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Research Article

When a name changes everything: taxonomy and conservation of the Atlantic bushmaster (*Lachesis* Daudin, 1803) (Serpentes: Viperidae: Crotalinae)

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The South American bushmaster *Lachesis muta* is currently considered a valid taxon with no recognized subspecies. However, its wide distribution with discontinuous populations, the restricted sampling of molecular data in previous studies, and the inherent difficulty in obtaining diagnostic data, preclude a detailed evaluation of the taxonomic status of this taxon. In this study, we use high-resolution genomics, as well as venomic, morphological, and ecological data to infer the speciation processes involved in the evolutionary history of *L. muta* populations. Our data recognize the Amazon populations as *L. muta* and the Atlantic Forest populations as *L. rhombeata*. *Lachesis rhombeata* has lower genetic diversity than *L. muta* and we recommend a critical evaluation to include this species in the national and international red lists of endangered species. Moreover, we propose classifying the *L. rhombeata* population from the Baturité massif (Ceará state) as an evolutionarily significant unit (ESU) for conservation and we also found genetic substructure within *L. muta* populations. We expect our results will contribute to the taxonomic stability of the

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Neotropical fauna and highlight the urgency of protecting Atlantic Forest populations of *Lachesis* species and, consequently, the landscape that this species and related biota inhabit.

Key words: endangered species, evolution, pit viper, systematics, viperid snakes

Introduction

The South American bushmaster, *Lachesis muta* (Linnaeus, 1766), is a medically important venomous snake widely distributed in South America, occurring in Surinam (type locality), Peru, Colombia, Ecuador, Venezuela, French Guiana, Guyana, Trinidad and Tobago, Bolivia (Campbell & Lamar, 2004; Uetz et al., 2023), and Brazil (Hoge, 1965; Nogueira et al., 2019; Pereira-Filho et al., 2020; Rodrigues et al., 2013). Until the early 2000s, two subspecies were recognized: *L. muta muta* (Linnaeus, 1766), which occurs in the equatorial forests of the Amazon region, and *L. muta rhombeata* (Wied-Neuwied, 1824), which is endemic to the Atlantic Forest of Brazil (Hoge, 1965). Unlike *L. muta muta*, *L. muta rhombeata* is rare and was classified as vulnerable by the Martins & Marques (2000) and as data deficient in the past by the 2008 Brazilian Red List (Martins & Molina, 2008).

Lachesis muta muta was described by Linnaeus (1766) as a large snake with connecting black dorsal diamond spots, dark postocular stripes, and tail without a rattle and full of rows of small spiky spines. The Atlantic forest subspecies, *L. m. rhombeata* was described by Wied-Neuwied (1824) and, according to Hoge (1965) and Hoge and Romano (1978), this taxon can be distinguished from *L. muta muta* by the combination of the following characters: very large and distinct spots on the top of the head, a wider postocular black stripe, no wide and well-contrasted light stripe between the black postocular stripe and the upper surface of the head, a rather triangular rostral shield, a very bright reddish ground colour, and supraoculars strongly contrasted by the surrounding black spots.

Lachesis muta shares a preference for primary undisturbed forests (Argôlo, 2004; Cunha & Nascimento, 1993). However, human exploitation began in the 16th century mainly in the Brazilian Atlantic Forest and has substantially increased in the last 50 years (de Lima et al., 2020), restricting the potential occurrence areas of this taxon. Even so, this biome is still recognized as a global biodiversity hotspot for conservation (Myers et al., 2000). This scenario has led *L. muta* to be categorized as facing severe threats throughout its distribution in the Atlantic Forest. In Ceará state, for example, the species is classified as critically endangered (SEMACE, 2022) and is restricted to the Baturité massif, one of the mountainous remnants of Atlantic Forest in north-eastern Brazil within the Caatinga morphoclimatic domain. In the north-eastern states of Bahia and Pernambuco (north-eastern Brazil), *L. muta* is classified as vulnerable (SEMA, 2017; SEMAS, 2017). In addition, the first finding of *L. muta* in Paraíba state after decades of faunal inventories raised concerns about the species' conservation status in the region (Rodrigues et al., 2013).

Based on genetic differences, Zamudio and Greene (1997) kept the South American populations of *L. muta* at the subspecific level as *L. m. muta* and *L. m. rhombeata*, although the Amazonian populations of Mato Grosso were recognized as more closely related to the Atlantic forest populations. However, this evaluation was based on a limited number of loci ($n = 2$) and individuals of *L. m. muta* ($n = 9$) and *L. m. rhombeata* ($n = 3$) (see Zamudio & Greene, 1997). Fernandes et al. (2004) conducted an extensive systematic review of the genus, employing phylogenetic analyses combining morphological and molecular data. The results robustly confirmed the taxonomy of *L. stenophrys* and *L. melanocephala* and found *L. m. muta* to be paraphyletic. These authors pointed out that the Atlantic forest population significantly differs from the Amazon and Mato Grosso state individuals, mostly on the number of ventral scales (see Fernandes et al., 2004, table 3). Despite these results, the phylogenetic analysis of Fernandes et al. (2004) recovered specimens from Mato Grosso state as more closely related to *L. m. rhombeata*, corroborating the findings of Zamudio and Greene (1997). Based on these results, Fernandes et al. (2004) considered *L. muta* as a full species, with no subspecies.

The following questions arise from observed differences in living individuals during fieldwork, from variations in specimens after exploratory examination in scientific collections, and from disparities noted by Fernandes et al. (2004): (i) a morphological trait distributed randomly across two distinct species, such as the postocular stripe found in Mato Grosso specimens from the Amazon resembling that in Atlantic specimens, may not indicate recent or historical hybridization. Instead, it could be an ancestral trait that has been retained (e.g., Meusel & Schwentner, 2017); (ii) morphological features traditionally regarded as diagnostic, such as the spots on the top of the head, may not hold taxonomic significance in *Lachesis*; and (iii) admixed specimens, such as the supposed hybrid individuals from Mato Grosso, are common in at least 10% of animal species

(Mallet, 2005). Rather than precluding speciation, such admixture often represents an essential component of the speciation process (Schluter, 2009). Thus, the differences among the populations of the Atlantic Forest, Mato Grosso, and the Amazon, currently classified as *L. muta*, need clearer species delimitation. We aim to reassess their identities by integrating molecular, ecological, physiological, and morphological data (e.g., Grobler et al., 2023; Melville, Chaplin, Hipsley, et al., 2019; Vacher et al., 2017).

This study aimed to conduct a comprehensive evolutionary study seeking the identity of the Atlantic Forest populations of *L. muta.* Therefore, we employed an integrative taxonomic approach using high-resolution data on thousands of loci of single nucleotide polymorphisms (SNPs) as molecular markers, venomics profile, morphology, and ecology. SNPs can be used successfully in vertebrates at the interface between population genetics and phylogenetics, exploring species boundaries and gene exchange (see Melville, Chaplin, Hutchinson, et al., 2019). Additionally, we evaluate the evolutionary position of the populations from the Mato Grosso state and revisit the nomenclature of *L. muta* from the Amazon region and Atlantic Forest. We expect our results will help to assess the taxonomic status of the Atlantic populations of the bushmaster, demonstrating the urgency of protecting it.

Materials and methods

Taxon sampling

DNA data. We obtained ethanol-preserved tissue samples from zoological collections (see Acknowledgements). Genomic DNA was extracted from shed skin, liver, or muscle samples using PureLink Genomic DNA (Invitrogen) or DNeasy Blood & Tissue (Qiagen) kits. The DNA concentration was estimated using the NanoDrop™ One spectrophotometer (Invitrogen) or the Qubit fluorometer (Invitrogen). Genomic SNPs were obtained using the DArTseq™ hybridization-based sequencing technology (Heller-Uszynska et al., 2010; Georges et al., 2018; https://www.diversityarrays.com/products-and-services/) at the Diversity Arrays Technology lab (University of Canberra, Australia). The SNP calling and filtering steps of the 53 individuals was conducted into R using the dartR package (available at https://cran.r-project.org/web/packages/dartR/index.html). The SNP calling and filtering steps of the 53 individuals was conducted into the R program. We filtered SNP matching the following criteria: (1) average read depth < 4, (2) average repeatability $< 90\%$, (3) loci call rate $< 70\%$, (4) individual call rate $< 80\%$, and (5) remove secondaries, which are multiple SNP loci within a fragment likely to be linked.

Species delimitation analyses

We adopted the general lineage concept of species (DE Queiroz, 2007), considering species as a separately evolving metapopulation lineage. We defined four operational taxonomic units (OTUs) based on the literature (Fernandes et al., 2004; Zamudio & Greene, 1997): Amazon Forest populations, Atlantic Forest populations, and Mato Grosso state populations. Additionally, we further considered specimens from Baturité massif, state of Ceará, as the fourth OTU since this population is disjunctively distributed from the others and this biogeographic region is rich in endemic taxa (Roberto & Loebmann, 2016). We evaluated the following set of parameters as criteria for species delimitation (*sensu* DE Queiroz, 2007):

Population structure. The test for population structure with the SNP dataset was determined using the model-based clustering method implemented in fastSTRUCTURE v. 1.0 (Raj et al., 2014) and the non-model-based principal coordinate analysis (PCoA) (Gower, 1966). We ran fastSTRUCTURE assuming the OTUs, for K ranging from 1 to 5 to obtain a reasonable range of values for the appropriate model complexity required to explain the structure in the data. The output files included the mean Q value for each individual, defining the mean probability of belonging to one of the populations K1 to K5 (Raj et al., 2014). The best K was determined by considering the estimates predicted by fastSTRUCTURE and by assessments of whether the retrieved populations occur in unique and distinct biogeographic regions, are allopatrically or continuously distributed, and coincide with previous taxonomic arrangements. To elucidate the genetic relationships between the samples, we used PCoA as a suitable complementary method without relying on model-based assumptions nor the OTUs from the codominant genetic distance matrix generated from the SNPs. The first principal components related to a large amount of inertia produce the structuring of the genetic data (Jombart et al., 2010). The levels of population structure retrieved by fastSTRUCTURE and PCoA were assessed using 1,000 bootstrap iterations across loci for pairwise genetic distance using Euclidean Distance of allele frequencies and the fixation index (FST) using the dartR package (Gruber et al., 2018).

We complemented the population genetic structure analysis by analysing the sequenced specimens' geographic distribution data with the SNPs data. We used

the TESS3 approach (Caye et al., 2016) and the tess3r R package (http://bcm-uga.github.io/TESS3_encho_sen) using default parameters for $K = 1$–10, with 10 repetitions for each value of K, to estimate individual ancestry coefficients and genetic groups considering the sample geographic coordinates. We also calculate individual admixture coefficients using the sparse non-negative matrix factorization algorithm (sNMF) implemented in the LEA R package (Frichot & François, 2015; Frichot et al., 2014). The sNMF algorithm estimates ancestry independently for each individual in a map and does not require a priori assumptions about population membership. We used EEMS v0.0.0.9 (Estimated Effective Migration Surfaces) to characterize gene flow in a spatial context (Petkova et al., 2016). We generated a genetic distance matrix using the program bed2diffs of the same package. We used five EEMS runs, each with an MCMC chain of 20,000,000 iterations with 10,000,000 iteration burn-in and 9999 thinning iterations. The number of geographic demes was set at 200 (Petkova et al., 2016). Surface maps were estimated using rEEMSplots in R v4.3.1 (R Development Core Team, 2010).

Population genetic parameters. Populations were assigned based on the outcomes of "fastStructure" and "PCoA" population structure analyses. We estimated the observed heterozygosity (Ho), the genetic diversity or expected heterozygosity (He) which describes the proportion of heterozygous genotypes expected under Hardy–Weinberg equilibrium (Nei, 1973), and the mean absolute allele frequency differences between loci using the dartR package (Gruber et al., 2018).

Bayesian coalescent-based species delimitation analyses. This analysis was performed in BPP 4.2.9 using the method proposed by Yang and Rannala (2010). Conceptually, coincident splits at multiple loci in gene trees for a sample of individuals (reciprocal monophyly) can support the existence of genetically isolated subpopulations and recognize groups that have experienced no recent gene flow as potential species. The analysis named A10 uses the multispecies coalescent (MSC) model and the reversible jump Markov chain Monte Carlo (rjMCMC) algorithm to move between different species-delimitation models compatible with a fixed guide tree (Rannala & Yang, 2013; Yang & Rannala, 2010). Therefore, we used the output tree from the SNP-based phylogeny and assigned all individual sequences to their OTUs. In BPP, the parameters population size (θ) and root age (τ at the root node) were assigned gamma priors $\theta \sim G\ (3, 0.002)$ and $\tau \sim G\ (4, 0.0015)$, respectively. We also explored the influence of different gamma priors on the posterior probabilities, and the results were similar to the prior assignment.

Tree topology with divergence times. A Bayesian tree with posterior probabilities values was inferred in BEAST2 v2.6.3 (Bouckaert et al., 2019) using the SNPs entire sequences of the final concatenated loci (a total matrix of 543,174 bp). Running parameters were the following: HKY substitution model with estimated frequencies; strict clock; coalescent, constant tree prior; MCMC chain with 10,000,000 generations, the first 10% discarded as burn-in, and 10,000 trees saved. We used the generalized squamate substitution rate of 2.4×10^{9}/year/site (Green et al., 2014; Pasquesi et al., 2018) to estimate the posterior density of divergence times.

Lineages characterization. We submitted the separately evolving metapopulation lineages, ultimately identified by the species delimitation approach, to further analyses using high-resolution data on ecology, venomics, and morphology in order to better characterize and compare other biological aspects of such lineages.

Niche modelling and niche overlap data. Considering the results of species delimitation analyses, we performed species distribution modelling based on geo-referenced species records compiled from the literature (Barrio-Amorós et al., 2020; Citeli et al., 2020 – except Colombia and Ecuador data; Díaz-Ricaurte et al., 2017, 2020; Machado, 1956; Nogueira et al., 2019; Pereira-Filho et al., 2020). We gathered 758 unique records for *L. muta*, assigned to the results of Amazon and Atlantic Forest genetic lineages. To address potential sampling bias, we reduced the records for each lineage using a 50 km spatial filter. For both lineages, we had enough occurrence records to compute species distribution models (SDMs). For these, we used 210 unique records for the Amazon sample and 49 unique records for the Atlantic Forest sample (Supplemental Files S4).

We obtained 19 bioclimatic variables from CHELSA v1.2b (Karger et al., 2017), as provided by the PaleoClim database (www.paleoclim.org; Brown et al., 2018). The current climate dataset represents average climatic conditions between 1979 and 2013 at a spatial resolution of 2.5 arc minutes. To address the sensitivity of correlative SDMs to multicollinearity among environmental predictors, we computed pairwise Spearman rank correlations between all possible predictors under current conditions across the species background area. We then selected only one variable among pairs with $rho^2 > 0.75$. The final dataset included isothermality (bio3), annual temperature range (bio7), mean temperature of

the wettest quarter (bio8), precipitation of the wettest quarter (bio16), precipitation of the driest quarter (bio17), precipitation of the warmest quarter (bio18), and precipitation of the coldest quarter (bio19).

To estimate the impact of past climatic fluctuations on the potential distributions, we used the downscaled palaeoclimatic scenarios provided by Brown et al. (2018) for the following Pleistocene periods: late-Holocene, Meghalayan (4.2–0.3 ka); mid-Holocene, Northgrippian (8.326–4.2 ka), v1.0; early-Holocene, Greenlandian (11.7–8.326 ka), v1.0; Younger Dryas Stadial (12.9–11.7 ka), v1.0; Bølling-Allerød (14.7–12.9 ka), v1.0; Heinrich Stadial 1 (17.0–14.7 ka), v1.0; and Last Interglacial (ca. 130 ka), v1.0. Previous studies (Fordham et al., 2017; Otto-Bliesner et al., 2006) provided the original data downscaled and reformatted by Brown et al. (2018).

Next, we compared the realized niches of the Amazon and Atlantic Forest lineages using n-dimensional hypervolumes (Blonder et al., 2014). Because this technique requires an orthogonal niche space, we first computed a principal component analysis based on the environmental conditions at the species locality records. Only principal components (PCs) with eigenvalues > 1 were considered. As an algorithm for niche space delimitation, we used support vector machines, as implemented in the hypervolume package for R (Blonder et al., 2018). Niche overlaps were quantified using the Jaccard and Sørensen indices. Then, we estimated the potential distribution of the clades using Maxent 3.4.4 (Phillips & Dudík, 2008; Phillips, Anderson, et al., 2017; Phillips, Dudík, et al., 2017). Maxent is a presence-pseudoabsence approach that estimates the potential geographic distribution of a species based on environmental conditions at the specimens' locality recording and randomly selected background sites. Given the almost continent-wide distribution of the species, we selected an area defined by circular buffers of 500 km around the records of each clade as the environmental background. When modelling the potential distribution of a species using Maxent, model performance may be optimized by testing different regularization multipliers and combinations of feature classes (Warren & Seifert, 2011). Therefore, for model fitting, we tested various regularization multipliers (ranging from 0.5 to 2.5, in 0.1 steps, 5 and 10) and combinations of feature classes (L, LP, LQ, LH, LT, LQP, LQH, LQT, LPH, LPT, LHT, LQPT, LQHT, LPHT, and LQPHT; where L = linear, P = product, Q = quadratic, H = hinge, and T = threshold) (see Ginal et al., 2022). Each combination was used to compute 25 single Maxent models, each time using 80% of occurrence records model fitting and 20% used for model testing using a bootstrap approach (see below). Using Maxent's raw output, we calculated the corrected Akaike Information Criterion (AICc; Warren & Seifert, 2011) for each replicate and used the average performance per combination to guide our decisions. Finally, the most suitable combination of settings was determined based on the lowest average AICc and AUCTest above 0.7 (AUC = Area under the ROC curve; Swets, 1988). We computed the final SDMs using a bootstrap approach with an 80:20 split in 100 replicates for model training and testing using the optimal settings. The predictions in clog log format were averaged across 100 replicates to compute the final maps. As a presence-absence threshold, we applied the average omission threshold of 5%, assuming a 5% error rate in the species records.

Projections of potential distributions across space and time may require extrapolation beyond the environmental training range of the models, leading to increased uncertainty (Elith et al., 2010; Engler & Rödder, 2012). To address this issue, we computed MESS (Multivariate Environmental Similarity Surfaces) maps to identify areas prone to extrapolations and where the results should be interpreted cautiously. All areas that required extrapolation in each geological time scale were then masked from the respective potential distributions. All model tuning was automated using customized R scripts and the following packages: raster (Hijmans, 2016), dismo (Hijmans et al., 2017), and ENMeval (Kass et al., 2021).

Venomics data. Venom samples of *L. muta* from the Amazon (Pará and Acre states of Brazil), Atlantic Forest (Pernambuco and Alagoas states of Brazil), and Mato Grosso state were individually collected. Snakes were milked by massaging the venom glands, and the venom was collected into vials. Crude venoms were centrifuged at low speed to remove cells and debris, lyophilized, and stored at −20 °C until use. Crude venoms (1.8–2.0 mg) were suspended in a 5% acetonitrile solution, centrifuged again to remove debris, and separated by reverse-phase HPLC using a Teknokroma Europa C18 column (0.4 × 25.0 cm, 5 mm particle size, 300 Å pore size). Venom fractions were eluted using 0.1% TFA (solution A) and 0.1% TFA in acetonitrile (solution B) as follows: 5% B for 5 min, 5–25% B for 10 min, 25–45% B for 60 min, 45–70% B for 10 min, and 70% B for 5 min, with protein detection at 214 nm. Protein fractions were compared with previously identified toxins from *L. muta* proteomes (Madrigal et al., 2012; Otero et al., 1998; Pla et al., 2013; Sanz et al., 2008).

Morphological data. We collected skull data from six Brazilian alcohol-preserved specimens in different localities in the Amazon, Mato Grosso, and the Atlantic Forest. These specimens are housed in the Coleção de Serpentes Instituto Vital Brazil (IVB), Universidade Federal do Acre (UFAC), and Museu de História Natural do Ceará Prof. Dias da Rocha (MHNCER): IVB001 (Santa Maria Madalena, Rio de Janeiro), IVB2908 (Valença, Bahia), UFAC665 (Rio Branco, Acre), IVB3636 (Colíder, Mato Grosso), and MHNCER558 and 559 (Pacoti, Ceará). Skull data were used to evaluate putative diagnostic characters, as these morphological data were not used in previous studies. The skull examination was conducted using computed tomography (CT) at CENABIO/UFRJ employing a high-energy microCT scan (microPET/SPECT/CT LabPET8, Gamma Medica). To avoid preparation and fixation artefacts, all images were enhanced, and the main bones were isolated with 3D Slicer software (Kikinis et al., 2014). Skull terminology follows Cundall and Irish (2008). Additional data on squamation, biometrics, and colour pattern were obtained from the literature, mostly from Fernandes et al. (2004) and Ganança and Hingst-Zaher (2015).

Results

Species delimitation analyses

DNA data. A total of 14,853 binary SNPs were obtained from 53 specimens of *L. muta* by DArTseq™ sequencing (Supplemental File S1). After performing quality control and filtering with dartR (see Materials and methods), 53 genotypes (Fig. 1; Supplemental File S2) and 7926 binary SNPs were subjected to further analyses.

Population structure. SNP-based PCoA axis 1 explained 29.3% of the total variance, and PCoA axis 1 and 2 combined explained 39.3% of the total variance (Fig. 2A). The distribution of the two dimensions created by PCoA on all 53 specimens supported the population structure into three well-defined clusters. Group 1 included 17 individuals from the Atlantic Forest (blue colour), group 2 consisted of 17 individuals from the Amazon (red colour) and nine from the Mato Grosso (pink colour), and group 3 included 10 individuals from the Baturité massif, in the Brazilian state of Ceará (green colour) (Fig. 2A). FastSTRUCTURE showed the highest likelihood values for $K = 2$ (Fig. 2B). The 17 individuals from the Atlantic Forest and 10 from the Baturité massif were homogeneous for a second component (Fig. 2B, in blue). On the other hand, 17 individuals from the Amazon and nine from Mato Grosso were homogeneous for a single genetic component (Fig. 2B, in red). Four individuals from the Amazon in Pará state and one individual in Mato Grosso state presented admixture proportions varying from 12% to 87% (Q) with the blue cluster, although other individuals from the same sites showed no admixture (Fig. 2B). We retrieved well-defined Atlantic Forest and Amazon populations in a scenario with $K = 3$, with a substructure within the Amazon population (Supplemental Fig. S1).

The TESS approach to visualize the spatial distribution of ancestries and inferred genetic groups in the *L. muta* population at $K = 2$ and $K = 3$ agreed with the samples from Amazon and Mato Grosso belonging to the same genetic group and indicates a larger-scale spatial pattern associated with their spatial proximity, but distinct from the Atlantic sample at $K = 2$ (Fig. 3A) and both are distinct from Ceará sample at $K = 3$ (Fig. 3B). The TESS predicting modelling indicates that this result reaches beyond the geographic coverage of our sample. Similarly, the map of the admixture composition of sNMF shows that the genetic clusters were partitioned in the geographic space of Amazon and Atlantic Forest for $K = 2$ (Fig. 3C) and Amazon, Atlantic Forest, and Ceará state for $K = 3$ (Fig. 3D), with some admixture. The best-supported number of clusters for sNMF admixture analysis was $K = 5$, which does not match any biological population.

Spatial population genetic structure was also supported by the Estimated Effective Migration Surfaces analysis, which detected reduced gene flow between the Atlantic Forest samples (including the Ceará samples) and Amazon samples (including the Mato Grosso samples) in an area corresponding to the South American diagonal of open formations comprising the Caatinga, Cerrado, and Chaco biomes (Fig. 4A). The analysis further shows a higher effective diversity within the Amazon sample (Fig. 4B). All the above analyses are in accordance, showing a strong population structure between Amazonian and Atlantic Forest populations, with restricted gene flow between these two populations.

Population genetic parameters. The overall within-sample FST was 0.36, suggesting that the allele frequencies within our sample differed moderately. Populations were assigned based on the outcomes of the population structure analyses: the Amazon population, including the Mato Grosso samples, and the Atlantic Forest population, excluding the samples from the Baturité massif. We observed that the Baturité massif population discriminated by the PCoA was retrieved as monophyletic in

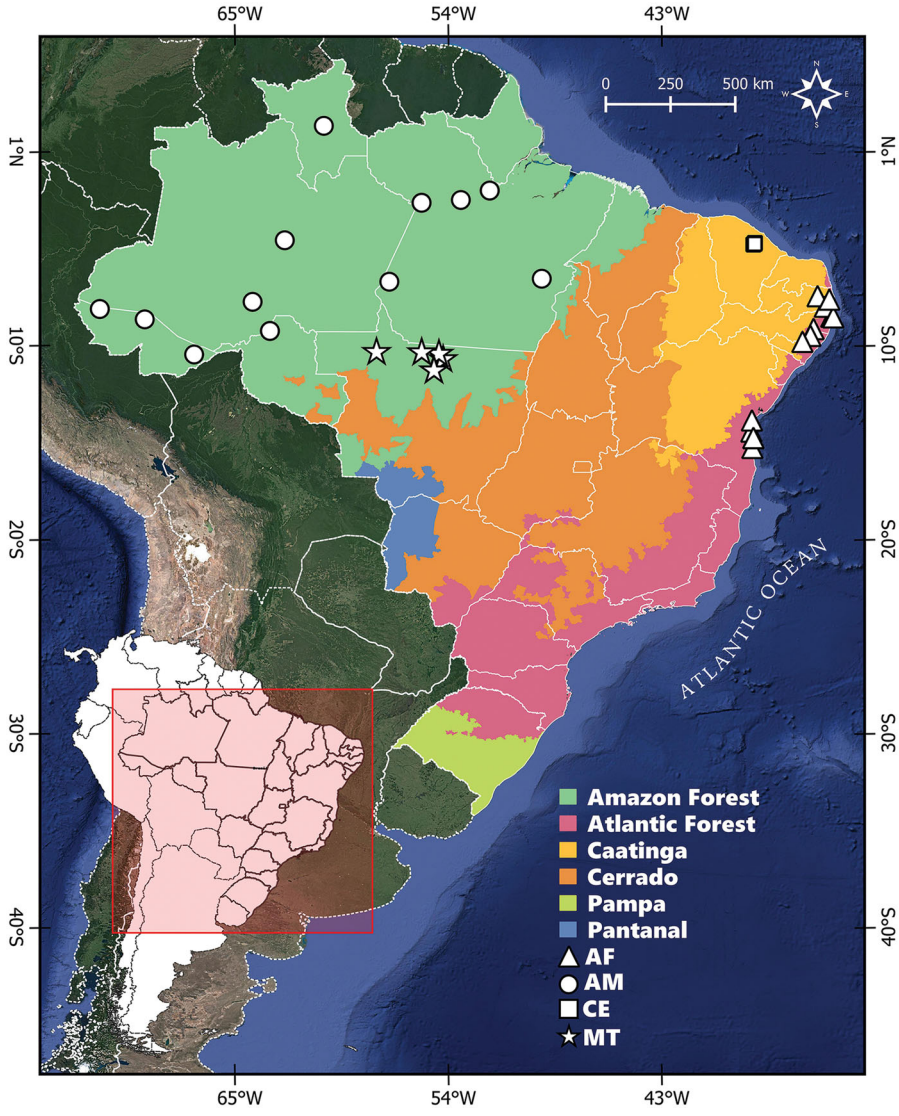

Fig. 1. The South America map depicting the sampling sites for tissue samples used in this study. The six distinct colours display the major biomes found along the species distribution. Symbols represent the specimens' assignment: AF: Atlantic Forest; AM: Amazon Forest; CE: Baturité massif in Ceará state; MT: Mato Grosso state.

the time tree. Additionally, species delimitation analysis (see below) revealed it as a distinct lineage. It is important to highlight that this population had a small sample size ($n = 10$), which is insufficient for presenting robust data on population genetics parameters.

The expected heterozygosity (He) was higher than the observed heterozygosity (Ho) values when we considered the Amazon and Atlantic Forest as a single population unit, referred to as "pooled *L. muta*" in Table 1. The observed genotypic frequencies were incompatible with those expected under Hardy–Weinberg equilibrium, suggesting some structuring when all samples are treated as a single population. Nevertheless, when considering the Amazon and Atlantic Forest as separate lineages, He and Ho revealed similar values to those of the Atlantic Forest population, showing compatibility with those expected under Hardy–Weinberg equilibrium. Moreover, the Amazon sample showed He still higher than Ho (see Table 1 and Fig. 4B), suggesting even more structuring within. Population genetic parameters revealed that the Amazon population harboured higher levels of genetic diversity (He) than the Atlantic population (Table 1). The genetic distance (18%) and FST (0.341) between them indicate distinct allele frequencies among populations. Additionally, these populations had 53 loci from which no alleles are shared with one another (fixed alleles), the Amazon sample having 6941 private alleles and the Atlantic Forest population 538 (Table 1).

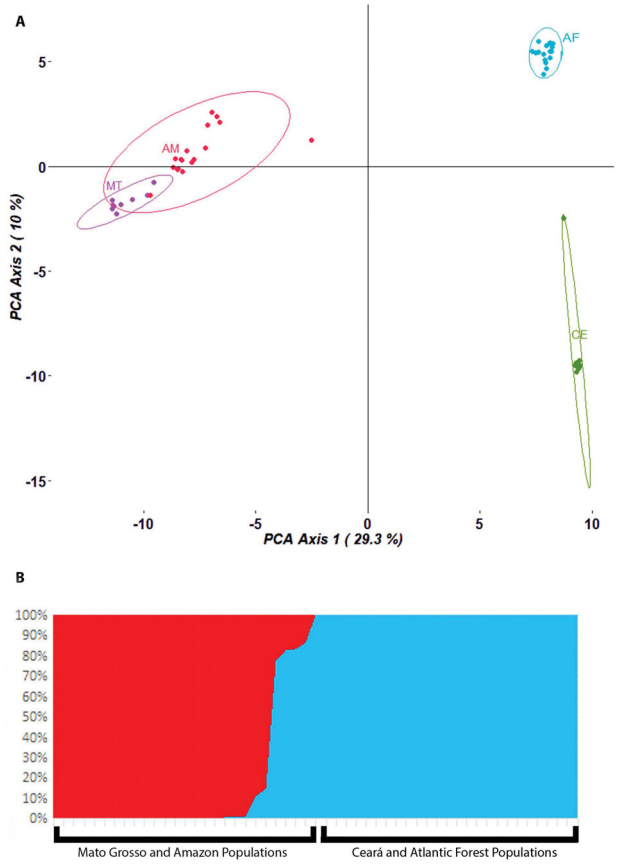

Fig. 2. Population structure analyses of 7926 SNPs of 53 individuals of *Lachesis*. (A) A distance-based (PCoA) plot of genetic distance between samples. The percentage of variation explained by each PCoA axis (PC) is in parentheses. (B) A fastSTRUCTURE plot of the Bayesian framework at $K = 2$. Atlantic Forest (AF), Amazon (AM), Baturité massif, Ceará state (CE), and Mato Grosso state (MT).

Bayesian coalescent-based species delimitation (BPP). The species arrangement with the highest posterior probability (0.93) grouped the OTU Mato Grosso within the OTU Amazon and consisted of three distinct major lineages for the populations currently assigned to *L. muta*: (1) the Amazon *L. muta*, with samples from the Amazon rainforest in the Brazilian states of Acre, Amazonas, Rondônia, Pará, Mato Grosso, and Roraima; (2) the Atlantic Forest *L. muta*, with samples from the states of Alagoas, Bahia, Paraíba, and Pernambuco; and (3) *L. muta* from the Baturité massif in Brazilian state of Ceará (Supplemental File S3).

Tree topology with divergence times. The tree obtained by aligning 543,174 bp from the 7926 SNPs retrieved all samples from the Atlantic Forest (composed of representatives from the Brazilian states of Alagoas, Bahia, Paraíba, and Pernambuco) and the Baturité massif individuals (Figs 1, 3) as sister clades with full support (Fig. 5). This arrangement is consistent with the population structure assigned by PCoA and Bayesian species delimitation analyses. The tree retrieved samples from the Amazon, including Mato Grosso (Figs 1, 3), with a more complex evolutionary substructure, with one individual from Pará more closely related to the clade Atlantic Forest + Baturité massif. The divergence between the Atlantic Forest and the "Ceará" clade was estimated at 125,000 years. The origin of the Baturité massif + Atlantic Forest clades dates back to 210,000 years, whereas the oldest split of the Amazon lineages occurred 280,000 years ago.

Circumscription of lineages

Niche modelling. The statistical test for the SDMs assessed their discriminatory ability in terms of AUCtest and AUCtrain (Supplemental Table S1; Fig. 7). In the SDM computed for the Atlantic Forest population, the environmental factor that most contributed to explaining the distribution pattern was the precipitation of the driest quarter (bio17, 34.1%), followed by precipitation of the warmest quarter (bio18, 21.4%) and temperature annual range (bio7, 21.2%). For the Amazon *L. muta*, the most influential factor was precipitation of the coldest quarter (bio19, 16.9%) and the second-largest contributing factor was isothermality (bio3, 23.619.6%). The full summary of variable contributions to the model can be found in Supplemental Table S1.

Principal component analysis for the hypervolume revealed three principal components with eigenvalues > 1 (Supplemental Fig. S2; Supplemental Table S2). PC1 was correlated mainly with isothermality (bio3), temperature annual range (bio7), and precipitation of the driest and coldest quarters (bio17 and bio19). PC2 correlated most strongly with mean temperature of the wettest quarter (bio8) and precipitation of the wettest quarter (bio18). PC3 was driven by the mean temperature of the wettest quarter (bio8). The hypervolume analysis revealed that Amazon *L. muta* occupies the largest realized niche (Fig. 6, during the last 130 ky and Fig. 7), whereas Atlantic Forest *L. muta* occupies a much smaller realized niche, which is almost entirely nested within the former (Figs 6, 7).

Venomics data. We compared venom data between Atlantic Forest ($n = 3$) and Amazon populations ($n = 2$) (Fig. 8). Unfortunately, we had no venom samples from the population of the Baturité massif. Our chromatograms indicated that the venom of adult *L. muta* from the Amazon and Atlantic Forest shares a conserved venom profile. This profile corresponds to key molecules previously identified by specific literature,

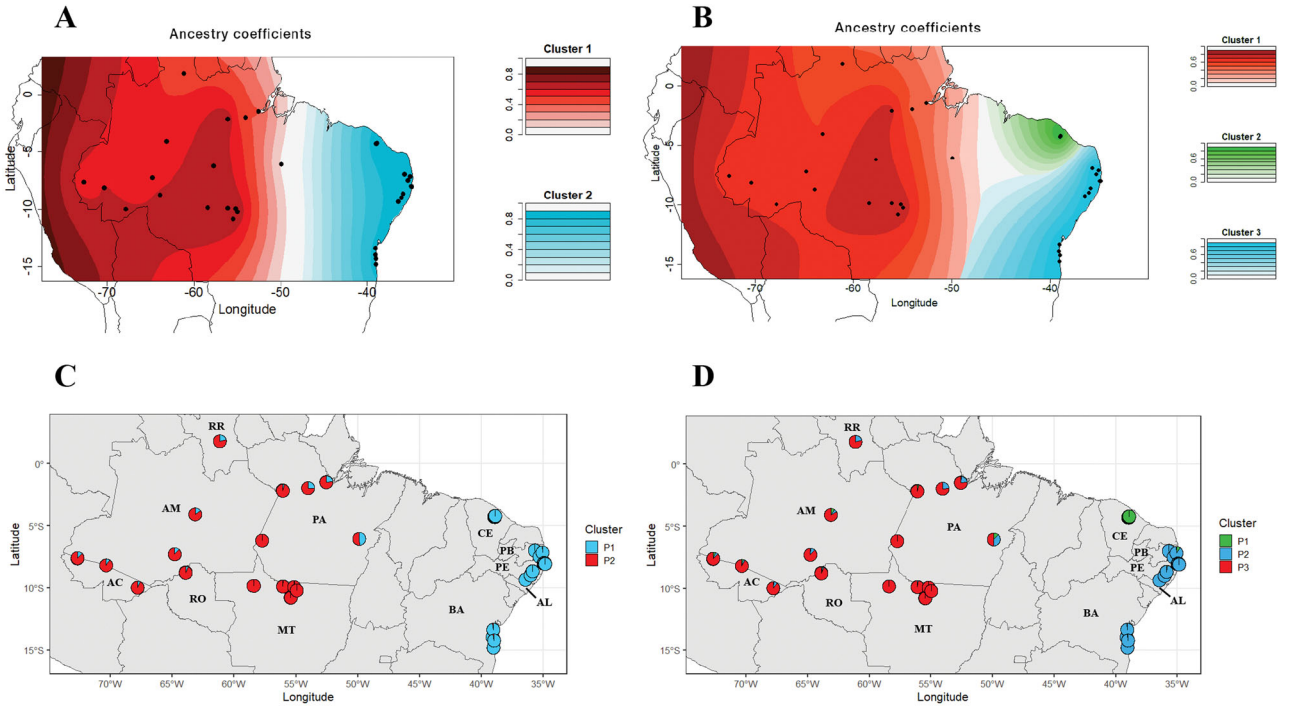

Fig. 3. The spatial population structure estimated with TESS3, the gradients illustrate the interpolated membership coefficients for each cluster, using $k = 2$ (3 A) and $k = 3$ (3B). Below are pie plots of sNMF admixture proportions for each individual for $k = 2$ (3 C) and $k = 3$ (3D). They are the 53 individuals sampled in our study. Points are coloured based on the predominant population assignment from admixture analysis. Brazilian states of Acre (AC), Amazonas (AM), Roraima (RR), Pará (PA), Rondônia (RO), and Mato Grosso (MT) in the Amazon; and the Brazilian states of Bahia (BA), Alagoas (AL), Pernambuco (PE), Paraíba (PB) and Ceará (CE) in the Atlantic Forest.

including bradykinin-potentiating peptides (BPPs) eluted between 10 and 30 min; phospholipases A_2 (PLA_2) and of the D49 subfamily (40–50 min); snake venom vascular endothelial growth factors (svVEGFs), 45–55 min; snake venom serine proteases (SVSP), C-type lectin-like proteins (CTL), cysteine-rich secretory proteins (CRISPs) between 55 and 70 min; class PI and PIII snake venom metalloproteinases (SVMPs), and L-amino acid oxidases (LAO) from 70 to 90 min. In particular, a large quantity of bradykinin-potentiating peptides (BPPs) was observed in *L. muta* venom. Our chromatographic data showed a considerable overlap of venom profiles, as expected for individuals of the genus *Lachesis* (Fig. 8H). The major venom variations observed show ontogenetic shift, since our sampled juvenile *L. muta* with 74 cm in total length from the Atlantic Forest showed evident quantitative variations, especially in the elution region of SVMPs and LAOs (70–90 min). Our data also indicated quantitative ontogenetic changes in the bradykinin-enhancing peptides (BPPs) concentration in *L. muta* venom. Compared with adult venom, the concentration of BPPs in the juvenile was lower at 15 min and higher at 25 min of elution. Furthermore, the amount of serine proteases and PI metalloproteinases (SVSPs and SVMPs) was lower at 80–90 min of elution. In fact, variation in lachesis venoms has been observed mainly in ontogenetic changes rather than in geographic and interspecies (Madrigal et al., 2012; Sanz et al., 2008). A second peak in the chromatogram, corresponding to a PLA_2 (Fig. 8A) in the venom of the Amazonian specimen from Rio Branco (Acre state), at an elution time of 40–45 min, demands further investigation.

Morphological data. All specimens ($n = 6$, Fig. 9) examined showed similar skull morphology, with minor variations related to the differences in body sizes and/or the positioning of the skull and mandibles during preparation (e.g., the position of suspensorium and mandibles during fixation may rotate articulation points, changing the anatomical position of the bones).

General morphology. Although we found no distinct differences, we present the first general description of the skull of *Lachesis* using specimens from the Atlantic Forest (IVB001 from Santa Maria Madalena, Rio de Janeiro; IVB2908 from Valença, Bahia) (Fig. 9). The snout is positioned anteromedially relative to the maxilla, with no overlap. The anterior portion of the braincase is widened, encompassing the maxilla, prefrontals,

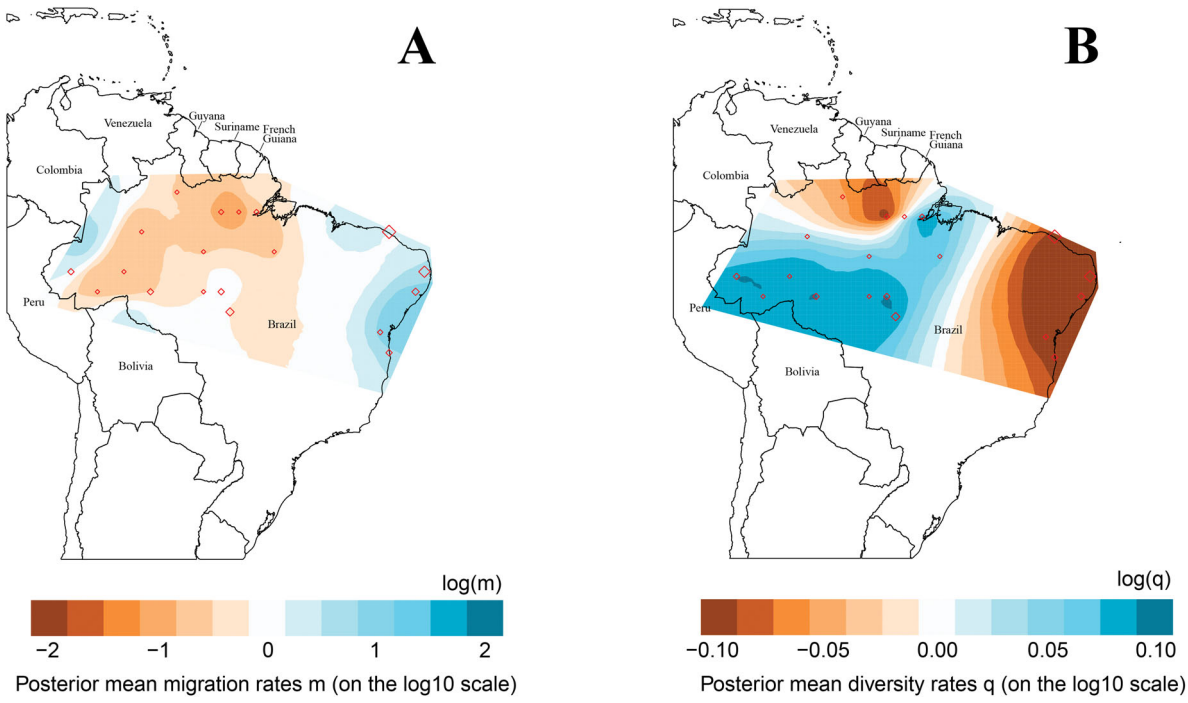

Fig. 4. (A) The estimated effective migration surfaces (EEMS) plot shows the effective migration rates (m) on a log10 scale for *Lachesis muta*. Geographic regions of reduced gene flow are presented in orange-brown, representing hypothesized migration barriers. Areas in blue represent geographic regions where samples are connected with gene flow higher than that expected under isolation by distance. Dots represent the sampled localities, with dot sizes proportional to sample sizes. (B) Effective diversity rates (q), with orange-brown representing areas of lower diversity in the Atlantic Forest region whereas blue representing higher diversity in the Amazon region.

Table 1. Population genetics data for the main groups of *Lachesis muta* assigned by fastSTRUCTURE and PCoA using the 7926 SNPs.

Group	N	He	Ho	PA	FA	GD	FST
Pooled *L. muta*	52	0.09	0.04				
Amazon	25	0.11	0.06	6941	53	18%	0.341
Atlantic Forest	17	0.02	0.01	538			

Pooled *L. muta:* individuals from both populations indifferently; N: sample size; He: expected heterozygosity; Ho: observed heterozygosity; PA: private alleles; FA: fixed allelic difference; GD: genetic distance. Samples from the Baturité massif (Ceará state) were not included in this analysis.

frontals, and postorbitals; the latter are located perpendicular to the longitudinal axis of the skull and represent the widest region of the braincase. The posterior portion of the braincase is progressively tapered from the descending process of the postorbital to the mesodorsal portion of the parietal; the latter being the narrowest portion of the braincase. Supratemporals are parallel to the longitudinal axis of the skull and with the rear portion connected to elongated quadrates obliquely oriented and with the rearmost portion laterally rotated. The orbits are relatively short, bounded by the lateral margins of the frontals and postorbitals. In ventral view, the anterior and posterior portions of the skull are wider, and the middle portion is narrower. Palatines and pterygoids are medially positioned relative to the maxilla, extending throughout the oral cavity roof, while the parabasisphenoid and basioccipital form the floor of the braincase. Mandibles are slightly arched with prominent pre-articular crests. Retroarticular process of the compound bone is expanded, facing the medial portion of the skull.

Discussion

Setting snake species boundaries is often challenging because morphological data may not reveal species-level units (e.g., Hamdan et al., 2017). However, our inability to identify unambiguous morphological diagnostic characters should not hamper species recognition. Species arise due to the dynamic nature of the entire landscape, and in this process, speciation with some polymorphism in morphological traits is predictable (Wiens & Servedio, 2000). Indeed, phenotypic conservatism may be common among representatives of the herpetofauna (despite strong genetic structure), sometimes indicating

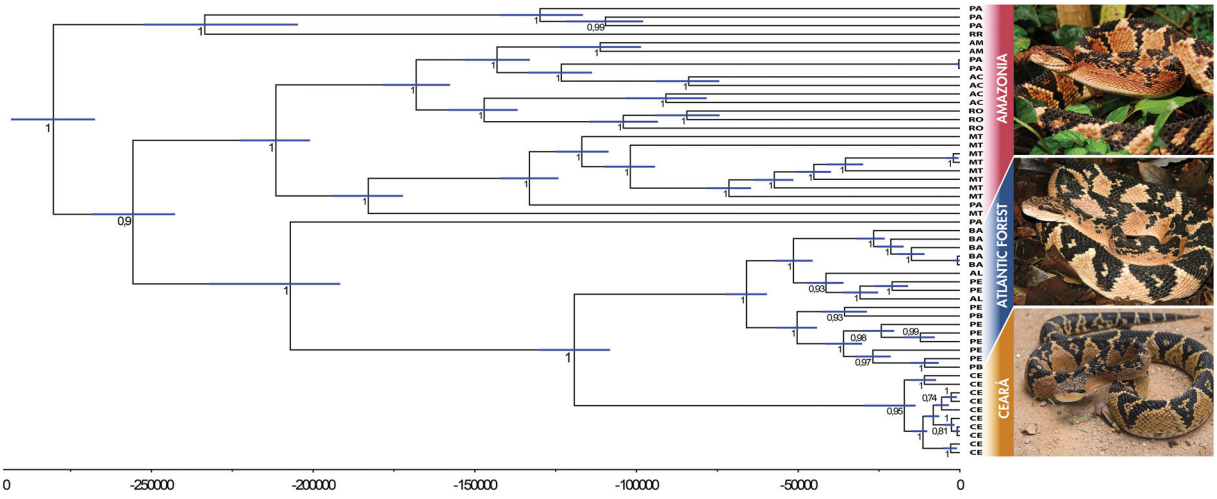

Fig. 5. Bayesian dated tree of 53 specimens of *Lachesis* using 543,174 bp from 7796 SNPs. Branches supported by posterior probability. The scale axis shows time in thousands of years, and the blue bars represent 95% confidence intervals. Brazilian states of AC: Acre; AM: Amazonas; AL: Alagoas; BA: Bahia; CE: Ceará; MT: Mato Grosso; PA: Pará; PE: Pernambuco; PB: Paraíba; RO: Rondônia; RR: Roraima. Photos by Carlos Cintra (Amazonia), Thabata Cavalcante (Ceará), and Marco Freitas (Atlantic Forest).

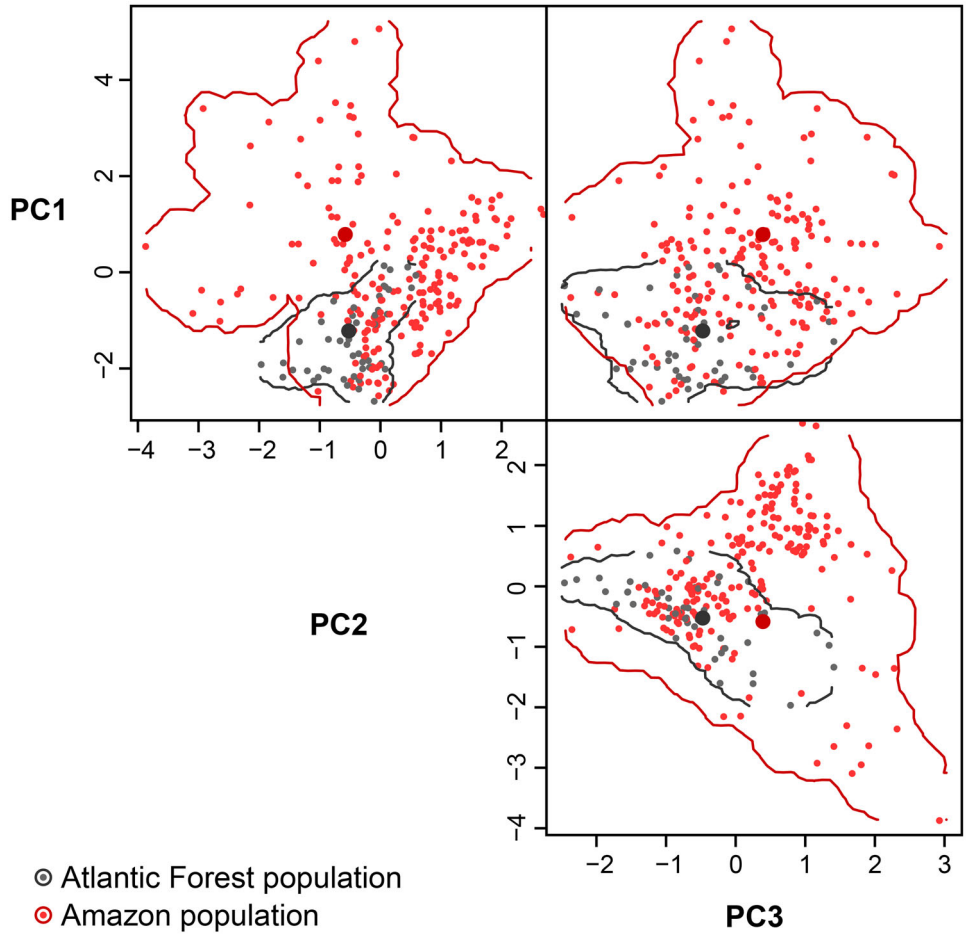

Fig. 6. Comparison of the realized niche expansion of the Atlantic Forest (grey) and South America Amazon (red) populations of *Lachesis* in the environmental space. Black and red lines indicate the estimated boundaries of the realized niche spaces, large points their respective centroids and small points indicate the species records used for computation. Comp. 1–3 refer to the first three principal components (for details see Supplemental Table S2).

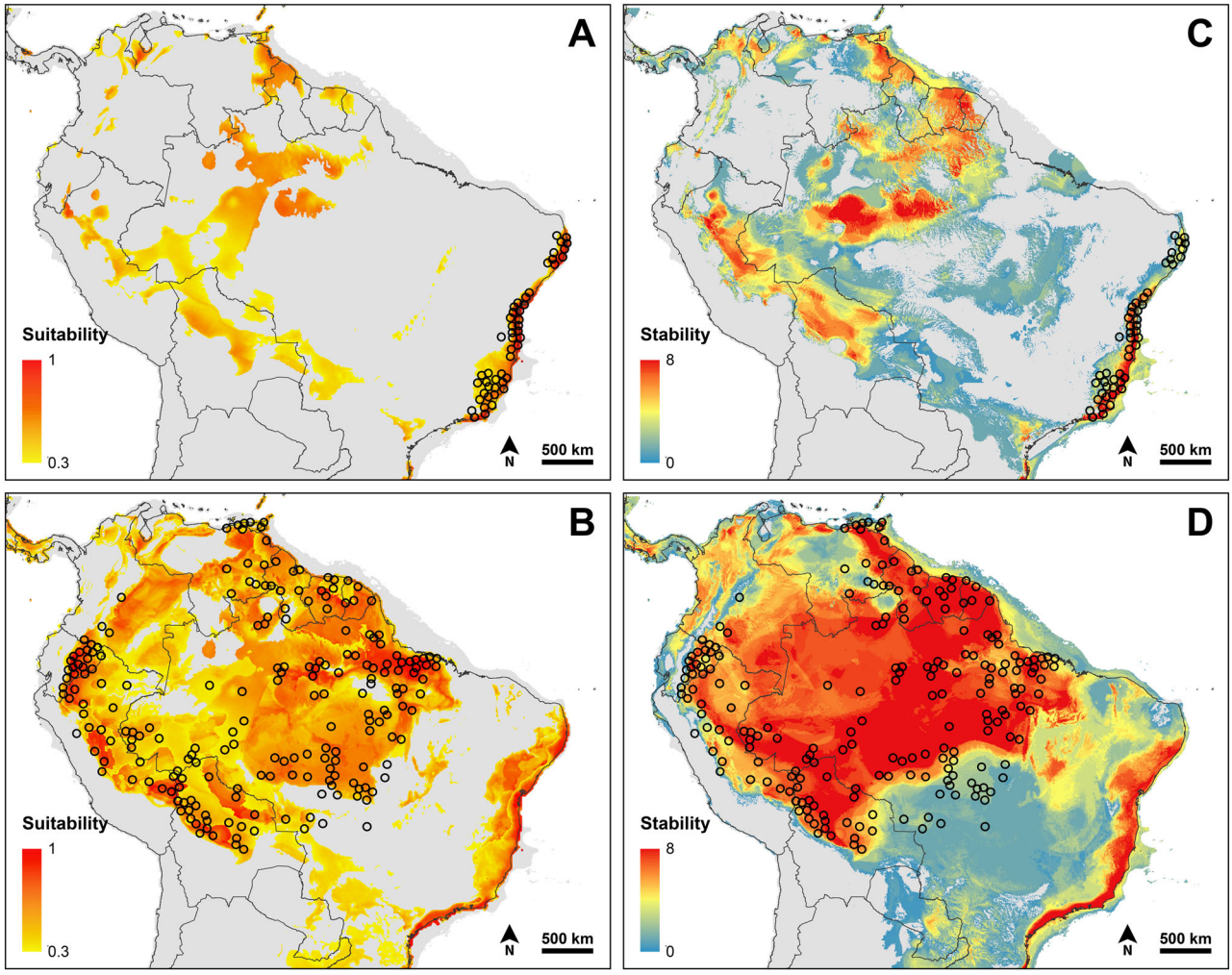

Fig. 7. Current potential distribution (A, B) and stability through time (C, D) for *Lachesis* lineages from the Atlantic Forest (A, C) and Amazon (B, D). The colour range (C, D) represents the areas providing suitable environmental conditions during the time slices in the Pleistocene: late-Holocene, Meghalayan (4.2–0.3 ka); mid-Holocene, Northgrippian (8.326–4.2 ka); early-Holocene, Greenlandian (11.7–8.326 ka); Younger Dryas Stadial (12.9–11.7 ka); Bølling-Allerød (14.7–12.9 ka); Heinrich Stadial 1 (17.0–14.7 ka); and Last Interglacial (ca. 130 ka). Stability scores indicate how many time slices from a given area were suitable. A score of eight (super red) suggests suitability in all time slices while a stability in only one time slice is indicated in blue.

a dominant role of stabilizing selective forces (e.g., Kaefer et al., 2013).

These mentioned issues are corroborated when one observes our species delimitation results, which unambiguously support the hypothesis that *Lachesis* populations from the Amazon (including the specimens from the Mato Grosso state) and the Atlantic Forest are two separately evolving lineages (Figs 2–5, Table 1, BPP results), even though our data on skull morphology were unable to find differences to distinguish them. The significantly large genetic distance and FST values highlight and deepen the distinct genetic structure in the Amazon and Atlantic Forest populations. The high number of private alleles – those exclusive from a single population – although there may be other alleles for the same locus, added to the 53 fixed loci in a single population (Table 1), also support this finding (Georges et al., 2018). Typically, species populations do not mate randomly across the landscape. Barriers, ranging from physical (such as mountains and arid regions between pairs of vicariant forest-dwelling species) to ecological and morphological (such as differentiation in reproductive cycles and differentiated anatomy of copulatory organs) may prevent gene flow, leading to genetic drift or mutation (Montgomery, 1987). As populations become isolated, allelic frequencies differ due to drift, selection, and mutation that are eventually fixed, acquiring a fixed allele difference (Haldane, 1927). Finally, the importance of distinct bioclimatic variables such as the precipitation of the driest quarter for Atlantic

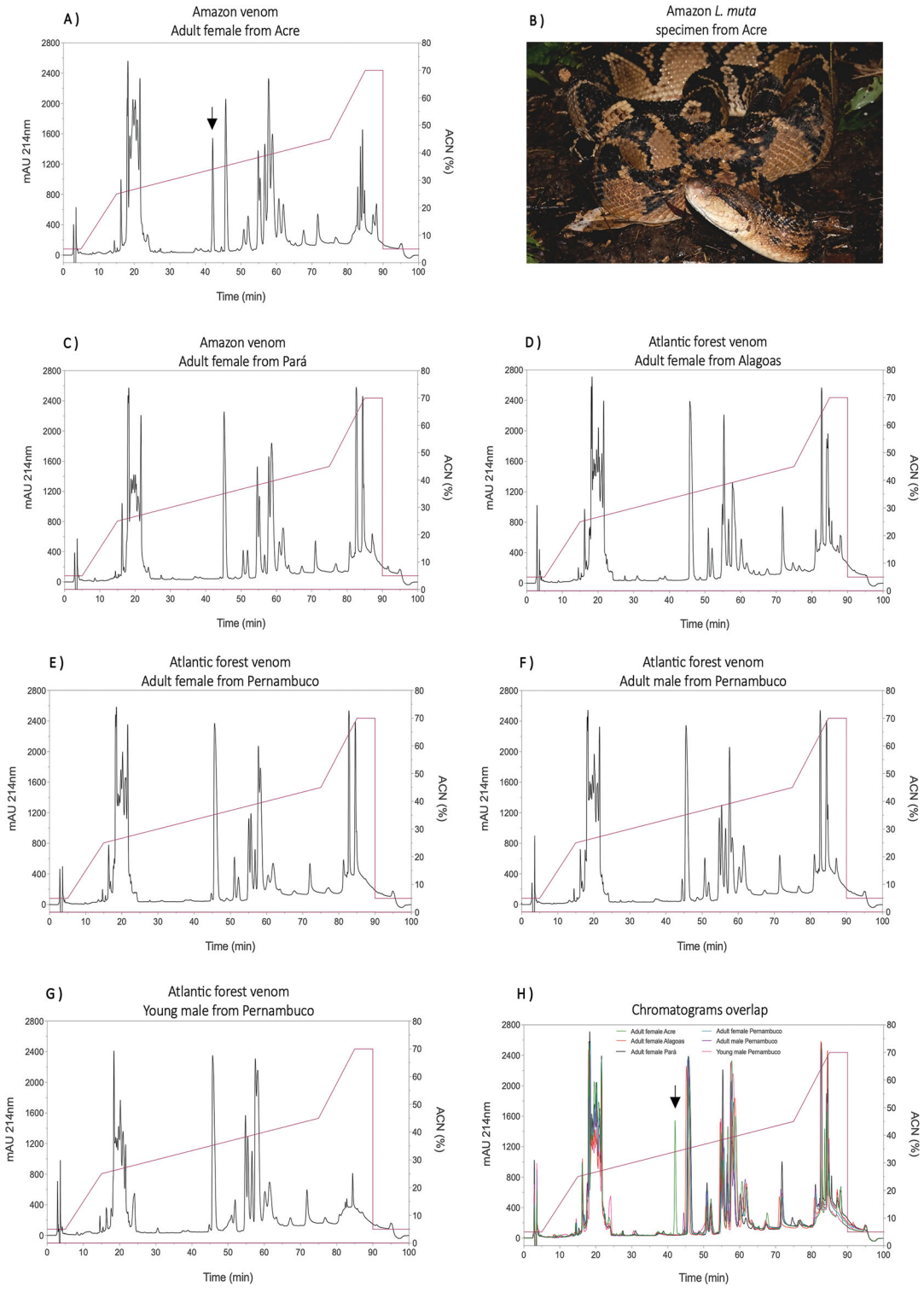

Fig. 8. Chromatographic profile of venoms from adults and juvenile of *Lachesis*, male and female, from different states of Brazil. The venoms (1.8–2 mg) were fractionated by reverse phase liquid chromatography (RP-HPLC) using a C18 column. (A) adult female venom from Acre; (B) Amazonian *L. muta* specimen from Acre; (C) adult female venom from Pará; (D) adult female venom from Alagoas; (E) adult female venom from Pernambuco; (F) adult male venom from Pernambuco; (G) young male venom from Pernambuco; and (H) all the chromatograms overlapped. Black arrows on panels A and H indicate a fraction that is observed only on the venom from the Acre specimen. Comparing our chromatographic profile with Sanz et al. (2008), we hypothesized that this fraction is a PLA2. Fig. 8B: *Lachesis muta* from the Parque Nacional da Serra do Divisor, Mâncio Lima, Brazilian state of Acre; photo by Marllus Rafael Almeida.

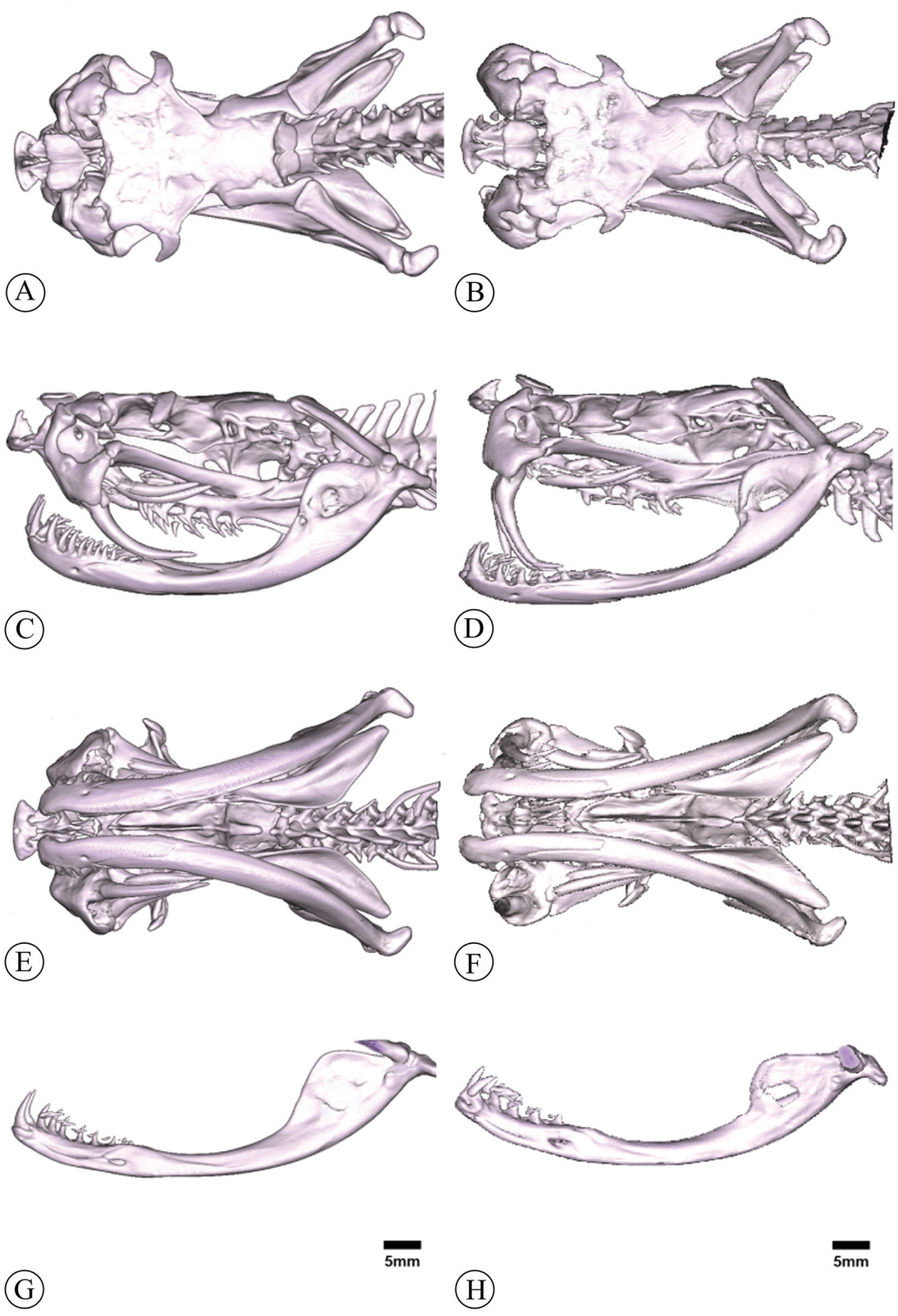

Fig. 9. Dorsal (A, B), lateral (C, D), ventral (E, F) and right mandible in dorsal (G, H) views of the skull of *Lachesis* from the Atlantic Forest of Santa Maria Madalena, Brazilian state of Rio de Janeiro (IVB001, A, C, E, G) and from the Amazon Forest of Rio Branco, Brazilian state of Acre (UFAC665, B, D, F, H).

population and the precipitation of the coldest quarter for Amazon population characterizing bioclimatic drivers of range boundaries (Supplemental Table S2), together with a second peak in the chromatogram only in an Amazon specimen (Fig. 8*A*), reinforces the distinct boundaries of these two evolutionary lineages.

Based on the results of exclusive coalescence of alleles, well-supported and distinct genetic cluster, together with the morphological data on the number of ventral scales and the width of the postocular stripe (Fernandes et al., 2004; Hoge, 1965; Table 3), reproductive biology (Souza & Almeida-Santos, 2020; Souza, 2020), and venom composition (Otero et al., 1998; Sanz et al., 2008), we here recognize the Amazonian populations and Mato Grosso specimens as *L. muta* (Linnaeus, 1766) and revalidate *L. rhombeata* (Wied-Neuwied, 1824) as a full species distributed in the Atlantic Forest.

We highlight that the SNP-based genealogy had no external groups, making rooting and confirming the closest relationships challenging to reach. There is also an admixture individual and an Amazon lineage closer to that of the Atlantic Forest, possibly due to incomplete lineage sorting, retention of ancestral polymorphism, hybrid phenomenon or even failure sequencing issues. An additional hypothesis is that we may have cryptic species within Amazon populations, as indicated by the genetic population analyses (see observed and expected heterozygosity data, faststructure Supplementary Material data) closest to the clade Atlantic Forest + Baturite, and this clade as the sister group of another Amazonian clade.

The existence of vicariant sister species in the Amazon and Atlantic Forest is common (e.g., *Chironius scurrulus* in the Amazon and *C. laevicollis* in the Atlantic Forest; Hamdan et al., 2017). Additionally, there is a large list of snake species endemic to the Amazon, including *Apostolepis striata*, *Atractus aboiporu*, *Bothrocophias microphthalmus*, *Bothrops oligobalius*, *Chlorosoma dunupyana*, *Epictia striatula*, *Erythrolamprus aenigma*, *Erythrolamprus carajasensis*, *Eutrachelophis papilio*, *Helicops hagmanni*, *Leptomicrurus collaris*, *Dryophylax ramonriveroi*, *Typhlophis squamosus*, and *Xenoxybelis boulengeri*. The list of snake species endemic to the Atlantic Forest is also extensive, comprising *Atractus serranus*, *B. muriciensis*, *Caaeteboia gaeli*, *Calamodontophis ronaldoi*, *Corallus cropanii*, *Tropidophis grapiuna*, and *Amerotyphlops paucisquamus* (Nogueira et al., 2019, Costa et al., 2022). Therefore, *L. muta* from the Amazon region and *L. rhombeata* from the Atlantic Forest represent an additional case corroborating the strong evolutionary identity of these two biogeographic regions.

Taxonomic account

***Lachesis muta* (Linnaeus, 1766). Synonyms.** 1766, *Crotalus mutus* Linnaeus, Systema Naturae, Ed. 12:373. Type-locality: Surinam; 1789, *Coluber crotalinus* Gmelin, Systema Naturae, Ed. 13(1):1094. Type-locality: not given; 1801, *Scytale catenatus* Latreille, In Sonnini and Latreille, Hist. Nat. Rept. 3:162. Type-locality: Surinam; 1802 *Scytale ammodytes* Latreille, In Sonnini and Latreille, Hist. Nat. Rept. 3:165. Type-locality: Ceylon [In error]; 1802, *Coluber alecto* Shaw, Gen. Zoology, Amphibians, 3(2):405. Type-locality: Ceylon [In error]; 1803, *Lachesis mutus*; Daudin, Hist. Nat. Rept. 5:351 1803, *Lachesis ater* Daudin, Hist. Nat. Rept. 5:354. Type-locality: Surinam; 1811, *Trigonocephalus ammodytes*; Oppel, Ann. Mus. Hist. Nat., Paris 16:390; 1820, *Cophias crotalinus*; Merrem, Tent. Syst. Amph., 191:144; 1822, *Trigonocephalus crotalinus*; Schinz, Das Thierreich Grund. Naturgesch. Ver. Anat. 2:144; 1822, *Lachesis muta*; Schinz, Das Thierreich Grund. Naturgesch. Ver. Anat. 2:144; 1822, *Lachesis atra*; Schinz, Das Thierreich Grund. Naturgesch. Ver. Anat. 2:144; 1822, *Scytale catenata*; Schinz, Das Thierreich Grund. Naturgesch. Ver. Anat. 2:144; 1824, *Bothrops surucucu* Wagler, in Spix, Spec. Nov. Serp. Brazil:59, pl. 23. Type-locality: Brazil; 1825, *Crasedocephalus crotalinus*; Gray, Ann. Philos. (2)10:205; 1854, *Lachesis mutus*; Duméril et al., Erp. Gén. 7(2):1486; 1859 *Crotalus mutus*; Jan, Rev. Mag. Zool. (2)11:1531896 *Lachesis mutus*; Boulenger, Cat. Sn. Brit. Mus. 3:534; 1898, *Lacheis muta*; Boettger, Kat. Rept. Samml. Mus. Senckenb. Naturforsch. Ges. 2:160; 1951 *Lachesis muta muta*; Taylor, Univ. Kansas Sci. Bull. 34:184. Type-locality: Surinam.

Taxonomic notes. *Crotalus mutus* was described by Linnaeus (1766) as a large-sized snake with connecting black dorsal diamond spots, dark postocular stripe, tail without a rattle, and full of rows of small spiky spines. Daudin (1803) placed this taxon in the genus *Lachesis*. Later, Duméril and Bibron (1854) presented more detailed morphological characteristics, highlighting that the species was found under the foliage of dense forests, feeding on mammals, birds, and reptiles. Boulenger (1896) presented detailed pholidosis, biometry, and colouration data, based on specimens from the Atlantic Forest and from the Amazon region to redescribe the species. Hoge (1965) showed a more accurate description, in which he indicates that specimens from the Amazon region would be easily distinguishable from those from the Atlantic Forest by having small spots on the top of the head; a much narrower postocular stripe, which is dorsally bordered by a light stripe; a truncated snout; a trapezoidal rostral; a more greyish ground colour, and supraoculars not strongly contrasted by the

Fig. 10. General view of adult (A–F) of *Lachesis muta* (Linnaeus, 1766) in life from Peru (A, C–D), Suriname, the type-locality (B), Guyana (E) and the Brazilian state of Pará, Amazonia (F). Photos by Andres Novales (A), Gerrit Jan Verspui (B), Marisa Ishimatsu (C–D), Andrew Snyder (E) and Carlos Cintra (F).

surrounding black spots. Based on 30 specimens from eastern Pará and north-western Maranhão states, Cunha and Nascimento (1975, 1982, 1993) presented detailed data on colouration, pholidosis, and habitat use. These authors also stated the species was not common in these regions at that time.

Diagnosis. *Lachesis muta* (Fig. 10) is distinguished from *Lachesis melanocephala* by having a brown top of head, with or without black markings and postocular stripe separate from head colouration (*vs.* dorsum of the head completely black with postocular stripe not distinguishable from black head colouration); from *L.*

stenophrys by having ventrals 219–236 and 95% confidence interval = 224–228, top of head with black markings and without lateral vertical bars (*vs.* ventrals 191–209 and top of head without black markings or with only a few small dots or speckles in the frontal and parietal regions; dark blotches forming vertical bars laterally); from *L. acrochorda* by having dorsal blotches distinctly diamond shaped, the intraspaces of each lateral triangular portion of the dorsal blotches frequently pale and about the same hue as ground colour, ventral surface of tail mostly pale (*vs.* dorsal blotches irregular, not distinctly diamond shaped, dorsal blotches with intraspaces dark brown, conspicuously darker than ground colour, ventral surface of tail mostly with large, dark spots or bars, sometimes extending almost to ventral midline (Campbell & Lamar, 2004; Fernandes et al., 2004). *Lachesis muta* is distinguished from *L. rhombeata* (Fig. 11) by having ventrals with a 95% confidence interval of 224–228, ranging from 219 to 236, postocular stripe thinner (Fernandes et al., 2004), average snout-vent-length (SVL) 1953.31 ± 229.18 mm, tail length (TL) 178 ± 26.56 mm, SVL/TL ratio of 9.1%, head length (HL) 68.53 ± 5.64 mm, head width (HW) 43.20 ± 5.34, HL/HW ratio of 63% (Ganança & Hingst-Zaher, 2015), and spermatogenesis between November and April (Souza, 2020; Souza & Almeida-Santos, 2020) [*vs.* ventrals with a 95% confidence interval 218–222, ranging from 213 to 231, postocular stripe thicker, except for specimens from Mato Grosso state (Fernandes et al., 2004); average SVL 1802.64 ± 193.49 mm, TL 160.85 ± 18.76 mm, SVL/TL ratio of 8.9%, HL 64.81 ± 5.54, HW 38.69 ± 7.18 mm, HL/HW ratio of 59.7% (Ganança & Hingst-Zaher, 2015), and spermatogenesis between March and August (Souza, 2020; Souza & Almeida-Santos, 2020)].

Description. An accurate morphological description with data on natural history of *Lachesis muta* can be obtained from Abuys (1987); Barrio-Amorós et al. (2020); Cañas et al. (2023); Campbell and Lamar (1989); Cunha and Nascimento (1975, 1982, 1993); Díaz-Ricaurte et al. (2020); Hoge (1965); Harvey et al. (2005); Jorge da Silva (1993); Pommer-Barbosa et al. (2023).

Evolution and natural history. *Lachesis muta* is genetically well-defined, forming a clear genetic cluster with low evidence of individual admixture (Figs 2–4), owning 6941 private alleles, and a 93% posterior probability of no gene flow with the Atlantic population (BPP analysis). It also has higher genetic diversity (He) (Table 1) and a broader realized niche compared with the Atlantic species (Figs 4, 5, 7; Supplemental Table S3), with temperature annual range and precipitation of the coldest quarters as the most critical environmental factors to explain its potential distribution (Supplemental Table S1).

Genetic data from the Amazon population samples reveal within-population structure, as indicated by the discrepancy between observed heterozygosity (0.06) and expected heterozygosity (0.11), the PCoA spread profile, the fastSTRUCTURE result with $K = 3$ (Supplemental Fig. S1), and the evolutionary relationship within *L. muta* in our time tree (Fig. 5, Table 1). Considering our sample distribution, a discussion based on these data is complex, but our sample size allows an insight into population genetics results. The Amazon region is extremely rich in biodiversity, exhibiting a wide heterogeneous landscape and a complex biogeographic history of diversification (e.g., Hamdan et al., 2019). For example, the Rio Amazonas is 6400 km long, and its origin and evolution are associated with a genetic substructure of many taxa that occur along its banks (Hoorn & Wesselingh, 2010). Fouquet et al. (2015) observed spatial genetic structure within *Adenomera andreae* and concluded that the species has a restricted dispersal ability that is probably tied to its life-history traits. Moreover, population structure is also found in species with higher dispersal potential, such as primates (Lecompte et al., 2017). Thus, the taxonomic status and distinct genetic lineages of *L. muta* should be more deeply investigated by sampling different biogeographic regions (see Morrone, 2014) throughout its range. Combining a morphology-based analysis and the sequencing of *Lachesis* specimens from eastern Pará and adjoining Maranhão, states of Brazil, would also shed light on admixture's influence on the genus speciation process (e.g., Schluter, 2009; Wüster et al. 1996). Unfortunately, only one tissue sample from these regions is available, and further studies filling this sample gap will better address the genus evolutionary history. Our venomic analysis confirmed the reported characteristics of the venom in this genus: the protein profile has high similarity with quantitative variations on the protein families (Sanz et al., 2008, Madrigal et al., 2012). The venom from the Amazonian specimen from Acre shows a unique peak of PLA_2 (Fig. 8A). The presence of PLA_2 isoforms in three specimens from the Bolivian Amazon and two from the Peruvian Amazon was reported by Sanz et al. (2008) and could imply some specificity from venoms from north-western South America. Otero et al. (1998) showed that the venom of a *L. muta* specimen from the Amazonian lineage, specifically from Mato Grosso, had a lower LD_{50} and higher haemorrhagic activity than the venom of a specimen from the Atlantic Forest, which showed a higher LD_{50}

Fig. 11. General view of *Lachesis rhombeata* (Wied-Neuwied, 1824) in life from the Brazilian Atlantic Forest states of Pernambuco (A), Bahia (B–D), Alagoas (E) and Paraíba (F). Figure 8*B* is a juvenile. Photos by Marco Freitas (A, D), Arthur Abegg (B), Cleiton Silva (C), Edson Taciano (E) and Diego Santana (F).

and higher coagulant activity in rodents. The observed differences in the biological activities may result from differences in the quantitative expression of the same pool of proteins (Margres et al., 2015). More individual venoms from each region should be analysed to confirm these observations.

Geographic distribution (Fig. 12). *Lachesis muta* occurs in equatorial forests in the Amazon biome of Bolivia, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname, Trinidad, Venezuela, and the Brazilian states of Acre, Amazonas, Amapá, Maranhão, Mato Grosso, Pará, Rondônia, and Roraima.

Fig. 12. Geographic distribution of *Lachesis muta* and *Lachesis rhombeata.*

***Lachesis rhombeata* (Wied-Neuwied, 1824).**
Synonyms. 1824, *Lachesis rhombeata* Wied-Neuwied, Abbild. Nat. Brazil, Lief. 5:pls. 5, 5a. Type-locality: Brazil; 1825, *Lachesis rhombeata* Wied-Neuwied, Beiträge zur Naturgeschichte von Brasilien, pg. 449. Type-locality: Bahia, Espirito Santo and Minas Gerais; 1872, *Trigonocephalus (Lachoesis) brasiliensis* Liais, Clim. Geol., Faune Geogr. Bresil: 306–307. Type-locality: not given, probably Rio Doce, MG, Brazil (Hoge & Romano, 1978); 1872, *Trigonocephalus rhumbeatus*; Liais, Clim. Geol., Faune Geogr. Brésil: 306; 1966 *Lachesis muta noctivaga* Hoge, Mem. Inst. Butantan, 32:162, pl. 20 (1965). Type-locality: Vitoria, ES, Brazil; 1978, *Lachesis muta rhombeata*; Hoge and Romano, Mem Inst. Butantan, 40/41:54 (1976/77). Type-locality: Restrict to Vitoria, ES, Brazil, in this study.

Taxonomic notes. The Atlantic subspecies *Lachesis muta rhombeata* was described by Wied-Neuwied (1824) with general morphological traits, characteristics of the snake-bite, in addition to indication that the species was often killed by humans. Wied-Neuwied (1825) presented a more detailed description, mainly through the analyses of specimens from the Atlantic Forests at the Iritiba, Itapemirim, Doce, and Peruíbe rivers in the states of Bahia, Espírito Santo, and Minas Gerais State. Liais (1872) describes aspects of the size, habitat, abundance, venom and behaviour of one specimen of *Lachesis*. Hoge (1965) described the population of the Atlantic Forest more meticulously as *L. muta noctivaga* (type locality: Vitória, Espírito Santo State, Brazil), and compared it to the Amazonian population, recognized as *L. muta muta* at that time. Later, *L. muta noctivaga* was synonymized with *L. muta rhombeata* by Hoge and Romano (1978). According to Hoge (1965), it would be distinguishable from *L. muta muta*, by having "very large and distinct spots on the top of the head, a wider postocular black stripe, the absence of wide and well contrasted light stripe between black post ocular stripe and the upper surface of the head, a rather triangular rostral shield, a very bright reddish ground colour and supra-oculars strongly contrasted by the surrounding black spots".

Diagnosis. *Lachesis rhombeata* (Fig. 11) is distinguished from *Lachesis melanocephal*a by having top of head brown, with or without black markings and postocular stripe separate from head colouration (*vs.* dorsum of the head completely black with postocular stripe not distinguishable from black head colouration); from *L. stenophrys* by having ventrals 219 to 236 and 95% confidence interval = 224–228, top of the head with black markings and without lateral vertical bars (*vs.* ventrals 191–209 and top of the head without black markings or with only a few small dots or speckles in the frontal and parietal regions; with dark blotches forming vertical bars laterally); from *L. acrochorda* by having dorsal blotches distinctly diamond shaped, the intraspaces of each lateral triangular portion of the dorsal blotches frequently pale, about the same hue as ground colour, ventral surface of tail mostly pale [*vs.* dorsal blotches irregular, not distinctly diamond shaped, dorsal blotches with intraspaces dark brown, conspicuously darker than ground colour, ventral surface of tail mostly with large, dark spots or bars, sometimes extending almost to ventral midline) (Campbell & Lamar, 2004; Fernandes et al., 2004)]. *Lachesis rhombeata* is distinguished from *L. muta* (Fig. 10) by having ventrals with a 95% confidence interval 218–222, ranging from 213–231, postocular stripe thicker, except for specimens from Mato Grosso state (Fernandes et al., 2004); average SVL 1802.64 ± 193.49 mm, TL 160.85 ± 18.76 mm, SVL/TL ratio of 8.9%, HL 64.81 ± 5.54, HW 38.69 ± 7.18 mm, HL/HW ratio of 59.7% (Ganança & Hingst-Zaher, 2015) and spermatogenesis between March and August (Souza, 2020; Souza & Almeida-Santos, 2020) [*vs.* ventrals with a 95% confidence interval of 224–228, ranging from 219–236, postocular stripe thinner, except for specimens from Mato Grosso state (Fernandes et al., 2004), average snout-vent-length (SVL) 1953.31 ± 229.18 mm, tail length (TL) 178 ± 26.56 mm, SVL/TL ratio of 9.1%, head length (HL) 68.53 ± 5.64 mm, head width (HW) 43.20 ± 5.34, HL/HW ratio of 63% (Ganança & Hingst-Zaher, 2015) and spermatogenesis between November and April (Souza, 2020; Souza & Almeida-Santos, 2020)].

Description. An accurate morphological description with data on the natural history of *Lachesis rhombeata* can be obtained by consulting Alves et al. (2014); Argôlo (2003, 2004); Campbell and Lamar (1989); Duque et al. (2023); Günter (1861); Hamdan et al. (2023); Hoge (1965); Hoge and Romano (1978); Liais (1872); Padrón et al. (2022); Souza (2007).

Evolution and natural history. *Lachesis rhombeata* is also genetically well-defined forming a genetic cluster (Figs 2–4). It has 538 private alleles and a 93% probability of no gene flow with *Lachesis muta. Lachesis rhombeata* shows lower genetic diversity (He) (Table 1, Fig. 4B) and a smaller realized niche than *L. muta* (Figs 6, 7; Supplemental Table S3), in which precipitation of the driest quarter and temperature annual range are the most important environmental factors (Supplemental Table S1). The venom does not differ substantially from that of *L. muta*, and our study reinforces the findings of

Sanz et al. (2008) and Pla et al. (2013), who showed that *Lachesis* venoms are conservative between species.

The Bayesian tree built using 543,174 bp recovered all samples from the Atlantic Forest in a monophyletic clade with full support (Fig. 5), consistent with the population structure analyses (Figs 2–4). Similar patterns have been observed in genetic studies of other forest-associated species in north-eastern Brazil (Carnaval & Bates, 2007). The oldest split of the Amazon specimens occurred 280,000 years ago, and our tree reveals a substructure within the Amazon population as previously mentioned. The Amazon and Atlantic forests are separated by the South American dry diagonal, which is formed by the Chaco, Cerrado, and Caatinga biomes (Ab'saber, 1977). It is believed that the Amazon and Atlantic Forest were connected several times in the past through lands that currently compose the dry diagonal region during periods of more humid climates (Auler et al., 2004). Thus, during these humid periods, forest routes would have been formed in these regions, allowing gene flow between these biotas. Fernandes et al. (2004) postulated that this could explain the clade recovered in their study, which included specimens from the Atlantic Forest and Mato Grosso Amazon. However, as aforementioned, our data do not support the Atlantic Forest + Mato Grosso clade. Additionally, the dry diagonal region currently shows no suitable environmental conditions for these taxa to establish, as indicated by our niche modelling results (Figs 6, 7).

The diversification process of biota throughout South America may have multifactorial causes at different temporal levels (e.g., Hamdan et al., 2019). Despite this, many examples of sister species pairs are distributed in the Amazon and Atlantic forests, such as *Phyllomys* spp. (mammals, Machado et al., 2018), *Chiasmocleis* spp. (amphibians, De Sá et al., 2019), *Neopelma* spp. (birds, Capurucho et al., 2018), *Bothrops* spp. (snakes, Pontes-Nogueira et al., 2021), *Enyalius* spp. (lizards, Rodrigues et al., 2014), and *Eulaema* spp. (orchid bees, Silva et al., 2014). Our results indicate a well-defined speciation process, in which *L. muta* in the Amazon and *L. rhombeata* in the Atlantic Forest reinforce the importance of the South American dry diagonal to explain diversification in the Neotropical region.

The conservation status of *Lachesis rhombeata*. Considering the high priority of biodiversity conservation, it is essential to promptly identify populations that are on the verge of extinction. The red lists of some Brazilian states published more than 15 years ago no longer accurately reflect the current conservation status of *Lachesis* in their territories. For example, the state of Espírito Santo has historical records of a wide distribution of *L. rhombeata* in the past, but these records are only from localities that are currently highly anthropogenically modified (Almeida et al., 2007). A similar situation is observed in Minas Gerais state. The species was categorized as critically endangered (Machado et al., 1998), but no records have been documented in Minas Gerais since then (Nogueira et al., 2019). Bergallo et al. (2000) classified *L. rhombeata* as endangered in Rio de Janeiro state, but the species had a broader geographic distribution, eventually not considered rare as reported until the 1940s in Tycho Ottilio de Siqueira Machado's field book housed at the Instituto Vital Brazil (Duque et al., 2023; Machado, 1956). The species experienced a marked decline, with the last collected specimen in 1986 in northern Rio de Janeiro (Oliveira et al., 2020). Melgarejo (2009) officially declared the species extinct in the wild. However, Oliveira et al. (2020) presented a photographic record from 2004 within the União Biological Reserve, representing, so far, the last documented record of the species in its native habitat in the state of Rio de Janeiro.

Lachesis rhombeata harbours lower genetic diversity (He) compared with *L. muta*, indicating a small effective population size (Table 1, Fig. 4B). This condition, combined with the historical lack of species records from several Brazilian states (e.g., Rio de Janeiro, Espírito Santo, and Minas Gerais), and the uninterrupted cycle of destruction of the Atlantic Forest raises the question of whether this species should be included in the national red list of fauna. Since the distribution criteria (extent of occurrence and area of occupancy) exceed the maximum required by the IUCN (2022) for inclusion in any threat category, historical context and investigations on population size and decline are essential for accurately assessing its conservation status.

The South American bushmaster in the Brazilian state of Ceará corresponds to areas of high humidity and mild climate in the middle of a semi-arid region with a long history of human exploitation. Agriculture and logging are the two most important factors driving local habitat loss (Brandão & Freitas, 2014). In this case, fast action for effective conservation involves identifying and delimiting meaningful conservation units (e.g., Mace, 2004). As *L. rhombeata* from the Baturité massif of Ceará shows monophyly, ancient divergence, and deep diversity, we are qualifying it here as an evolutionarily significant unit (ESU) (Moritz, 1994).

Distribution (Fig. 12). *Lachesis rhombeata* (Fig. 11) is an endemic clade from Brazil, distributed in the Atlantic Forest biome in Alagoas, Bahia, Ceará, Espírito Santo, Minas Gerais, Paraíba, Pernambuco, and Rio de Janeiro states (Hoge, 1965, Fig. 5B).

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Supplemental material

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