Geographic restriction, genetic divergence, and morphological disparity in the Brazilian Atlantic Forests: Insights from *Leposoma* lizards (Gymnophthalmidae, Squamata)

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ABSTRACT

Lineage differentiation, long-term persistence, and range limitation promote high levels of phylogenetic and phylogeographic endemisms and likely underlie the abundant morphologically cryptic diversity observed in the Brazilian Atlantic Forests (AF). We explore lineage differentiation and range restriction in the AF and ask if genetic divergence and morphological disparity are correlated by integrating coalescent-based species delimitation, molecular phylogenetic, and morphological analyses in the lizard genus *Leposoma*. We present the first species tree for *Leposoma* and of their tribe, the Ecpleopodini. The analyses are based on the largest dataset ever assembled for *Leposoma* in terms of number of species (all represented), genetic markers (12 loci), and geographic coverage (~2,500 km). The exercise allows us to robustly delimit species within the genus and phylogeographic lineages within all species. We find support for the monophyly of the genus and for the recognition of a yet undescribed species around the Baía de Todos-os-Santos, in the state of Bahia; this form is distinct from all other congeners, both genetically and morphologically. We find that *L. baturitensis*, from the northeastern state of Ceará, is basal to the genus – and sister to a clade of six species restricted to the AF across the eastern coast of Brazil. Relationships within this coastal clade are ((((*L. annectans*, *Leposoma* sp.), *L. scincoides*), *L. puk*) (*L. nanodactylus*, *L. sinepollex*)). Phylogenetic and phylogeographic analyses, together with precise distribution data, allowed us to update the ranges of species and phylogeographic lineages. We reveal pervasive geographic restriction of divergent lineages in *Leposoma* at and below species level and discuss how forest refuges and rivers might have contributed to it. We find that morphological disparity lags behind genetic divergence in the genus because although they are correlated, the first accumulates at a much slower rate than the latter. We hope to encourage new studies in the area of AF north of the Doce river; phylogeographic sampling in that region has been much less common relative to southern sites, yet it may hold the key to several important processes defining biodiversity patterns in eastern Brazil. This appears to specially apply to processes underlying geographic restriction of morphologically cryptic, yet genetic divergent lineages, as the case of *Leposoma*.

1. Introduction

Stretching along 1.3 million square kilometers of the eastern coast of South America, the Brazilian Atlantic Forests (AF) are known for their high levels of species diversity and endemism (Myers et al., 2000; Peres et al., 2020). Geographic restriction of evolutionary diversity also seem

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to be high in this ecosystem, and at multiple levels of the tree of life: recent studies of higher rank phylogenies showed relatively small areas concentrating disproportionally high phylogenetic diversity and therefore high phylogenetic endemism (Vale et al., 2018; Azevedo et al., 2020; Varzinczak et al., 2020; Brown et al., 2020), and recently evolved and genetically divergent lineages - either phylogeographic lineages or closely related species - are range restricted, meaning that phylogeographic endemism is also high in some AF regions (e.g. Carnaval et al., 2014; Gehara et al., 2017).

Lineage differentiation, lineage persistence over time, and range limitation due to abiotic or biotic factors are among the processes known to contribute to such geographic restriction of divergent lineages (Rosauer et al., 2009; Carnaval et al., 2014). In the AF, these processes may also underlie the abundant levels of morphologically cryptic diversity observed (Carnaval et al., 2014). Several studies have documented how lineage persistence, lineage differentiation, and range restriction correlate with environmental drivers along the AF. One hypothesis to explain this relationship, for instance, advocates that rivers have been acting as barriers to dispersal of terrestrial organisms, therefore limiting ranges, reducing gene flow, and promoting or maintaining lineage divergence (Wallace, 1852; Peres et al., 2020). Indeed, rivers coincide with areas of species turnover as well as of genetic breaks in many AF taxa (Pellegrino et al., 2005; Thomé et al., 2010; Rodrigues et al., 2014; Cazé et al., 2016; Paz et al., 2019; but see Batalha-Filho et al., 2012; 2019, Mascarenhas et al., 2019). On the other hand, former climatic fluctuations also have been identified as triggers of forest expansion, contraction, and fragmentation (Behling, 2002; Ledru et al., 2005; Cruz et al., 2009; Cheng et al., 2013), potentially altering the ranges of forest-dependent species (e.g. Carnaval et al., 2009; Batalha-Filho and Miyaki, 2016; Prates et al., 2016). For those species, it has been hypothesized that lineage persistence has been enabled within climatically stable refuges, and lineage divergence promoted across forest refuges (Moraes-Barros et al., 2006; Carnaval et al., 2009; Maldonado-Coelho, 2012, but see Thomé et al., 2010; Batalha-Filho et al., 2012; Cabanne et al., 2016). Less known, however, are the links between geographic restriction of divergent lineages and morphological disparity of the local biota.

We explore lineage differentiation and range restriction in the Atlantic Forests, asking if and how genetic divergence and morphological disparity are coupled in a group of lizards whose taxonomy has been changing with the use of molecular phylogenetic tools. *Leposoma* is a genus of small gymnophthalmid lizards (maximum body size around 5 cm) that live in the leaf litter (e.g. Pellegrino et al., 2001; Rodrigues, 1997). *Leposoma* species are typical examples of AF endemics known for a challenging taxonomy; their number more than doubled in the last 20 years (Rodrigues 1997; Rodrigues and Borges 1997; Rodrigues et al., 2002, 2013), and there is still undescribed diversity in the genus (e.g. the candidate species *Leposoma* sp. proposed by Pellegrino et al., 2011). As of now, the genus includes six described species: *L. annectans*, *L. nanodactylus, L. sinepollex, L. puk, L. scincoides,* and *L. baturitensis*. All but *L. baturitensis* occur in forests across the coasts of Bahia and northern Espírito Santo states, a large area thought to have remained forested throughout climatic oscillations in the Quaternary (the Bahia refuge; Carnaval et al., 2009). While the first four species have rather small distributions restricted to the mountains of coastal Bahia, *L. scincoides* has an extensive range across highlands and lowlands, from Bahia to Rio de Janeiro states. *L. baturitensis* shows a disjunct distribution relative to the other species and is restricted to highland forest enclaves in the semiarid Caatinga (a.k.a. *brejo de altitude* or *brejos nordestinos*) in Serra de Baturité (Northern Ceará state; Rodrigues and Borges, 1997) and in Pedra Talhada, (Alagoas state; Roberto et al., 2015). Hypotheses about *Leposoma* biogeography and species divergence invoke AF climate-driven forest expansion and retraction, and niche tracking. Specifically, it has been hypothesized that the *Leposoma* lineage is physiologically adapted to colder climates and that its species diverged allopatrically in distinct mountaintops due to erosion of AF highlands

and historical climate change, leading populations to track forests along mountains (Rodrigues et al., 2002, 2013). Such range shifts are akin to climate-driven movement across elevational gradients reported for other montane species in the southern portion of the AF, especially in Serra do Mar during the Quaternary (e.g. Amaro et al., 2012; Paz et al., 2019; Thom et al., 2020). But because *Leposoma* diversification started much earlier, around 16 Mya (Pellegrino et al., 2011), divergence among species might have responded to environmental shifts that predate the Quaternary.

We use extensive geographic sampling, precise distribution information, and new multilocus data (three mitochondrial and nine nuclear markers) to reveal the geographic restriction of divergent lineages within the genus *Leposoma*, and to test if and how morphometric disparity correlates with genetic divergence among species. To that end, we first test if the genus is monophyletic, identify phylogeographic lineages in all species, test if all described and one candidate species (*Leposoma* sp.; Pellegrino et al., 2011) are distinct lineages according to a species delimitation test based on the multispecies coalescent, and estimate a species tree for the genus. To evaluate levels of geographic restriction of *Leposoma* lineages, we then use that data to update lineage ranges both at the specific and the intraspecific levels. By combining these molecular data with newly collected morphometric information, we test if morphological disparity, measured as differences in body size and shape, is correlated with genetic divergence among *Leposoma* species. We discuss the results in the light of proposed hypotheses about the biogeographical history of *Leposoma* and the Atlantic Forests.

2. Methods

2.1. Sampling

From our own fieldwork, and based on data carefully vetted from Brazilian museum collections, we gathered a total of 229 tissue samples, 206 distribution records, and 325 vouchers of all described *Leposoma* species: *L. annectans*, *L. baturitensis*, *L. nanodactylus*, *L. puk*, *L. scincoides*, and *L. sinepollex* (Tables S1, S4, S5). Specimen identification was based on species descriptions (Rodrigues, 1997; Rodrigues and Borges, 1997; Rodrigues et al., 2002; Rodrigues et al., 2013). We also included 28 samples of the candidate species *Leposoma* sp., which were identified based on head scalation traits normally used in the *Leposoma* species descriptions. Specifically, *Leposoma* sp. specimens were diagnosed from *L. annectans* by the following features: (1) in *L. annectans* the interparietal projects beyond parietals, forming a jagged, irregular, almost indented posterior margin; in *Leposoma sp.*, no jagged posterior margin exists and the posterior part of the head is gently rounded. (2) *L. annectans* has a large scale separating the third pair of chinshields from the infralabials; in *Leposoma sp.*, this scale is smaller. (3) The third pair of chinshields is smaller in *L. annectans* and never reaches the external level of the second pair. In *Leposoma* sp. the external margin of the third and second pairs of chinshields reach approximately the same level.

2.2. Genetic datasets

We extracted genomic DNA from tissue samples (liver or tail) using a high-salt method (Aljanabi and Martinez, 1997), and sequenced regions from both mitochondrial (mtDNA) and nuclear (nuDNA) genomes. Specifically, we targeted two mtDNA genes, NADH dehydrogenase subunit 4 (ND4) and the 12S ribosomal RNA (12S), and four nuDNA genes - including the KIAA1217 ortholog, the KIAA2018 ortholog, oocyte maturation factor Mos (cmos), and Neurotrophin-3 (NT3). Additionally, we developed and sequenced five anonymous nuDNA regions from high throughput shotgun sequences (F8HR5, FP43A, FS94R, FYE1T, and GVX95), using low coverage sequencing obtained with 454 Roche technology, as described in Bertozzi et al. (2012). We amplified DNA with polymerase chain reaction (PCR) essays using primers and

reaction conditions reported in Table S2. We used Geneious Pro v.11.1.4 (Biometters Ltd.) to edit chromatograms, align sequences, and translate protein-coding loci into their amino acid sequences. The gametic phase of nuclear marker sequences was resolved analytically with seqPHASe (Flot, 2010) and PHASE v.2.1.1 (Stephens et al., 2001). To supplement our dataset, we gathered published sequenced data from the regions we sequenced and of the mitochondrial gene Cytochrome *b* (CYTB) from GenBank (Table S3). To perform a monophyly test (described below), we also complemented our nuclear dataset by sequencing the region KIAA2018 from samples of other Ecplepodini genera: samples 907989 (*Anotosaura vanzolinia*), 977924 (*Arthrosaura kocki*), 976977 (*Arthrosaura reticulata*), LG2129 (*Colobosauroides carvalhoi*), MTR09984 (*Kaieteurosaurus hindsi*), MRT6290 (*Loxopholis osvaldoi*), PK1409 (*Yanomamia guianensis*), and PK2031 (*Yanomamia hoogmoedi*). Genbank accession numbers of the newly generated sequences are: MT897845-MT897849 (region 12S); MT905412, MT905413, MT901943-MT902138 (ND4); MT918432-MT918450 (NT3); MT918451-MT918559 (KIAA2018); MT918560-MT918659 (cmos); MT918660-MT918760 (KIAA1217); MT922478-MT922565 (F8HR5); MT936123-MT936198 (FP43A); MT936199-MT936289 (FS94R); MT945428-MT945520 (FYE1T); MT945521-MT945603 (GVX95). Each set of analysis used different datasets (described below and in Tables S3, S4) because multispecies-coalescent based methods demand at least one sequence of all loci for each species included in the analysis. For each dataset, we used PartitionFinder v.2.1.1 (Lanfear et al., 2016) to infer the best models of nucleotide substitution per partition based on Bayesian Information Criterion values.

2.3. Testing monophyly

We tested if *Leposoma* is monophyletic with a multispecies coalescent-based species tree approach. The monophyly of *Leposoma* is supported in most previous work based on multi-locus, concatenated gene trees (Pyron et al., 2013; Goicoechea et al., 2016). However, Pellegrino et al. (2018) recently recovered low support for the genus in maximum likelihood and in Bayesian analyses (maximum parsimony analyses based on the same dataset nonetheless returned high support for *Leposoma*). To verify the monophyly of the genus, we used 2,616 bp of DNA sequence data from three mitochondrial and three nuclear markers. The mtDNA dataset included the genes 12S (333 bp), CYTB (303 bp), and ND4 (569 bp); the nuDNA dataset included cmos (331 bp), KIAA2018 (519 bp), and NT3 (561 bp) (Tables S3, S4). By complementing our sequence datasets, we were able to sample all genera in the tribe Ecplepodini, except for *Marinussaurus*, *Pantepuisaurus*, and *Adercosaurus*. This resulted in a dataset with samples of all described and the candidate *Leposoma* species, as well as samples of *Amapasaurus tetradactylus*, *Anotosaura vanzolinia*, *Arthrosaura kockii*, *Ar. reticulata*, *Colobosauroides carvalhoi*, *Kaieteurosaurus hindsi*, *Loxopholis guianense*, *Lo. osvaldoi*, *Lo. parietalis*, *Lo. percarinatum*, *Lo. rugiceps*, *Yanomamia guianensis*, and *Y. hoogmoedi*. Using the software StarBEAST2 (v.2.5.2; Bouckaert et al., 2019), we performed two runs with 100 million generations each, discarding 10% as burn-in and sampling every 10,000 steps. We applied a Yule prior on the tree, a lognormal relaxed clock model, and the population size model was set to linear, with constant root. We set the clock.rate of the marker 12S as 0.02, corresponding to an mtDNA rate of 2.0E-2 substitutions/site/million years, as proposed for squamates (Macey et al., 1998). This is extensively used in analyses of Gymnophthalmidae and other lizards (e.g. Pellegrino et al., 2011; Werneck et al., 2012; Olave et al., 2016; Marques-Souza et al., 2019). We estimated the clock rates of all other markers as relative to 12S. We used Tracer v.1.8.2 (Rambaut and Drummond, 2007) to assess if effective sample sizes (ESS) reached at least 200 in each run, and to compare the posterior summaries of both runs with the full prior distributions. We combined log and trees files with LogCombiner (Rambaut and Drummond, 2007), discarding 10% as burn-in. By combining two independent runs, we achieved most ESSs > than 200, but ESS of the prior was

20. In order to improve chain mixing, we ran similar analyses but simplified models of substitution. We used the HKY model with empirical base frequencies for the markers 12S, NT3, cmos, and KIAA2018, instead of GTR that was the best-fit model according to PartitionFinder. We used TreeAnnotator v.1.8.2 (Drummond et al., 2012) to annotate the Maximum Clade Credibility tree with posterior probability limit of 50%, and used FigTree (v.1.4.3; Rambaut, 2016) to visualize and edit the tree.

2.4. Identifying phylogeographic lineages within Leposoma species

Aiming to identify lineage divergence and range restriction within species, and taking advantage of our extensive geographic and genetic sampling, we delimited phylogeographic lineages as intraspecific phylogroups recovered in gene trees that showed geographic orientation. This exercise informed the sub-sampling for phylogenetic analyses on account of intraspecific structure and the delimitation of species ranges. We estimated concatenated gene trees from mtDNA and nuDNA datasets separately. We used RAxML v.7.2.8 (Stamatakis, 2006) to construct maximum likelihood trees with GTR-GAMMA model for all partitions, 1,000 rapid bootstrap inferences, and thorough maximum likelihood search. The mtDNA dataset consisted of three partitions (12S: 469 bp; CYTB: 352 bp; ND4: 808 bp) and sampled 244 individuals. The nuDNA dataset consisted of nine partitions (cmos: 356 bp, F8HR5: 456 bp, FP43A: 406 bp, FS94R: 413 bp, FYE1T: 422 bp, GVX95: 407 bp, KIAA1217: 486 bp, KIAA2018: 623 bp, and NT3: 558 bp) and included 163 individuals (Tables S3, S4). As outgroups we used sequences of *Loxopholis percarinatum* (mtDNA tree) and of *Yanomamia guianensis* (nuDNA tree). For each dataset, we ran two independent runs and selected the one with higher log-likelihood value to present.

2.5. Testing if Leposoma species are independently evolving units

To test for lineage divergence at the species level, we used a multispecies coalescent-based species delimitation method to test if all *Leposoma* species are evolutionarily independent lineages (*sensu* Sukumaran and Knowles, 2017). Our analysis included *Leposoma* sp., as hypothesized by Pellegrino et al. (2011) and given its morphological distinctiveness. The expectation that distinct species are genetically isolated (Yang and Rannala, 2014) is compatible with the idea that species are independently evolving units as in the general lineage concept (de Queiroz, 2007). We used the software Bayesian Phylogenetics and Phylogeography (BP&P, v.3.4; Yang and Rannala, 2014) and the multilocus datasets to delimit species and to estimate their posterior probabilities without the constraints of a guide tree (A11 analysis). The dataset was composed by seven nuDNA markers (cmos: 356 bp, FS94R: 414 bp, FYE1T: 422 bp, GVX95: 407 bp, KIAA1217: 486 bp, KIAA2018: 624 bp, and NT3: 558 bp; Tables S3, S4).

As a prior on divergence time at the root of the tree (τ) we used an inverse gamma (IG) distribution with parameters (3, 0.2) and mean = 0.1. Considering that the *Leposoma* diversification started around 16.3 Mya (Pellegrino et al., 2011), and a mtDNA rate of 2.0E-2 substitutions per site/million years proposed for squamates (Macey et al., 1998), we would expect approximately 0.326 (32.6%) divergence between the root and the tips of the species tree. To reflect this same order of magnitude in divergence, we set the mean of τ prior = 0.1 with a shape parameter of 3 in the inverse gamma distribution, specifying a diffuse prior. We used two priors for ancestral population size: "large" (θ = IG(3, 0.2), mean = 0.1) and "small" (θ = IG(3, 0.002), mean = 0.001). The means 0.01 and 0.001 represent, respectively, 10 and 1 difference(s) per kb. As demonstrated by empirical and simulation studies, a prior of large population size is conservative, tending to delimit fewer species than the prior of small size (e.g. Leaché and Fujita, 2010; Yang and Rannala, 2014). For each prior configuration and for each algorithm (0 and 1), we performed two replicates, starting with different seed values. As a starting tree, we used the maximum likelihood tree topology estimated in the phylogeographic analyses with nuDNA. In each run, the reversible

jump MCMC was sampled 250,000 times every 2 generations, using 8,000 generations as burn-in. In all runs, acceptance rates were between 20 and 70% to ensure good chain mixing. Replicate runs were summarized with the command print −1.

We calculated pairwise genetic distances between all delimited species as Dxy (average number of nucleotide substitutions per site; Cruickshank and Hahn, 2014) in the software DNAsp (v.5; Librado and Rozas, 2009). For that, we used only the ND4 data, given that this marker provided the most sequence representation.

2.6. Leposoma species tree estimation

Although all phylogenetic hypotheses available for *Leposoma* have recently shifted from being based on external morphology traits to molecular genetic data, they still rely on concatenated gene trees (Pellegrino et al., 2011; Rodrigues et al., 2013). Aiming at a robust hypothesis of *Leposoma* phylogenetic relationships, including all delimited species, we estimated a species tree with the software StarBEAST2 (v.2.5.2; Bouckaert et al., 2019) and multi-locus data including three mtDNA partitions (12S: 333 bp, CYTB: 303 bp, and ND4: 838 bp) and seven nuDNA partitions (cmos: 356 bp, NT3: 561 bp, FS94R: 414 bp, FYE1T: 422 bp, GVX95: 407 bp, KIAA1217: 486 bp, and KIAA2018: 624 bp; Tables S3, S4). We considered all described *Leposoma*, plus *Leposoma* sp. as distinct species, following BP&P results (described in section 3.3). We set up four runs similarly to those used in the *Leposoma* monophyly test (described in section 2.3). Combining the independent runs was not sufficient to yield ESSs < 200 for some parameters, and the ESS of the prior was particularly low (=16). To improve chain mixing, we ran similar analyses but simplified models of substitution. We used the HKY model with empirical base frequencies for the markers 12S, NT3, cmos, and KIAA2018, instead of GTR model, which had been flagged as the best fitting model to those markers.

2.7. Correlations between morphological disparity and genetic divergence

To test if body shape and size are correlated with genetic divergence among *Leposoma* species, we measured 10 morphometric traits in 325 preserved specimens. We used a digital caliper to the nearest 0.01 cm and following Rodrigues et al. (2013) collected data on snout–vent length (SVL), trunk length (TRL), arm length (AL, end of longest finger-to-elbow length), humerus length (HUL), foot length (FTL), tibia length (TL), femur length (FL), head length (HL), head width (HW), and head height (HH; Table S5). We log-transformed all measurements to reduce deviation from multivariate normal distributions and unequal ranges in the original variables (Ricklefs and Miles, 1994; McCune and Grace, 2002), and ran a Principal Components Analysis (PCA) with the function "prcomp" in R (v.3.1.1; R Core Team, 2013).

To visualize how related species occupy the morphometric space, we projected the species tree into the principal components axis, such that tree tips are located at the species centroid, and branch lengths represent morphometric change (phylomorphospace approach; Sidlauskas, 2008).

We then tested for correlation between morphometric and genetic distances with a Multiple Matrix Regression with Randomization (MMRR; Wang, 2013a) approach, in R, using the MMRR function R script available from Dryad (Wang, 2013b). MMRR provides outputs in the form of a multiple regression equation and performs randomized permutations, estimating regression coefficients (β) with significance values (P-values) and the overall fit of the model (coefficient of determination; Wang, 2013a). To represent morphometric distances, we calculated (multivariate) Mahalanobis distances (McCune and Grace, 2002) based on the 10 measurements, across all species pairs, using the R package HDMD (McFerrin, 2013), function "pairwise.mahalanobis". To represent multi-locus genetic divergence, we used the *Leposoma* species tree (Fig. S1) and calculated tree distances as half of the sum of branch lengths connecting each species pair, divided by tree height.

3. Results

3.1. Genus Leposoma is monophyletic

Our species tree analyses of the Ecpleopodini, including most genera in the tribe and all *Leposoma* species, show strong support for the monophyly of *Leposoma* (Fig. 1). Runs in which we substituted the GTR model for the HKY model with empirical base frequencies yielded all ESSs much higher than 1,000. Tree topology and posterior probabilities of nodes were consistent across all runs, either using GTR or not. Relationships among species and genera are well resolved, except for the most basal nodes and the position of *Arthrosaura kockii*. *Leposoma baturitensis* is sister to all other *Leposoma* species, which form two clades: (((*L. annectans, Leposoma* sp.), *L. scincoides*), *L. puk*) and (*L. nanodactylus, L. sinepollex*), although the support for this sister relationship was around 0.50. The *Loxopholis* species included are monophyletic and are sister to *Amapasaurus tetradactylus*. The clade (*Amapasaurus, Loxopholis*) is sister to *Yanomamia* spp.. *Anotosaura vanzolinia* is sister to *Colobosauroides carvalhoi* and both are sister to *Kaieteurosaurus hindsi*. *Arthrosaura reticulata* is sister to the clade including *Anotosaura, Colobosauroides*, and *Kaieteurosaurus*.

3.2. Multiple phylogeographic lineages within Leposoma species

Concatenated mtDNA and nuDNA gene trees strongly support all described *Leposoma* species, as well as the candidate species *Leposoma* sp., as monophyletic; bootstrap values ranged from 75 to 100 (Figs. S2, S3). In the mtDNA tree, the position of one sample of the candidate species sampled in Serra da Jibóia (sample FJ224) is undefined, but all other *Leposoma* sp. samples form a clade (bootstrap = 100). The same individual (sample FJ224) groups with all other individuals of *Leposoma* sp. in the nuDNA tree, with bootstrap value 98.

There is geographic restriction of divergent lineages within four *Leposoma* species: all strongly supported intraspecific lineages are circumscribed geographically. There are three mitochondrial divergent lineages within *Leposoma* sp. (Lsp-1, Lsp-2, and Lsp-3), with bootstrap of 96 or higher (Figs. S2, S4) - but the sample from Mata de São João (MRT5779) does not cluster to any of the three. In *L. puk*, two lineages were recovered: Puk-1 and Puk-2 (both with bootstrap = 100; Figs. S2, S4). For the first time, we report genetic data for *L. baturitensis* from Alagoas; the only sample from there does not belong to and is divergent from the lineage formed by Ceará samples (bootstrap = 100; Fig. S2). *Leposoma scincoides* shows two deeply divergent mtDNA phylogeographic lineages, northern and southern, each of them composed by several smaller lineages: Sci-1 through Sci-5, in a northern clade, and lineages Sci-6 through Sci-15, in a southern clade; all have bootstrap equal to 85 or higher (Figs. S2, S5). Most smaller-ranged lineages contain all samples from a single or a few neighboring localities. Six *L. scincoides* individuals from Una (MZUSP87875, MZUSP87882, MZUSP87884, MD909, LG1409, and LG1777) grouped with lineage Sci-3 (northern clade), while all other samples from the same locality were recovered in the southern clade (Sci-6). Precise latitude and longitude information revealed that the first four individuals were sampled in Olivença, 22 km to the north of Una. We were unable to obtain latitude and longitude information for the last two samples. Samples from Una are monophyletic, and the range limits of the northern and southern clades of *L. scincoides* are located between Olivença and Una, in the state of Bahia. We did not detect strongly supported mitochondrial lineages within *L. annectans*, *L. nanodactylus*, or *L. sinepollex*.

The nuDNA tree shows much less spatial structure within species than the mtDNA tree, partly due to a smaller number of samples and localities (Fig. S3). We only detected intraspecific lineages in one species, *L. scincoides*. Within it, there is a southern clade (bootstrap = 99), corresponding with the one in the mtDNA tree, but northern localities do not form a clade. The nuclear dataset supports only three phylogeographic lineages: Sci-2 (bootstrap = 100), one lineage (bootstrap = 97)
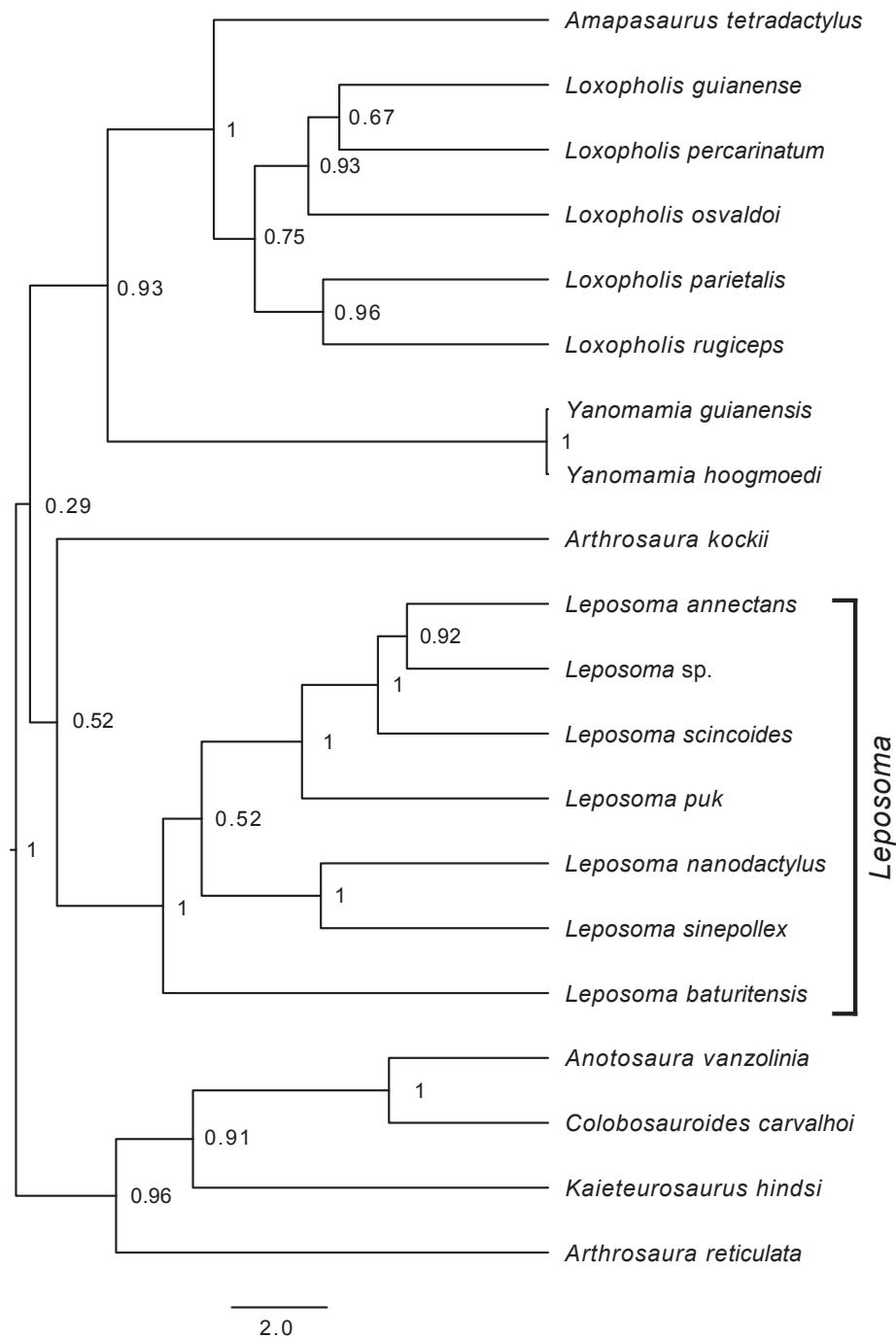
Fig. 1. Ecpleopodini species tree based on mtDNA and nuDNA datasets. Next to nodes are shown posterior probabilities from analysis in which GTR models were substituted by HKY model with empirical base frequencies.

corresponding to Sci-11 through 14 and part of Sci-10 (nuclear data of samples from Barrolândia were not available), and one lineage corresponding to Sci-8 and part of Sci-7 (nuclear data from Canavieiras and Belmonte were not available). Nuclear data were unavailable from Parque Estadual do Desengano (Sci-15) in the state of Rio de Janeiro, and from Parque Nacional do Alto Cariri (Sci-9), at the border between the states of Bahia and of Minas Gerais.

3.3. All Leposoma species, including Leposoma sp., are independently evolving lineages

BP&P analyses with nuDNA support seven distinct *Leposoma* species:

L. annectans, *L. baturitensis*, *L. nanodactylus*, *L. puk*, *L. scincoides*, *L. sinepollex*, and *Leposoma* sp.. All species show posterior probabilities (PP) equal to 1.0, in all runs, regardless of the prior on ancestral population size (Table 1). In downstream analyses, we therefore treated the seven species as distinct. Genetic distances (Dxy) among species based on ND4 are large, ranging from 12.7% (between *L. annectans* and *L. scincoides*) and 24.2% (between *L. baturitensis* and *Leposoma* sp.; Table S6).

3.4. Update of Leposoma species distributions

Our extensive geographic sampling and detailed distribution records

Table 1

Species posterior probabilities estimated by Bayesian Phylogenetics & Phylogeography (BP&P) analyses with nuDNA. θ = Mean of population size prior; τ = mean of divergence time at the root of the tree prior; Algorithm = BP&P algorithm.

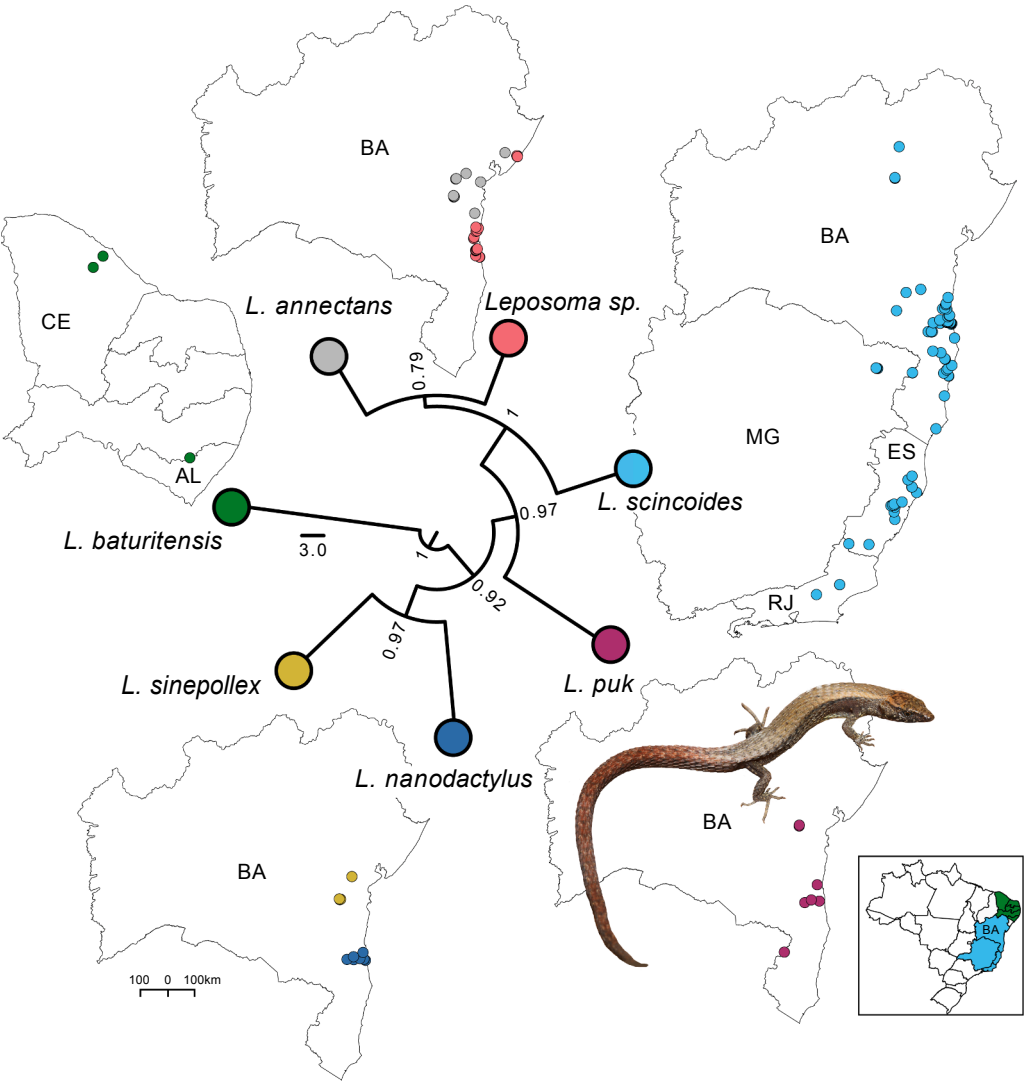|			Species posterior probabilities						
θ	τ	Algorithm	*L. baturitensis*	*L. sinepollex*	*L. nanodactylus*	*L. puk*	*Leposoma* sp.	*L. annectans*	*L. scincoides*
0.001	0.1	0	1	1	1	1	1	1	1
0.001	0.1	1	1	1	1	1	1	1	1
0.1	0.1	0	1	1	1	1	1	1	1
0.1	0.1	1	1	1	1	1	1	1	1

allow us to update the distribution and to assess geographic restriction of *Leposoma* species and phylogeographic lineages. The data expand the distribution of *L. baturitensis* to include the Serra do Maranguape (Northern Ceará) and the Reserva Biológica de Pedra Talhada (Alagoas; Roberto et al., 2015), which are located 43 km and 620 km, respectively, from the type locality in Serra do Baturité, Pacoti (Northern Ceará, Fig. 2). We report new records of *L. nanodactylus* in Serra das Lontras, of *L. sinepollex* in Serra da Jibóia, and of *L. puk* in Parque Nacional do Alto Cariri and in Amargosa, all in Bahia. The new data extend the range of *L. sinepollex* 70 km northward, and *L. puk* 150 km southward.

L. annectans was collected in Una, Ilhéus, Uruçuca, Itacaré, in southern Bahia, as well as in Mata de São João, to the north of Baía de Todos-os-Santos, without any records in between. *Leposoma* sp. is present in Camamu, Wenceslau Guimarães, Jaguaripe, Ubaíra, and also in

Mata de São João, where it is syntopic with *L. annectans*. The new records expand the distribution of *Leposoma* sp. and show that most of the candidate species' range coincides with the *L. annectans* distribution gap. We also report several new records of *L. scincoides*, which ranges from Serra do Mar in the state of Rio de Janeiro (township of Teresópolis) to inland mountains in northern Bahia (townships of Miguel Calmon and Campo Formoso; Fig. S5). *Leposoma* species are syntopic in many localities, but most notably in the state of Bahia, particularly in Ilhéus, Olivença, and Una, where three or even four species can occur together.

3.5. The Leposoma species tree

The *Leposoma* species tree (Fig. 2) shows a topology similar to the

Fig. 2. Species tree and distribution records of *Leposoma* species in eastern Brazil. In the tree, values next to nodes are posterior probabilities. The insert shows Brazil's map and highlights states relevant to *L. scincoides'* range (in cyan) and to *L. baturitensis'* range (in green); all the other species ranges are restricted to the state of Bahia (=BA; ES = Espírito Santo, RJ = Rio de Janeiro, MG = Minas Gerais, CE = Ceará, AL = Alagoas). Picture shows *Leposoma puk*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ecpleopodini species tree (Fig. 1), placing *L. baturitensis* as sister to all other *Leposoma* species with PP = 1. Apart from *L. baturitensis*, there are two *Leposoma* clades: one clade is (((*L. annectans*, *Leposoma* sp.), *L. scincoides*), *L. puk*) and the other is (*L. nanodactylus*, *L. sinepollex*).

Combining the independent runs of the HKY model with empirical base frequencies, instead of GTR, resulted in ESSs higher than 200 for the majority of the parameters, although the ESS of the prior was still 166. Tree topology and node support were similar among runs either using GTR or not. All nodes show PP >= 0.91, except for the sister relationship between *Leposoma* sp. and *L. annectans* (PP = 0.79), which is high but lower than in the Ecpleopodini tree.

3.6. Morphological disparity is correlated with genetic divergence among Leposoma species

Most of the morphometric variation in *Leposoma* represents variation in body size. The first principal component (PC1) explained 81.7% of the variance and represents all measurements with positive loadings, varying from 0.264 to 0.381 (Table S7). Larger individuals show larger scores in PC1 (Fig. 3a). PC2 and PC3 explained 5.55% and 3.91% of the variance, respectively, and represent variation in body shape. Individuals with large scores in PC2 show larger heads, shorter feet, and shorter trunks compared to individuals with low scores. In PC3, large scores represent individuals with shorter trunks and longer humerus.

Conspecifics do not form distinct clusters in morphometric space, because there is partial overlap among species, especially between the sister species *Leposoma* sp. and *L. annectans*. However, species centroids are located in distinct parts of morphometric space, showing some degree of differentiation (Fig. 3b). *L. scincoides* and *L. puk* showed the largest body sizes and *L. sinepollex* is smaller than the majority of samples (Fig. 3a). *L. puk* is the most distinct, overlapping only with *L. scincoides*.

We observe a positive relationship between tree distance and morphometric distance. In the MMRR test, tree distance explained 63.8% of the morphometric variation within the genus ($R^2 = 0.638$, F = 33.518, $P = 0.002$), but the regression coefficient (β) was 0.171, showing that morphological distance changes at a much slower rate than tree distance (Fig. 4). Variance in morphometric distance also increases with increased tree distance.

4. Discussion

4.1. Geographic restriction of divergent lineages in the Atlantic forests and Leposoma biogeography

There is pervasive geographic restriction of divergent lineages in *Leposoma*. Species are deeply divergent and, except for *L. scincoides*, have small ranges (Fig. 2). Moreover, there are several divergent intraspecific lineages that are geographically circumscribed, especially within *L. scincoides* (Figs. S4, S5). Geographically restricted species are numerous in the AF, as demonstrated by its high levels of species (micro) endemism (e.g. Fouquet et al., 2012a; Firkowski et al., 2016; Pie et al., 2018; Thomé et al., 2020). However, small-ranged and divergent lineages are less documented in the coastal forests in Bahia and northern Espírito Santo, where most *Leposoma* species are distributed. One assemblage-level study with 25 terrestrial vertebrate species, based on one mtDNA marker, showed that levels of phylogeographic endemism in this region are amongst the highest in the entire AF (Carnaval et al., 2014). Other examples of range restriction in this region have been reported in intraspecific lineages of the lizard *Coleodactylus meridionalis* (Damasceno et al., 2014) and in frog species of the genera *Adelophryne* (Fouquet et al., 2012a), *Adenomera* (Fouquet et al., 2013), *Dendrophryniscus* (Fouquet et al., 2012b), and *Pristimantis* (Trevisan et al., 2020). Interestingly, they are all small-sized and inconspicuously colored, such as *Leposoma*. We hope this study will encourage the generation of data from other clades mostly distributed in Bahia and
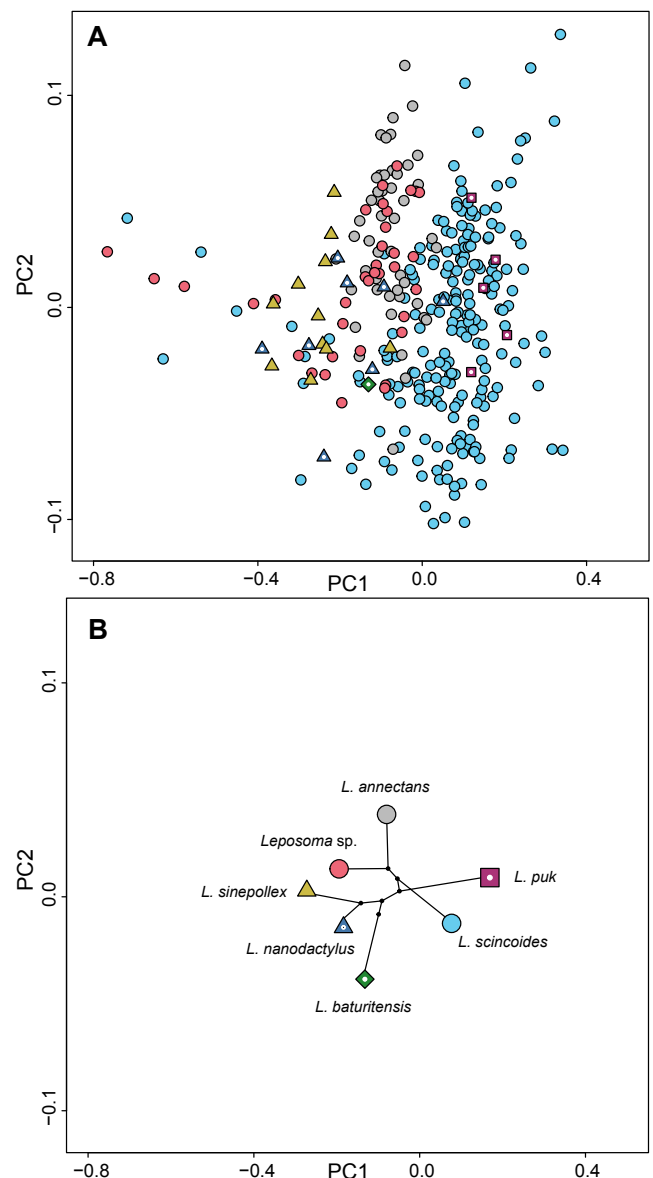
Fig. 3. Morphometric variation in *Leposoma* species across principal components (PC) 1 and 2, showing (A) all individuals and (B) the *Leposoma* species tree with tips representing species centroids in morphological space and branch lengths representing morphometric variation.

northern Espírito Santo; phylogeographic sampling there has been significantly less common relative to southern sites, yet this region may hold the key to several important processes defining biodiversity patterns in eastern Brazil.

Most of the ranges of the *Leposoma* species occur within AF forest refuges either inland, in *brejos de altitude*, or on the coast. Therefore, habitat stability might have promoted lineage maintenance through time, probably contributing to phylogeographic endemism in this group (Carnaval et al. 2014). However, range restriction and lineage differentiation are not concordant with the location of AF forest refuges. For instance, several intraspecific lineages of *L. scincoides* from the Bahian coast are divergent and geographically restricted, as expected if they were isolated in distinct forest refuges. But multiple AF modeling exercises, despite their differences, predict one large refuge in coastal Bahia, some even predict a much larger refuge also including coastal ES and Northern Minas Gerais (Carnaval and Moritz, 2008; Carnaval et al., 2009; Carnaval et al., 2014; Costa et al., 2017; Silveira et al., 2019), supporting a context for lineage maintenance but not for range
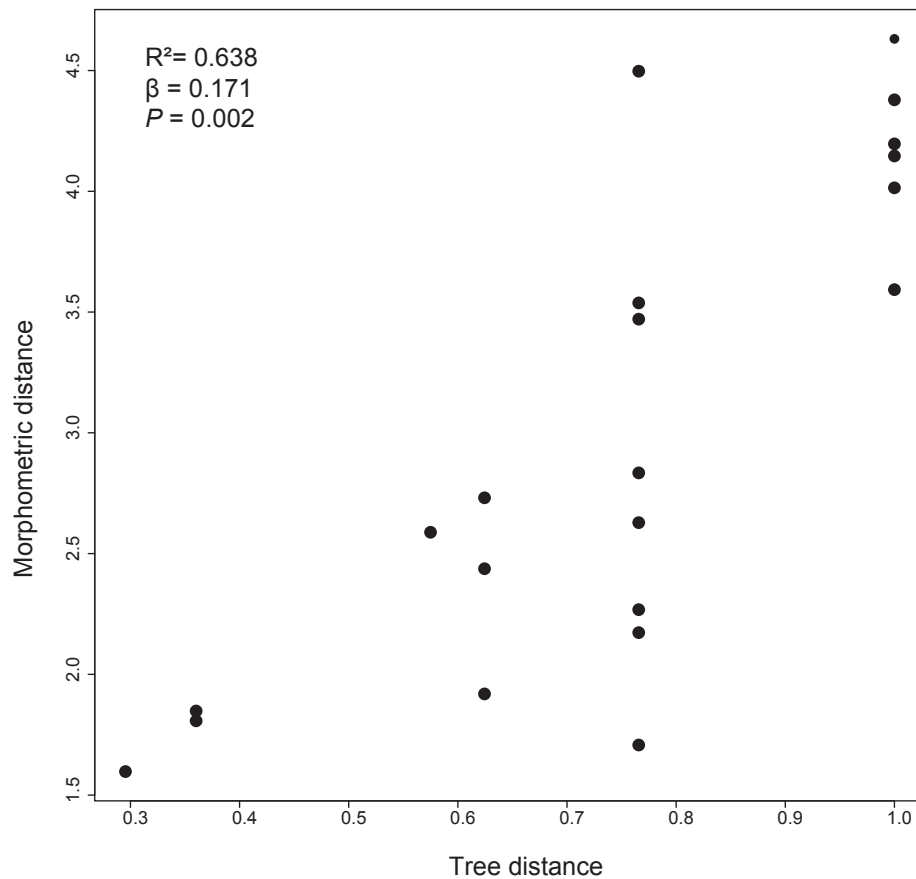
Fig. 4. Relationship between tree distance and morphometric distance among *Leposoma* species with points representing species pairwise comparisons. Results of the multiple matrix regression with randomization (MMRR) are presented in the upper left corner: R² = coefficient of determination, β = regression coefficient and *P* = probability.

restriction in that region. Similarly, intraspecific lineages within *Leposoma* sp. and within *L. puk* are divergent, yet are all within the Bahia refuge. Silveira et al. (2019) predicted a forest refuge at Chapada Diamantina, in inland Bahia, distinct from the coastal refuge, but their model also showed small forest patches in between the two refuges, in all time periods modeled during the Quaternary. They predicted that populations from both refuges would show weak genetic structure between them given the potential path of connectivity. Yet, the *L. scincoides* lineage we identified in the Chapada Diamantina (Sci-2) is divergent from coastal lineages. The only sites where lineages of *L. scincoides* might have diverged in distinct refuges is in the southern part of the species range, in southern Espírito Santo and northern Rio de Janeiro, where forest refuges showed a different dynamics compared to Bahia, as modeled by Carnaval et al. (2014). Tied to existing forest models under past conditions, our results suggest that lineage divergence within *Leposoma* species occurred in the absence of habitat barriers. We hypothesize that one possible mechanism contributing to range limitation in the genus is low migration capacity as suggested by *Leposoma*'s small body size.

Although AF forest refuge dynamics in the Pliocene and Late Pleistocene have been proposed as a driver of intraspecific diversification and narrow lineage ranges in *L. baturitensis* (Rodrigues et al. 2002), our data do not support this view. Rodrigues et al. (2002) proposed that this species may have had a much larger distribution in NE Brazil, and dwindled following retraction of forest habitat to high altitude refuges due to past climate change. Alternatively, the species may have had diverged in one isolated *brejo*, and later colonized other *brejos*. Opportunities for either scenario occurred since wet forest expanded and contracted across NE Brazil during the Pliocene and Late Pleistocene, as

suggested by phylogeographical, palaeopalynological, and speleothems studies (e.g. Oliveira et al., 1999; Auler and Smart, 2001; Wang et al., 2004; Batalha-Filho et al., 2013; Costa et al., 2017; Prates et al., 2017; Dal-Vechio et al., 2018; Silveira et al., 2019). However, the ND4 data presented here show that *L. baturitensis* samples from Ceará form a clade, excluding the divergent Alagoas sample, and that Dxy between CE and AL samples is 4.8% - suggesting long-term isolation between *brejos* that likely predates the Pliocene or Pleistocene. Consistent with our data, a recent modeling study proposed that the Northern Ceará *brejos*, where Baturité and Maranguape are located, remained isolated from the coastal forest and other *brejos* during the Quaternary glacial cycles (Silveira et al., 2019). That being said, a model-based approach is still needed to test if the genetic data support a scenario of recent colonization in either *brejo*, or one of longer-term isolation.

Rivers only coincide with major breaks among phylogeographic lineages in the southern clade of *L. scincoides*, potentially contributing to lineage range restriction (Fig. S5). These results are concordant with the riverine barrier hypothesis (Wallace, 1852) and records from other vertebrates in the AF (e.g. *Gymnodactylus darwinii* - Pellegrino et al., 2005, *Rhinella crucifer* - Thomé et al., 2010, *Enyalius* spp. - Rodrigues et al., 2014, *Vitreorana* spp. - Paz et al., 2019). The location of the Jequitinhonha river coincides with a break separating Sci-6 (Una), Sci-7 (Canavieiras, Belmonte, and Itapebi) and Sci-8 (Jequitinhonha) from all other southern phylogeographic lineages (Sci-9 through Sci-15). Further south, the Doce river coincides with the separation between southern Bahia (Sci-9, 10, and 11) and Espírito Santo, and Rio de Janeiro (Sci-12, 13, 14, and 15). This river is coincident with strong biological turnover, major genetic breaks in several taxa, and a large environmental shift (Carnaval et al., 2014; Martins, 2011; Silva et al., 2012). The Paraíba do

Sul river coincides with the break between Espírito Santo (Sci-12, 13, and 14) and Rio de Janeiro (Sci- 15). Yet, other genetic breaks within *L. scincoides* are not geographically congruent with rivers (Fig. S5). For instance, (1) the separation of lineages Sci-3, Sci-5, and Sci-6 in the Una-Ilhéus region, (2) the range limit of the northern and southern clades, between Una and Olivença, (3) the differentiation between lineages Sci-10 (Porto Seguro) and Sci-11 (Trancoso). Lack of concordance between genetic breaks and rivers are reported for several birds (e.g. *Basileuterus leucoblepharus* - Batalha-Filho et al., 2012, *Synallaxis* spp. - Batalha-Filho et al., 2019, *Mionectes rufiventris* - Mascarenhas et al., 2019).

Our analysis, tied to the new distribution data reported here, address previously unanswered questions about the biogeography of *Leposoma*. The newly delimited ranges of *L. annectans* and *Leposoma* sp. demonstrate that the genus occurs to the north of Salvador, in the state of Bahia, as questioned by Rodrigues and Borges (1997). The analyses also extend, by several kilometers, the ranges of *L. sinepollex* (northwards), *L. puk* (southwards), and *L. scincoides* (landwards). Additionally, we resolved range limits of the northern and southern clades of *L. scincoides*, by showing that samples from Una form a monophyletic lineage, differently from Pellegrino et al. (2011). The phylogenetic analyses confirm that the early diverging lineages of *Leposoma* now occur mostly in highland areas. *L. baturitensis* is restricted to *brejos de altitude*, and *L. sinepollex*, *L. nanodactylus*, and *L. puk* occur mostly in mountain complexes near the coast of central Bahia, although the latter two are also present in nearby lowlands around Ilhéus and Una. Rodrigues et al. (2013) hypothesized that, differently from these four species, *L. scincoides* occupies lowlands as well as high elevation areas, likely as a result of broadened thermal tolerances. Our new distribution data show that not only *L. scincoides* but also *L. annectans* and *Leposoma* sp. occur in lowlands and in highlands. It remains to be tested if Rodrigues et al.'s (2013) hypothesis of broader thermal tolerances applies to the lineage leading to the clade comprising those three species. Precise distribution data are as important as genetic data when describing spatial genetic structure, delimiting lineages' ranges, or testing phylogeographic and biogeographic scenarios.

4.2. Morphological disparity and genetic divergence among Leposoma species

Leposoma species are similar in body size and shape because they partially overlap in morphometric space (Fig. 3). A small and elongated body with well-developed limbs, as seen in *Leposoma*, may be well suited for a 'generalist' lifestyle on the leaf-litter of tropical forests (Wiens et al., 2006). But even if similar selection keeps the *Leposoma* body shape within an adaptive zone, body sizes are likely diverging according to genetic drift as 63.8% of the morphometric variation among species can be explained by species tree distance. It suggests that morphometric disparity is accumulating with genetic divergence, although at a much lower rate (regression coefficient = 0.171 in MMRR, Fig. 4). Among *Leposoma* species, morphological disparity lags behind genetic divergence.

We hypothesize that these differing rates in morphological disparity and genetic differentiation are related to the environmental conditions experienced by *Leposoma* species. It is well known that geographic isolation and persistence of genetically distinct lineages in different environments set up the context for adaptive or plastic divergence in phenotypes (e.g. Rabosky and Glor, 2010; Thorpe et al., 2010; Hoskin et al., 2011; Surget-Groba et al., 2012; Blom et al., 2016), leading to lineages that are divergent both genetically and phenotypically. On the other hand, persistence of divergent lineages in isolated but similar and environmentally stable areas are conducive to low morphological disparity (e.g. Phillips et al., 2004; Kozak et al., 2006; Singhal and Moritz, 2013), leading to high genetic divergence that is phenotypically cryptic. Phenotypes tightly associated with habitat structure are also expected to vary little between lineages that inhabit similar habitats, even if they are deeply divergent (Winger and Bates, 2015; Zamudio

et al., 2016; Trujillo-Arias et al., 2020). Because ecological shifts are seen as central to morphological diversification (Schluter, 2000), and given that morphological traits in lizards are labile at the intraspecific level (Vitt et al., 1997; Kolbe et al., 2014; Llewelyn et al., 2016) and can respond quickly to habitat shifts, even when genetic divergence is low (Vitt et al., 1997; Measey et al., 2009; Miller and Alexander, 2009; Sistrom et al., 2012; Muñoz et al., 2014), we propose that the discord observed here between levels of morphological disparity and genetic differentiation in *Leposoma* lizards is a reflection of long-term stability of the habitats they occupy. Given the latitudinal and altitudinal gradients *Leposoma* spans, it will be important to test if differences in environmental conditions experienced by its species are also correlated with morphometric variation (e.g. Vitt et al., 1997).

In the Gymnophthalmidae family, cryptic genetic diversification does not seem to be a process unique to *Leposoma*. For instance, significant morphological differentiation has been associated with major habitat shifts - such as transitions to fossorial or aquatic lifestyles, leading to recurrent convergence in body shapes (Wiens et al., 2006; Marques-Souza et al., 2018). While congeners distributed across different biomes show evident morphological differentiation concordant with genetic structure (Recoder et al., 2014; Arteaga et al., 2016; Rodrigues et al., 2017; Sturaro et al., 2017), genetically divergent conspecific populations occurring in similar biomes or habitats present low variation in external morphology (e.g. Siedchlag et al., 2010; Pellegrino et al., 2011; Nunes et al., 2012; Santos et al., 2014; Murphy et al., 2019). Given the high prevalence of cryptic lineages in other Atlantic Forests groups (Lara-Ruiz et al., 2008; Mata et al., 2009; Ventura et al., 2012; Fusinatto et al., 2013; Gehara et al., 2017; Rivera et al., 2020), it will be interesting to assess if and how strongly morphological disparity correlate with local levels and patterns of habitat stability.

4.3. Leposoma monophyly

We present the first species tree exercise with the genus *Leposoma* and the tribe Ecpleopodini (Fig. 1). Our tribe-wide species tree strongly supports the monophyly of the genus *Leposoma*, similarly to previous work (Pellegrino et al., 2011; Pyron et al., 2013; Goicoechea et al., 2016), and despite the large differences in number of loci and species included, phylogenetic scope, and approach used. Interestingly, our results disagree with maximum likelihood and with Bayesian results of Pellegrino et al. (2018), whose dataset is the most similar to ours - the only differences being that our dataset includes (1) sequences of *L. nanodactylus,* (2) one additional nuclear gene (KIAA2018), and (3) that our matrix is complete (in the sense that there is at least one sequence per species for all loci). We believe that the combination of (i) additional genetic data, composing a complete matrix, and (ii) a phylogenetic method that accounts for gene tree heterogeneity and incomplete lineage sorting allowed us to adequately test for the monophyly of the genus.

Because the phylogeny of the tribe did not include all genera, we were unable to test if *Ecpleopus* is the sister taxa to *Leposoma*, as some gene trees seem to suggest (Pyron et al., 2013; Goicoechea et al., 2016; Pellegrino et al., 2018). It is worth noting that our results do not support *Arthrosaura* as monophyletic (the position of *Arthrosaura kockii* is undetermined, and *Arthrosaura reticulata* groups with *Kaieteurosaurus hindsi, Colobosauroides carvalhoi*, and *Anotosaura vanzolinia*). Support to the monophyly of *Arthrosaura* was low in Goicoechea et al. (2016) but high in Pellegrino et al. (2018). Those two studies have similar taxon sampling and included a few more genera than ours. Such discordance highlights the need of future species tree analyses of the tribe based on multi-locus data that include all genera. This is especially difficult since tissue samples of the species *Adercosaurus vixadnexus* were never collected: this monotypic genus is described after a single specimen from the Cerro Yutajé, a hard-to-reach area in the Tepui region.

4.4. *Leposoma* sp., an undescribed species

Leposoma sp. is distinct from all other *Leposoma* species - genetically and morphologically. The monophyly of the *Leposoma* sp. is strongly supported in the concatenated mtDNA and nuDNA gene trees (Figs. S2, S3). Additionally, BP&P tests based on multi-locus data support this lineage as evolutionarily independent from *L. annectans* (Table 1) - from which it can also be distinguished morphologically by differences in head scalation as shown here. Genetic distances (Dxy) between *Leposoma* sp. and the other species are comparable to distances between described species (Table S6). Together, these results show unequivocally that *Leposoma* sp. should be separated from *L. annectans* and described as a new species. A formal description of the species is nevertheless beyond the scope of this study.

4.5. *Leposoma* phylogeny

The *Leposoma* phylogeny we present here is consistent with previously published *Leposoma* gene trees (e.g. Rodrigues et al., 2013, Goicoechea et al., 2016), and supports the arrangement (*L. puk*, (*L. scincoides*, (*L. annectans*, *Leposoma* sp.))). Our results, however, are different from the gene trees presented in Pellegrino et al. (2011) and in Pyron et al. (2013), which lacked data from *L. sinepollex* and recovered *L. baturitensis* as sister to *L. nanodactylus* (Fig. 2). They also differ from the gene tree presented in Pellegrino et al. (2018), which failed to include data from *L. nanodactylus*, recovering *L. sinepollex* as sister to the clade (*L. baturitensis*, (*L. puk*, (*L. scincoides*, (*L. annectans*, *Leposoma* sp.)))). Because we combine the largest genetic dataset so far assembled for *Leposoma* under a species tree approach that accounts for stochastic gene tree discordance, we believe that our *Leposoma* species tree is a strong hypothesis of the genus phylogeny and should be used in comparative and evolutionary studies.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106993.

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