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# Hidden in the DNA: How multiple historical processes and natural history traits shaped patterns of cryptic diversity in an Amazon leaf-litter lizard *Loxopholis osvaldoi* (Squamata: Gymnophthalmidae)

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## Abstract

**Aim:** To investigate the cryptic diversity and diversification timing in the putatively low-dispersal Amazonian leaf-litter lizard *Loxopholis osvaldoi*, and to ask how geography (rivers, isolation by distance, IBD), ecological drivers (isolation by environment, IBE) and historical factors (climatic refugia) explain intraspecific genetic variation.

**Location:** Central Amazonia, Brazil.

**Taxon:** Squamata; Gymnophthalmidae; *Loxopholis osvaldoi*.

**Methods:** We sequenced two mitochondrial and two nuclear markers in 157 individuals. Phylogeographic structure and the occurrence of independent evolving lineages where explored through phylogenetic and coalescent analyses. A species tree and divergence dates of lineages were inferred with *BEAST, employing multiple DNA substitution rates. The potential genetic impacts of geographical distance among localities, the environment and the position of localities in relation to main rivers were tested by redundancy analysis (RDA).

**Results:** We detected 11 independently evolving and largely divergent intraspecific lineages. Lineage distribution patterns are complex and do not match any conspicuous barrier to gene flow, except for the Amazon River. Most lineages appear to have originated in the lower Miocene and Pliocene, in disagreement with the Pleistocene refuge hypothesis. IBD, IBE and rivers appear to have acted in concert establishing and maintaining genetic structure. However, when controlling for other explanatory variables, IBD explains significantly more variation than rivers, IBE or historical factors.

**Main Conclusions:** Our results strongly suggest that *L. osvaldoi* is a species complex. Future taxonomic work should use an integrative approach to explore whether morphological variation is present and congruent with the genetic data. While the use of a sensitive dating analysis allowed us to better describe the diversification history of *L. osvaldoi*, the lack of a spatial model of Neogene river dynamics prevents the test of specific, more informative river barrier hypotheses. The data suggest that

  

nonlinear correlation analyses (e.g. RDA) should be preferred to detect factors that affect phylogeographic patterns in the Amazon, instead of linear multiple regressions (e.g. Mantel tests). Given the high level of cryptic diversity detected within this and other Amazonian species, we caution against hypothesis tests based solely on the distribution of nominal taxa, which can provide a rather incomplete view of the processes behind Amazonian diversity.

**KEYWORDS**

Amazonia, cryptic diversity, isolation-by-distance, lizard, phylogeography, refuge, rivers

## 1 | INTRODUCTION

The Amazon Rainforest is likely one of our planet's largest open evolutionary laboratory. It is environmentally heterogeneous (Tuomisto, Zuquim, & Cárdenas, 2014), distributed over a relatively flat area and crossed by some of the world's largest rivers (Goulding, Barthem, Ferreira, & Duenas, 2003). Accordingly, biological richness is heterogeneously distributed along the basin (Avila-Pires, 1995;Kress et al., 1998;ter Steege et al., 2013;Tuomisto & Ruokolainen, 1997), with distribution patterns still poorly known. Recent genetic assessments of Amazon vertebrates demonstrate that the evolutionary history of the region is far more complex than that indicated by alpha taxonomy. Specifically, several species once seen as widely distributed are, in fact, cryptic species complexes with intricate distribution patterns (e.g. Fernandes, 2013;Fouquet, Cassini, Haddad, Pech, & Rodrigues, 2014;Fouquet et al., 2012;Fouquet et al., 2016;Geurgas & Rodrigues, 2010;Kaefer, Tsuji-Nishikido, Mota, Farias, & Lima, 2013;Marques-Souza et al., 2018;Miralles & Carranza, 2010;Nunes, Fouquet, Curcio, Kok, & Rodrigues, 2012;Oliveira, Carvalho, & Hrbek, 2016;Simões, Lima, & Farias, 2010). By consequence, we are almost certainly underestimating the biological diversity of the Amazon region, even in extensively inventoried and well-studied groups. This posits a challenge in identifying the biodiversity hotspots, in understanding biogeographic processes that led to them, and the conservation of biological patterns and processes.

Over the last 30 million years (Ma), Amazon biodiversity evolved amidst extraordinary geomorphological dynamics. While the western sedimentary portion suffered several changes (Hoorn, Guerrero, Sarmiento, & Lorente, 1995;Hoorn et al., 2010;Rossetti, Toledo, & Góes, 2005;Vonhof, Wesselingh, & Ganssen, 1998), the eastern granitic shields experienced relative stability (Aleixo & Rossetti, 2007;Irion & Kalliola, 2010). How the evolution of the Amazonian biota reflects these environmental changes is still not fully understood (Leite & Rogers, 2013;Ribas, Aleixo, Nogueira, Miyaki, & Cracraft, 2012). Such responses are probably species-specific, being modulated by varying their natural history and ecological traits (Rull, 2011;Smith et al., 2014).

One important way by which natural history can drive microevolutionary processes, ultimately influencing macroevolutionary patterns, is through isolation by distance (IBD; Wright, 1943). IBD describes how allele frequencies change in a continuous population as a function of geographical distance among individuals, due to dispersal limitations. While its importance to explain the spatial distribution of genetic variation is widely accepted (Meirmans, 2012;Puebla, Bermingham, & Guichard, 2009), the IBD model assumes habitat homogeneity and thus does not take into consideration spatial variation of the environment. To expand on this topic, an association between environmental heterogeneity and genetic structure was coined as isolation by environment (IBE; Wang & Summers, 2010); it emerges when dispersal across the landscape is limited due to unfavourable conditions. Isolation by environment can also appear when there is natural or sexual selection against immigrants, or against hybrid offspring (Wang & Bradburd, 2014). In the Amazon Rainforest, given the large extent and significant environmental variation (Tuomisto et al., 2014), we would expect both IBD and IBE as important factors generating and maintaining genetic variation. However, their absolute or relative roles are seldom evaluated in this region.

Most of the diversification hypotheses proposed for the Neotropics have evoked processes where geological phenomena are the main drivers of speciation (Antonelli et al., 2010;Leite & Rogers, 2013). Among them, the Riverine Barrier Hypothesis (Ayres & Clutton-Brock, 1992;Ribas et al., 2012;Wallace, 1852) is one of the most influential. It postulates that the emergence of rivers in the Amazon Basin isolated previously continuous populations, leading to vicariance and speciation. This hypothesis predicts that sister species or lineages will occur on opposite river banks and that the boundaries of species distributions will match rivers courses (Da Silva & Patton, 1998;Gascon et al., 2000;Ribas et al., 2012). However, testing those predictions is not straightforward. A similar distribution pattern can be achieved by secondary dispersal after the river formation, as demonstrated for several bird species in the Rio Negro and Rio Branco basins (Naka & Brumfield, 2018). Additionally, new lines of evidence suggest that river configuration within the Amazon Basin was far more dynamic in the Neogene than previously thought (e.g. Hoorn et al., 2017;Hoorn et al., 1995;Rossetti et al., 2005;Ruokolainen, Moulatlet, Zuquim, Hoorn, & Tuomisto, 2018), potentially mixing up populations from opposite banks over thousands of generations.

Another influential diversification hypothesis in the Amazonia is the Pleistocene Refuge Hypothesis (Haffer, 1969;Vanzolini & Williams, 1970). It focuses on the role of glaciation cycles during

the Pleistocene, positing that the humid ombrophilous forest was restricted to refugia during periods of cool and dry conditions, when open or savanna environments expanded. Forest fragmentation would have then reduced gene flow across isolated populations of forest-adapted species, leading to allopatric speciation. Although different refuge dynamics have been associated with the maintenance of genetic diversity in other South American biomes (e.g. Atlantic Rainforest; Carnaval, Hickerson, Haddad, Rodrigues, & Moritz, 2009;Carnaval et al., 2014), it has been argued that Haffer's Pleistocene refuge hypothesis is inconsistent with estimated ages of Amazonian tetrapods (e.g. Ribas et al., 2012), and with the reconstruction of Amazonian paleoenvironments (Colinvaux, De Oliveira, Moreno, Miller, & Bush, 1996). However, the response of the Amazon rainforest to Pleistocene climatic fluctuations may have been heterogeneous in geography (e.g. Cohen, Rossetti, Pessenda, Friaes, & Oliveira, 2014), causing distributional shifts and/or local extinctions and influencing the genetic structure of species (e.g. Prates, Rivera, Rodrigues, & Carnaval, 2016).

Here, we focus on the spatial patterns of genetic diversity in the Amazonian lizard *Loxopholis osvaldoi*. This small (ca. 30 mm body size) leaf-litter dweller has a narrow thermal range (TOB and Agustín Camacho, ongoing study) and is strictly associated with forest habitat. Its small size and putative ecological restriction to forest environments suggest that dispersal limitations due to geographical distance, ecological and/or geographical barriers are important factors in the evolutionary history of *L. osvaldoi*. Previous studies tested the influence of main rivers of Central Amazon in the phenotype of this species, finding little support for the Riverine Barrier Hypothesis, but also showing a conservative morphology along its distribution (Marques-Souza, Rodrigues, & Cohn-Haft, 2013), which may conceal large genetic distances among distinct evolutionary units, typical of species complexes (Bickford et al., 2007). Additionally, previous studies of Amazonian leaf-litter lizards showed deep time divergences and cryptic diversity (e.g. Brunes, Silva, Marques-Souza, Rodrigues, & Pellegrino, 2019;Geurgas & Rodrigues, 2010;Nunes et al., 2012), indicating that this group of animals may disclose valuable insights on the landscape history of the Amazon Basin.

We first explored if *L. osvaldoi*, similarly to other leaf-litter vertebrates in the Amazon Basin, shows cryptic genetic diversity. For that, we implemented multilocus phylogenetic analyses, and described geographical structure via summary statistics and genetic distances. Using coalescence theory, we then tested if intraspecific lineages represent independent evolutionary units. Based on a sensitive dating analysis, we asked if intraspecificgenetic structure was shaped in the Pleistocene, consistent with the Refuge Hypothesis, or if it precedes it, falsifying the hypothesis that the glacial cycles led to diversification. Lastly, we applied a model selection approach to test the relative importance of three factors in explaining the current spatial genetic divergence of *L. osvaldoi*: geographical distance among localities (a proxy to measure IBD), environmental conditions (via IBE) and rivers.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling and laboratory procedures

*Loxopholis osvaldoi* is distributed in Central Amazonia, occupying the southern margin of the Amazon River along the Purus, Madeira and Tapajós river basins, with few populations occurring on the northern margin of the Amazon River, near Manaus (Oliveira, Marques-Souza, Frazão, Almeida, & Hrbek, 2014;Ribeiro-Junior & Amaral, 2017). Our molecular database included 157 individuals, with tissue samples from 47 localities (Figure 1; Tables S1 and S2 in Appendix S1). Of

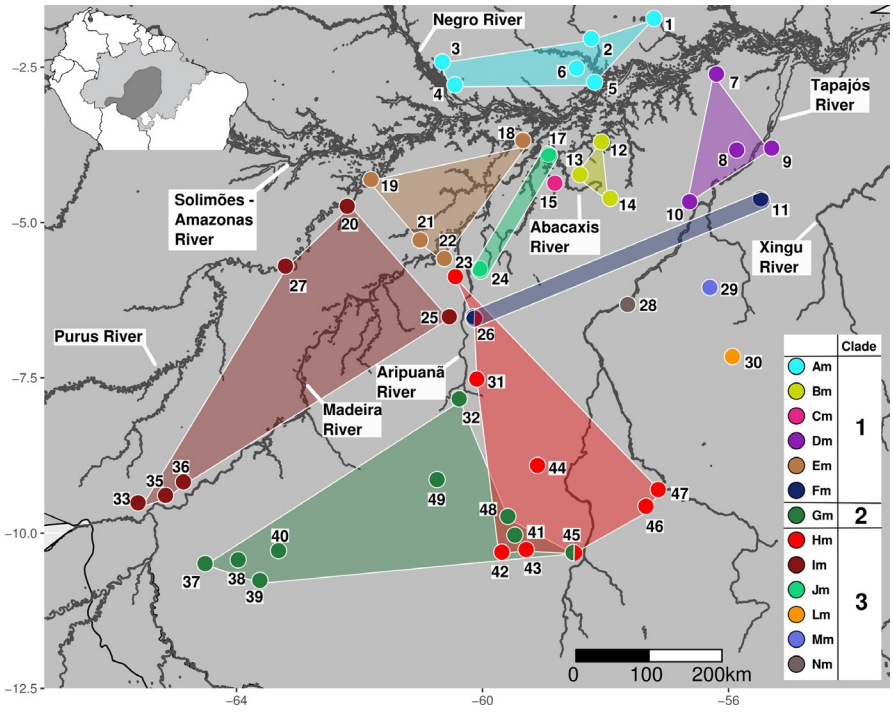


**FIGURE 1** Geographical location of all *Loxopholis osvaldoi* samples included in this study, and the distribution of mitochondrial lineages. On the inset, the Legal Brazilian Amazon (light grey) and the distribution of *L. osvaldoi* according to Ribeiro-Junior & Amaral, 2017 (dark grey). Numbers adjacent to points represent sampled localities [Colour figure can be viewed at wileyonlinelibrary.com]

those, we verified the morphology of 128 voucher specimens according to Avila-Pires (1995) and Marques-Souza et al. (2013) to confirm species identification. Our sampling covered most of the species range, except for the upper Purus River (Ribeiro-Junior & Amaral, 2017).

We extracted DNA from muscle and liver samples conserved in 100% ethanol and amplified and sequenced four loci per specimen (two mitochondrial and two nuclear genes). Extraction protocols followed Fetzner (1999). From the mitochondrial (mtDNA) genome, we amplified and sequenced fragments of the NADH dehydrogenase subunit 4 gene (*ND4)* and the Cytochrome b gene (*CYTB*). From the nuclear (nuDNA) genome, we sequenced two protein-coding gene fragments: the Neurotrophin-3 (*NT3*) and the Upstream Transcription Factor Family Member 3 (USF3) (*KIAA2018*). PCR conditions are listed in Table S3.

We phased nuclear heterozygous sequences and used them to build haplotype networks and gene trees (see below). Alignments were built in ClustalW 1.82 (Higgins, Bleasby, & Fuchs, 1992). We also used the sequence data to estimate haplotype and nucleotide diversity, the number of segregating sites, the genetic distance between mtDNA haplogroups, and to conduct neutrality tests (Table S4). To describe patterns of haplotype sharing among localities, we plotted pie charts over the species range, depicting the number of exclusive and shared haplotypes per locality (Figure 3). Additional details of the analyses and laboratory procedures are found in Appendix S1.

## 2.2 | Delimiting independent evolutionary units within *Loxopholis osvaldoi*

### 2.2.1 | Phylogenetic analyses: identifying intraspecific lineages

Aiming to delimit intraspecific lineages, we performed phylogenetic analyses using (a) each marker individually (gene trees), (b) concatenated mtDNA loci and (c) concatenated mtDNA and nuDNA datasets. We used phased haplotypes for (a), but unphased sequences for (b) and (c). We called an intraspecific lineage every monophyletic group recovered by both the mtDNA and the nuDNA datasets which included all samples from a given locality or from neighbouring localities (geographical coherence). We used two optimality criteria, maximum likelihood (ML) and Bayesian inference (BI). DNA substitution models and the best codon partition scheme were inferred with PartitionFinder v1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012), under a Bayesian Information Criterion (BIC). Sequences of the alopoglossid *Alopoglossus*, recurrently recovered as sister to all other gymnophthalmids (Goicoechea et al., 2016;Pellegrino, Rodrigues, Yonenaga-Yassuda, & Sites, 2001), were used as outgroups.

Our ML analyses were carried out in RAxML 8.2.10 (Stamatakis, 2014), through three independent searches. All searches used rapid Bootstrap and aimed at the best-scoring ML tree (-f a and -# 1,000), implementing the -m GTRCAT model of nucleotide substitution. MrBayes 3.2 (Huelsenbeck & Ronquist, 2001) was used for the BI analysis with two independent runs and four Markov Chain Monte Carlo (MCMC), starting with a random seed. Each run consisted of 10,000,000 generations, sampled at every 1,000th generation. Convergence between chains and effective sample size (ESS) values were verified in Tracer 1.6. The maximum probability posterior tree (MAP) and the posterior probability (PP) of nodes were estimated after considering a 25% value of burn-in.

### 2.2.2 | Lineage validation using Bayesian Phylogenetics and Phylogeography (BPP)

We then used BPP 3.4 (Yang & Rannala, 2010) to test whether the intraspecific lineages recovered by the phylogenetic analyses can be considered independently evolving lineages sensu Sukumaran and Knowles (2017), according to the multispecies coalescent model (MSC). Based on the nuDNA markers (NT3 and KIAA2018), we performed A11 analyses, which compare different species models while being unconstrained by a species tree topology.

Following Leaché and Fujita (2010), we ran BPP with a suite of priors on ancestral population size ($\theta$s) and divergence times ($\tau$s). Specifically, we used four combinations of priors: large ancestral population sizes and deep divergences ($\theta$s = $\tau$s = IG(3, 0.2)); large ancestral population sizes and shallow divergences ($\theta$s = IG(3, 0.2) and $\tau$s = IG(3,0.002)); small ancestral population sizes and deep divergence times ($\theta$s = IG(3, 0.002); $\tau$s = IG(3, 0.2)) and small ancestral population sizes and shallow divergence times ($\theta$s = $\tau$s = IG(3, 0.002)). For each prior configuration, we performed two separate runs with different seed values to check for congruence between results. The reversible jump MCMC was sampled 250,000 times every 2 generations, using a burn-in value of 8,000.

In addition to BPP, we tested whether candidate BPP lineages corresponded to genetic clusters using allele frequency information from the nuDNA markers in a STRUCTURE analysis (Pritchard, Stephens, & Donnelly, 2000). Detailed methods and outcomes of this test are available in Appendix S2.

## 2.3 | Species tree and dating analysis

We performed a likelihood ratio test (LRT) to verify whether the data fit a molecular clock model (null model), or if the rate on each branch varied independently (alternative model). Likelihoods of the null and alternative models were calculated in PAUP* 4.0 (Swofford, 2002), using the mtDNA ML tree. The significance of LRT statistic 2*(logL0-logLa), where logL0 is the likelihood of null hypothesis and logLa is the alternative hypothesis, was calculated through a chi-square test in R, with the function pchisq().

We used *BEAST (Heled & Drummond, 2010) to estimate a multilocus species tree, which accounts for incongruences among gene trees, and to estimate divergence times among *L. osvaldoi* lineages. We considered three partitions in this analysis: one mitochondrial (a

concatenated matrix of *CYTB* and *ND4*), and the other two nuclear partitions. We assigned samples to species according to the BPP results. Since the null hypothesis of molecular clock was strongly rejected given the data, we used an uncorrelated lognormal relaxed clock.

We explored how two different mtDNA substitution rates previously proposed for squamates affect our divergence time estimates. For that, we assumed a 'fast' and a 'slow' rate: respectively, 2.0E-2 substitutions/site/million year (Olave, Avila, Sites, & Morando, 2016;Pellegrino, Rodrigues, Harris, Yonenaga-Yassuda, & Sites, 2011;Werneck, Leite, Geurgas, & Rodrigues, 2015), and 6.5E-3 substitutions/site/million year (Guarnizo et al., 2016;Werneck, Gamble, Colli, Rodrigues, & Sites, 2012). For the 'slow' scenario, the prior on the mean rate of a relaxed clock (ucld.mean prior) for the mtDNA partition was implemented as a lognormal distribution, with mean 1.0E-2 and standard deviation of 0.2. For the 'fast' scenario, we only changed the mean of lognormal distribution to 2.0E-2. In both scenarios, the ucld.mean prior was defined as non-informative for *NT3* and *KIAA2018*, implemented as a gamma distribution with parameters shape = 0.001 and scale = 1,000.

We applied a Yule process prior to the species tree, and set the population size model as piecewise linear, with a constant root. To avoid over parameterization, we simplified the nucleotide substitution models estimated for the previous phylogenetic analyses, and used the HKY model for the three partitions (base frequencies were nonetheless estimated from the data). For each scenario, we performed two MCMCs with 50,000,000 of generations each, sampled every 10,000th generation, and combined the two log files with LogCombiner v1.8.1. We checked for convergence of model parameters and ESS values with Tracer 1.6 (Rambaut, Ho, Drummond, & Shapiro, 2008), and annotated the Maximum Clade Credibility tree with TreeAnnotator 1.8.1 (Rambaut & Drummond, 2010), after removing a 10% value of burn-in. Finally, we visualized all 50% majority-rule trees and edited them with FigTree v.1.4.2 (Rambaut, 2014) and InkScape 0.91 (www.inkscape.org).

## 2.4 | Correlates of genetic structure

Following Diniz-Filho et al. (2013) and Myers, Hickerson, and Burbrink (2017), we ran redundancy analysis (RDA) to determine how geography (distance among localities, D), environment (E) and main Amazonian rivers (R) correlate with genetic structure. We preferred RDA over the more frequently used Mantel tests (Mantel, 1967) for two reasons. First, the null hypothesis tested by Mantel analysis refers to an island model. As such, it leads to false positives (type 1 error) when the genetic data are hierarchically structured (Meirmans, 2012), which is the case for *L. osvaldoi* (see details in Results). Second, we wanted to test linear as well as nonlinear models, and Mantel tests assume linear and monotonic relationships among variables. Nonetheless, because Mantel correlograms are useful tools to explore how the genetic structure changes in different spatial scales of data (Diniz-Filho et al., 2013), we also used them to test whether genetic similarity decay differs from a null distribution. We compared the variation explained by each factor in the full model (i.e. the model containing the three variables), controlling for the other factors. Additionally, we explored whether the addition/removal of explanatory variables increased the fraction of explained genetic variance, comparing models of every possible combination of two and one explanatory variables. We compared models using the adjusted R-square, which penalizes the increase in explanatory power due to an increase in the number of explanatory variables. Additionally, we evaluated model fit in the relationship between genetic and geographical distance, using AIC. Models used in this comparison were a null model, a linear model, a second-order polynomial model, a third-order polynomial model, a logarithmic model and a Michaelis–Menten model.

Genetic structure (the response variable) was calculated from a pairwise matrix of genetic distances of nuDNA genes among all localities sampled, using Gst (Nei, 1973). We used only the nuDNA data rather than mtDNA to avoid issues related to differences in coalescence times, mutation rates and effective population size between the two genomes. Because Gst was originally developed to use alleles frequency instead of sequence data (Nei, 1973), we considered each single nucleotide polymorphisms (SNPs) of our two nuDNA datasets as a locus, using the function multidna2genind() on R package 'apex' (i.e. argument mlst = F). The pairwise Gst matrix was calculated with the R function pairwise_Gst_Nei() (package 'mmod'). The pairwise matrix of genetic distances was submitted to a principal coordinate analysis (PCoA) and the first 12 axes selected by the Broken-Stick criteria were used as the input to RDA (Diniz-Filho et al., 2013;Legendre & Legendre, 2012;Myers et al., 2017).

Explanatory variables were calculated as follows: a pairwise matrix of geographical distances was calculated using the function distgeo() in R, which we developed for this study (script available in Dryad Data Repository). Next, we extracted the eigenvectors of the geographic distance matrix, using a principal coordinates of neighbour matrices (PCNM, Borcard & Legendre, 2002), which detects patterns at different spatial scales (Borcard & Legendre, 2002;Diniz-Filho et al., 2013). Eigenvectors with positive eigenvalues were selected and composed the new geographical variable (i.e. D) in RDAs. Each locality was classified into four categories according to its position relative to main rivers crossing the distribution of *L. osvaldoi*: north of Solimões-Amazon River (n.ama), interfluve between Purus and Madeira rivers (pur.mad), interfluve between Madeira and Tapajós rivers (mad.tap) and interfluve between Tapajós and Xingú rivers (tap.xin).

For each locality, we extracted environmental data from 19 bioclimatic variables describing annual variability in precipitation and temperature, and solar radiation (WorldClim 2.0). We also extracted data from five vegetation variables from MODIS data (www.modis.gsfc.nasa.gov/data/): the fraction of photosynthetically active radiation (400–700 nm) absorbed by green vegetation (FPAR); the green leaf area per unit ground area (LAI); and three estimates of land cover, all available by vegetation continuous field (VCF) collection: percent tree cover, percent non-tree vegetation and percent non-vegetated. The

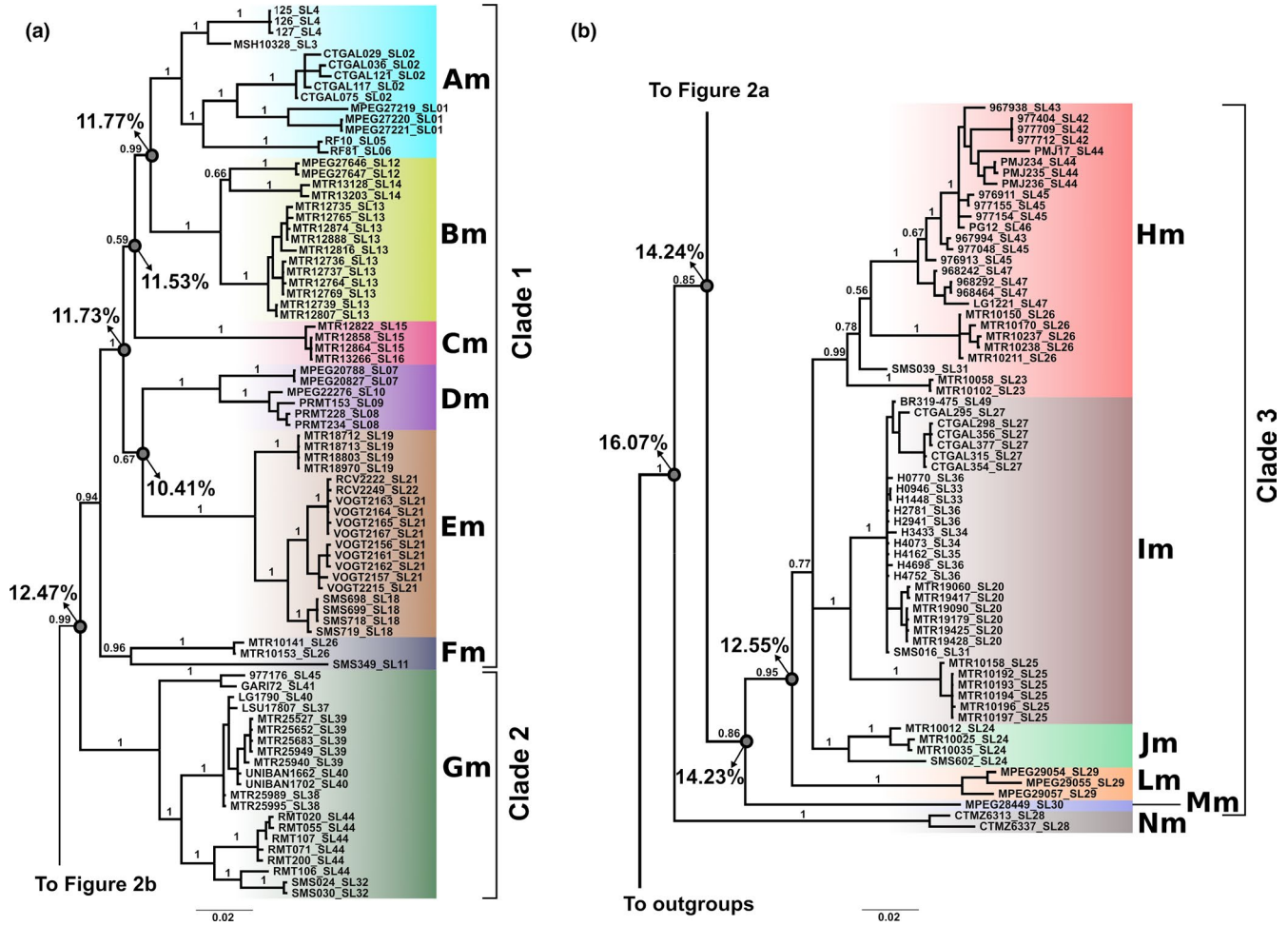


**FIGURE 2** (a and b) Maximum posterior probability tree of *Loxopholis osvaldoi* inferred with Bayesian inference from concatenated mitochondrial markers (*CYTB* and *ND4*). Numbers above nodes represent posterior probability values (left) and Bootstrap values (right) derived from maximum likelihood analysis, which recovered identical results. Terminals are named with voucher number and locality code (see Table S2 and Figure 1 for locality names and geographical locations). Colours and acronyms indicate the mitochondrial groups confirmed via Bayesian phylogenetics and phylogeography. Values next to the nodes marked with grey circles indicate pairwise average genetic distances for *CYTB* and *ND4* gene fragments [Colour figure can be viewed at wileyonlinelibrary.com]

spatial resolution of all datasets was 2.5 min. We then investigated possible correlations among environmental variables, excluding variables with variance inflation factor (VIF) greater than 3 (excluded variables: bio01, bio02, bio03, bio05, bio06, bio07, bio09, bio10, bio11, bio12, bio15, bio16, bio17 [WorldClim data], tree_dens, srad) (Figures S7 and S8). The final environmental matrices were used as inputs to the RDA.

## 3 | RESULTS

After sequence clean-up, we retained fragments of ND4 (799 base pairs [bp]), *CYTB* (411 bp), NT3 (648 bp) and KIAA2018 (618 bp), totalizing 2,476 bp. All fragments belong to protein-coding genes, except for the bases located after position 705 in the ND4 alignment, in which a stop codon ('AGA') indicates the presence of three tRNAs amplified with the primers used. Neutrality tests resulted in significant negative values only for *NT3* and KIAA2018 (Table S4 in Appendix S1). Haplotype diversity is high (0.98–0.99) in both mtDNA and nuDNA.

### 3.1 | Intraspecific lineages in *L. osvaldoi*

Phylogenetic analyses and genetic distances indicate deep phylogeographic structure within *L. osvaldoi* (Figure 2, Table S4 in Appendix S1). MtDNA trees are better resolved than nuDNA trees (Figure S4 in Appendix 1). BI and ML analyses using concatenated mtDNA data and the complete dataset (four gene regions concatenated; Figures S2 and S3) yielded trees of similar topology, structure and node support, however, differing in the position of samples MTR10141 and MTR10153 (which represent putative hybrids between distinct lineages, see Appendix S1). For the sake of simplicity, we show only the mtDNA tree (Figure 2). In general, samples from the same locality were assembled into monophyletic groups in the mtDNA tree, with few exceptions highlighted below. We identified three main mtDNA clades and 13 well-supported and geographically restricted intraspecific lineages (Figure 2): Clade 1 is composed by lineages Am, Bm, Cm, Dm, Em and Fm; Clade 2 is lineage Gm; Clade 3 is composed by lineages Hm, Im, Jm, Lm, Mm and Nm. Moreover,

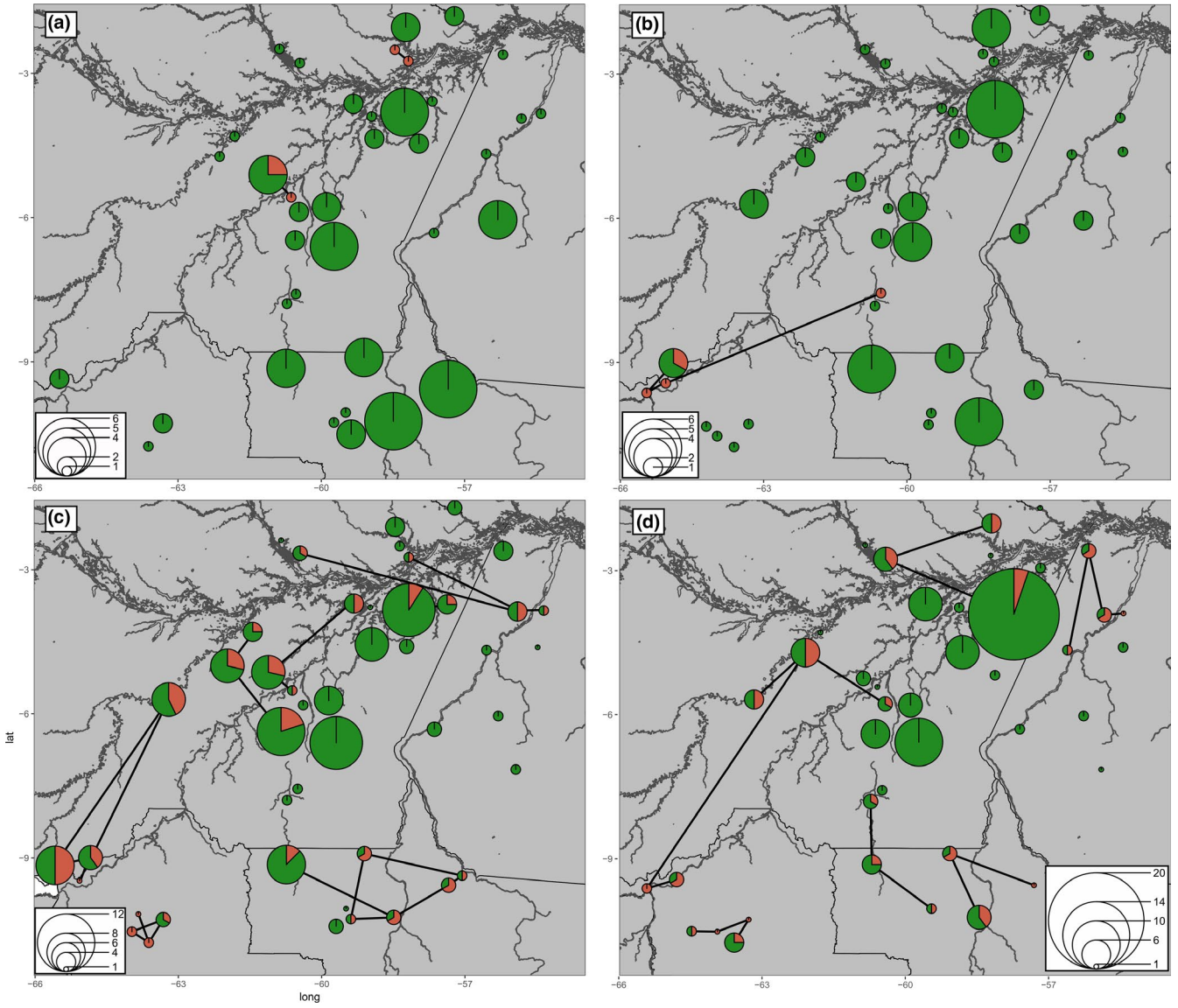


**FIGURE 3** (a–d) Pie charts with haplotype frequencies for localities sampled for mtDNA gene fragments *CYTB* (a), *ND4* (b) and nuDNA *NT3* (c) and *KIAA2018* (d). Green denotes haplotypes that are exclusive of that locality, red denotes shared haplotypes. Localities that share haplotypes are connected by lines. The figure shows higher haplotype sharing among localities in the nuclear DNA fragments. Noteworthy is the haplotype sharing of *CYTB* across the Madeira River [Colour figure can be viewed at wileyonlinelibrary.com]

significant sub-structure is observed within some lineages (e.g. Am; Figure 2). Despite the high support for lineage monophyly, most lineage relationships remain unresolved; genetic distances among lineages range from ~10% to ~16% (Figure 2).

Haplotype diversity is also high within lineages (Table S4 in Appendix S1). This result is in agreement with the observed low frequency of shared haplotypes among localities, for both mtDNA and nuDNA datasets (Figure 3). The genealogies reveal that shared haplotypes in mtDNA and nuDNA generally belong to the same lineage (Figure S1 in Appendix S1). Nucleotide diversity of nuDNA markers is about 10-fold smaller than that of mitochondrial fragments (Table S4 in Appendix S1).

Lineages are restricted geographically and also parapatrically distributed (Figure 1). However, ranges do not coincide with obvious geographical barrier, with the exception of group Am, which is delimited by the Amazon River (Figure 1) in the south. In a few cases (localities 26 and 45), samples from two lineages occur simpatrically (Figure 1). Lineages belonging to clade 1 are distributed mainly in the northeastern portion of *L. osvaldoi* range, while clades 2 and 3 are distributed to the southwest.

## 3.2 | Lineage validation

A BPP analysis supports the recognition of most intraspecific lineages (Am, Bm, Cm, Em, Gm, Hm, Im, Jm and Mm) as evolutionarily independent, regardless of prior scenarios (Table 1). However, the prior on ancestral population size (θ) strongly influenced the species delimitation results: large values of θ led to a smaller number of lineages, collapsing lineages Dm, Fm, Lm and Nm. For example, models

**TABLE 1** Summary of BPP results, showing the highest supported species delimitation model under each prior scenario

| Priors | PP | #sp | Collapsed species |
|---|---|---|---|
| Scenario 1 (↑θ and ↑T)) | 0.316 | 12 | DmFm |
| | 0.285 | 13 | — |
| | 0.152 | 11 | DmFm, LmNm |
| | 0.123 | 12 | LmNm |
| Scenario 2 (↑θ and ↓T) | 0.361 | 13 | — |
| | 0.316 | 12 | DmFm |
| | 0.108 | 12 | LmNm |
| | 0.090 | 11 | DmFm, LmNm |
| Scenario 3 (↓θ and ↑T) | 0.994 | 13 | — |
| Scenario 4 (↓θ and ↓T) | 0.994 | 13 | — |

*Note:* The geographical distribution of each collapsed species is presented in Figure 1.

Abbreviations: BPP, Bayesian phylogenetics and phylogeography; PP, posterior probability; #sp, number of species in each model.

with 13 (i.e. no lineages collapsed), 12 (Dm collapsed with Fm or Lm collapsed with Nm) and 11 lineages (Dm collapsed with Fm and Lm collapsed with Nm) received similar support values in a scenario of large θ values (Table 1). In contrast, models with small θ values largely supported the independence of all 13 lineages tested (Table 1). The τ (diversification time) prior did not influence the results (Table 1).

Sample assignment based on nuDNA allele frequency by STRUCTURE (Figure S5) was highly consistent with the previous phylogenetic and BPP results. The first round of analysis recovered the basal phylogenetic structure (clades 1, 2 and 3; Figure 2), and the second round recovered the genetic clusters, both in agreement with the intraspecific lineages described by the phylogenetic analyses and BPP. The few differences observed between phylogenies, BPP and sample assignment on STRUCTURE are detailed in Appendix S2.

### 3.3 | Species tree and timing of diversification

The lineage relationships recovered by the species tree in *BEAST were highly concordant with both mtDNA and concatenated (four markers) trees. In both scenarios, clade 1 (Am, Bm, DmFm, Cm and Em) and clade 3 (Im, Hm, Jm and Mm) were recovered with high support. The lineage Gm (clade 2) was placed as sister to clade 1, however with only moderate support (PP = 0.68 in the 'slow' scenario; 0.76 in the 'fast' scenario). The sister relationship between Am/Bm and Cm/Em received moderate support (PP = 0.7 and 0.6 respectively), and the close relationship among Im, Hm and Jm was highly supported (PP = 0.96; Figure 4).

Divergence times were highly affected by the 'slow' and 'fast' priors on mtDNA substitution rates; however, none of the scenarios are consistent with a Pleistocene Refuge Hypothesis. Under the 'slow' scenario (Figure 4a), events of lineage diversification are inferred to have occurred mostly in the mid-Miocene and Pliocene. Under the 'fast' scenario, most diversification events are estimated in the Pliocene and the Late Pleistocene (c.a. 2.0 Mya). Median values and 95% HPD dates are available in Table S5.

### 3.4 | Correlates of genetic structure

The main axis of environmental variation underlying *L. osvaldoi* lineages' range coincides with the latitudinal axis (south to north), with southern localities being subjected to more seasonality in precipitation and temperature, while northern localities underwent less seasonality, but experienced higher precipitation and temperature absolute values (Figure S6 in Appendix S3). Lineages occupy different areas of the e-space and it is reflected in the effect of IBE on genetic diversity (Table 2).

The relationship between genetic and geographical distance is best described by the third- and second-order polynomial (Table S6 in Appendix 3). Both models showed that the relationship between these variables changes with geographical distance among localities (Figure S10 in Appendix S3; Figure 5a, b). The second-order polynomial model describes a positive relationship between geographical and genetic distances at smaller geographical distances, and a negative relationship at larger geographical distances. The third-order polynomial shows similar relationships, but describes an increase in both distances at the largest geographical distances. Some localities that are very divergent genetically can be close geographically; this is observed for both intra- and inter-lineage comparisons (upper left portion of Figure 5a).

As for the correlates of genetic structure, the model with the largest adjusted R squared was the full model ($adjR^2$ = 66%, $p$ = .001) that included geographical distances, environment and rivers (Table 2, Figure S9 in Appendix S3). That being said, the most important factor to explain the genetic structure in *L. osvaldoi*, when controlling for the other variables, is geographical distance ($adjR^2$ = 40%, $p$ = .001; Table 2), supporting IBD. Rivers alone explained 21% ($p$ = .02) of the patterns observed, and the environment, 14% (although with $p$ = .08) of the genetic variation, supporting IBE. The interaction among the three factors explained 23% of the variance observed.

## 4 | DISCUSSION

Our study demonstrates that *L. osvaldoi* is composed of several independently evolving lineages. The mtDNA and nuDNA datasets support wide cryptic diversity and large genetic distances among clades and lineages, suggesting that *L. osvaldoi* is a species complex. Further integrative taxonomic studies should test whether the genetic lineages can be diagnosed by morphological characters. The high level of cryptic diversity found in *L. osvaldoi*, as observed in other leaf-litter vertebrates (e.g. Brunes et al., 2019;Geurgas & Rodrigues, 2010;Nunes et al., 2012), suggests that tests of biogeographical

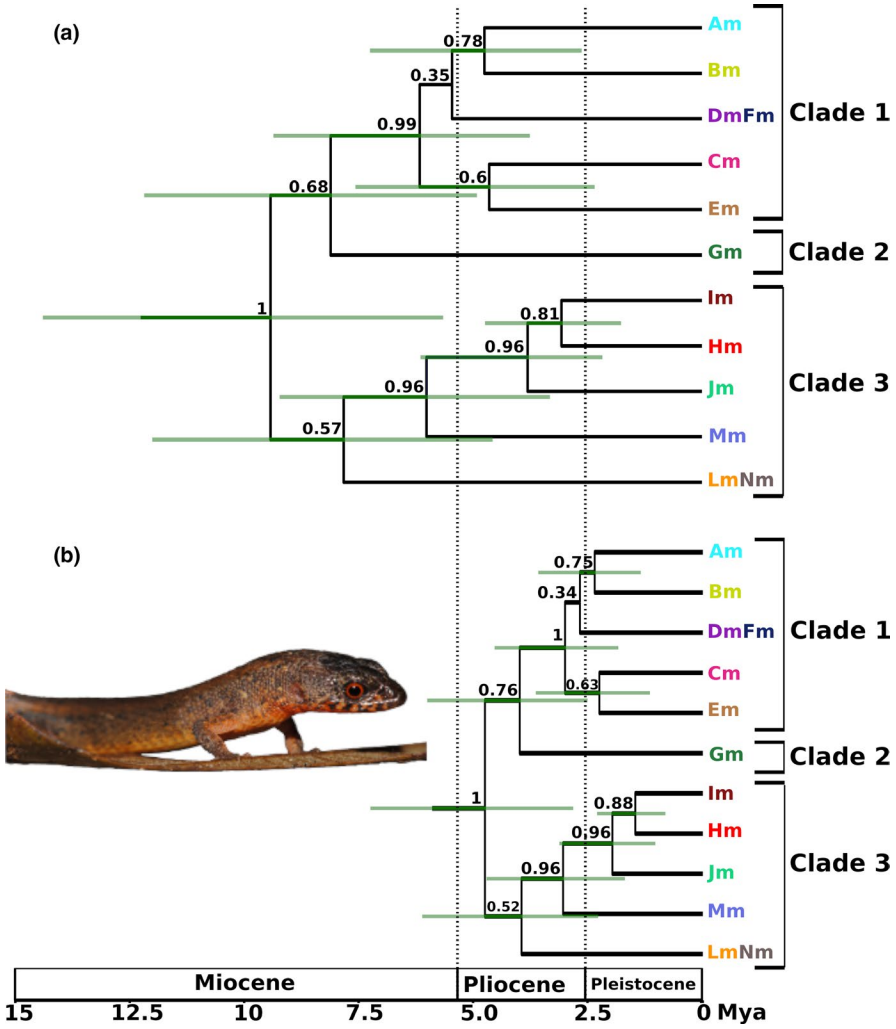


**FIGURE 4** Species trees of *Loxopholis osvaldoi* lineages under 'slow' (a) and 'fast' (b) mutation rate scenarios, showing that the diversification of *L. osvaldoi* started at least during the Pliocene. The species trees were inferred from two mtDNA and two nuDNA concatenated partitions; see text for details about the parameters used in both scenarios. Green bars at nodes represent divergence time estimates and 95% HPD intervals. Numbers above nodes represent posterior probability values. Mya, million years ago [Colour figure can be viewed at wileyonlinelibrary.com]

hypothesis based only on nominal Amazonian taxa – and hence ignoring possible levels of cryptic diversity within it (e.g. Castro-Godinho & Silva, 2018;Santorelli, Magnusson, & Deus, 2018) – are potentially prone to inaccurate and spurious conclusions.

Our datasets agree on the spatial context and levels of divergence among lineages, resulting in clear patterns, yet they are based on four loci only. Studies of high-throughput data, presently under way, should resolve phylogenetic uncertainties and provide further insight about the diversification dynamics and changes in lineage range, particularly in the Pleistocene.

## 4.1 | A matter of scale: ecological versus historical processes

### 4.1.1 | IBD and IBE

The fact that IBD explains so much of the genetic patterns observed suggests that limited dispersal plays a central role in defining phylogeographic structure in *L. osvaldoi*. Both morphological traits (small body size; Marques-Souza et al., 2013) and low level of haplotype sharing among localities indicate that dispersion and gene flow among local populations are limited. A similarly pronounced hierarchical genetic structure was found in low vagility and philopatric organisms such as the Californian Trapdoor Spiders (Bond & Stockman, 2008), small-bodied frogs in Madagascar and the Brazilian Atlantic

**TABLE 2** Summary of results of the redundancy analysis, ordered by the most supported model according to the adjusted $R^2$

| Dependent variable | Independent variables | Adjusted $R^2$ | $R^2$ | *p* value |
|---|---|---|---|---|
| G | D, E, R | 0.66 | .97 | .001 |
| G | D, E | 0.56 | .80 | .001 |
| G | D, R | 0.44 | .58 | .001 |
| G | D | 0.40 | .50 | .001 |
| G | R | 0.21 | .06 | .025 |
| G | E, R | 0.19 | .17 | .043 |
| G | E | 0.15 | .12 | .079 |

Abbreviations: D, geographical distance among localities; E, environmental distance among localities; G, genetic distance (Gst) among populations; R, geographical position of locality relative to main rivers (see text for details).

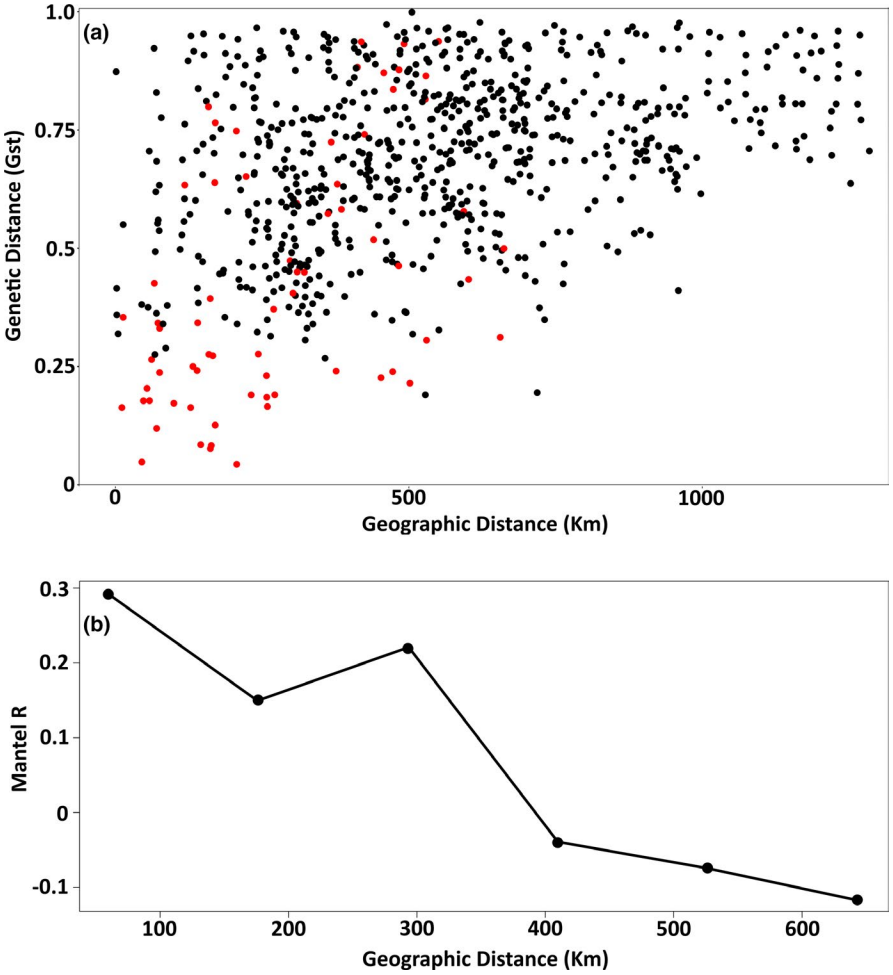


**FIGURE 5** (a) Scatter plot of pairwise genetic distances (Nei´s Gst) and geographical distance among localities. Red dots represent pairwise comparisons among localities recovered as belonging to the same lineage (see text for explanation). (b) Mantel correlogram presenting the R statistic of Mantel test calculated for each geographical distance class [Colour figure can be viewed at wileyonlinelibrary.com]

Rainforest (Fusinatto et al., 2013;Pabijan, Wollenberg, & Vences, 2012 respectively), South American land planarians (Álvarez-Presas, Sánchez-Gracia, Carbayo, Rozas, & Riutort, 2014) and rock-dwelling geckos (Werneck et al., 2012).

Our analyses also demonstrate that the correlation between genetic and geographical distances is not linear (see Supplementary Material). Mantel correlogram and model fit tests indicated that IBD exerted its greatest effect at small, local geographical scales. Yet very close localities can have extreme values of genetic distances, either very low or very high. This is due mainly to the parapatric distribution of divergent lineages. Additionally, in geographical distances larger than 400 km, the effect of geography is constant and very high.

How can IBD ultimately generate the parapatric distribution of lineages recovered here? One hypothesis is that dispersal limitation and selection against hybrids led to the appearance of stochastic phylogeographic breaks, in the presence of IBD and the absence of geographical barriers or environmental gradients (Hoelzer, Drewes, Meier, & Dorsat, 2008;Irwin, 2002;Kuo & Avise, 2005). We provisionally reject this hypothesis for *L. osvaldoi*, as the general congruence between patterns in mtDNA and multilocus nuDNA genomes, as shown by BPP and STRUCTURE analyses, suggest that historical or ecological factors defined the restricted and parapatric distribution of lineages. As pointed by Kuo and Avise (2005), the convergence to the same spatial pattern by independent markers is unlikely under a model of stochastic phylogeographic breaks.

## 4.2 | Rivers, refuges and natural history traits

Variance partitioning analysis pointed that rivers explain a significant portion of the genetic structure, after controlling for other explanatory variables – a result that may be driven by the apparently geographical restriction of the Am lineage to the north bank of the Solimões–Amazonas rivers. However, unlike birds and primates (Naka & Brumfield, 2018;Ribas et al., 2012;Roosmalen, Roosmalen, & Mittermeier, 2002), lineage turnover in *L. osvaldoi* fails to coincide perfectly with main river courses. It may, however, be explained by river dynamics in the Amazon. It has been shown that present-day courses of Amazon tributaries were unstable over geological time (Ruokolainen et al., 2018). Major changes in the Madeira river course were already elucidated (e.g. Rossetti et al., 2014), and new data reveal more dramatic changes, supporting the hypothesis that the Madeira and Purus rivers were connected between 13 and 25 Kyr (Ruokolainen et al., 2018). Due to erosion, evidence of avulsion events older than 50 Kyr in Central Amazonia may be unavailable. However, a similar dynamics of drainage capture could have occurred at different times, provoked by sporadic floods since the

Miocene–Pliocene (Ruokolainen et al., 2018). Considering the small differences in elevation that separates most of the drainage basins in the Amazonia, we argue that river capture caused by floods could have led to deep changes in the landscape, and carry with them small animals living in the forest floor, favouring passive dispersal, isolation and dramatically altering their distributions.

Our data revealed that most *L. osvaldoi* lineages originated between 10 and 2.5 Mya, which ca. 100 times older than the Madeira River avulsion (Ruokolainen et al., 2018). In this context, we hypothesize that lineage distribution within this species may reflect changes of paleo-river courses dating back to the Miocene and Pliocene, and that those spatial patterns have remained until today due to the poor dispersion capacity of the species. Alternatively, *L. osvaldoi* may have undergone a period of rapid diversification immediately after its divergence from its sister group (*Loxopholis guianense;* Marques de Souza unpublished data), which is distributed on the east portion of the Amazonia. This would explain the lack of support for the relationships among its main lineages, as recovered by the phylogenetic analyses.

Despite a possible scenario of turbulent drainage dynamics as predicted by Ruokolainen et al. (2018), the restricted distribution of lineage Am on the northern bank of Solimões–Madeira River indicates some stability in the course of this river in Central Amazonia since at least the Pliocene, when the Am lineage diverged from its sister group Bm. Considering that the evidence of rearrangement in paleo-courses of Amazon tributaries is still limited to tens of thousands of years (Ruokolainen et al., 2018), testing the above hypothesis for species complexes, which have originated during the Miocene–Pliocene, is a great challenge for Amazonian biogeographers.

Molecular dating does not show support for a Pleistocene divergence of *L. osvaldoi* lineages, as the period of major lineage formation was inferred to have occurred between 10 and 2.5 Mya (Miocene to Pliocene). This result is consistent with those reported for other Amazon leaf-litter lizards as *Chatogekko amazonicus* (Geurgas & Rodrigues, 2010), *Iphisa elegans* (Nunes et al., 2012), as well as birds (Ribas et al., 2012;Tilston-Smith et al., 2014) and several other groups (for a review, see Turchetto-Zolet, Pinheiro, Salgueiro, & Palma-Silva, 2013). It is nonetheless possible that *L. osvaldoi* underwent rage and population size reductions due to Pleistocene climatic fluctuations, especially given that the species is restricted to the forest environment. While the dataset utilized here does not have enough resolution to test for such Pleistocene population fluctuations (results not shown), novel analyses with high-throughput data, presently under way, may clarify the impact of Pleistocene fluctuations on *L. osvaldoi* and on the forests of the Central Amazon.

We reveal deep genetic structure within *L. osvaldoi* and three environmental factors that correlate with such structure. Other leaf-litter vertebrates are remarkably similar to it – especially regarding levels of genetic structure and spatial organization of lineages (e.g. Fouquet et al., 2014;Geurgas & Rodrigues, 2010;Nunes et al., 2012;Rojas et al., 2018). However, not all Amazonian reptiles share the same pattern: natural history traits appear to influence genetic structure since traits determine long-term dispersal capacity of species, and hence impact gene flow and admixture (Paz, Ibáñez, Lips, & Crawford, 2015;Zamudio, Bell, & Mason, 2016). For example, arboreal lizards, such as *Anolis fuscoauratus*, *Dactyloa punctata*, *Gonatodes humeralis* or even the arboreal pitviper *Bothrops bilineatus*, are not similarly structured. Arboreal species are much less structured genetically in the Amazon, and were able to colonize the Atlantic Rainforest (except *G. humeralis)*, showing much greater dispersion capacity (Dal Vechio, Prates, Grazziotin, Zaher, & Rodrigues, 2018;Pinto et al., 2019;Prates et al., 2016). Genetically structured leaf-litter (i.e. terrestrial) lizards have small body sizes, like *Loxopholis*, *Chatogekko* and *Iphisa*, while those arboreal species have larger body sizes and are less structured. Formal tests about the effects of natural history traits will further clarify how genetic structure of the Amazon herpetofauna was formed and modified.

## 5 | CONCLUSIONS

In the face of the deep levels of genetic divergence that we uncovered among seemingly cryptic lineages of *L. osvaldoi*, we argue that any meta-analysis of Amazon biogeographic patterns based solely on taxonomic data will run into the risk of telling us only part of the story behind the local and regional structuring of biodiversity. Focusing on nominal taxa ignores other layers of diversity that reveal non-straightforward distribution patterns and their underscoring processes; in Amazonia, these layers seem even more pervasive and important. A better understanding of Neogene river dynamics will be crucial to new biological studies in the region: coupled with dated phylogenies and historical demography studies, it will provide new perspectives and insights on the role of rivers as drivers of biotic diversification in the Amazon Basin. We suggest that future studies employing molecular information to understand the causes and timing of diversification of the Amazonian biota explore multiple substitution rates or dating mechanisms; the lesson from *L. osvaldo*i is that this prior can have a dramatic influence on estimated divergence dates. We hope to have illustrated that ecological, historical and landscape factors other than rivers may have had profound effects on the distribution of genetic diversity in the Amazon, and that modelling these impacts should not invoke a simple, linear relationship.

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## DATA AVAILABILITY STATEMENT

All R scripts used in this manuscript are available at Dryad Data Repository (https://doi.org/10.5061/dryad.fttdz08nj). The sequence data generated in this manuscript have been submitted to the GenBank and the acession numbers are available on the Table S2 in Appendix S1.

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## BIOSKETCH

**Sergio Marques-Souza** is a post-doc at Universidade de São Paulo, Brazil. His main research interest are focused on the biological processes by which the Neotropics achieved it outstanding biodiversity, using lizards as a focal organism, as well as investigate the distribution and systematics of Neotropical biota.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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