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Research paper

Phylogenetic relationships of diurnal, phytotelm-breeding *Melanophryniscus* (Anura: Bufonidae) based on mitogenomic data

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ABSTRACT

Melanophryniscus is a bufonid frog genus with a broad geographic distribution over southeastern South America. In recent years, several new species of *Melanophryniscus* have been discovered in southern Brazil showing a distinctive life-history strategy for the genus - breeding in phytotelmata - as well as a strong association with high-altitude regions. In this study, we use mitogenomic data to infer the phylogenetic relationships among diurnal, phytotelm-breeding *Melanophryniscus* and to determine the timing of their divergence. We obtained the mitochondrial genomes (not including the control region) for eight individuals of *Melanophryniscus* representing all three described species (*M. alipioi*, *M. milanoi*, and *M. xanthostomus*), as well as some recently-discovered and potentially new species. Gene order was conserved in all species and corresponded to the general order found in bufonids. Although the phylogenetic relationships among the studied species was poorly supported, dating confirmed that they diverged during the Pleistocene, suggesting that phytotelm breeding could have arisen during drier periods in the glacial/interglacial cycles due to a decrease in the availability of permanent streams or ephemeral/temporary streams or ponds in which *Melanophryniscus* species commonly breed.

1. Introduction

Melanophryniscus (Anura: Bufonidae) is a fascinating frog genus with a broad geographic distribution over southeastern South America. With 29 currently described species, the genus is distributed across the south and southeastern Brazilian Atlantic Forest, wetland and grassland regions of Brazil to the inter-Andean valleys in Bolivia, and areas across Paraguay and Uruguay down to central Argentina (Frost, 2016). The genus is a charismatic component of the anurofauna of the Neotropics, with many species with bright colours that advertise the alkaloid-containing defensive chemicals secreted by the granular glands in their skin, which are either sequestered from the arthropod in their diet or endogenously biosynthesized (Hantak et al., 2013; Jeckel et al., 2015). Although many species of *Melanophryniscus* are still poorly studied, there has been increasing concern regarding the conservation of its species. For instance, of all 23 species of *Melanophryniscus* currently assessed on the IUCN database, 11 are under some level of threat (IUCN, 2016).

Although most *Melanophryniscus* species breed either in small permanent streams or on ephemeral/temporary streams or ponds (see Baldo et al., 2014), it was recently discovered that some species evolved in association with plants for phytotelm breeding (Langone et al., 2008; Steinbach-Padilha, 2008; Bornschein et al., 2015). As a consequence, the distribution of phytotelm-breeding species is not constrained by the presence of "larger" bodies of water, thus allowing these species to occupy other types of habitat, such as *campos de altitude* (highland grasslands), in which there are no sources of accumulated water other than phytotelms (Langone et al., 2008). There are five currently recognized species of phytotelm-breeding *Melanophryniscus*, all which from the highlands of the Atlantic Forest of the states of Paraná and Santa Catarina, southern Brazil, namely *M. alipioi* (Langone et al., 2008), *M. vilavelhensis* (Steinbach-Padilha, 2008), and *M. biancae*, *M. milanoi*, and *M. xanthostomus* (Bornschein et al., 2015). Based on preliminary data on the phylogeny of the genus (Baldo et al., 2014), all five

Abbreviations: HPD, highest posterior density; BI, Bayesian inference; ML, maximum likelihood

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phytotelm-breeding species and the pond-breeding *M. moreirae* form a monophyletic group. Moreover, Firkowski et al. (2016) showed that *M. biancae* is phylogenetically close to *M. vilavelhensis* and that this group is phylogenetically distant from *M. alipioi*, *M. milanoi*, and *M. xanthostomus*. Although the phylogeny of Firkowski et al. (2016) does not include *M. moreirae*, we tentatively propose that *M. biancae* and *M. vilavelhensis* should be considered sister species given that, in addition to their phylogenetic proximity, both species live in the same habitat (grasslands; see below), breed in *Eriocaulon ligulatum*, seem to be nocturnal, and have similar morphology (e.g. small snout-vent length and general colour pattern) (Steinbach-Padilha, 2008; Bornschein et al., 2015; MRB per. obs. regarding the species used *M. vilavelhensis* during the breeding season). On the other hand, the remaining phytotelm-breeding *Melanophryniscus* are diurnal, have larger body size, and reproduce in bromeliads (on rare occasions also on fallen dead bamboo), both in forests and in grasslands (Langone et al., 2008; Bornschein et al., 2015). It is important to note that kind of habitat in the grasslands with marshes where that pair of species occurs is distinct from that in the grasslands at the top of the mountains that harbour *M. alipioi* (Langone et al., 2008) and some other newly discovered related populations, which are classified as "Refúgio Vegetacional", whereas *M. biancae* and *M. vilavelhensis* are found in "Estepe Gramíneo Lenhosa" (sensu Veloso et al., 1991). Although the fact that these different classifications do not necessarily imply that they have a different phytogeographic origin, the "Refúgios Vegetacionais" of the mountains of the Serra do Mar of Paraná and Santa Catarina do not contain the marshes with *E. ligulatum* where *M. biancae* and *M. vilavelhensis* reproduce (MRB pers. obs.). Therefore, based on ecological and morphological traits, one can define two groups phytotelm-breeding of species of *Melanophryniscus*: the nocturnal *M. biancae* and *M. vilavelhensis*, which breed on *Eriocaulon*, and the diurnal *M. alipioi*, *M. milanoi*, and *M. xanthostomus*, which breed on bromeliads. However, based on the results of Firkowski et al. (2016), it is possible that these three species are in fact complexes of highly endemic species.

An important step towards understanding diversification in *Melanophryniscus* is to assess the timing of recent speciation events, so that they can be interpreted in relation to alternative divergence mechanisms. In this study, we investigate the evolution of diurnal, phytotelm-breeding species of *Melanophryniscus* using mitogenomic data. In particular, our goals are threefold: (1) to describe the structure of the mitochondrial genome of phytotelm-breeding *Melanophryniscus*; (2) to infer the phylogenetic relationships among populations of the three recently described species of *Melanophryniscus*, as well as some potentially undescribed new species, based on their mitogenomes; and (3) to infer the timing of their diversification.

2. Methods

Tissue samples were obtained from field-collected specimens in seven locations for *Melanophryniscus*, including both described and potentially undescribed species (Table 1). Given that, although our datasets involve mitogenomes, they only involve a single locus and therefore is limited regarding species delimitations. However, given the evidence in Firkowski et al. (2016), we tentatively called them either by the available name when the sample was obtained from the type locality, or as "affinis", when data from Firkowski et al. (2016) indicated that it could be a distinct species. Voucher specimens were deposited in the herpetological collection of the Department of Zoology of the Universidade Federal do Paraná (DZUP) and Museu de História Natural Capão da Imbuia (MHNCI), Curitiba, Brazil. Total genomic DNA was extracted using PureLink™ Genomic DNA kit (Invitrogen™, USA), according to the manufacturer's instructions. mtDNA sequences were obtained as off-target regions from another study (Pie et al., unpublished results) using target capture of Ultraconserved Elements (see Faircloth et al., 2012). We used the resulting reads from the assemble performed by Trinity (Grabherr et al., 2011) and executed in Phyluce (Faircloth, 2013). As expected (Hung et al., 2013), the longest contig included the entire mtDNA genome - except for the control region. mtDNA genome notation was carried out using Mitos (Bernt et al., 2013). Sequence reads for this project are available from NCBI BioProject PRJNA391191.

We obtained additional mtDNA genome sequences from GenBank for the only other *Melanophryniscus* mitogenome available to date (*M. simplex*, Machado et al., 2016), and six other bufonid species (four *Bufo* species, one *Duttaphrynus*, and one *Leptophryne*), as well as 10 species of Nobleobatrachia (as defined by Frost et al. (2006:196)), representing other six families (Table 1). Sequence alignment of coding genes was carried out using Mafft v. 5 (Katoh et al., 2005) using the TranslatorX server (Abascal et al., 2010), which first translated sequences into aminoacids prior to the alignment and back-translated into nucleotides for later analyses. Non-coding genes (*rrnS*, *rrnL*, tRNAs) were aligned directly in Mafft 7.2 (Katoh and Standley, 2013). The best partitioning scheme was then determined using PartitionFinder v. 2 (Lanfear et al., 2016) based on its greedy algorithm (Lanfear et al., 2012), with models selected according to the Bayesian Information Criterion (Schwarz, 1978). We performed different analyses to choose the models to be used in tree estimation methods by Bayesian inference (BI) (constrained to the 24 models of evolution available in MrBayes or models available in Beast v. 1.8) and maximum likelihood (ML) (constrained to the GTR + Γ model for RAxML).

We estimated the phylogeny using RAxML v. 8.2.4 (Stamatakis, 2014), using the selected partitioning scheme for the ML analysis, and accessed nodal support with 1000 bootstrap replicates. We inferred the phylogenetic relationships among the studied species using BI in MrBayes v. 3.2.6 (Ronquist et al., 2012), selecting the partitioning scheme and models selected with PartitionFinder (with two independent replicates of 10^7 generations, with one cold and seven heated chains, with sampling every 1000th generations). We examined the convergence of both runs in Tracer v. 1.6 (Rambaut and Drummond, 2013; Rambaut et al., 2013) and Awty (Wilgenbusch et al., 2004; Nylander et al., 2008). As both runs appeared to converge, we combined the trees of both replicates after discarding the first 25% samples of each, as burn-in.

To test if the same molecular clock model could be implemented for all the partitions defined for the Bayesian analyses, we estimated ML trees in RAxML under the GTR + Γ model for all the defined subsets, and provided these trees as input to estimate a minimum branch-score distance matrix and the optimal number of clock partitions using ClockstaR2 (Duchêne et al., 2014) in R v. 3.3 (R Development Core Team, 2016). The timing of lineage divergence was then inferred using Beast v. 1.8 (Drummond and Rambaut, 2007; Drummond et al., 2012), with two independent runs of 50×10^6 generations with a Yule prior, sampled every 1000th generation, with the first 25% omitted as burn-in. Given that there are no known *Melanophryniscus* fossils, we employed a relaxed log-normal molecular clock (Drummond et al., 2006) to calibrate the age of the split between *Melanophryniscus* and the remaining Bufonidae, set as a normal prior with a mean age of 67.92 million years ago (Mya) and distribution between 52.7 and 92.7 Mya (see Van Bocxlaer et al., 2010; Firkowski et al., 2016). Convergence of the runs was examined in Tracer v. 1.6. As the runs with the complete dataset failed to converge, possible due unstable topology at the root of the tree, we performed the dating analysis including only the Bufonidae. As the two runs with the Bufonidae-only dataset converged, we combined both runs (after removal of a 25% burn-in), and analyzed the combined dataset to obtain a majority rule consensus tree, using TreeAnnotator v. 1.8 (Drummond et al., 2012). We then used DensiTree v. 2.2 (Bouckaert, 2010; Bouckaert and Heled, 2014) to explore the differences between the topologies obtained in the different analyses. We performed all phylogenetic analyses at the Cipres server (Miller et al., 2010).

Table 1
Sampled taxa used for phylogenetic analyses and corresponding GenBank accession numbers. Localities are provided for the species sequenced in the present study.

Taxa	Locality	Family	GenBank accession
Melanophryniscus sp. aff. *alipioi* DZUP345	Apiaí, São Paulo	Bufonidae	KY260875
Melanophryniscus sp. aff. *alipioi* DZUP321	Itapiroca, Paraná	Bufonidae	KY260872
Melanophryniscus alipioi DZUP344	Capivari Grande, Paraná	Bufonidae	KY260870
Melanophryniscus sp. aff. *alipioi* DZUP201	Torre da Prata, Paraná	Bufonidae	KY260871
Melanophryniscus sp. aff. *alipioi* DZUP323	Morro dos Padres, Paraná	Bufonidae	KY260873
Melanophryniscus sp. aff. *xanthostomus* MHNCI 9806	Condomínio Vale dos Lagos, Santa Catarina	Bufonidae	KY260876
Melanophryniscus milanoi DZUP200	Morro do Baú, Santa Catarina	Bufonidae	KY260874
Melanophryniscus sp. aff. *milanoi* DZUP437	Morro do Cachorro, Santa Catarina	Bufonidae	KY260877
Melanophryniscus simplex	–	Bufonidae	KT221611
Bufo gargarizans	–	Bufonidae	NC008410
Bufo japonicus	–	Bufonidae	NC009886
Bufo stejnegeri	–	Bufonidae	KR136211
Bufo tibetanus	–	Bufonidae	NC020048
Duttaphrynus melanostictus	–	Bufonidae	NC005794
Leptophrynine borbonica	–	Bufonidae	JX564876
Espadarana prosoblepon	–	Centrolenidae	JX564857
Hyalinobatrachium fleischmanni	–	Centrolenidae	JX564869
Ceratophrys ornata	–	Ceratophrydae	JX564858
Telmatobius bolivianus	–	Telmatobidae	NC020002
Rhinoderma darwinii	–	Rhinodermatidae	JX564891
Hyla japonica	–	Hylidae	NC010232
Leptodactylus melanonotus	–	Leptodactylidae	JX564873
Phyllomedusa tomopterna	–	Hylidae	JX564887
Dendrobates auratus	–	Dendrobatidae	JX564862
Mannophryne trinitatis	–	Dendrobatidae	JX564878

3. Results

We successfully obtained eight mitogenomes, varying between 15,206 and 15,210 base pairs without the control region (Table 1), with an average coverage of 30.7 (range = 8.44–65.82). These genome sizes are shorter than in the African clawed frog *Xenopus laevis* (c. 17,500 bp, GenBank accession NC001573) due to the control region not being computed. All the typical 37 genes are present (Fig. 1) and the overall nucleotide composition is biased against guanine and cytosine (G + C = 38.9%). Only the *nad6* gene and eight tRNAs (*trnE*, *trnS2*, *trnY*, *trnC*, *trnN*, *trnA*, *trnQ* and *trnP*) are encoded in the heavy strand. The *trnV* gene separates the ribosomal RNAs (*rrnS* and *rrnL*). The length of *rrnS* is the same for the three species, 934 bp, while the length of *rrnL* slightly fluctuate from 1596 to 1599 bp. Both RNAs sequences are A (adenine) and T (thymine) rich with proportions around 56.1% for *rrnS* and 63.5% for *rrnL*.

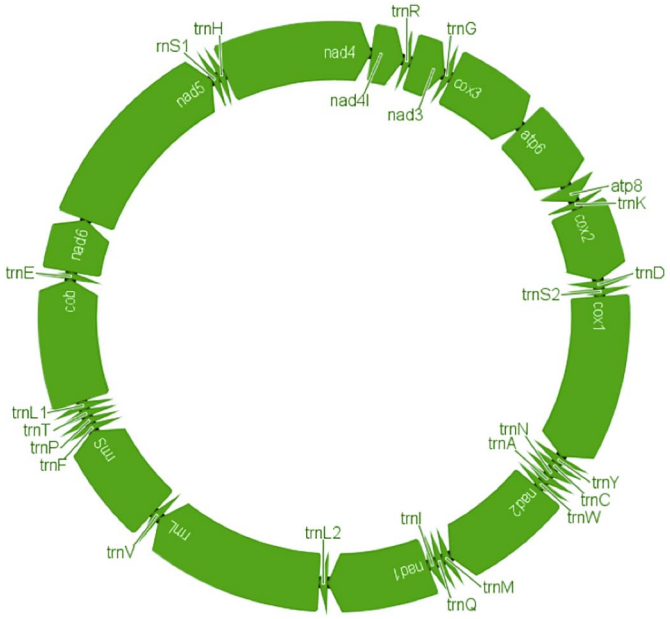

Fig. 1. The structure of the mitochondrial genome of diurnal, phytotelm-breeding *Melanophryniscus*.

The aligned dataset had 15,319 bp. The partitioning analyses realized in PartitionFinder returned a scheme with 13 partitions for MrBayes (Table S1), 14 for Beast (Table S2) (10 for the Bufonidae-only dataset; Table S3), and 11 for RAxML (Table S4). The phylogenies inferred by BI and ML using the nucleotide alignment presented similar results (Figs. 2 and 3), with *Melanophryniscus* being recovered as sister to other analyzed bufonids (*Bufo*, *Duttaphrynus*, and *Leptophryne*). Both analyses recovered the family Centrolenidae (represented by *Hyalinobatrachium* and *Espadarana*) as sister to the Bufonidae, but this relationship was not well supported in the ML tree. A sister group relationship between Telmatobidae and Ceratophrydae, and their close relationship with Rhinodermatidae were recovered by both methods with high support. The two analyses recovered Dendrobatidae as sister to a group including Hylidae and Leptodactylidae, but this topology was not well supported in the ML phylogeny. Both methods recovered *M. simplex* as sister to all other sampled *Melanophryniscus*. All phylogenetic analyses place *M. alipioi* and *M.* spp. aff. *alipioi* as most closely related to *M. milanoi* and *M.* sp. aff. *milanoi*, whereas *M.* sp. aff. *xanthostomus* would be their sister lineage (Figs. 2 and 3).

The ClockstaR2 analysis returned an optimal number of clock partitions of one, as would be expected for mitochondrial sequences (Fig. S1). The dated tree we obtained using Beast for the Bufonidae-only dataset showed a different topology than that obtained by the analysis in MrBayes and RAxML (Fig. 4A–B). The topology obtained by MrBayes and RAxML represented the second most common topology in the Beast analysis (Fig. 4C–D). The dated tree returned a Late Cretaceous age for the Bufonidae (median 67.99 Mya, 95% HPD 52.74–85.06 Mya; Fig. 4A), with a Miocene split between *M. simplex* and the sampled phytotelm *Melanophryniscus* (median 21.5 Mya, 95% HPD 33.6–12.75 Mya). A much younger age was recovered for the diversification of the phytotelm *Melanophryniscus* (Fig. 4C), estimated to have started in the Pleistocene (median 1.68 Mya, 95% HPD 0.95–2.76 Mya), whereas the split between *M. alipioi* + *M.* sp. aff. *alipioi* and *M.* sp. aff. *xanthostomus* was also dated as of Pleistocene age (median 1.31 Mya, 95% HPD 0.74–2.16 Mya). Our results suggest that the diversification of *Bufo* was already ongoing during the Late Miocene (median 9.02 Mya, 95% HPD 5.19–14.39 Mya).

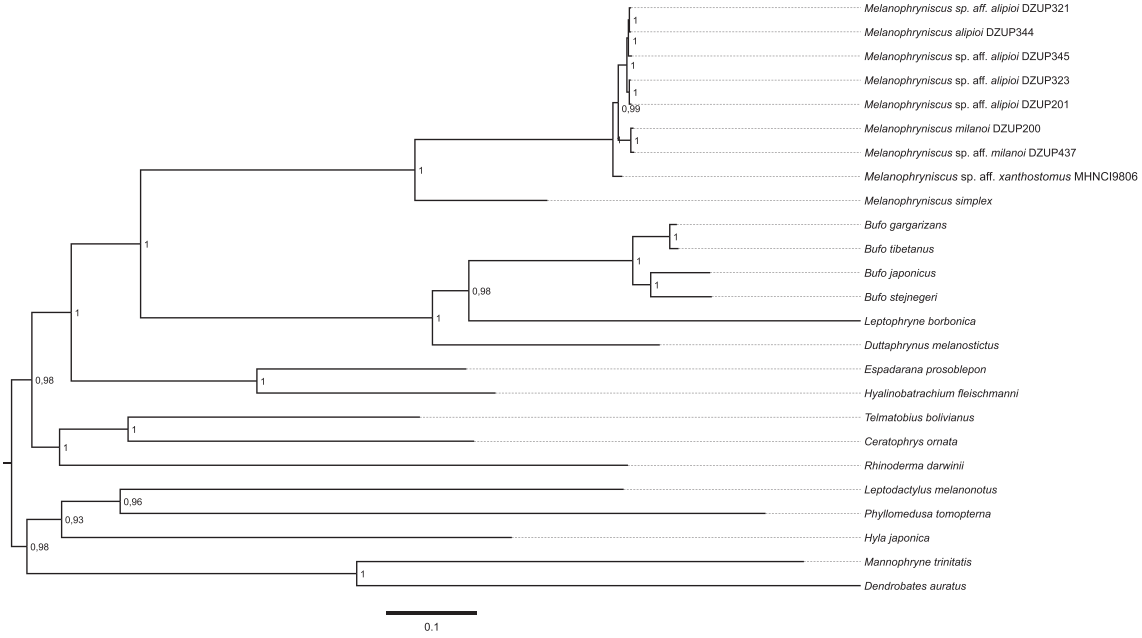

Fig. 2. Phylogenetic relationships among the studied mitogenomes based on the consensus of two Bayesian inference runs in MrBayes v.3.2.4. of 10^7 generations sampled each 1000th (burn-in of 25%).

4. Discussion

The genome content and gene order are consistent among the examined *Melanophryniscus* species, and with other Bufonidae and Nobleobatrachia lineages (see Xia et al., 2014). Several studies have recovered Dendrobatidae as the sister group of Bufonidae, in general with low support (e.g. Hedges and Maxson, 1993; Frost et al., 2006; Bossuyt and Roelants, 2009; Pyron and Wiens, 2011). Other authors inferred a sister relationship between Bufonidae and Hylidae (Irisarri et al., 2010; Kakehashi et al., 2013; Li et al., 2014; Xia et al., 2014; Carr et al., 2015). Our results using the nucleotide mitochondrial dataset suggests that the sister group of Bufonidae is the family Centrolenidae, as also recovered by Zhang et al. (2013). The sister group relationship between Ceratophryidae and Telmatobidae recovered in the present study is consistent with the founding of previously published studies (e.g. Frost et al., 2006; Bossuyt and Roelants, 2009; Zhang et al., 2013). However, it is important to emphasize that inferences regarding the relationships among the studied families should be done with caution, given the low level of taxon sampling in our analyses.

As expected, given the calibration used in the present study, the estimated Paleocene age for the split between *Melanophryniscus* and the other Bufonidae is similar to those obtained in studies that used similar calibrations (e.g. Maciel et al., 2010; Van Bocxlaer et al., 2010), but younger than the Late Cretaceous ages obtained by other authors that

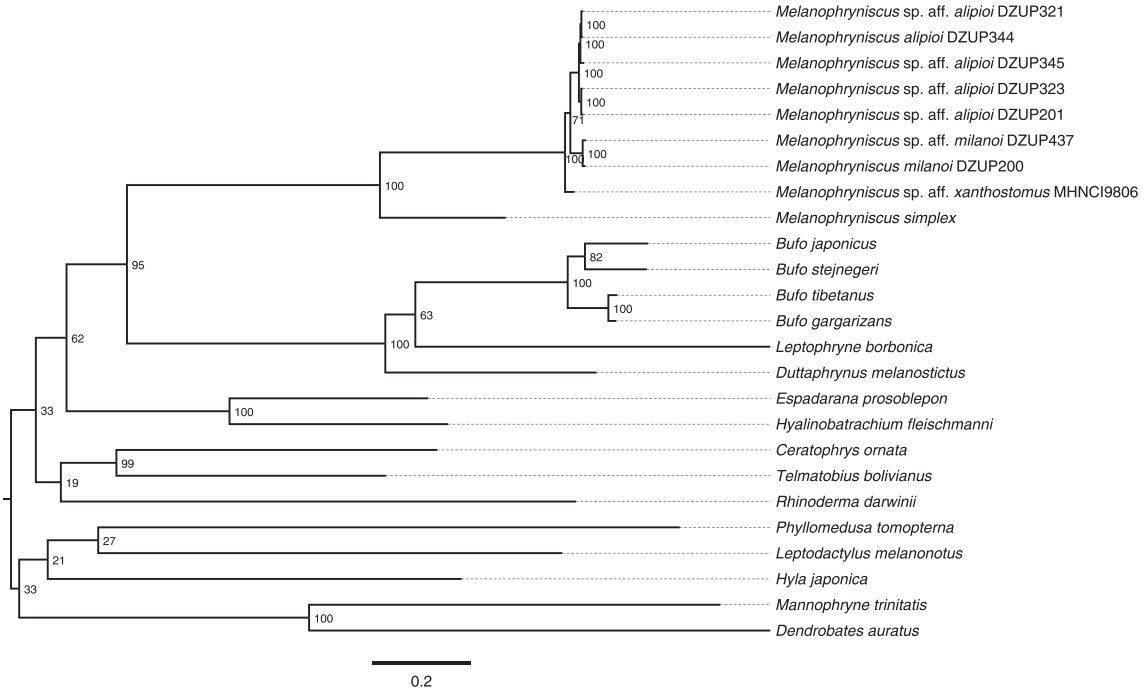

Fig. 3. Phylogenetic relationships among the studied mitogenomes based on the majority rule consensus of 1000 maximum likelihood bootstrap replicates using RAxML v.8.2.6.

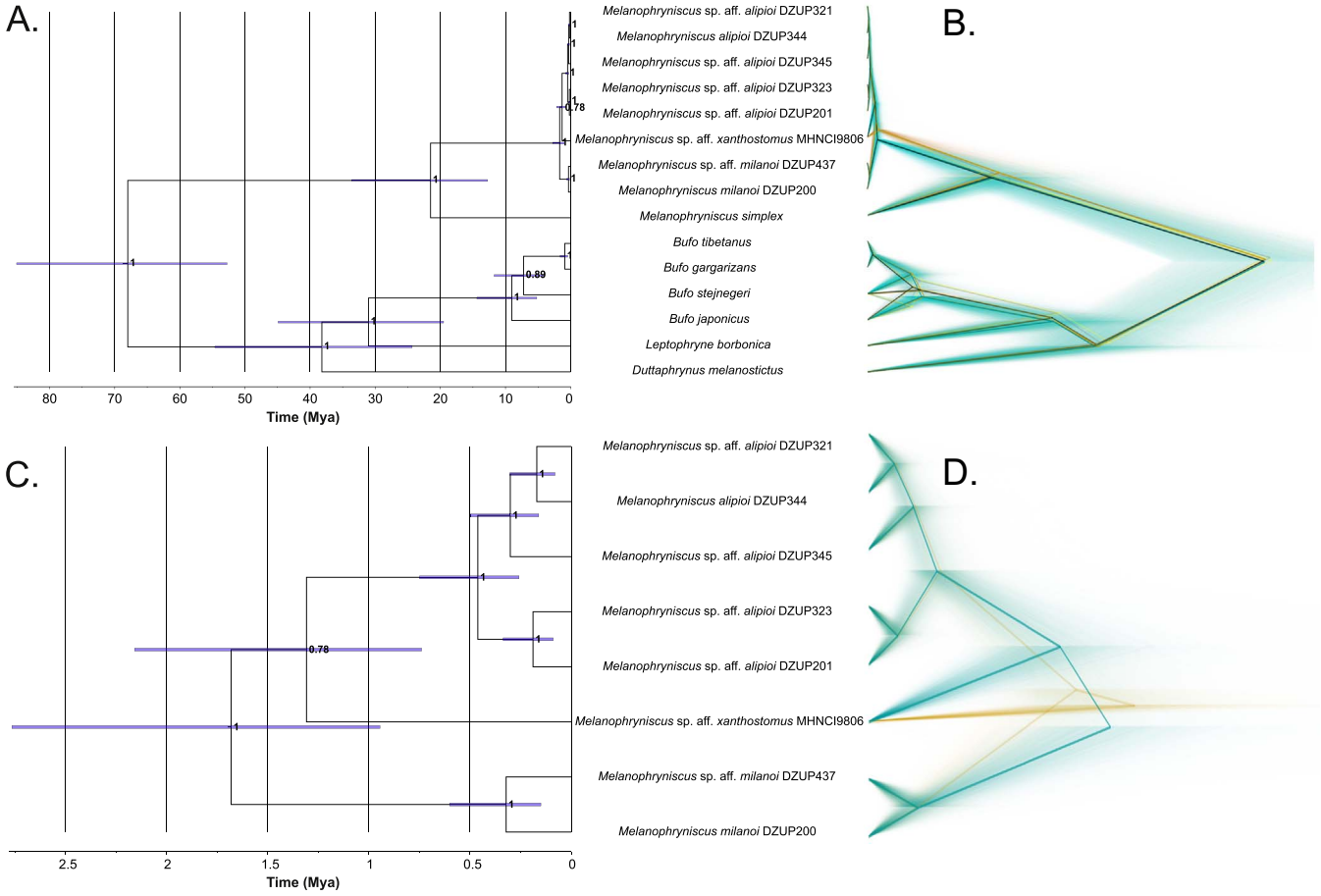

Fig. 4. A. Dated BEAST chronograms of the studied mitogenomes. Node values are of posterior probability; blue bars indicate the 95% HPD for the node age estimates. B. Representation of the set of 15,001 postburn-in trees evidencing the three most common topologies (first most common - green, second most common - orange, third most common - black). C. Detail of the topology for the *Melanophryniscus* species from "A". D. Detail of the set of 15,001 postburn-in trees, from "B", evidencing the three most common topologies for the sampled *Melanophryniscus* species. Colours are the same as in "B". (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used different calibration points (Pramuk et al., 2008; Fouquet et al., 2012; Portik and Papenfuss, 2015). Such deep divergence between *Melanophryniscus* and other bufonids could be compared to those found among some other anuran families (see Bossuyt and Roelants, 2009; Zhang et al., 2013), with estimates of an Oligocene age (c. 33 Mya) for the onset of the *Melanophryniscus* diversification (Pyron, 2014), with such old divergence and diversification suggesting the possibility that this clade could represent a Family level taxon. Our results also recovered a Pleistocene age for the splits between the sampled populations of phytotelm *Melanophryniscus*, in agreement with the results of Firkowski et al. (2016), despite the different datasets and methods used in our study (concatenated analysis of mitogenomes) and that of Firkowski et al. (2016) (three mitochondrial and three nuclear genes, species tree analysis), with both using the same calibration.

Within *Melanophryniscus*, our results were partially consistent with the species tree topology recovered by Firkowski et al. (2016). Our MrBayes and RAxML analyses recovered the topology in which *M. xanthostomus* is the earliest-diverging species ((*M. alipioi*, *M. milanoi*), *M. xanthostomus*), but with low support, as in Firkowski et al. (2016). On the other hand, our Beast analysis recovered a topology where *M. milanoi*, the southernmost diurnal, phytotelm-breeding species, was the first to diverge, but also with low support (the first mentioned topology representing the second most common tree in the Beast analysis). Although it is possible that these topological differences were due to the different partitioning schemes used for each inference method, the analyses in MrBayes and RAxML used different partitioning schemes and returned the same topology. Therefore, it seems probable that the differences in the obtained topologies in our study could be related to tree rooting, most specifically to the exclusion of the non-bufonid taxa for the dating analysis.

Such young divergence between the sampled populations, within a small geographical scale (see map in Bornschein et al., 2015) seems consistent with models that propose that endemism patterns in southern part of the Atlantic Forest are related to climatic heterogeneity (Carnaval et al., 2014). It is possible that *Melanophryniscus* evolved phytotelm-breeding during the drier periods in the Pleistocene cycles of glacial and interglacial climate (see Bowen, 2009 and Langone et al., 2008), a period in which one would expect a decrease in the availability of permanent streams or ephemeral/temporary streams or ponds where the genus commonly breeds. Likewise, Langone et al. (2008) suggested that the reduction in the area of suitable habitat due to the past climate change could have isolated populations and promoted speciation, but also believe that the isolation of populations in patch of habitat with inadequate topography for water to accumulate on the ground forced the emergence of reproduction in phytotelmata.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.07.048.

Conflict of interest

The authors declare they have no conflicts of interest.

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