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Phylogenomics of montane frogs of the Brazilian Atlantic Forest is consistent with isolation in sky islands followed by climatic stability

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Despite encompassing a relatively small geographical area, montane regions harbour disproportionately high levels of species diversity and endemism. Nonetheless, relatively little is known about the evolutionary mechanisms that ultimately lead to montane diversity. In this study, we used target capture of ultraconserved elements to investigate the phylogenetic relationships and diversification patterns of *Melanophryniscus* (Bufonidae) and *Brachycephalus* (Brachycephalidae), two frog genera that occur in sky islands of the southern Atlantic Forest of Brazil. Specifically, we tested whether diversification of montane species in these genera could be explained by a single climatic shift leading to isolation in sky islands, followed by climatic stability that maintained populations in allopatry. In both genera, the topologies inferred using concatenation and coalescent-based methods were concordant and had strong nodal support, except for a few recent splits, which nevertheless tended to be supported by more informative loci. Estimation of divergence time of a combined dataset using both genera is consistent with a concordant timing of their diversification. These results support the scenario of diversification by isolation in sky islands and suggest that allopatry attributable to climatic gradients in montane regions is an important mechanism for generating species diversity and endemism in these regions.

ADDITIONAL KEYWORDS: *Brachycephalus* – coalescent – *Melanophryniscus* – target enrichment – ultraconserved elements.

INTRODUCTION

It has long been recognized that montane habitats tend to display disproportionately high levels of species diversity and endemism, but relatively little is known about the processes underlying this phenomenon (Kessler & Kluge, 2008; Fjeldså *et al.*, 2012). For example, diversification of montane lineages is often attributed to the direct effects of mountain uplift on the interruption of gene flow (e.g. Roy *et al.*, 1997; Toussaint *et al.*, 2014; Xing & Ree, 2017). However, the initial isolating mechanism may not simply result from the creation of a new, physical barrier. Rather, isolation could result from the intrinsic spatiotemporal heterogeneity in lineage persistence that subsequently produces high species turnover (Roy *et al.*, 1997). Likewise, some researchers have argued that newly formed species arise in montane habitats and subsequently migrate into the lowlands, creating a situation where lowland habitats are 'sinks' of species accumulation rather than centres of species diversification (e.g. Fjeldså, 1994). For example, Roy *et al.* (1997) used distributional records of bird species in South America

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and Africa to suggest that avian diversification in these regions was driven by a dynamic process of local isolation in stable montane forests with occasional dispersal to other montane forest patches and that new species gradually expanded into other habitats, finally accumulating in the extensive tracts of lowland forest and woodland savannas. These examples demonstrate that we are still far from a comprehensive understanding of the relative contributions of different mechanisms in generating and maintaining species diversity in montane regions and the importance of montane ecosystems as drivers of diversity across the landscape (Condamine *et al.*, 2018). An important tool to investigate montane diversification is the careful use of case studies, particularly of co-distributed but ecologically distinct species, because these allow researchers to distinguish lineage-specific idiosyncrasies and common driving mechanisms.

An intriguing model system for investigating montane diversification is the Brazilian Atlantic Forest along the Serra do Mar mountain range. These mountains run parallel to the Atlantic coast of Brazil (de Almeida & Carneiro, 1998) and form a barrier to moisture from the Atlantic (Safford, 1999a). This means that the South Atlantic anticyclone, a semi-permanent high-pressure system that transports moist tropical air masses inland all year round (Behling, 2008), provides a constant source of precipitation, which is likely to be responsible for the formation of montane and cloud forests throughout the Serra do Mar mountain range (Behling, 2008). Along the Serra do Mar range, the interaction between geography and climate also produces dry environments caused by strong winds, with a thin soil layer and high levels of water runoff, leading to the formation of high-elevation grasslands ('campos de altitude') on many mountaintops (Safford, 1999a, b). These habitats are home to two anuran genera: *Melanophryniscus* (Bufonidae) and *Brachycephalus* (Brachycephalidae) (Fig. 1).

Melanophryniscus is widely distributed throughout southeastern South America, including parts of Brazil, Bolivia, Paraguay, Uruguay and Argentina (Frost, 2017). The species of *Melanophryniscus* that occur in the Serra do Mar are montane endemics with restricted and isolated distributions in montane forests, cloud forests, campos de altitude and inland grasslands (Langone *et al.*, 2008; Steinbach-Padilha, 2008; Bornschein *et al.*, 2015) and include five of the 29 currently described *Melanophryniscus* species (Frost, 2017): *Melanophryniscus alipioi* Langone, Segalla, Bornschein & de Sá, 2008, *Melanophryniscus biancae* Bornschein, Baldo, Pie, Firkowski, Ribeiro & Corrêa, 2015, *Melanophryniscus milanoi* Baldo, Bornschein, Pie, Firkowski, Ribeiro & Belmonte-Lopes, 2015, *Melanophryniscus vilavelhensis* Steinbach-Padilha, 2008, and *Melanophryniscus xanthostomus* Baldo, Bornschein, Pie, Ribeiro, Firkowski & Morato, 2015. These five species are unique among their congeners owing to their reproduction in phytotelmata, as opposed to the other species that reproduce in temporary streamlets or temporary ponds (Baldo *et al.*, 2014). Two of these species (*M. biancae* and *M. vilavelhensis*) represent a distinct lineage within montane *Melanophryniscus*, given their phylogenetic distinctiveness (Firkowski *et al.*, 2016), their ecology (nocturnal vs. diurnal), the unique type of vegetation in which they occur and the plant species in which they reproduce (Bornschein *et al.*, 2015). The number of the remaining montane *Melanophryniscus* is probably underestimated (Firkowski *et al.*, 2016), and phylogenomic species delimitation analyses suggest that some nominal species might be species complexes (Pie *et al.*, 2017).

*Brachycephalus*is endemic to the Brazilian Atlantic Forest, with a distribution extending nearly 1700 km along the biome (Bornschein *et al.*, 2016a). Most *Brachycephalus* species are found in montane and cloud forests on isolated mountaintops, from the Brazilian states of Bahia in northeastern Brazil to Santa Catarina in southern Brazil (Pie *et al.*, 2013; Pie & Ribeiro, 2015; Ribeiro *et al.*, 2015, 2017; Bornschein *et al.*, 2016a, b). The most remarkable feature of this genus is their extreme level of miniaturization (snout–vent length ≈ 1–1.5 cm), which has led to severe modifications of their body plan, such as a reduction in the number of digits (Hanken & Wake, 1993; Yeh, 2002; da Silva *et al.*, 2007). The distributions of montane species of *Melanophryniscus* and *Brachycephalus* overlap broadly across the southern Serra do Mar, with many pairs of species of each genus being found on the same mountains (Pie *et al.*, 2013; Bornschein *et al.*, 2015, 2016a; Firkowski *et al.*, 2016). Although the virtual lack of sympatry between congeners and their reduced geographical ranges (Bornschein *et al.*, 2016a) lead to severe challenges to the application of traditional methods of comparative biogeography (e.g. Ree & Smith, 2008), these species still provide a unique opportunity to investigate the mechanisms driving their evolution. In particular, concordant timing of diversification would be strong evidence for common vicariance processes.

A previous study of montane *Brachycephalus* and *Melanophryniscus* (Firkowski *et al.*, 2016) used a small sample of loci to propose a two-step scenario explaining the diversification of montane species in the southern Serra do Mar. First, a climatic shift led cold-adapted species to track their ancestral niches (see Pie *et al.*, 2013) to the mountaintops, creating a distributional arrangement commonly referred to as sky islands (McCormack *et al.*, 2009). Thereafter, climatic stability in the region maintained populations in isolation, leading to their diversification. The main sources of

Figure 1. Examples of the species of *Melanophryniscus* and *Brachycephalus* investigated in the present study. A, *Melanophryniscus alipioi*. B, *Melanophryniscus* sp. Boa Vista. C, *Melanophryniscus milanoi*. D, *Melanophryniscus* sp. Morro do Boi. E, *Brachycephalus brunneus*. F, *Brachycephalus izecksohni*. G, *Brachycephalus fuscolineatus*. H, *Brachycephalus auroguttatus*. Photographs by L.F.R.

evidence for this scenario were twofold: the virtual lack of sympatry of microendemic species of each genus (Pie *et al.*, 2013, Bornschein *et al.*, 2015, 2016a) and the relative concordance in the timing of their diversification (Firkowski *et al.*, 2016). However, the limited dataset used by Firkowski *et al.* (2016) left uncertainty in the estimated species trees upon which the inferred diversification scenario depended. Here, we investigate the phylogenomic relationships and diversification patterns of *Melanophryniscus* and *Brachycephalus* in sky islands of the southern Serra do Mar to test whether they share a similar timing in their diversification, which would be consistent with common vicariance mechanisms leading to their isolation.

MATERIAL AND METHODS

We obtained tissue samples from field-collected specimens of seven species of *Melanophryniscus* and 16 species of *Brachycephalus* (Supporting Information, Table S1; for a detailed account of the species delimitation, including still undescribed species, see Pie *et al.*, 2017). We deposited voucher specimens in the herpetological collection of the Department of Zoology of the Universidade Federal do Paraná (DZUP) and in the Museu de História Natural Capão da Imbuia (MHNCI), both in Curitiba, Brazil. More information on specimen collection methods and localities is given by Firkowski *et al.* (2016).

We extracted genomic DNA using a PureLink Genomic DNA kit (Invitrogen, USA) and fragmented the DNA obtained using a BioRuptor NGS (Diagenode). We prepared Illumina libraries using KAPA library preparation kits (Kapa Biosystems) and ligated adapters to each sample that included unique, custom indexes (Faircloth & Glenn, 2012). To enrich targeted ultraconserved element (UCE) loci, we followed an established workflow (Gnirke *et al.*, 2009; Blumenstiel *et al.*, 2010), while incorporating several modifications to the protocol detailed by Faircloth *et al.* (2012). In particular, we pooled eight indexed libraries at equimolar ratios before enrichment, we enriched each pool using a set of 2560 custom-designed probes (MYcroarray, Inc.) targeting 2386 UCE loci (see Faircloth *et al.*, 2012; and http://ultraconserved.org for details on probe design), and we blocked the Illumina TruSeq adapter sequence using custom blocking oligos (inosine at each index position in the blocking oligo). Before sequencing, we qPCR-quantified enriched pools, combined enriched pools at equimolar ratios, and sequenced the combined libraries using two, partial (50%) runs of a MiSeq PE250 (Cofactor Genomics). Coverage varied among libraries between 10.4 and 34.7 X. We performed species delimitation analyses using these data in a separate manuscript (Pie *et al.*, 2017). Such analyses were a necessary first step to ensure that we are investigating species that are well supported, both by phenotypic and molecular data. In the present study, in turn, we build on those inferences by exploring a variety of methods for phylogenetic inference and divergence time estimation to gain a better understanding of the evolution of montane *Brachycephalus* and *Melanophryniscus*. Sequence reads for this project are available from NCBI BioProject PRJNA391191.

We demultiplexed reads using automated procedures of the BaseSpace platform, and we filtered reads for adapter contamination, low-quality ends and ambiguous bases using an automated pipeline (https://github.com/faircloth-lab/illumiprocessor) that incorporates TRIMMOMATIC (Bolger *et al.*, 2014). We assembled reads for each individual using TRINITY (Grabherr *et al.*, 2011). We used the PHYLUCE software package (Faircloth, 2015) to align assembled contigs back to their associated UCE loci, remove duplicate matches, create a taxon-specific database of contig-to-UCE matches and extract UCE loci for all *Brachycephalus* and *Melanophryniscus* individuals. We also used PHYLUCE to harvest UCE loci from the *Xenopus* and *Rana* genomes to use as outgroup sequences. We then generated three sets of data for phylogenetic and divergence time analyses: (1) all *Brachycephalus* species, using *Melanophryniscus* sp. (collected in Morro dos Padres, Serra da Igreja, municipality of Morretes, Paraná) as the outgroup; (2) all *Melanophryniscus* species, using *Brachycephalus sulfuratus* Condez, Monteiro, Comitti, Garcia, Amaral & Haddad, 2016 as the outgroup; and (3) a combined dataset including both *Brachycephalus* and *Melanophryniscus* species, using *Xenopus* and *Rana* as outgroups. The main use of the combined dataset was divergence time estimation, given that the same loci were analysed in both genera and therefore their corresponding divergences are directly comparable. We filtered the loci included in each dataset to ensure there were no missing data. We aligned data for each individual in each dataset using MAFFT (Katoh, 2013), and we trimmed resulting alignments using GBlocks (Castresana, 2000) with default parameters.

After alignment, we carried out phylogenetic inference using both concatenated and coalescent-based methods. Concatenated analyses were carried out in RAxML 8.2.8 (Stamatakis, 2014) using a single GTRGAMMA model across the entirety of the concatenated data, and we performed 1000 rapid bootstrap replicates as a measure of branch support. We then inferred individual gene trees using the same parameters in RAxML, and we performed coalescent-based phylogenetic inference using ASTRAL-II 5.0.3 (Mirarab & Warnow, 2015), a statistically consistent approach under the multispecies coalescent model and therefore capable of handling potential incomplete lineage sorting (Mirarab *et al.*, 2014). Branch support was estimated using local posterior probabilities (LPPs; Erfan & Siavash, 2016). Given that some nodes showed relatively low support values (see Results), we investigated them further by comparing three statistics (average within-locus bootstrap support, proportion of informative sites, and fragment length) of sets of loci that were either consistent or inconsistent with a particular node. Comparisons were obtained with Wilcoxon rank sum tests using the STATS 3.5.0 package in R 3.3.2 (R Core Team, 2018). We determined whether each gene tree was consistent with those nodes using the is.monophyletic function and obtained the corresponding locus statistics using the APE 4.1 (Paradis *et al.*, 2004) package.

We used RelTime (Tamura *et al.*, 2012), as implemented in MEGA (Kumar *et al.*, 2016), to estimate

divergence times for the combined dataset, using a GTR+Γ5 model of evolution and a calibration between *Rana* and the ingroup at 147–162 Mya (Hedges *et al.*, 2006). RelTime first transforms an evolutionary tree with branch lengths, in the units of number of substitutions per site, into an ultrametric tree with relative times by estimating branch-specific relative rates for descendants of each internal node. This procedure is based on the fact that the time elapsed from the most recent common ancestor of two sister lineages is equal when all the taxa are contemporaneous (Tamura *et al.*, 2012). RelTime then converts the ultrametric tree into a timetree using one or more calibration points (Kumar & Hedges, 2016). Comparisons between RelTime and a variety of large-scale datasets showed high correlations in divergence time estimates when compared with alternative dating approaches, such as MCMCTree and BEAST, but at a small fraction of the computation time (Mello *et al.*, 2017). We also estimated divergence times using BEAST v. 2.5.6 (Drummond *et al.*, 2012; Bouckaert *et al.*, 2014). We used an uncorrelated log-normal relaxed clock model (UCLN) with a calibrated Yule model for the tree prior and default priors for the remaining parameters and empirical nucleotide frequencies (tests using other clock models and priors generated similar results). To minimize computational demands and to ensure convergence, we used an unpartitioned GTR model, and we included only one individual for each species. We used the same secondary calibration point indicated above, yet we emphasize that these estimates should be interpreted with caution, given that they can cause unrealistically narrow confidence intervals (Drummond & Bouckaert, 2014). We ran two replicates of each analysis for 100 million generations, sampling every 10 000 generations, and we combined separate runs using LOGCOMBINER v. 2.4.7 (Bouckaert *et al.*, 2014). We examined the combined log files in TRACER v. 1.6 (Rambaut & Drummond, 2007) to assess convergence and burn-in, and all estimated parameters had effective sample sizes (ESS) > 400. We then pruned the timetrees to include only one individual per species in each genus, and we visualized the timing of lineage splits in each genus using lineage-through-time plots (200 trees for each analysis) as implemented

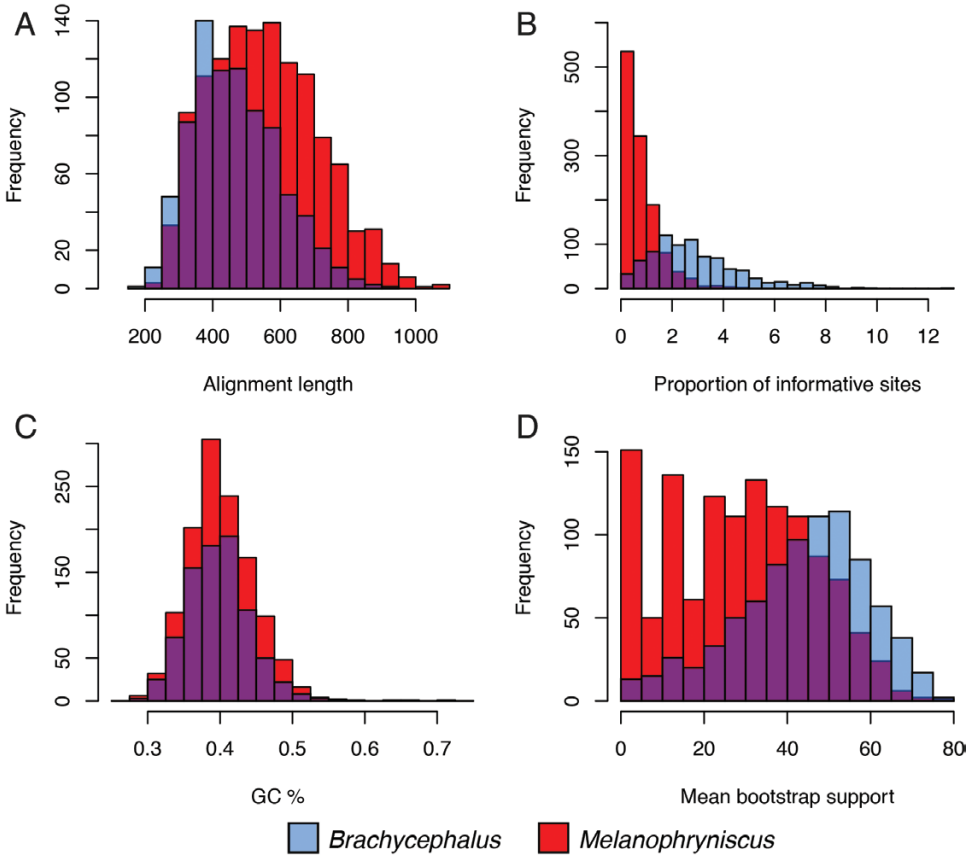

Figure 2. Properties of the ultraconserved element loci obtained for *Brachycephalus* and *Melanophryniscus*. Frequency distributions summarize the properties of the phylogenomic datasets on a per locus basis, including alignment length (A); proportion of parsimony-informative sites (B); GC content (C); and mean bootstrap support (D).

in APE. Finally, we analysed the spatial distribution of the studied lineages with respect to their topography and divergence times using geophylogenies, as implemented in GenGIS v. 2.5.3 (Parks *et al.*, 2013).

RESULTS

After filtering loci with missing data, our final datasets included 820, 1227 and 303 loci (total of 385 080, 677 667 and 155 683 bp) for the *Brachycephalus*, *Melanophryniscus* and combined datasets, respectively, with 610 loci in common between the first two datasets. There was an increase in variability towards the flanking regions of the UCE core (Supporting Information, Fig. S1), with only 1.6 and 13.2% of the loci being invariant in the *Brachycephalus* and *Melanophryniscus* datasets, respectively (Supporting Information, Fig. S2). Basic properties of the UCE loci obtained in the present study are shown in Figure 2.

All phylogenetic analyses supported the same topologies (Fig. 3), with slight differences in nodal support. Phylogenetic analyses of the concatenated *Brachycephalus* and *Melanophryniscus* datasets using maximum likelihood (ML) provided 100% bootstrap support for all nodes, except for the clade including *M. milanoi* and *Melanophryniscus* sp. 'Azul', which had a bootstrap support of 97%. The concatenated ML analysis of the combined dataset produced the same topology as the previous datasets, but with decreased support at some of the nodes (Supporting Information, Fig. S3). Coalescent-based analyses also tended to show high support (LPP = 0.94–1.0) for most nodes, except for three clades: *Brachycephalus auroguttatus* Ribeiro, Firkowski, Bornschein & Pie, 2015 + *Brachycephalus quiririensis* Pie & Ribeiro, 2015 (LPP = 0.58), *Brachycephalus verrucosus* Ribeiro, Firkowski, Bornschein & Pie, 2015 + *Brachycephalus olivaceus* Bornschein, Morato, Firkowski, Ribeiro & Pie, 2015 (LPP = 0.87) and *M. milanoi* + *M.* sp. 'Azul' (LPP = 0.88). To explore this issue further, we compared three statistics (average within-locus bootstrap support, proportion of informative sites and fragment length) of the loci that were consistent with those clades in relationship to the remaining loci, namely *B. auroguttatus* + *B. quiririensis* (supported by 126 loci, not supported by 694 loci), *B. verrucosus* + *B. olivaceus* (supported by 136 loci, not supported by 684 loci) and *M. milanoi* + *M.* sp. 'Azul' (supported by 595 loci, not supported by 225 loci). In all three cases, the loci supporting those clades had significantly higher proportions of informative sites and mean bootstrap values ($P = 0.0015–6.11 \times 10^{-6}$) than the loci conflicting with these relationships, but not larger fragment lengths ($P = 0.53–0.053$).

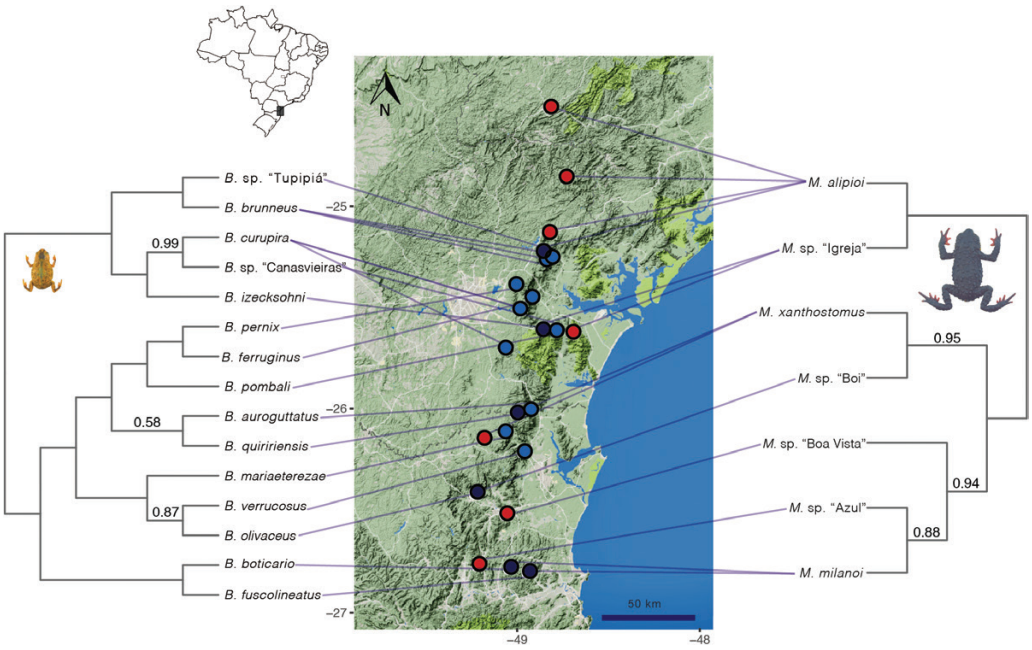

Figure 3. Relationships among the studied species of *Brachycephalus* (left) and *Melanophryniscus* (right). The topologies were identical in all concatenated and species tree analyses. Nodes above branches correspond to local posterior probabilities (LPPs) based on ASTRAL-II species tree analyses. Nodes without annotation are supported with LPP = 1. All nodes were received 100% bootstrap in concatenated analyses (RAxML and BEAST). Outgroup lineages were omitted to facilitate visualization. Light blue and red circles indicate the presence of *Brachycephalus* and *Melanophryniscus*, whereas dark blue circles indicate locations where both genera were sampled.

RelTime and BEAST provided congruent estimates of divergence times (Fig. 4; Supporting Information, Fig. S4), indicating that most species in both genera appear to have originated abruptly during the Pliocene (Fig. 5). Estimates for *Melanophryniscus* were slightly older in RelTime in relationship to BEAST, possibly because the latter were based on a matrix in which all but one tip per species was sampled before estimation. The concordant timing of the recent divergence times in each genus was consistent with a scenario in which their speciation events were driven by the same isolation mechanisms (Fig. 4). On the contrary, despite the strong temporal congruence in the diversification of both genera, they did not share a common geographical distribution. For instance, *Melanophryniscus* species were distributed across a simple north–south axis, whereas there was some overlap between the distributions of the two major clades of *Brachycephalus* in the state of Paraná (Fig. 3). Interestingly, this overlap is associated with some of the most recent speciation events in *Brachycephalus* (Fig. 3).

DISCUSSION

We observed substantial differences in the phylogeny inferred using UCE data compared with that of Firkowski *et al.* (2016). *Melanophryniscus xanthostomus* and *Melanophryniscus* sp. 'Boi' were shown to be more closely related to the clade including *M. milanoi* and related lineages than to *M. alipioi* and *Melanophryniscus* sp. 'Igreja'. In addition, we

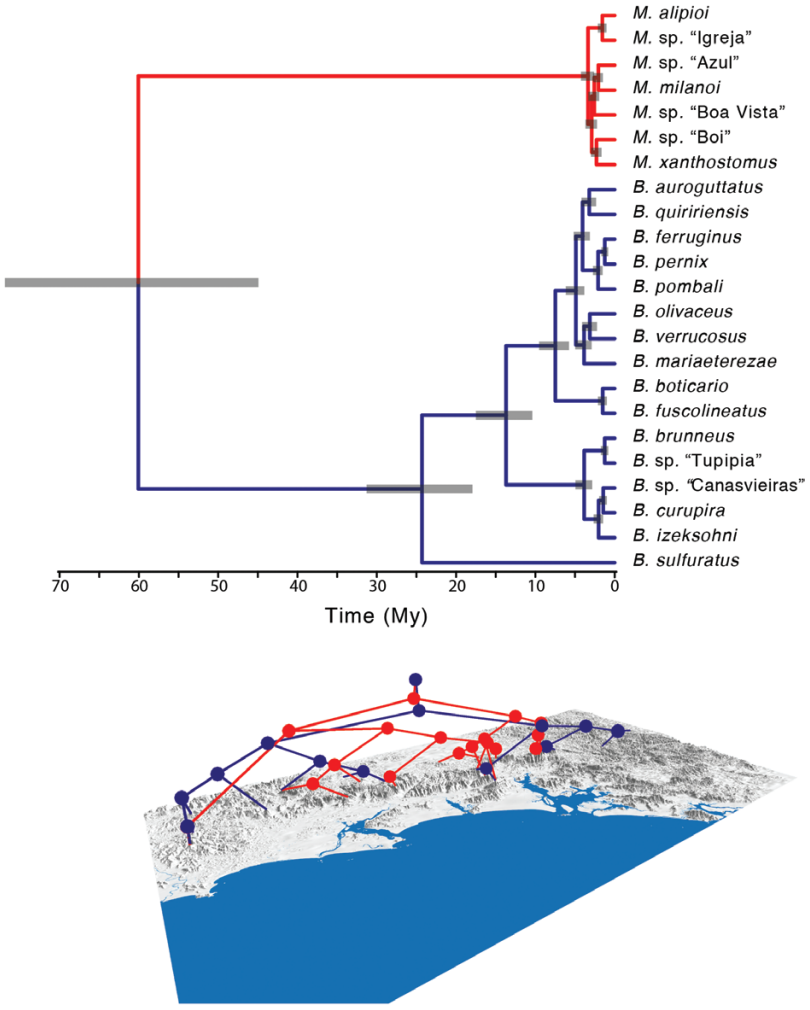

Figure 4. Timing of diversification of *Brachycephalus* (blue) and *Melanophryniscus* (red) and their distribution over geographical space. Divergence time estimates are based on 150 million Markov chain Monte Carlo generations of an unpartitioned matrix (303 UCE loci, 155 683 bp) under a GTR+Γ4 model of evolution. Divergence estimates based on RelTime are indicated in the Supporting Information (Fig. S4). The distribution of the studied lineages and their relationships are shown in relationship to the topography of the region (below; see Fig. 3 for scale).

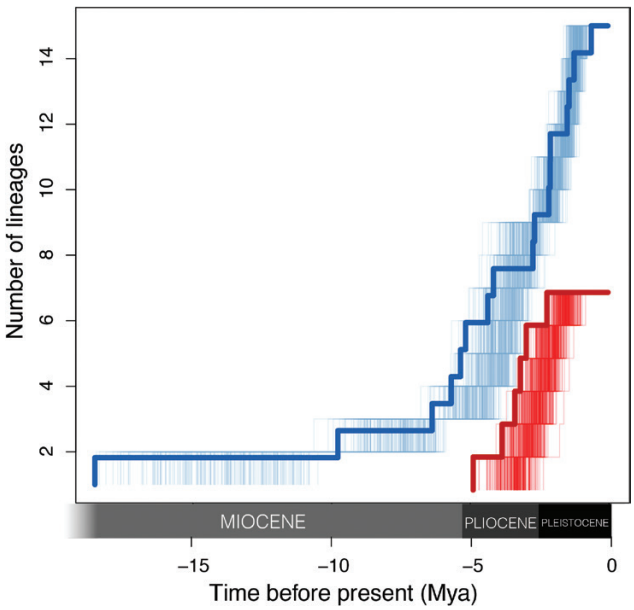

Figure 5. Lineages-through-time plots indicating the timing of diversification of *Brachycephalus* (blue) and *Melanophryniscus* (red). Thin lines correspond to 200 post-burn-in trees from BEAST analyses, whereas thick lines indicate divergence times based on the RelTime method.

detected many differences in the southern clade of *Brachycephalus*, particularly with respect to the phylogenetic position of *B. auroguttatus* and *B. quiririensis*. These differences might have resulted from the rapid diversification of these lineages (Fig. 4), which would require large-scale datasets to be resolved (Smith *et al.*, 2015). The consistency in the topologies obtained across methods indicates that our results are likely to be a solid basis for further interpretation of the diversification patterns in these anurans.

Estimates of divergence times in this study are older than those from a previous study that used anuran *ND2* mutation rates (Firkowski *et al.*, 2016). Indeed, our estimates bring the speciation events in *Brachycephalus* and *Melanophryniscus* closer to divergences found in other montane lineages (e.g. Toussaint *et al.*, 2014). Although it is often difficult to establish a cause–effect relationship between geological and biological events (see Rull, 2015), geological processes that gave rise to the Serra do Mar predate these speciation events considerably, given that the Serra do Mar was formed by the differential erosion of rocks of varying levels of resistance that took place from the Palaeogene and throughout the Miocene (de Almeida & Carneiro, 1998). The inference of older divergence times for both lineages creates another conundrum. How was it possible for these species to have retained microendemism and allopatry, given habitat change and potential connectivity of mountains over that time? It seems unlikely that each lineage remained isolated without either dispersing or colonizing nearby areas over this long period of time, especially given that some are only a few kilometres distant from one another.

According to the scenario proposed by Firkowski *et al.* (2016), this could have been achieved through climatic stability, which prevented colder climates from expanding into lowlands, thus hampering the possibility of secondary contact and sympatry (for a general discussion of how niche conservatism might lead to population isolation and divergence, see: Wiens, 2004; Kozak & Wiens, 2006, 2010; Cadena *et al.*, 2012). Although stability in montane habitats in this region might seem unlikely, given that grasslands covered many areas of the Atlantic Forest during glacial times (e.g. Behling, 2008), areas of palaeoecological stability might have persisted in small pockets within mountains (Roy *et al.*, 1997), thus acting as microrefugia (*sensu* Brown & Ab'Saber, 1979). Indeed, the concordant timing of the lineage accumulation of *Brachycephalus* and *Melanophryniscus* (Fig. 5) is remarkable given the marked ecological differences between them (e.g. direct/indirect development, breeding in litter/phytotelmata) and is therefore strong evidence for a common mechanism underlying their diversification.

The new phylogenies presented here immediately reveal some fascinating evolutionary scenarios that could be followed up by future studies. For instance, most of the species in the northern clade of *Brachycephalus* (Fig. 4A, C) are characterized by highly cryptic coloration, including a dark brown dorsum (e.g. *Brachycephalus brunneus* Ribeiro, Alves, Haddad & Reis, 2005; Fig. 1E). However, one species (*Brachycephalus izecksohni* Ribeiro, Alves, Haddad & Reis, 2005; Fig. 1F), and many other *Brachycephalus*, are highly aposematic, with bright coloration patterns warning of the presence of a powerful neurotoxin (e.g. Pires *et al.*, 2002). The phylogenetic distribution of coloration patterns suggests that aposematism was lost at the origin of this clade, and later regained with the evolution of *B. izecksohni* (Ribeiro *et al.*, 2017).

It is also noteworthy that there are exceptions to the rule that most of the species of *Brachycephalus* in this study are found only on one or a few adjacent mountaintops; namely, *B. brunneus* and *Brachycephalus curupira* Ribeiro, Blackburn, Stanley, Pie & Bornschein, 2017, which are precisely the species with cryptic coloration. Even broader geographical distributions are found in more distantly related lineages in the *didactylus* group (*sensu* Ribeiro *et al.*, 2015), such as *Brachycephalus didactylus* (Izecksohn, 1971), *Brachycephalus hermogenesi* (Giaretta & Sawaya, 1998) and *B. sulfuratus*, all of which are cryptic. The wider distribution found in all cryptic *Brachycephalus* lineages might indicate that skin coloration patterns are one of the factors driving endemism in the genus.

Another important area for future studies involves tests of the diversification scenario provided by other montane endemics, such as rhinocryptid birds of the genus *Scytalopus* (Mata *et al.*, 2009; Pulido-Santacruz *et al.* 2016). Alternatively, organisms without such stringent environmental requirements might still show the signature of our hypothesized scenario in their corresponding patterns of intraspecific genetic variability in the form of phylogeographical endemism, as found in the southern Atlantic Forest (Carnaval *et al.*, 2014). Explicit tests with multiple lineages, such as those based on approximate Bayesian computation (e.g. Overcast *et al.*, 2017), are a particularly promising area for future research to elucidate the mechanisms underlying montane diversification in the southern Atlantic Forest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Samples used in the present study. Unnamed species are indicated by 'sp.', followed by a code indicating the first recorded location.

Figure S1. Increase in variability flanking the ultraconserved regions of the studied datasets.

Figure S2. Proportion of parsimony-informative, parsimony-uninformative and constant sites per locus in each dataset. Loci were ranked by the number of variable sites to facilitate visualization.

Figure S3. Phylogenetic relationships among the studied species, as inferred by the concatenated maximum likelihood analysis of the combined dataset. Values on branches indicate bootstrap support values (based on 1000 replicates). Branches without values are supported by 100% bootstrap support.

Figure S4. Phylogenetic relationships between studied species based on RelTime estimates of concatenated sequences of 303 ultraconserved element (UCE) loci (155 683 bp).