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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, Irissari 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  across 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+ Γ 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+ Γ 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”) were
220 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 acceptance; 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 3rd 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 (ω). The software PAML v.3.15
383 (Yang, 1997) was used to estimate the likelihood and the ω 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 ω for the whole tree, and they were fixed when
387 all other parameters were estimated under alternative models. The null model had a single ω
388 value for all branches, and it was compared against four alternatives, which allowed a second
389 ω 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 ω constant across the whole tree (single- ω model) with a “three- ω
465 model” assuming independent ω 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 ω value estimated with the
467 single- ω 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- ω model, for all individual mt genes (except *atp8* but consists of a short
471 159 bp locus), ω 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 ω estimate for stem *Pipa*
473 was also about twice higher than the overall rate estimated with the single- ω model (Table 1).
474 The three- ω model significantly outperformed ($\Delta AIC < 2$) the single- ω model for seven genes
475 (*cob*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*), and the single- ω model significantly
476 outperformed the three- ω model for two genes (*atp8* and *cox1*), as shown in Table 1. When all
477 mitochondrial genes were considered jointly, the three- ω model was favoured
478 overwhelmingly ($\Delta AIC = 124$, 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- ω model never significantly outperformed ($\Delta AIC < 2$) the single- ω model, and the
481 single- ω 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 ω 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) variation of ω 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 Irissari 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 km²; 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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788 **References**

- 789 Acosta-Galvis, A.R., Lasso, C.A., Morales-Betancourt, M.A., 2016. Ranas del género *Pipa*
790 (Anura: Pipidae) de la Orinoquia colombiana: nuevos registros y comentarios sobre su
791 taxonomía, distribución e historia natural. *Biota Colomb.* 17, 105–116.
- 792 Albert, J.S., Val, P., Hoorn, C., 2018. The changing course of the Amazon River in the
793 Neogene: center stage for Neotropical diversification. *Neotrop. Ichthyol.* 16(3),
794 e180033.
- 795 Aleixo, A., de Fátima Rossetti, D., 2007. Avian gene trees, landscape evolution, and geology:
796 towards a modern synthesis of Amazonian historical biogeography?. *J. Ornithol.*
797 148(2), 443–453.
- 798 Anisimova, M., Bielawski, J.P., Yang, Z., 2001. Accuracy and power of the likelihood ratio
799 test in detecting adaptive molecular evolution. *Mol. Biol. Evol.* 18, 1585–1592.
- 800 Antoine, P.O., Yans, J., Castillo, A.A., Stutz, N., Abello, M.A., Adnet, S., Custódio, M.A.,
801 Benites-Palomino, A., Billet, G., Boivin, M., Herrera, F., 2021. Biotic community and

802 landscape changes around the Eocene–Oligocene transition at Shapaja, Peruvian
803 Amazonia: Regional or global drivers? [Glob. Planet. Change](#) 202, p.103512.

804 Antonelli, A., Zizka, A., Carvalho, F.A., Scharn, R., Bacon, C.D., Silvestro, D., Condamine,
805 F.L., 2018. Amazonia is the primary source of Neotropical biodiversity. *PNAS*
806 115(23), 6034–6039.

807 Báez, A.M., Scanferla, C.A., Agnolin, F.L., Cenizo, M., Reyes, M.D.L., 2008. Pipid frog
808 from the Pleistocene of the Pampas of southern South America. *J. Vertebr. Paleontol.*
809 28(4), 1195–1198.

810 Bandelt, H., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific
811 phylogenies. *Mol. Biol. Evol.* 16(1), 37–48.

812 Batalha-Filho, H., Fjeldså, J., Fabre, P.H., Miyaki, C.Y., 2013. Connections between the
813 Atlantic and the Amazonian forest avifaunas represent distinct historical events. *J.*
814 *Ornithol.* 154(1), 41–50.

815 Beheregaray, L.B., Cooke, G.M., Chao, N.L., Landguth, E.L., 2015. Ecological speciation in
816 the tropics: insights from comparative genetic studies in Amazonia. *Front. Genet.* 5,
817 477.

818 Bessa-Silva, A., Vallinoto, M., Sampaio, I., Flores-Villela, O.A., Smith, E.N., Sequeira, F.,
819 2020. The roles of vicariance and dispersal in the differentiation of two species of the
820 *Rhinella marina* species complex. *Mol. Phylogenet. Evol.* 145, 106723.

821 Bewick, A.J., Chain, F.J., Heled, J., Evans, B.J., 2012. The pipid root. *Syst. Biol.* 61(6), 913–
822 926.

823 Bicudo, T.C., Sacek, V., de Almeida, R.P., Bates, J.M., Ribas, C.C., 2019. Andean tectonics
824 and Mantle Dynamics as a Pervasive Influence on Amazonian ecosystem. *Sci. Rep.*
825 9(1), 1–11.

826 Blanco-Torres, A., Báez, L., Patiño-Flores, E., Renjifo, J.M., 2013. Herpetofauna del valle
827 medio del río Ranchería, La Guajira, Colombia. *Rev. Biodivers. Neotrop.* 3(2), 113–
828 122.

829 Böhme, M., 2003. The Miocene climatic optimum: evidence from ectothermic vertebrates of
830 Central Europe. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 195, 389–401.

831 Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C-H., Xie, D., Suchard, MA.,
832 Rambaut, A., & Drummond, A.J., 2014. BEAST 2: A Software Platform for Bayesian
833 Evolutionary Analysis. *PLoS Comput. Biol.* 10(4), e1003537.

834 Burki, F., Roger, A.J., Brown, M.W., Simpson, A.G., 2020. The new tree of eukaryotes.
835 *Trends Ecol Evol.* 35(1), 43–55.

836 Buschiazzo, E., Ritland, C, Bohlmann, J., Ritland, K., 2012. Slow but not low: genomic
837 comparisons reveal slower evolutionary rate and higher dN/dS in conifers compared to
838 angiosperms. *BMC Evol. Biol.* 12, 8.

839 Caminer, M.A., Ron, S.R., 2020. Systematics of the *Boana semilineata* species group (Anura:
840 Hylidae), with a description of two new species from Amazonian Ecuador. *Zool. J.*
841 *Linnean Soc.* 190(1), 149–180.

842 Cannarozzi, G. M., Schneider, A., 2012). *Codon evolution: mechanisms and models.* Oxford
843 University Press.

844 Cannatella, D., 2015. *Xenopus* in space and time: fossils, node calibrations, tip-dating, and
845 paleobiogeography. *Cytogenet. Genome Res.* 145(3–4), 283–301.

846 Cannatella, D.C., Trueb, L., 1988. Evolution of pipoid frogs: intergeneric relationships of the
847 aquatic frog family Pipidae (Anura). *Zool. J. Linnean Soc.* 94(1), 1–38.

848 Carvalho, T.R., Moraes, L.J., Lima, A.P., Fouquet, A., Peloso, P.L., Pavan, D., Drummond,
849 L., Rodrigues, M.T., Giaretta, A., Gordo, M., Neckel-Oliveira, S., Haddad, C.F., 2021.
850 Systematics and historical biogeography of Neotropical foam-nesting frogs of the

851 *Adenomera heyeri* clade (Leptodactylidae), with the description of six new
852 Amazonian species. Zool. J. Linnean Soc. 191(2), 395–433.

853 Castellana, S., Vicario, S., Saccone, C., 2011. Evolutionary patterns of the mitochondrial
854 genome in Metazoa: exploring the role of mutation and selection in mitochondrial
855 protein coding genes. Genome Biol. Evol. 3, 1067–1079.

856 Castroviejo- Fisher, S., Guayasamin, J.M., Gonzalez- Voyer, A., Vilà, C., 2014. Neotropical
857 diversification seen through glassfrogs. J. Biogeogr. 41(1), 66–80.

858 Cooke, G.M., Landguth, E.L., Beheregaray, L.B., 2014. Riverscape genetics identifies
859 replicated ecological divergence across an Amazonian ecotone. Evolution 68(7),
860 1947–1960.

861 Cracraft, J., Ribas, C.C., d’Horta, F.M., Bates, J., Almeida, R.P., Aleixo, A., Boubli, J.P.,
862 Campbell, K.E., Cruz, F.W., Ferreira, M. and Fritz, S.C., Grohmann, C.H., Latrubesse,
863 E.M., Lohmann, L.G., Musher, L.J., Nogueira, A., Sawakuchi, A.O., Baker, P., 2020.
864 The origin and evolution of Amazonian species diversity, in: Rull, V., Carnaval A.C.
865 (Eds.), Neotropical diversification: patterns and processes. Springer, Cham, pp. 225–
866 244.

867 Cundall, D., Fernandez, E., Irish, F., 2017. The suction mechanism of the pipid frog, *Pipa*
868 *pipa* (Linnaeus, 1758). J. Morphol. 278(9), 1229–1240.

869 Cuvier, G.L.C.F.D., 1831. The animal kingdom arranged in conformity with its organization.
870 Translated from the French, with notes and additions by H. M’Murtrie. Volume 2. G.
871 & C. & H. Carvill, New York.

872 da Fonte, L.F.M., Latombe, G., Gordo, M., Menin, M., de Almeida, A.P., Hui, C., Lötters, S.,
873 2021. Amphibian diversity in the Amazonian floating meadows: a Hanski core-
874 satellite species system. Ecography 44, 1–16.

875 Dal Vechio, F., Prates, I., Graziotin, F.G., Zaher, H., Rodrigues, M.T., 2018.
876 Phylogeography and historical demography of the arboreal pit viper *Bothrops*
877 *bilineatus* (Serpentes, Crotalinae) reveal multiple connections between Amazonian
878 and Atlantic rain forests. J. Biogeogr. 45(10), 2415–2426.

879 Dantas, S.P., Tavares, H.D., Pascoal, W., Saviato, M.J., Ávila, R.W., Vasconcelos, T.S., Oda,
880 F.H., 2019. New distribution records from the Brazilian Cerrado and species
881 distribution modelling of *Boana crepitans*, *Lithobates palmipes*, *Pipa pipa*, and
882 *Micrurus h. hemprichii*. Biodiversity 20(4), 149–160.

883 Delfino, M., Sánchez-Villagra, M.R., 2018. A Late Miocene Pipine Frog from the Urumaco
884 Formation, Venezuela. Ameghiniana 55(2), 210–214.

885 Delsuc, F., Brinkmann, H., Philippe, H., 2005. Phylogenomics and the reconstruction of the
886 tree of life. Nat. Rev. Genet. 6(5), 361–375.

887 Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., (2012) Bayesian phylogenetics with
888 BEAUti and the BEAST 1.7 Mol. Biol. Evol. 29, 1969–1973.

889 Donoghue, P.C., Yang, Z., 2016. The evolution of methods for establishing evolutionary
890 timescales. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 371(1699), p.20160020.

891 Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling
892 trees. BMC Evol. Biol. 7, 214.

893 Evans, B.J., Gansauge, M.T., Stanley, E.L., Furman, B.L., Cauret, C.M., Ofori-Boateng, C.,
894 Gvoždík, V., Streicher, J.W., Greenbaum, E., Tinsley, R.C., Meyer, M., 2019.
895 *Xenopus fraseri*: Mr. Fraser, where did your frog come from?. PloS one 14(9),
896 p.e0220892.

897 Ezard, T., Fujisawa, T., Barraclough, T.G., 2009. Splits: species' limits by threshold statistics.
898 R package version 1, 29.

899 Feng, Y.J., Blackburn, D.C., Liang, D., Hillis, D.M., Wake, D.B., Cannatella, D.C., Zhang,
900 P., 2017. Phylogenomics reveals rapid, simultaneous diversification of three major
901 clades of Gondwanan frogs at the Cretaceous–Paleogene boundary. PNAS 114(29),
902 E5864–E5870.

903 Fernandez, E., Irish, F., Cundall, D., 2017. How a frog, *Pipa pipa*, succeeds or fails in
904 catching fish. Copeia 105(1), 108–119.

905 Fouquet, A., Loebmann, D., Castroviejo-Fisher, S., Padial, J.M., Orrico, V.G., Lyra, M.L.,
906 Roberto, I.J., Kok, P.J., Haddad, C.F., Rodrigues, M.T., 2012a. From Amazonia to the
907 Atlantic forest: Molecular phylogeny of Phyzelaphryninae frogs reveals unexpected
908 diversity and a striking biogeographic pattern emphasizing conservation challenges.
909 Mol. Phylogenet. Evol. 65(2), 547–561.

910 Fouquet, A., Recoder, R., Teixeira Jr, M., Cassimiro, J., Amaro, R. C., Camacho, A.,
911 Damasceno, R., Carnaval, A.C., Moritz, C., Rodrigues, M.T., 2012b. Molecular
912 phylogeny and morphometric analyses reveal deep divergence between Amazonia and
913 Atlantic Forest species of *Dendrophryniscus*. Mol. Phylogenet. Evol. 62(3), 826–838.

914 Fouquet, A., Blotto, B.L., Maronna, M.M., Verdade, V.K., Juncá, F.A., de Sá, R., Rodrigues,
915 M.T., 2013. Unexpected phylogenetic positions of the genera *Rupirana* and
916 *Crossodactylodes* reveal insights into the biogeography and reproductive evolution of
917 leptodactylid frogs. Mol. Phylogenet. Evol. 67(2), 445–457.

918 Fouquet, A., Cassini, C.S., Haddad, C.F.B., Pech, N., Rodrigues, M.T., 2014. Species
919 delimitation, patterns of diversification and historical biogeography of the Neotropical
920 frog genus *Adenomera* (Anura, Leptodactylidae). J. Biogeogr. 41(5), 855–870.

921 Fouquet A., Leblanc K., Framit M., Réjaud A., Rodrigues M.T., Castroviejo-Fisher S., Peloso
922 P.L.V., Prates I., Manzi S., Suescun U., Baroni S., Moraes L.J.C.L., Recoder R., de
923 Souza S.M., Dal Vecchio F., Camacho A., Guellere J.M., Rojas-Runjaic F.J.M.,

924 Gagliardi-Urrutia G., de Carvalho V.T., Gordo M., Menin M., Kok P.J.R., Hrbek T.,
925 Werneck F.P., Crawford A.J., Ron S.R., Mueses-Cisneros J.J., Rojas Zamora R.R.,
926 Pavan D., Simões P.I., Ernst R., Fabre A.C., 2021a. Species diversity and
927 biogeography of an ancient frog clade from the Guiana Shield (Anura: Microhylidae:
928 *Adelastes*, *Otophryne*, *Synapturanus*) exhibiting spectacular phenotypic
929 diversification. Biol. J. Linn. Soc. 132(2), 233–256.

930 Fouquet, A., Marinho, P., Réjaud, A., Carvalho, T. R., Caminer, M. A., Jansen, M., Rainha,
931 R.N., Rodrigues, M.T., Werneck, F.P., Lima, A.P., Hrbek, T., Giaretta, A.A.,
932 Venegas, P.J., Chavez, G. Ron, S., 2021b. Systematics and biogeography of the *Boana*
933 *albopunctata* species group (Anura, Hylidae), with the description of two new species
934 from Amazonia. Syst. Biodivers. 19(4), 375–399.

935 Funk, W.C., Caminer, M., Ron, S.R., 2012. High levels of cryptic species diversity uncovered
936 in Amazonian frogs. Proc. R. Soc. B: Biol. Sci. 279(1734), 1806–1814.

937 Galvis, P.A., Mejía-Tobón, A., Rueda-Almonacid, J.V., 2011. Fauna silvestre de la reserva
938 forestal protectora, Montes de Oca, La Guajira, Colombia. Corpoguajira, Riohacha,
939 Colombia. pp. 822.

940 Garda, A.A., Biavati, G.M., Costa, G.C., 2006. Sexual dimorphism, female fertility, and diet
941 of *Pipa arrabali* (Anura, Pipidae) in Serra do Cachimbo, Pará, Brazil. South American
942 J. Herpetol. 1(1), 20–24.

943 Gazoni, T., Lyra, M.L., Ron, S.R., Strüssmann, C., Baldo, D., Narimatsu, H., Pansonato, A.,
944 Schneider, R.G., Giaretta, A.A., Haddad, C.F., Parise-Maltempi, P.P., 2021. Revisiting
945 the systematics of the *Leptodactylus melanonotus* group (Anura: Leptodactylidae):
946 [Redescription of *L. petersii* and revalidation of its junior synonyms](#). Zool. Anz. 290,
947 117–134.

948 Gehara, M., Crawford, A.J., Orrico, V.G., Rodriguez, A., Loetters, S., Fouquet, A.,
949 Barrientos, L.S., Brusquetti, F., De la Riva, I., Ernst, R., Urrutia, G.G., 2014. High
950 levels of diversity uncovered in a widespread nominal taxon: continental
951 phylogeography of the Neotropical tree frog *Dendropsophus minutus*. PloS one 9(9),
952 p.e103958.

953 Gissi, C., San Mauro, D., Pesole, G., Zardoya, R., 2006. Mitochondrial phylogeny of Anura
954 (Amphibia): a case study of congruent phylogenetic reconstruction using amino acid
955 and nucleotide characters. Gene 366,228–237.

956 Gómez, R.O., 2016. A new pipid frog from the Upper Cretaceous of Patagonia and early
957 evolution of crown-group Pipidae. Cretac. Res. 62, 52–64.

958 Gómez, R.O., Pérez-Ben, C.M., 2019. Fossils reveal long-term continuous and parallel
959 innovation in the sacro-caudo-pelvic complex of the highly aquatic pipid frogs. Front.
960 Earth Sci. 7, 56.

961 Greven, H., 2011. Maternal adaptations to reproductive modes in amphibians, in: Norris,
962 D.O., Lopez, E.M. (Eds.), *Hormones and reproduction of vertebrates*, Vol. 2.
963 Academic Press pp. 117–141.

964 Hedtke, S.M., Morgan, M.J., Cannatella, D.C. and Hillis, D.M., 2013. Targeted enrichment:
965 maximizing orthologous gene comparisons across deep evolutionary time. PloS one
966 8(7), p.e67908.

967 Hemmi, K., Kakehashi, R., Kambayashi, C., Du Preez, L., Minter, L., Furuno, N.,
968 Kurabayashi, A., 2020. Exceptional enlargement of the mitochondrial genome results
969 from distinct causes in different rain frogs (Anura: Brevicipitidae: *Breviceps*). Int. J.
970 Genomics, 2020.

971 Hime, P.M., Lemmon, A.R., Lemmon, E.C.M., Prendini, E., Brown, J.M., Thomson, R.C.,
972 Kratovil, J.D., Noonan, B.P., Pyron, R.A., Peloso, P.L., Kortyna, M.L.  1.

973 Phylogenomics reveals ancient gene tree discordance in the amphibian tree of life.
974 Syst. Biol. 70(1), 49–66.

975 Hoon, C., Wesselingh, F.P., Ter Steege, H., Bermudez, M.A., Mora, A., Sevink, J., Sanchez-
976 Meseguer, A., Anderson, C.L., Figueiredo, J.P., Riff, D., Negri, F.R., Hooghiemstra,
977 H., Lundberg, J., Stadler, T., Sarkinen, T.S., Antonelli, A., 2010. Amazonia through
978 time: Andean uplift, climate change, landscape evolution, and biodiversity. Science
979 330(6006), 927–931.

980 Hoon, C., Bogotá-A, G. R., - 41 -Romero-Baez, M., Lammertsma, E. I., Flantua, S. G.,
981 Dantas, E. L., Dino, R., do Carmo, D.A., Chemale Jr, F., 2017. The Amazon at sea:
982 Onset and stages of the Amazon River from a marine record, with special reference to
983 Neogene plant turnover in the drainage basin. Glob. Planet. Change 153, 51–65.

984 Hubert, N., Duponchelle, F., Nunez, J., Garcia- Davila, C., Paugy, D., Renno, J.F., 2007.
985 Phylogeography of the piranha genera *Serrasalmus* and *Pygocentrus*: implications for
986 the diversification of the Neotropical ichthyofauna. Mol. Ecol. 16(10), 2115–2136.

987 Igawa, T., Kurabayashi, A., Usuki, C., Fujii, T., Sumida, M., 2008. Complete mitochondrial
988 genomes of three neobatrachian anurans: a case study of divergence time estimation
989 using different data and calibration settings. Gene 407(1–2), 116–129.

990 Irisarri, I., Baurain, D., Brinkmann, H., Delsuc, F., Sire, J.Y., Kupfer, A., Petersen, J., Jarek,
991 M., Meyer, A., Vences, M., Philippe, H., 2017. Phylotranscriptomic consolidation of
992 the jawed vertebrate timetree. Nat. Ecol. Evol. 1(9), 1370–1378.

993 Irisarri, I., San Mauro, D., Abascal, F., Ohler, A., Vences, M., Zardoya, R., 2012. The origin
994 of modern frogs (Neobatrachia) was accompanied by acceleration in mitochondrial
995 and nuclear substitution rates. BMC Genomics 13(1), 1–19.

996 Irisarri, I., Vences, M., San Mauro, D., Glaw, F., Zardoya, R., 2011. Reversal to air-driven
997 sound production revealed by a molecular phylogeny of tongueless frogs, family
998 Pipidae. *BMC Evol. Biol.* 11(1), 1–10.

999 Kakehashi, R., Kurabayashi, A., 2021. Patterns of natural selection on mitochondrial protein-
1000 coding genes in lungless salamanders: relaxed purifying selection and presence of
1001 positively selected codon sites in the family Plethodontidae. *Int. J. Genomics*, 2021.

1002 Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A., Flouri, T., 2017.
1003 Multi-rate Poisson tree processes for single-locus species delimitation under
1004 maximum likelihood and Markov chain Monte Carlo. *Bioinformatics* 33, 1630–1638.

1005 Katoh, K., Rozewicki, J., Yamada, K.D., 2017. MAFFT online service: multiple sequence
1006 alignment, interactive sequence choice and visualization. *Brief. Bioinformatics* 20,
1007 1160–1166.

1008 Klaus, K.V., Matzke, N.J., 2020. Statistical comparison of trait-dependent biogeographical
1009 models indicates that podocarpaceae dispersal is influenced by both seed cone traits
1010 and geographical distance. *Syst. Biol.* 69, 61–75.

1011 Landis, M.J., Matzke, N.J., Moore, B.R., Huelsenbeck, J.P., 2013. Bayesian analysis of
1012 biogeography when the number of areas is large. *Syst. Biol.* 62, 789–804.

1013 Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., Calcott, B., 2017. PartitionFinder 2:
1014 new methods for selecting partitioned models of evolution for molecular and
1015 morphological phylogenetic analyses. *Mole. Biol. Evol.* 34, 772–773.

1016 Ledo, R.M.D., Colli, G.R., 2017. The historical connections between the Amazon and the
1017 Atlantic Forest revisited. *J. Biogeogr.* 44(11), 2551–2563.

1018 Leigh, J.W., Bryant, D., 2015. PopART: Full-feature software for haplotype network
1019 construction. *Methods Ecol. Evol.* 6(9), 1110–1116.

1020 Lescure, J., Marty, C., 2000. Atlas des amphibiens de Guyane. Collection patrimoines
1021 naturels. MNHN, Paris.

1022 Lima, L.R., Bruschi, D.P., Do Nascimento, F.A.C., Scherrer De Araujo, P.V., Costa, L.P.,
1023 Thomé, M.T.C., Garda, A.A., Zattera, M.L., Mott, T., 2020. Below the waterline:
1024 cryptic diversity of aquatic pipid frogs (*Pipa carvalhoi*) unveiled through an
1025 integrative taxonomy approach. Syst. Biodivers. 18(8), 771–783.

1026 Linnaeus, C., 1758. Systema naturae (Vol. 1, No. part 1). Laurentii Salvii, Stockholm, p. 532.

1027 Silva, S.M., Peterson, A.T., Carneiro, L., Burlamaqui, T.C.T., Ribas, C.C., Sousa-Neves, T.,
1028 Miranda, L.S., Fernandes, A.M., d'Horta, F.M., Araújo-Silva, L.E., Batista, R., 2019.
1029 A dynamic continental moisture gradient drove Amazonian bird diversification. Sci.
1030 Adv. 5(7), p.eaat5752.

1031 Massemin, D., Bordage, D., Kuntz, K., 2007. Report on the occurrence of *Pipa snethlageae*
1032 (Anura: Pipidae) in French Guiana, with notes on its natural history. Salamandra 43,
1033 139–147.

1034 Matzke, N.J., 2013. BioGeoBEARS: biogeography with Bayesian (and likelihood)
1035 evolutionary analysis in R scripts. R Package, Version 0.2, 1, 2013.

1036 Mendoza, Á.M., Ospina, O.E., Cárdenas-Henao, H., García-R, J.C., 2015. A likelihood
1037 inference of historical biogeography in the world's most diverse terrestrial vertebrate
1038 genus: Diversification of direct-developing frogs (Craugastoridae: *Pristimantis*) across
1039 the Neotropics. Mol. Phylogenet. Evol. 85, 50–58.

1040 Mezzasalma, M., Glaw, F., Odierna, G., Petraccioli, A., Guarino, F.M., 2015. Karyological
1041 analyses of *Pseudhymenochirus merlini* and *Hymenochirus boettgeri* provide new
1042 insights into the chromosome evolution in the anuran family Pipidae. Zool. Anz. 258,
1043 47–53.

- 1044 Molak, M., Ho, S.Y., 2015. Prolonged decay of molecular rate estimates for metazoan
1045 mitochondrial DNA. PeerJ 3, e821.
- 1046 Molina-Zuluaga, C., Cano, E., Restrepo, A., Rada, M., Daza, J.M., 2017. Out of Amazonia:
1047 the unexpected trans-Andean distribution of *Cochranella resplendens* (Lynch and
1048 Duellman, 1978)(Anura: Centrolenidae). Zootaxa 4238(2), 268–274.
- 1049 Monaghan, M.T., Wild, R., Elliot, M., Fujisawa, T., Balke, M., Inward, D.J., Lees, D.C.,
1050 Ranaivosolo, R., Eggleton, P., Barraclough, T.G., Vogler, A.P., 2009. Accelerated
1051 species inventory on Madagascar using coalescent-based models of species
1052 delineation. Syst. Biol. 58, 298–311.
- 1053 Mora, A., Baby, P., Roddaz, M., Parra, M., Brusset, S., Hermoza, W., Espurt, N., 2010.
1054 Tectonic history of the Andes and sub-Andean zones: implications for the
1055 development of the Amazon drainage basin, in: Hoorn, C. Wesselingh, F. (Eds.),
1056 Amazonia, landscape and species evolution: a look into the past. Wiley-Blackwell, pp.
1057 38–60.
- 1058 Motta, J., Menin, M., Almeida, A. P., Hrbek, T., Farias, I.P., 2018. When the unknown lives
1059 next door: a study of central Amazonian anurofauna. Zootaxa 4438(1), 79–104.
- 1060 Oliveira, J.A., Farias, I.P., Costa, G C., Werneck, F.P., 2019. Model- based riverscape
1061 genetics: disentangling the roles of local and connectivity factors in shaping spatial
1062 genetic patterns of two Amazonian turtles with different dispersal abilities. Evol. Ecol.
1063 33, 273–298.
- 1064 Padial, J.M., Miralles, A., De la Riva, I., Vences, M., 2010. The integrative future of
1065 taxonomy. Front. Zool. 7(1), 1–14.
- 1066 Pellegrino, K.C., Rodrigues, M.T., Harris, D.J., Yonenaga-Yassuda, Y., Sites Jr, J.W., 2011.
1067 Molecular phylogeny, biogeography and insights into the origin of parthenogenesis in

1068 the Neotropical genus *Leposoma* (Squamata: Gymnophthalmidae): Ancient links
1069 between the Atlantic Forest and Amazonia. *Mol. Phylogenet. Evol.* 61(2), 446–459.

1070 Pinheiro, L.C., da Cunha Bitar, Y.O., Galatti, U., Neckel-Oliveira, S., dos Santos-Costa, M.
1071 C., 2012. Amphibians from southeastern state of Pará: Carajás Region, northern
1072 Brazil. *Check List* 8(4), 693–702.

1073 Pirani, R.M., Peloso, P.L., Prado, J.R., Polo, É.M., Knowles, L.L., Ron, S.R., Rodrigues,
1074 M.T., Sturaro, M.J., Werneck, F. P., 2020. Diversification history of clown tree frogs
1075 in neotropical rainforests (Anura, Hylidae, *Dendropsophus leucophyllatus* group).
1076 *Mol. Phylogenet. Evol.* 150, 106877.

1077 Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun,
1078 S., Sumlin, W.D., Vogler, A.P., 2006. Sequence-based species delimitation for the
1079 DNA taxonomy of undescribed insects. *Syst. Biol.* 55, 595–609.

1080 Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD, Automatic Barcode Gap
1081 Discovery for primary species delimitation. *Mol. Ecol.* 21, 1864–1877.

1082 Prates, I., Penna, A., Rodrigues, M.T., Carnaval, A.C., 2018. Local adaptation in mainland
1083 anole lizards: Integrating population history and genome–environment associations.
1084 *Ecol. Evol.* 8(23), 11932–11944.

1085 Rabb, G.B., Rabb, M.S., 1960. On the mating and egg-laying behavior of the Surinam toad,
1086 *Pipa pipa*. *Copeia* 1960(4), 271–276.

1087 Ree, R.H., Sanmartín, I., 2018. Conceptual and statistical problems with the DEC +J model of
1088 founder-event speciation and its comparison with DEC via model selection. *J.*
1089 *Biogeogr.* 45, 741–749.

1090 Ree, R.H., Smith, S.A., 2008. Maximum Likelihood inference of geographic range evolution
1091 by dispersal, local extinction, and cladogenesis. *Syst. Biol.* 57, 4–14.

1092 Réjaud, A., Rodrigues, M.T., Crawford, A.J., Castroviejo- Fisher, S., Jaramillo, A.F.,
1093 Chaparro, J.C., Glaw, F., Gagliardi- Urrutia, G., Moravec, J., De la Riva, I.J., Perez,
1094 P., 2020. Historical biogeography identifies a possible role of Miocene wetlands in the
1095 diversification of the Amazonian rocket frogs (Aromobatidae: *Allobates*). *J. Biogeogr.*
1096 47(11), 2472–2482.

1097 Rojas, R.R., Fouquet, A., Ron, S. R., Hernández-Ruz, E. J., Melo-Sampaio, P. R., Chaparro,
1098 J. C., Vogt, R.C., De Carvalho, V.T., Pinheiro, L.C., Avila, R.W., Farias, I., Gordo,
1099 M., Hrbek, T., 2018. A Pan-Amazonian species delimitation: high species diversity
1100 within the genus *Amazophrynella* (Anura: Bufonidae). *PeerJ* 6, e4941.

1101 Ronquist, F., 1997. Dispersal-vicariance analysis: a new approach to the quantification of
1102 historical biogeography. *Syst. Biol.* 46, 195–203.

1103 Ruokolainen, K., Moulatlet, G.M., Zuquim, G., Hoorn, C., Tuomisto, H., 2018. River network
1104 rearrangements in Amazonia shake biogeography and civil security. [Preprints](#).

1105 Santos, J.C., Coloma, L.A., Summers, K., Caldwell, J.P., Ree, R., Cannatella, D.C., 2009.
1106 Amazonian amphibian diversity is primarily derived from late Miocene Andean
1107 lineages. *PLoS Biol.* 7(3), e1000056.

1108 Silva, L.A.D., Carvalho, P.S., Pereira, E.A., Fadel, R.M., Dantas, S.P., Brandão, R.A.,
1109 Santana, D.J., 2020. Richness, diversity patterns, and taxonomic notes of amphibians
1110 from the Tocantins state. *Biota Neotrop.* 20(1).

1111 Smith, B.T., McCormack, J.E., Cuervo, A.M., Hickerson, M.J., Aleixo, A., Cadena, C.D.,
1112 Perez-Eman, J., Burney, C.W., Xie, X., Harvey, M.G., Faircloth, B.C., Glenn, T.C.,
1113 Derryberry, E.P., Prejean, J., Fields, S., Brumfield, R.T., 2014. The drivers of tropical
1114 speciation. *Nature* 515(7527), 406–409.

1115 Sokol, O.M., 1977. The free swimming *Pipa* larvae, with a review of pipid larvae and pipid
1116 phylogeny (Anura: Pipidae). *J. Morphol.* 154(3), 357–425.

- 1117 de Souza, L.S., Armbruster, J.W., Willink, P.W., 2020. Connectivity of neotropical river
1118 basins in the central Guiana Shield based on fish distributions. *Front. For. Glob.*
1119 *change* 3, 8.
- 1120 Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
1121 large phylogenies. *Bioinformatics* 30, 1312–1313.
- 1122 Steinhorsdottir, M., Coxall, H.K., De Boer, A.M., Huber, M., Barbolini, N., Bradshaw, C.D.,
1123 Burls, N.J., Feakins, S.J., Gasson, E., Henderiks, J., Holbourn, A.E., 2021. The
1124 Miocene: The future of the past. *Paleoceanogr. Paleoclimatology* 36(4),
1125 p.e2020PA004037.
- 1126 Streicher, J.W., Miller, E.C., Guerrero, P.C., Correa, C., Ortiz, J.C., Crawford, A.J., Pie,
1127 M.R., Wiens, J.J., 2018. Evaluating methods for phylogenomic analyses, and a new
1128 phylogeny for a major frog clade (Hyloidea) based on 2214 loci. *Mol. Phylogenet.*
1129 *Evol.* 119, 128–143.
- 1130 Thom, G., Xue, A. T., Sawakuchi, A. O., Ribas, C. C., Hickerson, M. J., Aleixo, A., Miyaki,
1131 C., 2020. Quaternary climate changes as speciation drivers in the Amazon floodplains.
1132 *Sci. Adv.* 6(11), eaax4718.
- 1133 Torsvik, T.H., Müller, R.D., Van der Voo, R., Steinberger, B., Gaina, C., 2008. Global plate
1134 motion frames: toward a unified model. *Rev. Geophys.* 46(3), 1–44.
- 1135 Trueb, L., 1984. Description of a new species of *Pipa* (Anura: Pipidae) from Panama.
1136 *Herpetologica* 40, 225–234.
- 1137 Trueb, L., Cannatella, D.C., 1986. Systematics, morphology, and phylogeny of genus *Pi*
1138 (Anura: Pipidae). *Herpetologica* 42, 412–449.
- 1139 Trueb, L., Maselein, D., 2001. The osteology and relationships of *Pipa*era (Amphibia:
1140 Anura: Pipidae), with notes on its natural history in French Guiana. *Amphibia-Reptilia*
1141 22(1), 33–54.

- 1142 Trueb, L., Ross, C.F., Smith, R., 2005. A new pipoid anuran from the Late Cretaceous of
1143 South Africa. *J. Vertebr. Paleontol.* 25(3), 533–547.
- 1144 Vacher, J.P., 2017. Diversification in the Guiana Shield as seen through frogs (Doctoral
1145 dissertation, Université de Toulouse, Université Toulouse III-Paul Sabatier).
- 1146 Vacher J.-P., Chave J., Ficetola F., Sommeria-Klein G., Tao S., Thébaud C., Blanc M.,
1147 Camacho A., Cassimiro J., Colston T.J., Dewynter M., Ernst R., Gaucher P., Gomes
1148 J.O., Jairam R., Kok P.J.R., Dias Lima J., Martinez Q., Marty C., Noonan B.P., Nunes
1149 P.M.S., Ouboter P., Recoder R., Rodrigues M.T., Snyder A., de Souza S.M., Fouquet
1150 A. 2020. Large scale DNA-based survey of Amazonian frogs suggest a vast
1151 underestimation of species richness and endemism. *J. Biogeogr.* 47, 1781–1791.
- 1152 Vargas-Ramírez, M., Caballero, S., Morales-Betancourt, M.A., Lasso, C.A., Amaya, L.,
1153 Martínez, J.G., Viana, M.D.N.S., Vogt, R.C., Farias, I.P., Hrbek, T., Campbell, P.D.,
1154 Fritz, U., 2020. Genomic analyses reveal two species of the matamata (Testudines:
1155 Chelidae: *Chelus* spp.) and clarify their phylogeography. *Mol. Phylogenet. Evol.* 148,
1156 106823.
- 1157 Wallace, A.R. 1854. On the monkeys of the Amazon. *Ann. Mag. Nat. Hist.* 14, 451–454.
- 1158 Vaz-Silva, W., de Andrade, T.A., 2009. Amphibia, Anura, Pipidae, *Pipa pipa*: Distribution
1159 extension, new state record and geographic distribution map. *Check List* 5(3), 507-
1160 509.
- 1161 Weygoldt, P., 1976. Beobachtungen zur biologie und ethologie von *Pipa (Hemipipa)*
1162 *carvalhoi* Miranda Ribeiro 1937 (Anura, Pipidae) 1. *Zeitschrift für Tierpsychologie*
1163 40(1), 80–99.
- 1164 Yang, Z., 1997. PAML: a program package for phylogenetic analysis by maximum
1165 likelihood. *Comput. Appl. Biosci.* 13, 555–556.
- 1166 Yang, Z., 2006. *Computational molecular evolution*. Oxford University Press, New York.

1167 Zhang, J., Miao, G., Hu, S., Sun, Q., Ding, H., Ji, Z., Guo, P., Yan, S., Wang, C., Kan, X.,
1168 Nie, L., 2021. Quantification and evolution of mitochondrial genome rearrangement in
1169 [Amphibians](#). *BMC Ecol. Evol.* 21(1), 1–14.

1170

1171

1172

1173 **Table 1**

1174 Results from the CodeML analyses for each locus and mitochondrial and nuclear loci
1175 combined. The highest ω 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

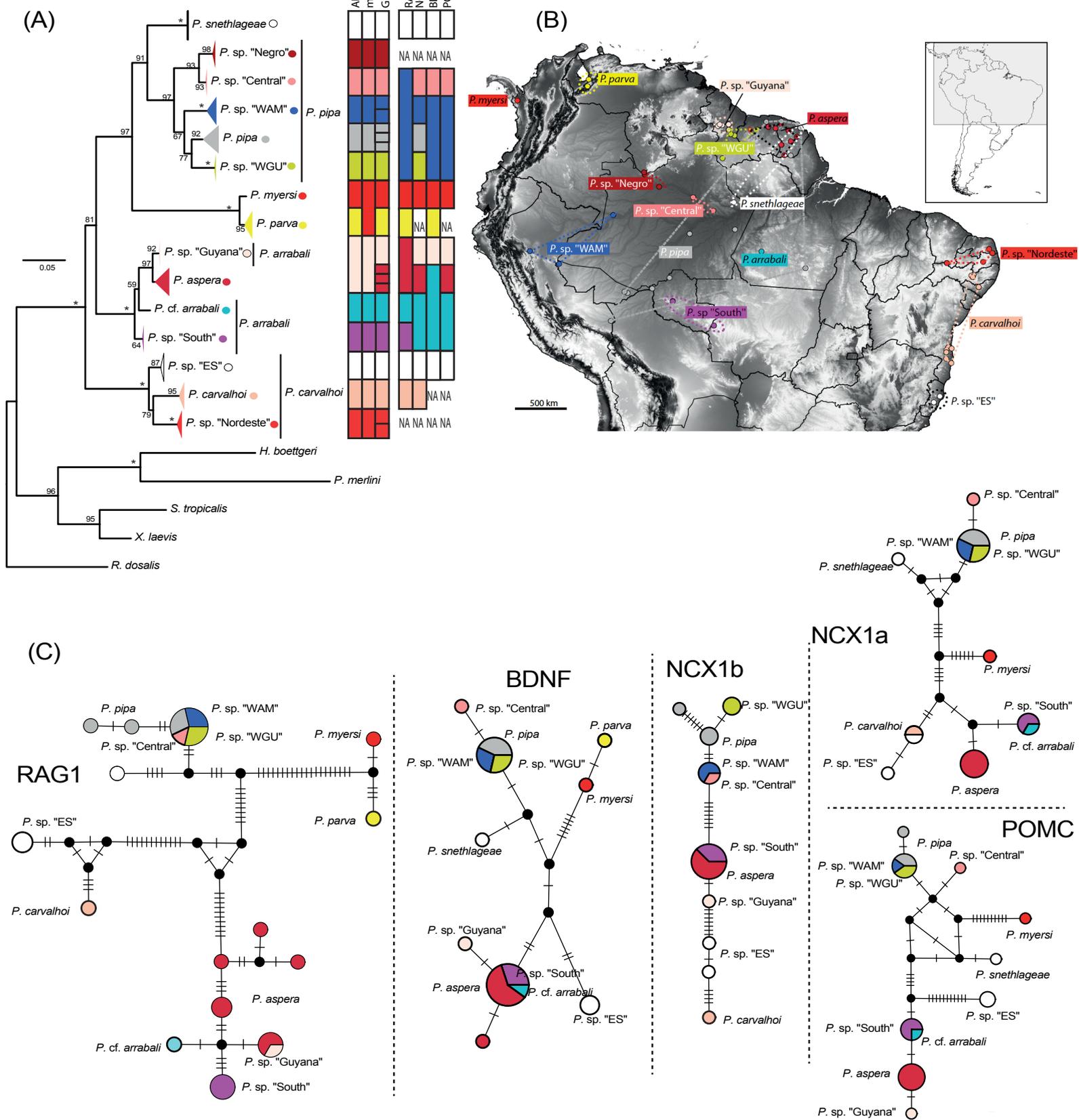


Figure 2

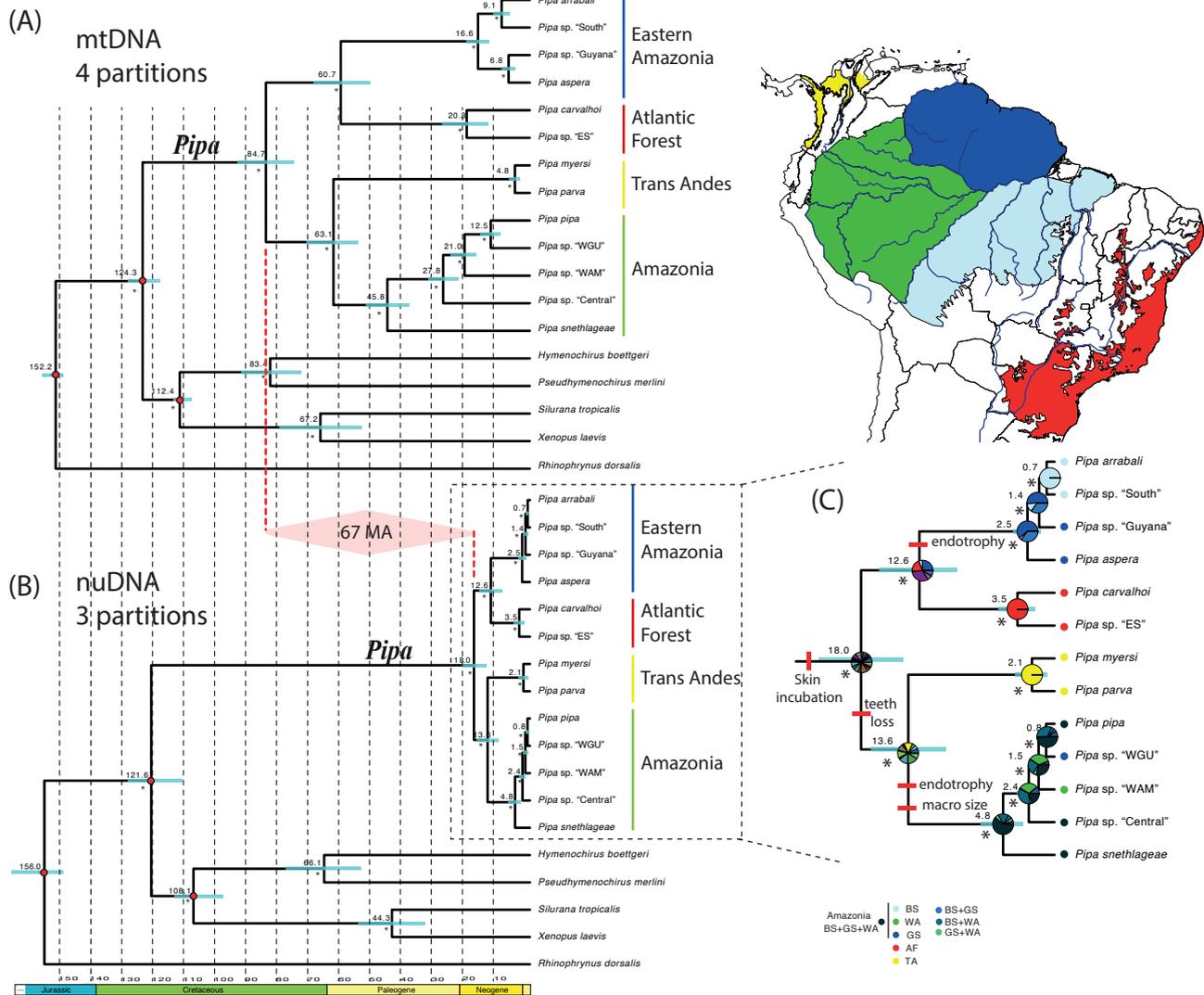
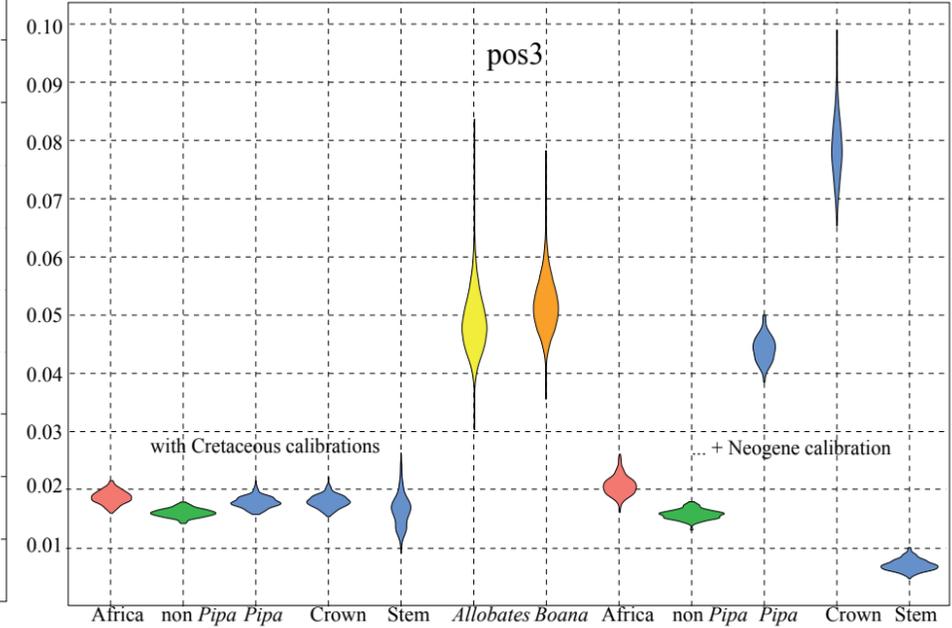
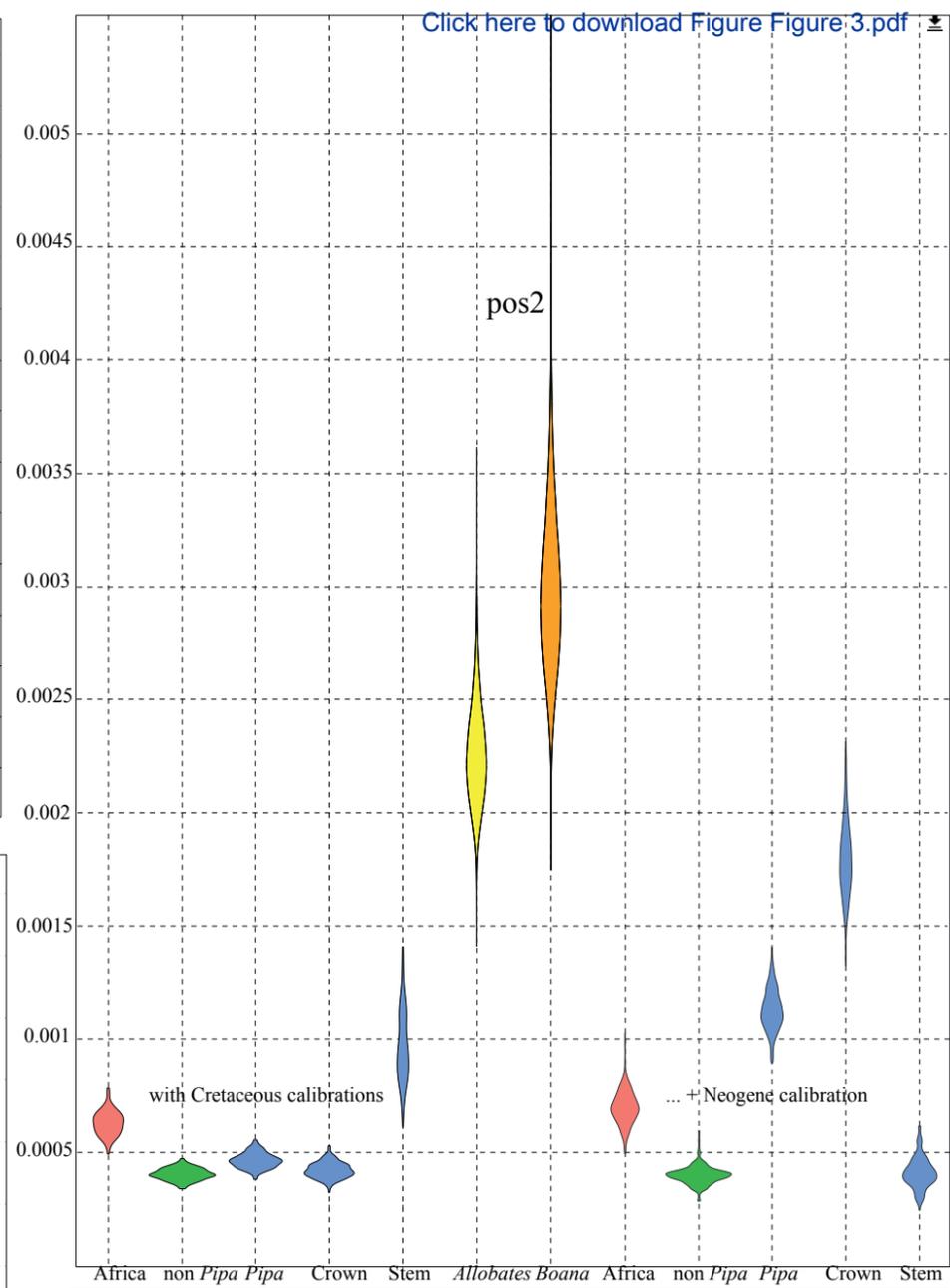
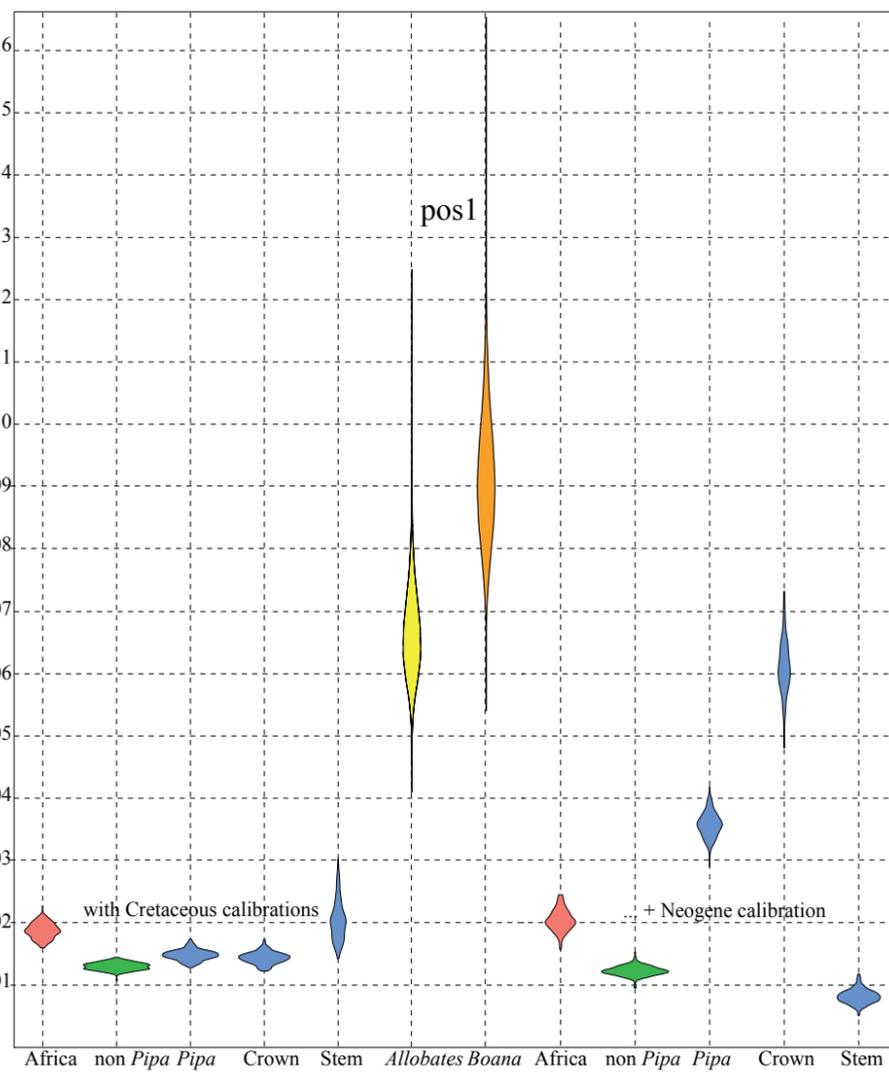
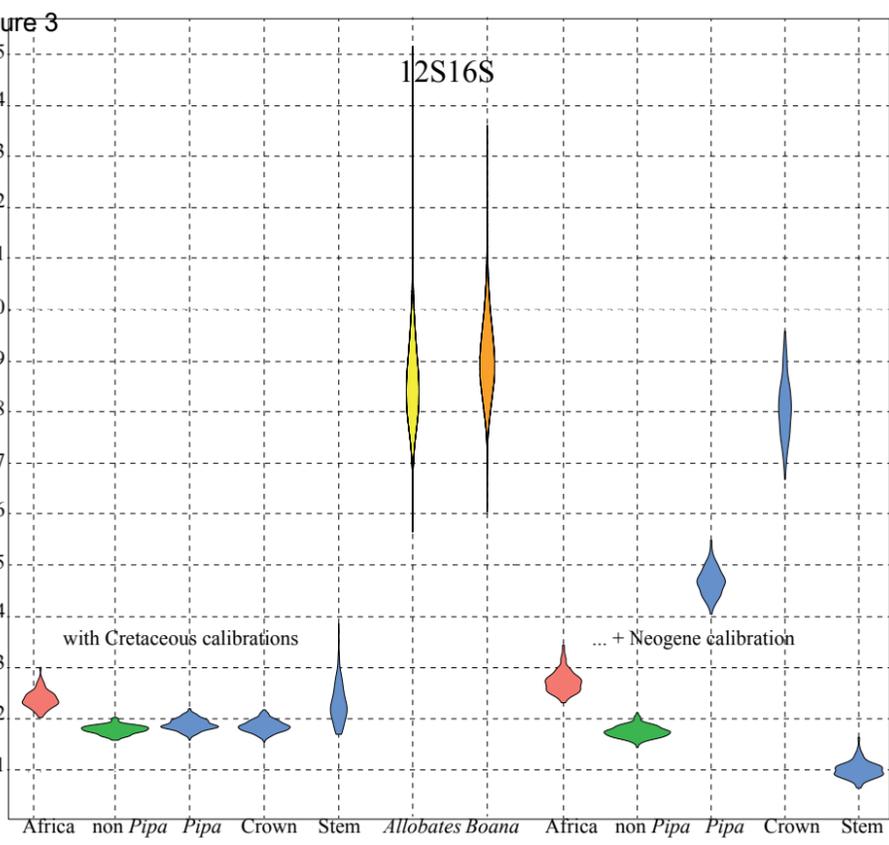
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Figure 3

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Genes	Single- ω model (null)	Three- ω model			Delta AIC null	Delta AIC three- ω 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>	0.16524	0.16417	0.17029	0.12765	0.00	3.79
<i>cob</i>	0.05246	0.05421	0.04754	0.16684	6.74	0.00
<i>cox1</i>	0.01312	0.0131	0.0125	0.0272	0.00	2.25
<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	0.083716	0.061665	0.168248	6.39	0.00
<i>nad2</i>	0.1235	0.14276	0.10034	0.2643	20.46	0.00
<i>nad3</i>	0.1159	0.14233	0.0899	0.21379	3.84	0.00
<i>nad4</i>	0.09141	0.08673	0.08854	0.21757	9.24	0.00
<i>nad4L</i>	0.09169	0.11485	0.06896	0.12563	2.43	0.00
<i>nad5</i>	0.09683	0.10923	0.08168	0.20561	23.26	0.00
<i>nad6</i>	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
<i>bdnf</i>	0.0518	0.05395	0.01992	0.06853	0.00	2.63
<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>	0.1939	0.20566	0.13296	0.16237	0.00	2.46
<i>rho</i>	0.09248	0.0968	0.12761	0.04355	0.00	2.50
<i>slc8a3</i>	0.03126	0.02816	0.03978	0.04252	0.00	3.11
<i>tyr</i>	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



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