these  
609 species tightly linked to large bodies of water that are currently widespread in the seasonal  
610 floodplains of the region, ~~contrary to~~ the Guiana and the Brazilian Shields. Moreover, the  
611 historic presence of large lacustrine systems (Acre and Pebas systems; see Hoorn et al., 2010)  
612 in western Amazonia and the increasing eastward expansion of the flooded ecosystems since  
613 late Pleistocene (Aleixo and Rossetti, 2007, Bicudo et al., 2019) also support that hypothesis.  
614 However, the existence of five OTUs recovered within what has been so far considered ~~as~~ *P.*  
615 *pipa* is more surprising since it suggests limited connectivity across main amazonian  
616 tributaries whereas one would expect these large aquatic frogs to efficiently disperse along the  
617 vast hydrological network of the Amazon basin. In fact, there is an apparent paradox in *P.*  
618 *pipa* because it does display a shallow genetic structure throughout eastern Amazonia (Purus,  
619 Madeira, Tapajos, Xingu), but also including the easternmost part of the Guiana Shield (from  
620 Amapa, Brazil, to a western limit in the Suriname River in Suriname), while distinct lineages  
621 occupy western Amazonia (*P.* sp. “WAM”) and the Negro River (*P.* sp. “Negro” and *P.* sp.  
622 “Central”). The rivers of the Guiana Shield are not directly connected to the Amazon basin  
623 (although recent connection through the Rio Branco existed; de Souza et al., 2020) but the  
624 rivers of western Amazonia and the Negro River are. This pattern, along with the existence of  
625 two diverging lineages along the Negro river, is particularly noteworthy since spatially similar  
626 genetic structures are observed in fish (Piranhas: Hubert et al., 2007), in *Chelus fimbriata*  
627 (Vargas-Ramirez et al., 2020), with which *Pipa pipa* may actually be ecologically closer than  
628 to other frogs; but also in birds associated with flooded forests (Thom et al., 2020). Therefore,  
629 we can hypothesize that this pattern originates from either historical or ecological processes.  
630 Avulsion between the Japura and the course of the lower Negro river during Pleistocene  
631 (Ruokolainen et al., 2018) may explain this pattern; i.e., with ancient barriers that no longer  
632 exist; but also major paleo-mega-wetlands that were scattered in Amazonia during late

633 Neogene (Albert et al., 2018). Currently the strong ecological differences between the main  
634 types of floodplains (white waters in western Amazonia vs. black waters of the Negro, Cooke  
635 et al., 2014; Beheregaray et al., 2015; Oliveira et al., 2019) may also play a role in  
636 maintaining ecological isolation of the Rio Negro populations from the rest of the Amazon  
637 basin. Nevertheless, the nuDNA ~~only~~ support these difference for the Negro River OTUs ~~and~~  
638 ~~caution should be taken given that the genetic structure observed on mtDNA may not be a~~  
639 ~~reliable proxy of contemporaneous lack of interconnectivity among populations.~~ Therefore,  
640 more spatial and genomic data are needed to investigate at finer scale the phylogeography  
641 within *Pipa pipa*.

642 In contrast, the genetic structure in the *P. aspera/arrabali* clade was expected to be  
643 more pronounced than within *Pipa pipa* since these species are associated with smaller water  
644 bodies in terra firme forest of the Brazilian and the Guiana Shields. However, even if a  
645 marked genetic structure exists, divergences remain relatively low among these lineages and  
646 even across the Amazon River. Nevertheless, the distribution of the different OTUs is more  
647 similar to the one found in terrestrial anurans i.e., distribution breaks matching major  
648 Amazonian tributaries and bisecting the Guiana Shield (Vacher et al., 2020). Interestingly, we  
649 recovered a mtDNA/nuDNA topological incongruence in the *Pipa aspera/arrabali* clade with  
650 the Brazilian Shield OTUs being nested within Guiana Shield ones according to nuDNA vs.  
651 ~~forming two subclades~~ according to mtDNA. These topological differences suggest either (1)  
652 introgression with mtDNA capture among GS lineages after the dispersal of *P. arrabali* from  
653 the GS or (2) introgression between BS and Guyana populations after dispersal from the BS,  
654 or (3) different coalescent gene histories. The origin of the group in the Guiana Shield was  
655 supported by the biogeographic analysis and the lower diversity among Brazilian Shield  
656 populations on the different nuDNA loci supports the first hypothesis. However, both  
657 topologies imply trans-amazon dispersals, nuDNA suggesting relatively a recent event (<1.4

658 Ma) when the transcontinental Amazon river was already established which is surprising  
659 given the ecology of the species.

660

#### 661 4.4. Biogeography

662 The geographic center of diversification of most Neotropical groups remains difficult to infer  
663 not only because of putative extinctions but also because subsequent dispersals and intense  
664 landscape dynamics reshuffled the spatial distribution of organisms throughout the Cenozoic  
665 (e.g. Antonelli et al., 2018). Consequently, our ability to investigate the spatial origins of focal  
666 groups is usually restricted to more recent periods (Smith et al., 2014; Marques Silva et al.,  
667 2019; Cracraft et al., 2020). ~~The case of *Pipa*~~ is no different, with little signal for the early  
668 ancestral range of the main groups. Nevertheless, since (1) two of the main *Pipa* lineages  
669 occupy Amazonia and that they possibly originated in the eastern (*P. aspera*/*P. arrabali*) and  
670 the western portions (*P. pipa*/*P. snethlageae*) of that region, and that (2) their respective sister  
671 groups occupies the Atlantic Forest (*P. carvalhoi*) on the east and the Trans-Andean region  
672 (*P. myersi*/*P. parva*) on the west, a likely scenario consists of an Amazonian origin (14–22  
673 Ma) and subsequent dispersal/vicariance some 12–13 Ma towards neighbouring regions. Such  
674 a scenario seems particularly plausible since several co-occurring amphibians have similar  
675 trajectories with amazonian origin dating back to the early Neogene also displaying an east-  
676 west pattern in Amazonia as well as concomitant dispersal/vicariance with Atlantic Forest and  
677 Trans-Andean regions (see below).


678 The main split in *Pipa* separates an eastern Amazonia and the Atlantic Forest clade  
679 from a panamazonian (with a likely western Amazonian origin see above) and a Trans-  
680 Andean clades. This East-West split dating back to early Neogene (14–22 Ma) coincides with  
681 the Pebas system, a large freshwater system initially connected to the Caribbean Sea, that may

682 have covered a surface of up to 13% of current Amazonia (800,000 km<sup>2</sup>; Albert et al., 2018).  
683 The Pebas system supposedly formed during the early Miocene (23 Mya) and occupied most  
684 of the Western Amazonian lowlands until around 10–9 Ma, when this system was  
685 progressively drained eastward into the Atlantic Ocean and transitioned into the modern  
686 Amazon watershed (Albert et al., 2018; Hoorn et al., 2017). Recent advances in Amazonian  
687 biogeography of amphibians (Rojas et al., 2018; Réjaud et al. 2020; de Carvalho et al., 2021;  
688 Fouquet et al. 2021a,b) and also other groups of terrestrial vertebrates recovered this east-west  
689 pattern within Amazonia. Interestingly, the edentate species of *Pipa* correspond to this  
690 western clade, which suggests a single loss, a much simpler scenario than what was  
691 hypothesised by Trueb and Cannatella (1986). The origin of edentulism in this group of *Pipa*  
692 remains highly speculative but may be linked with feeding habits in an ancestral habitat in a  
693 large lacustrine system. How these frogs actually feed has been investigated by Fernandez et al.  
694 (2017) and Cundall et al. (2017) but only in *P. pipa*. A comparison between edentate and  
695 dentate species may provide some insights about the evolution of feeding habits.

696 The Andes represent a formidable barrier to dispersal between Amazonia and the  
697 Trans-Andean region. The Andean orogeny has been an ongoing process over the last 40 Ma  
698 punctuated by several intensive phases notably 12 Ma (Mora et al., 2010; Hoorn et al., 2010).  
699 The 12–13 Ma dispersal vicariance between Amazonia and the Trans-Andean region in *Pipa*  
700 coincides with this middle Miocene phase of orogeny. Moreover, similar Trans-Andean  
701 events occurred in other forestial groups such as *Allobates* (Réjaud et al., 2020),  
702 *Engystomops* (Funk et al., 2012) and several lineages of Dendrobatidae (Santos et al., 2009),  
703 *Dendropsophus* (Pirani et al., 2020) and *Pristimantis* (Mendoza et al., 2015). Conversely, in  
704 amphibian taxa with higher dispersal abilities, notably having frequently adapted to altitude,  
705 such as glassfrogs or toads, some species have been isolated by the Andes (Bessa-Silva et al.,  
706 2020; Castroviejo-Fisher et al., 2014), while other species even display a cross-Andean

707 distribution (e.g. *Boana boans*, Caminer and Ron, 2020; *Cochranella resplendens*; Molina-  
708 Zuluaga et al., 2017). Given that *Pipa* spp. are exclusively found in lowlands, we hypothesize  
709 that the Andes acted as a barrier to dispersal 12 Ma onward since the intense orogeny phase.

710 Similarly, the savannas of the Cerrado that today separates Amazonia and the Atlantic  
711 Forest represent a barrier for most forest amphibians. Middle Miocene divergence appears  
712 relatively concomitant with those other Neotropical frog and lizard complexes occurring in  
713 both regions (*Adenomera*: Fouquet et al., 2014; *Adelophryne*: Fouquet et al., 2012a;  
714 *Dendropsophus*: Pirani et al., 2020; *Leposoma*: Pellegrino et al., 2011), as well as birds  
715 (Batalha-Filho et al., 2013). The occurrence of successive dispersal routes between Amazonia  
716 and the Atlantic Forest has been largely recognized since this pattern has been recovered for  
717 frogs and other vertebrates (Ledo and Colli, 2017). However, both timing and approximate  
718 location of these connections remain largely debated. Nevertheless, there is little doubt that  
719 recurrent phases of forest extension have allowed recurrent connectivity and exchanges of  
720 fauna between these forests notably during the Miocene. The Middle Miocene Climatic  
721 Optimum (MMCO) (17–15 Ma) was a warm and wet period followed by periods of stronger  
722 seasonality associated with open vegetation expansion (Steinhorsdottir et al., 2021). Such  
723 environmental changes may have isolated forest-adapted species. Batalha-Filho et al. (2013)  
724 hypothesized a southern pathway between these biomes during the middle Miocene (Por,  
725 1992; Costa, 2003). Widespread vegetation opening is documented subsequent to the MMCO  
726 (Flower & Kennett, 1994) with drastic climatic changes linked to major uplift of the Andes  
727 (Hoorn et al., 2010) and sea-current modifications (Herold et al., 2009; Le Roux, 2011).

728 The *Pipa* species with endotrophic development, which correspond to the two  
729 amazonian clades, are  thus paraphyletic. Furness and Capellini (2019) demonstrated that  
730 complex parental investment such as brooding and “viviparity” are very unlikely to undergo  
731 reversals. Therefore, assuming that skin incubation predates endotrophic development, this

732 implies two independent acquisitions of endotrophy: one in macropipa and one in the eastern  
733 amazonian *P. aspera/arrabali* clade. ~~The fact that endotrophy was probably acquired twice~~  
734 ~~only in Amazonia may not be a simple coincidence but implying any causality, such as more~~  
735 ~~stable climatic conditions in Amazonia providing long suitable periods for incubation or~~  
736 ~~stronger selective pressure from predators remains highly speculative. Moreover, some~~  
737 reversals from endotrophy to exotrophy have been documented in *Anomalogossus* (Vacher et  
738 al., 2017), and in *Adenomera* (Fouquet et al., 2014) and since the reproductive mode of many  
739 species remain undocumented, the occurrence of endotrophy-exotrophy shifts may ~~have in~~  
740 ~~fact~~ be more common than currently considered. Given that there are only two pairs of  
741 lineages with both modalities in *Pipa*, reversals from endotrophy to exotrophy in the Atlantic  
742 Forest and Trans-Andes clades remain thus plausible.

743

## 744 **5. Conclusion**



745 These findings shed new light on the diversity, genomic evolution, historical biogeography  
746 and the evolution of morphology and reproductive mode in *Pipa*. Much still remains to be  
747 investigated on each of these aspects, notably improving the sampling in Amazonia and  
748 combining morphology and bioacoustic with molecular data to delimit species and ~~describe~~  
749 the actual diversity in the genus. Our understanding of the biogeography and the  
750 morphological evolution of *Pipa* also harbor large gaps that fossils from Amazonia (Antoine  
751 et al., 2021) and microtomography could help to fill. We hope that this work will foster these

752

## 753 **Declaration of Competing Interest**

754 The authors declare that they have no known competing financial interests or personal  
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756

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1170

1171

1172

1173 **Table 1**

1174 Results from the CodeML analyses for each locus and mitochondrial and nuclear loci  
1175 combined. The highest  $\omega$  values are in red and values in bold indicate models significantly  
1176 outperforming the others according to the AIC.

1177

1178 **Fig. 1.** (A) Maximum Likelihood phylogenetic tree obtained from the analysis of 115  
1179 sequences of 16S (595 bp) of *Pipa*. Bootstraps > 50 % are indicated on the left side of the  
1180 nodes and depicted with \* when >99%. For sake of clarity, terminal branches are collapsed  
1181 according to the results of the DNA-based species delimitation (ABGD, mPTP, GMYC)  
1182 (complete tree given in Supplementary figure S6). Congruence across DNA-based species  
1183 delimitation methods and with nuDNA networks (absence of allele sharing) is illustrated by  
1184 the use of colored columns on the right. Absence of available data for a given lineage is  
1185 indicated with “NA”. (B) Maps of northern South America showing the distribution of the  
1186 sampled material color-coded as in the ML tree according to the species delimitation. (C)  
1187 Median Joining networks based on four loci (NCX1 being divided in two, because parts of the  
1188 individual sequences mostly overlapped either on 3’ or the 5’ ends) with corresponding colour  
1189 code.

1190

1191 **Fig. 2.** Maximum clade credibility chronogram inferred in BEAST 2 based on (A)  
1192 mitogenomic and (B) nuDNA, and (C) ancestral areas for *Pipa* inferred in BioGeoBEARS  
1193 under the DEC model (results of the DEC+J model are available in Supplementary table S8).  
1194 Nodes with maximum posterior probability are indicated with an asterisk under the branches.  
1195 Calibrated nodes are indicated with a red circle. Node bars indicate the 95 % highest posterior  
1196 distribution of node dates. Colored circles on the tips of the tree indicate the geographical  
1197 distribution of sampled OTUs. Pie charts on nodes show the proportion of most likely

1198 ancestral areas. Colors of node pie charts correspond to the geographic areas shown in the  
1199 map. Changes in teeth, body size and larval development states are indicated with red bars.

1200

1201 **Fig. 3.** Comparison among posterior distributions, depicted as mustache plots, of the rates  
1202 across the four mtDNA partitions either when mtDNA is analysed with calibrations dating  
1203 back to the Cretaceous or when a Neogene calibration for the *Pipa* crown age is added (from  
1204 nuDNA results). “Pipa” includes both “crown” and “stem”. Comparative rates for two groups  
1205 of Neobatrachia (*Allobates* and *Boana* are also depicted for comparative purposes).

1206

Figure 1

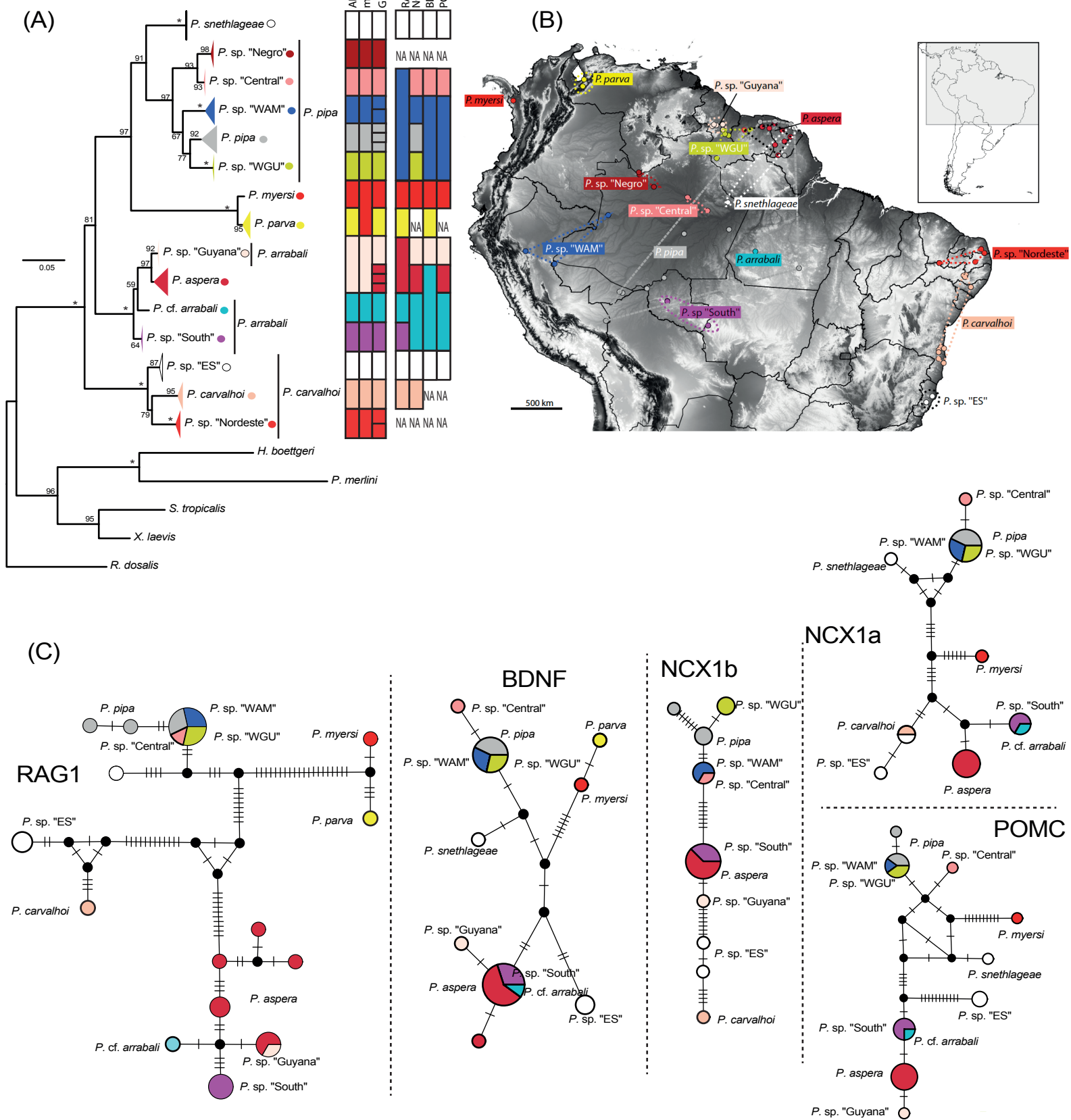
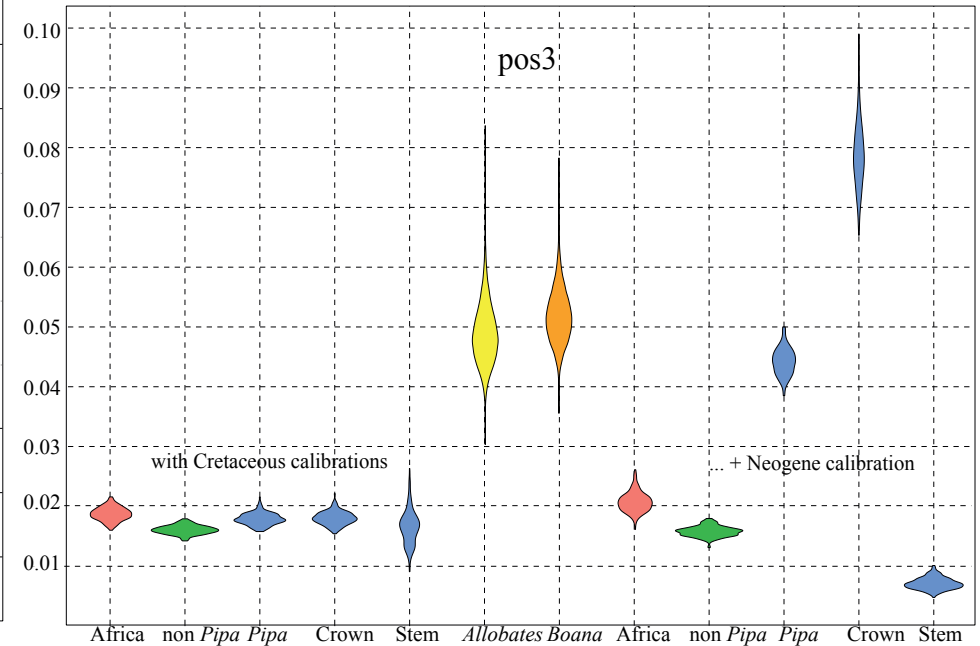
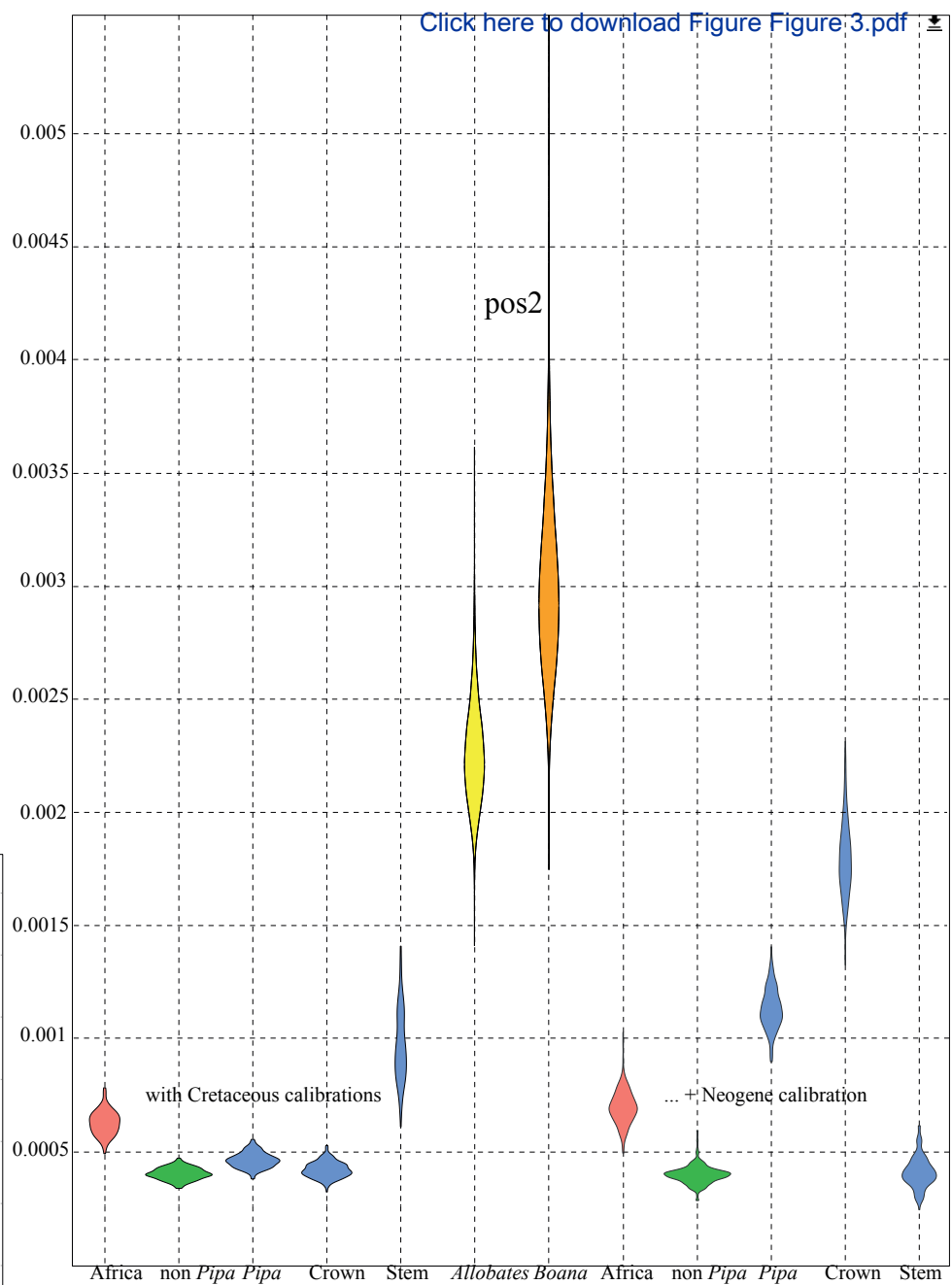
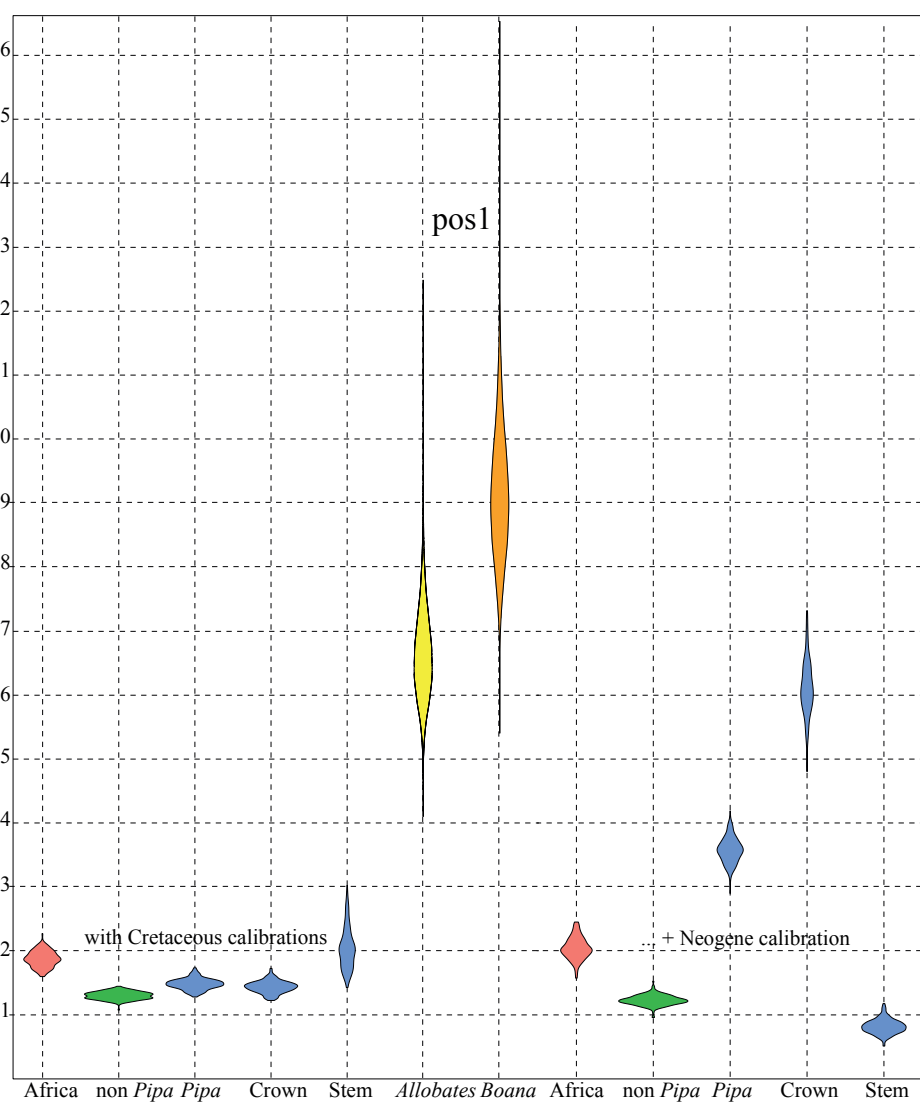
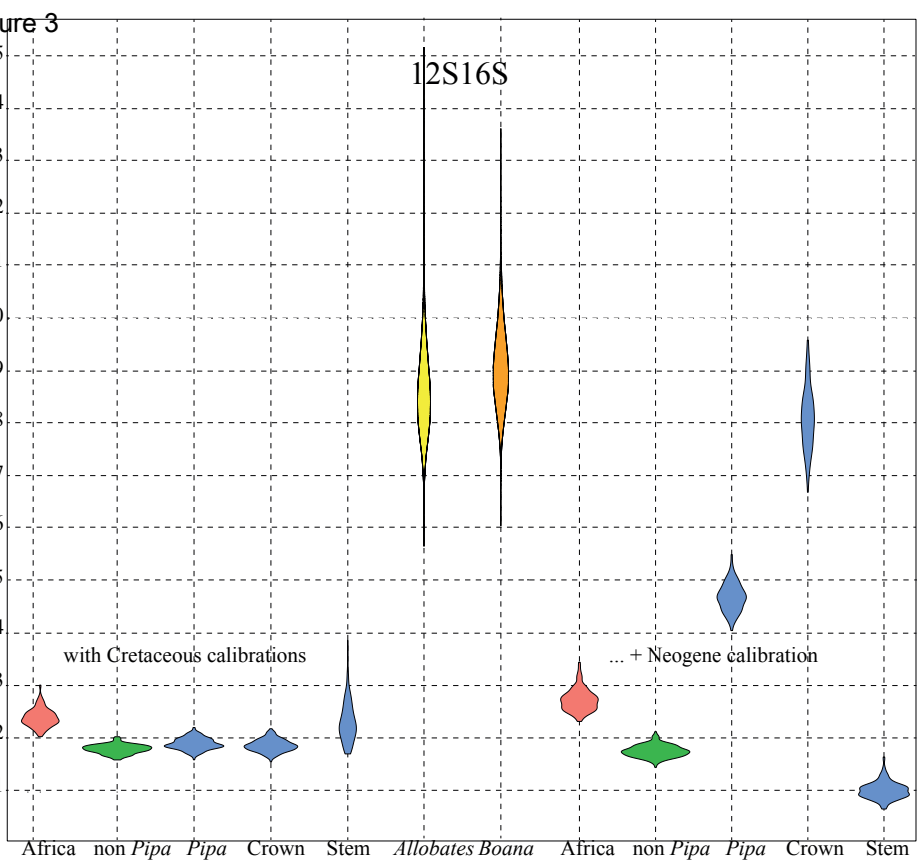




Figure 3

[Click here to download Figure Figure 3.pdf](#)



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



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