ORIGINAL ARTICLE

Pleistocene expansion and connectivity of mesic forests inside the South American Dry Diagonal supported by the phylogeography of a small lizard

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Traditionally focused on Amazonian and Atlantic rainforests, studies on the origins of high Neotropical biodiversity have recently shifted to also investigate biodiversity processes in the South American dry diagonal, encompassing Chaco, Cerrado savannas, and Caatinga seasonally dry tropical forests. The plateau/depression hypothesis states that riparian forests in the Brazilian Shield in central Brazil are inhabited by Pleistocene lineages, with shallow divergences and signatures of population expansion. Moreover, riparian forests may have acted as a vegetation network in the Pleistocene, allowing gene/species flow across the South American dry diagonal. We tested these hypotheses using *Colobosaura modesta*, a small gymnophthalmid lizard from forested habitats in the Cerrado savannas and montane/submontane forests in the Caatinga. We conducted phylogeographic analyses using a multi-locus dataset, tested alternative demographic scenarios with Approximate Bayesian Computation, and also employed species delimitation tests. We recovered a history of recent colonization and expansion along riparian forests, associated with Pleistocene climate shifts, and the existence of a new species of *Colobosaura* restricted to the Serra do Cachimbo region. We also present evidence that riparian forests have provided an interconnected network for forest organisms within the South American dry diagonal and that Pleistocene events played an important role in their evolutionary history.

KEY WORDS: brejos de altitude, Cerrado, Gymnophthalmidae, Neotropics, riparian forest, species delimitation.

Documenting and explaining the amazing biodiversity of the South American tropics has fascinated naturalists for centuries (Spix and Martius 1823, 1828, 1831; Wallace 1853; Bates 1863; Prance 1982). Although traditionally focused on Amazonia and Atlantic Forest, in recent years Neotropical research has gradu-ally shifted to also investigate biodiversity processes in Cerrado savannas and Caatinga seasonally dry tropical forests (Oliveira and Marquis 2002; Werneck 2011; da Silva et al. 2017; Antonelli et al. 2018a). Cerrado and Caatinga are characterized by more open vegetation and drier climates relative to rainforests,

and along with the Chaco, they form the "savanna corridor" (Schmidt and Inger 1951), "diagonal of open formations" (Vanzolini 1963), or "dry diagonal" (Prado and Gibbs 1993), which spans from southwestern to northeastern South America and separates Amazonia from the Atlantic Forest. The Cerrado and Caatinga "dry diagonal" also harbor numerous mesic vegetation types, such as montane/submontane forests (Santos et al. 2007; Moro et al. 2014) and riparian forests (Oliveira-Filho and Ratter 1995; Oliveira-Filho and Fontes 2000; Bueno et al. 2018). These forests have allowed the dispersal into and persistence of rainforest lineages within "open formation" biomes, suggesting an intimate history between moist and relatively drier biomes in South America (Ledo and Colli 2017; de Sá et al. 2019).

Research on the mechanisms that have promoted high biodiversity in Cerrado and Caatinga has emphasized a mixed history of geological events from the Paleogene/Neogene and Quaternary climatic fluctuations (reviewed in Werneck 2011). Occupying a central position in South America, the Brazilian Shield comprises a landscape of alternating ancient plateaus and younger inter-plateau depressions. The plateaus reach up to 1676 m at Chapada dos Veadeiros National Park (de Carvalho Júnior et al. 2015) are largely covered by savanna vegetation, and have enjoyed relative climatic stability through time (Werneck et al. 2012b; Burbridge et al. 2004). The inter-plateau depressions were carved by the headwaters of three large river basins—Tocantins-Araguaia, São Francisco, and Paraná—and are covered by a mosaic of savannas and forests, which have shifted during Quaternary climatic cycles (Ab'Saber 1983; Silva 1996; Bueno et al. 2017). The Caatinga landscapes of northeastern Brazil also includes plateaus, but restricted to interior and coastal inselbergs and covered by savanna on the plateau tops or by montane and submontane mesic forests on tops and along slopes. In contrast, the depressions are broadly extended and typically covered by seasonally dry tropical forests and by riparian forests (Prado 2003). Paleoenvironmental studies in these regions suggest moister conditions and profound changes in the Caatinga vegetation cover during the Quaternary (De Oliveira et al. 1999; Pessenda et al. 2004; Pessenda et al. 2010), which were imprinted in the genetic structure of several depression and montane lineages (Carnaval and Bates 2007; Thomé et al. 2016; Gehara et al. 2017).

The highly dynamic landscapes of Cerrado and Caatinga putatively favored the assembly of (1) older lineages (paleoendemics) on Brazilian Shield's plateaus, characterized by strong genetic structure, high demographic stability, and high genetic diversity; and (2) younger lineages (neoendemics) confined to inter-plateau depressions, characterized by weaker genetic structure, lower genetic diversity, and signatures of demographic changes, hereafter called the "plateau/depression hypothesis" (Werneck 2011). Furthermore, riparian forests in Cerrado and Caatinga were likely connected during favorable periods in the

Quaternary, allowing species and intraspecific gene flow across river basins and biomes (Oliveira-Filho and Ratter 1995; De Oliveira et al. 1999). Other studies suggest that the Brazilian Shield can also act as a barrier to gene flow for forest-dwelling organisms inhabiting inter-plateau depressions (Silva 1997; Silva and Bates 2002; Werneck 2011). For instance, altitude is an important predictor of anuran (Valdujo et al. 2013), bird (Silva 1996), and lizard (Nogueira et al. 2009) species turnover in Cerrado.

Statistical phylogeography is a powerful approach to understanding the processes that have shaped spatiotemporal patterns of genetic variation (Knowles 2009; Knowles and Alvarado-Serrano 2010). Using model-based inference, statistical phylogeography offers a framework for testing competing hypotheses, providing greater explanatory power compared to classic diversification studies (i.e., biogeographical or paleoecological evidence; Beaumont et al. 2010). The few phylogeographic studies of species typical of forest habitats in the Brazilian Shield depressions (de Lima et al. 2014; Bartoleti et al. 2018; Vasconcellos et al. 2019) show that these taxa represent "recent" lineages, whose genetic structure and distributions were primarily influenced by Pleistocene climatic fluctuations. In these cases, riparian forests have acted as interconnected networks, promoting dispersal and gene flow across the Cerrado savanna-like landscapes.

Lizards are considered model organisms for ecological (Huey et al. 1983) and speciation studies (Camargo et al. 2010). The lizard *Colobosaura modesta* (Gymnophthalmidae: Gymnophthalminae) (Reinhardt and Lütken 1862) is widely distributed in forested habitats throughout the Cerrado, primarily in riparian forests and "cerradão" (Colli et al. 2002; Nogueira et al. 2009), and also in eastern Amazonia (Cunha 1977; Ávila-Pires 1995). Isolated populations of *C. modesta* occur in Restinga habitats from the Atlantic Forest of northern Bahia (Couto-Ferreira et al. 2011) and in forest enclaves ("brejos de altitude") of Chapada do Araripe, Crateús, and Planalto do Ibiapaba (Freire et al. 2012) (Fig. 1), suggesting that Pleistocene climatic changes might have played an important role in shaping the current distribution of the species. Despite its patchy distribution, *C. modesta* is considered the only valid species within the genus. Previously, *C. (Perodactylus) kraepelini* (Werner 1910) and *C. landii* (Cunha 1977) were described respectively from Puerto Max (Paraguay) and from primary forests of the eastern Amazonia, in different habitats from the previous known records of *Colobosaura*. However, both were synonymized with *C. modesta* in later revisions (Amaral 1932; Cunha and Nascimento 1982, 1983; Nascimento et al. 1987). Hence, considering its broad distribution crossing the South American dry diagonal, and the fact that it prefers forest habitats, this species is an ideal candidate to investigate broad-scale biogeographic and biodiversity generating hypotheses in the Neotropical region.
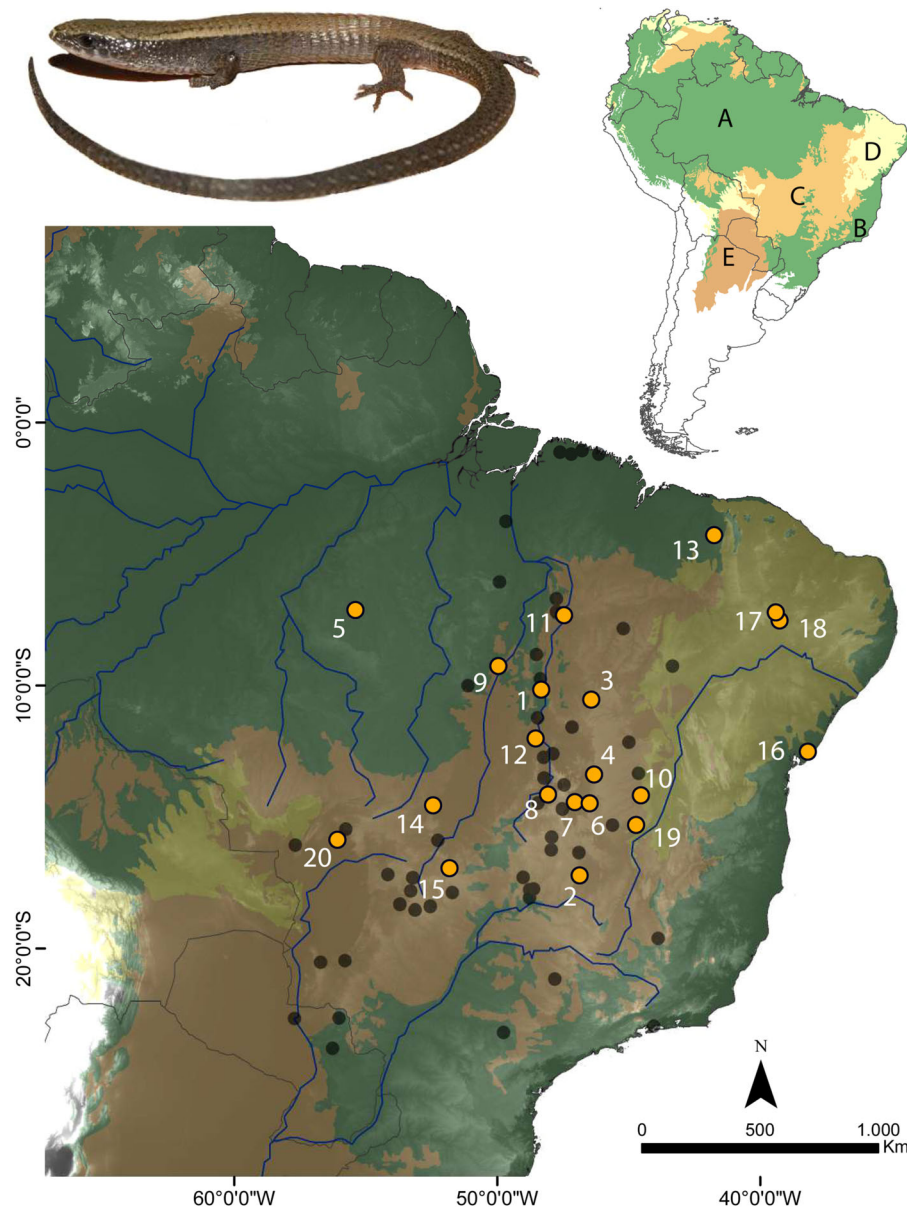
Figure 1. Upper left: a specimen of *Colobosaura modesta* (photo modified from Carlos Cândido). Upper right: South America evidencing rainforests (A, Amazonia; B, Atlantic Forest) and the "Dry Diagonal" (C, Cerrado; D, Caatinga; E, Chaco). Center: distribution of *Colobosaura modesta* (gray dots) and localities sampled in this study (orange dots). Locality numbers: 1, Palmas – TO; 2, Paracatu – MG; 3, Mateiros – TO; 4, São Domingos – GO; 5, Novo Progresso – PA; 6, Alvorada do Norte – GO; 7, Flores de Goiás – GO; 8, Colinas do Sul – GO; 9, Caseara – TO; 10, Cocos – BA; 11, Carolina – MA; 12, Peixe – TO; 13, Piripiri – PI; 14, Nova Xavantina – MT; 15, Caiapônia – GO; 16, Mata de São João – BA; 17, Jardim – CE; 18, Crato – CE; 19, Bonito de Minas – MG; 20, Santo Antônio do Leverger – MS. Brazilian states: BA, Bahia; CE, Ceará; GO, Goiás; PA, Pará; MA, Maranhão; MG, Minas Gerais; MT, Mato Grosso; PE, Pernambuco; RN, Rio Grande do Norte; TO, Tocantins.

Herein, we studied the phylogeographic history of *Colobosaura modesta* and tested several hypotheses concerning the evolution of this widespread species and its enclosing ecosystems. Given that *C. modesta* is mainly associated with riparian vegetation in central Brazil, but also marginally distributed in eastern Amazonia, and with isolated populations in Caatinga and Atlantic Forest, we tested the hypothesis of Pleistocene climatic events shaping the diversity and population structure of this species. If this hypothesis is correct, we predict that: (a) *C. modesta* would be characterized by shallow among-population divergence, depicting a pattern of recent colonization history and population expansion; (b) divergence times among lineages would be more closely associated with Pleistocene climatic changes than to pre-Pleistocene geological events; and (c) have a genetic

signature of population expansion, associated with forest shifts in the Cerrado valleys during the Quaternary. We also tested the hypothesis that riparian forests have acted as an interconnected network permitting gene flow across the Cerrado, versus the hypothesis of the Cerrado plateaus acting as barriers to gene flow. If riparian forests were interconnected, we predict populations of *C. modesta* will show: (d) genetic similarity among headwaters of the main Cerrado river basins, and (e) patterns of isolation-by-distance as important explanations for genetic differences among populations. However, if the Cerrado plateaus act as barriers to gene flow, genetic breaks will probably be associated with river basin limits, and other features (i.e., altitude) will be important in explaining genetic structure. In parallel, we used species delimitation methods based on multiple independent markers to test whether *C. modesta* is a single species, or a species complex.

Material and Methods
COLLECTION OF SPECIMENS AND LABORATORY PROTOCOLS

We obtained 76 tissue samples of *Colobosaura modesta* from 20 different localities, representing populations from the Cerrado, Amazonia, Atlantic Forest, and Caatinga (Chapada do Araripe, CE) (Table S1; Fig. 1). Tissue samples were primarily obtained from specimens deposited at Coleção Herpetológica da Universidade de Brasília - CHUNB, but we also obtained samples through fieldwork led by the authors, and loans from colleagues and other museums (Zoological collections at Universidade Federal de Goiás - ZUFG, Universidade Federal do Rio Grande do Norte - UFRN, and Universidade Católica de Salvador - UCSAL). We used *Acratosaura mentalis* (AAGARDA 4227, 5689, 5690, 6874, and 8631) and *Vanzosaura rubricauda* (CHUNB 58600) as outgroups in phylogeographic reconstructions.

We extracted genomic DNA with the DNAeasy Qiagen® and Invitrogen® extraction kits, following the manufacturers' protocol. We sequenced all individuals for a fragment of the mitochondrial cytochrome B (cytb), and a subset of the individuals for fragments of five nuclear genes: the prolactin receptor (PRLR), beta-fibrinogen (B-FIB), matrix remodeling associated 5 (MXRA5), alpha-cardiac actin gene, intron 3 and exon 4 (ACA), and synuclein alpha interacting protein gene (SINCAIP) in forward and reverse directions. PCR protocols varied for each locus (Table S1). We vacuum-purified PCR products using MANU 30 PCR Millipore plates, and subsequently resuspended the DNA with ultra-pure water. Sequencing reactions used the ABI Big-Dye Terminator version 3.1 Cycle Sequencing Kit in an ABI GeneAmp PCR 9700 thermal cycler. DNA products were purified with Sephadex G-50 Fine (GE Healthcare) and sequenced on an ABI 3730xl DNA Analyzer at the Brigham Young University DNA Sequencing Center (http://dnasc.byu.edu/). Some samples were also sent to Macrogen® for sequencing. Individual chromatograms in forward and reverse directions for each marker were first assembled with highest sensitivity in GENEIOUS R 8.1 (Geneious Co., Wellington, New Zealand), generating larger consensus sequences. We aligned these sequences with MUSCLE (Edgar 2004) in GENEIOUS R 8.1, using default settings, concatenated, and inspected by eye using the same program.

Nuclear genetic data were phased using PHASE (Stephens et al. 2001) in DNASP 5 (Librado and Rozas 2009). Haplotype and nucleotide diversity, and other general genetic summary statistics were also obtained with DNASP 5. To analyze cytb haplotype relationships among populations, we employed a median-joining network (Bradlet et al. 1999) with POPART 1.0 (http://popart.otago.ac.nz), using the default epsilon = 0 to avoid excessive median vectors.

SPECIES DELIMITATION ANALYSES

Prior to any phylogeographic analysis, we tested whether or not *C. modesta* is a single species, by identifying divergent genetic groups as candidate species, and used this information to guide subsequent phylogeographic reconstructions. For this, we investigated the genetic structure with Bayesian mixture and admixture analyses implemented in BAPS 6.0 (Corander et al. 2013). We conducted mixture analyses using a phased concatenated alignment of the six genes, and designated individuals and localities as priors. We ran BAPS with the maximal number of groups (*K*) set as a vector ranging from 1 to 10 and ran all analyses five times for each group (total of 50 runs). After this mixture analysis of individuals, we conducted the admixture analysis with the same data, implementing 1000 simulations to estimate posterior allele frequencies.

We used the software BPP 3.2 (Yang and Rannala 2010, 2014) to test alternative species delimitation hypotheses within *C. modesta*, based on the genetic groups obtained in BAPS 6.0. Different from previous versions, BPP 3 estimates a species tree while running the reversible-jump Markov chain Monte Carlo (MCMC) species delimitation algorithm, eliminating the concern of over-estimating species limits (Leaché and Fujita 2010; Caviedis-Solis et al. 2015). Briefly, the software estimates a species tree using a Subtree Pruning and Regrafting algorithm (Rannala and Yang 2017), while species hypotheses are tested by collapsing branches of the different possible phylogenetic hypotheses (species trees) and comparing their posterior probabilities.

We separated individuals of *Colobosaura modesta* into candidate species based on the groups generated in BAPS 6.0. First, we ran initial trials using different parameters and checking for convergence among repeated runs, which denote prior quality and run consistency in BPP. We then used a gamma prior of $\sim$ G (1,1000) for population size (θ), $\sim$ G (1,100) for the age of the

root in the species tree (τ_0), and the Dirichlet prior (Yang and Rannala 2010: equation 2) for other divergence time parameters. The gamma prior G (α, β) has mean α/β, so the $\theta \sim$ G (1,1000) corresponds to one difference per kilobase (0.001), while the $\tau_0 \sim$ G (1,100) corresponds to 1% sequence divergence (0.01). Therefore, these priors assume large population sizes and a relatively shallow divergence times. They returned similar results after different runs (indicating consistency), while other priors delivered different results for each run.

We ran all analyses for 500,000 MCMC generations, taking samples every five generations, and using 10,000 burn-in generations. We used both available reversible-jump MCMC species delimitation algorithms (algorithms 0 and 1, Yang and Rannala 2010), excluding or not alignment gaps (*cleandata* = 1, the program removes all columns with gaps or ambiguous characters, and *cleandata* = 0 means that these edited data will be used in the likelihood calculation). To check for consistency of results, we conducted at least two independent runs starting at random tree models for each analysis type.

PHYLOGENETIC RELATIONSHIPS AND DIVERGENCE TIMES IN *Colobosaura modesta*

To reconstruct the phylogeographic history of *Colobosaura modesta*, and also to look for shallow divergences and short branch lengths in this riparian forest-dwelling species, we estimated gene genealogies and divergence times based on single-genes and partitioned concatenated alignments (mitochondrial and nuclear DNA). Gene genealogies were based on Bayesian inference (BI) and maximum likelihood (ML) methods. We implemented the Bayesian Information Criterion (BIC) in PARTITION FINDER 1.1.1 (Lanfear et al. 2012) to select the optimal nucleotide substitution model defining one data block for each codon position in each gene for BI (cytb: HKY+G; PRLR: HKY+I+G; BFIB: HKY+I+G; MXRA5: HKY+G; ACA: HKY+I+G; SINCAIP: HKY+I+G). We used MRBAYES 3.2.5 (Ronquist et al. 2012) to generate 10 million generations with the Metropolis-coupled MCMC algorithm. Each run contained four incrementally heated Markov chains, sampled every 1,000 generations. Convergence of the two runs was assumed when the average standard deviation of the split frequencies was <0.01. We contrasted our BI analysis with a partitioned maximum likelihood inference (ML) using RAXML version 8.0 (Stamatakis 2014), using the GTRGAMMAI model of evolution and a standard heuristic search with 1000 pseudoreplicates (bootstrap).

Divergence times within *Colobosaura modesta* were based on the mitochondrial and the five phased loci under a coalescent exponential model implemented in BEAST version 2.3.1 (Bouckaert et al. 2014). We used a coalescent exponential prior, uncorrelated lognormal relaxed clocks, and given the lack of fossil information for the group, calibrated the time estimates using a normal prior distribution on the mtDNA global substitution rate (mean = 0.0065 substitutions/million years), following the estimate for lizards of 0.65% changes/million years (Macey et al. 1998). Substitution rates for the five nuclear markers were estimated relative to the mtDNA rate using a uniform prior for *ucld.mean* with default values, and uniform prior for *ucld.stev*, with a mean of 0.5. We performed five independent runs of 100 million generations each, sampled at every 2000 steps, totaling a posterior distribution of 50,000 trees/run. We accessed stationary posterior distributions, effective sample sizes (ESS above 200), and convergence between runs with TRACER version 1.6 (Bouckaert et al. 2014). We combined trees after removing a burn-in of 25% with LOG COMBINER version 1.7.5 and subsequently annotated the combined tree file with TreeAnnotator version 1.7.5 (Bouckaert et al. 2014) to calculate the maximum clade credibility (MCC) species tree. We accessed the tree and divergence times with FIGTREE 1.4.2 (Bouckaert et al. 2014).

GEOGRAPHIC STRUCTURE AND POPULATION HISTORY

We evaluated patterns of isolation-by-distance within *Colobosaura modesta* using a Mantel test with pairwise genetic and geographic distances among all pairwise combinations of cytb haplotypes. We conducted this analysis with the functions *dist.gene* and *mantel.rtest*, using the packages APE (Popescu et al. 2012) and ADE4 (Dray and Dufour 2007) respectively, in R 2.3.1 (R Development Core Team 2015), employing 9999 Monte Carlo permutations. To evaluate the hypothesis of riparian forests acting as an interconnected net versus the hypothesis of central Brazilian plateaus acting as barriers to gene flow among riparian forest-dwelling species, we used circuit theory to calculate the environmental cost of all possible routes connecting pairs of localities, and identified the corridor with the lowest resistance using CIRCUITSCAPE 4.0 (McRae and Beier 2007). We used an altitude raster (2.5 arc-minutes resolution) as a resistance map and calculated a resistance matrix among localities, and then conducted a partial Mantel test using genetic distance, resistance matrix, and isolation-by-distance as a covariate. We conducted this analysis using the *mantel.partial* (VEGAN) (Dixon 2003) function in R 2.3.1, employing 9999 Monte Carlo permutations.

We also used Monmonier's (1973) maximum difference algorithm implemented in Barrier 2.2 (Manni et al. 2004) to identify genetic barriers among *Colobosaura modesta* haplotypes. If riparian forests act as interconnected networks for gene flow, genetic breaks will not be associated with the central distribution of the species, which correspond to river basin limits. However, if the Central Brazilian plateau is a barrier for gene flow, maximum genetic breaks will probably be associated with river basins limits, as river basins are positioned and flow in
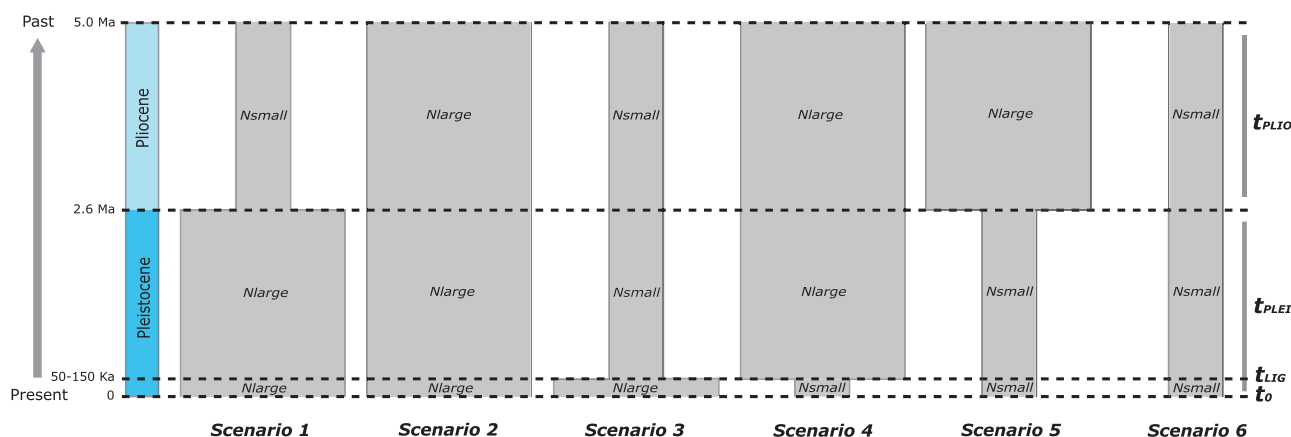
Figure 2. Different scenarios tested using approximate Bayesian computation (ABC) in DIY-ABC. Scenario 1: Pleistocene population expansion; scenario 2: constant large population size; scenario 3: population expansion during the Last Interglacial (LIG); scenario 4: LIG bottleneck; scenario 5: Pleistocene bottleneck; scenario 6: constant small population size. Time variations (minimum − maximum) used in each scenario: t_{LIG} (50,000 to 150,000 years ago), t_{PLEI} (10,000 to 2.6 million years ago), and t_{PLIO} (2.6 to 5.0 million years ago); Variations in population size: 100,000 to 1,000,000. Scenario conditions: $t_{LIG} < t_{PLEI}$; $Nsmall < Nlarge$.

different directions from the plateau. We conducted this analysis subsampling one haplotype per locality and setting the algorithm to identify three barriers, considering it sufficient to contrast both hypotheses in a regional geographic scenario of the Cerrado.

To test the hypothesis that recent events have shaped the genetic structure of *Colobosaura modesta*, we conducted Bayesian Skyline Plots (BSP, Drummond et al. 2005) implemented in BEAST 2.3.1 (Bouckaert et al. 2014). Considering the "structure effect" due to violation of panmixia, already reported for EBSP/BSP analyses (Heller et al. 2013), we ran BSP analyses based on the subpopulations generated from BAPS. We estimated the optimal nucleotide substitution model for each gene using PARTITION FINDER. For each subpopulation we ran five independent analyses using the following parameters: HKY+G (cytb), HKY+I (PRLR and SINCAIP), HKY+I+G (BFIB, MXRA5, and ACA) evolution models, gamma category count = 4, relaxed log normal clock model with a clock rate of 0.0065 for cytb, estimating the nuclear clock rates based on cytb; priors: coalescent Bayesian skyline, starting with a UPGMA tree, MarkovChained-PopSizes.t = "Jeffreys" (*Shape* = 1). *gammaShape.s* = log normal (initial = 1), *kappa.s* = log normal (initial = 2); proportion *invariant.s* = normal (initial 0.1); *ucldstdev.c* = gamma (initial 0.1); chain length of 100 million generations, sampled every 10,000 steps. We checked parameter convergence among runs and performance (ESS values above 200) with TRACER 1.6 (Rambaut et al. 2014).

To test the hypothesis of the influence of Pleistocene events on the demographic history of *Colobosaura modesta*, specifically looking for a signature of population expansion, as expected for riparian forest-dwelling species (Werneck 2011), we generated

six competing population history scenarios and submitted them to Approximate Bayesian Computation (ABC), implemented in DIY-ABC 2.0 (Cornuet et al. 2014). These six scenarios were: constant population size (two models), Pleistocene population expansion, population expansion during the Last Interglacial (LIG), Pleistocene bottleneck, and LIG bottleneck. The time ranges of effective population size changes in the models included the Pliocene (5 to 2.6 million years ago), the entire Pleistocene (2600 to 10,000 years ago), and the Last interglacial (LIG; 150,000 to 50,000 years ago; Fig. 2). We considered the LIG in our analysis instead of considering just the Pleistocene because the former contains explicit information of warm and wet conditions, and signs of lowland rainforest expansion toward the South America dry diagonal (Gosling et al. 2008; Ledru et al. 2009; Cheng et al. 2013). Moreover, this approach can also improve our understanding of the evolutionary processes that likely impacted *C. modesta*, including information on population size changes in the late Pleistocene. This is important because the Pleistocene was characterized by several climatic shifts throughout its duration for more than two million years (Pessenda et al. 2010; Hanselman et al. 2011; Hermanowski et al. 2012). We included all sequence data, used an HKY evolution model for each marker, selected all within-population summary statistics in the program, and a prior of equal probability to each scenario (16.67%). We used a generation time of 1 year for *C. modesta*, which is consistent with studies of closely related gymnophthalmids (Garda et al. 2014; Sousa et al. 2015). Six million datasets were simulated for each model. Logistic regression was used to estimate the posterior probability of each model based on 1% of simulated datasets for each scenario, producing scenario summary statistics closest to the observed summary statistics.

To infer possible distributional shifts in *Colobosaura modesta* associated with Pleistocene climatic changes, we modeled its current distribution and projected the results onto the Last Glacial Maximum (LGM) and Last Interglacial (LIG). We downloaded high resolution bioclimatic layers (2.5 min) for the Current (1960–1990), LGM (21,000 years ago; three different models), and LIG (120,000–140,000 years ago) (Otto-Bliesner et al. 2006) from the WorldClim project version 1.4 (http://www.worldclim.org/) (Table S3) and cropped them based on a South America shapefile. To reduce any bias in species distribution modelings (SDMs), we submitted the occurrences of *C. modesta* to the function envSample (Varela et al. 2014) in R, using BIO1 (Annual Mean Temperature) and BIO12 (Annual Precipitation) as filters and selected 40 equidistant points in the climatic space. We also performed pairwise correlation analyses with the current climatic variables to avoid redundancy in our SDM. Highly correlated variables ($r > 0.9$) were excluded from the model, based on their biological relevance and ease of interpretation, following a procedure described by Rissler and Apodaca (2007), and used in other studies (Werneck et al. 2011; Werneck et al. 2012b; Ledo and Colli 2017). Based on this approach, we excluded five of the 19 bioclimatic variables, and used the following: BIO1 (annual mean temperature), BIO2 (mean diurnal range), BIO3 (isothermality), BIO4 (temperature seasonality), BIO5 (max temperature of warmest month), BIO6 (min temperature of coldest month), BIO7 (temperature annual range), BIO8 (mean temperature of wettest quarter), BIO12 (annual precipitation), BIO13 (precipitation of wettest month), BIO14 (precipitation of driest month), BIO15 (precipitation seasonality), BIO18 (precipitation of warmest quarter), and BIO19 (precipitation of coldest quarter).

We modeled the current distribution of the filtered points of *Colobosaura modesta* with 10 SDM algorithms available in the package BIOMOD2 (Thuiller et al. 2009) in R, and selected the best algorithm based on results of the area under the curve (AUC) from the receiver operating characteristic (ROC), true skill statistic (TSS), accuracy, and bias. We then projected the results of our best model to all available AOGCMs for the LGM and LIG. We also transformed the projections of each time period into binary maps, based on the lowest presence threshold of TSS results. Considering that there is more than one AOGCM available for the LGM, we generated a consensus map based on the sum of the binary projections, and only considered areas with 100% of concordance of the consensus map in our interpretations and discussions.

Results

SPECIES DELIMITATION METHODS

Our BAPS cluster analysis recovered five groups within *Colobosaura modesta* (Fig. 3A and B). Group I included a sample (CHUNB34981) from a population from Novo Progresso–PA (locality #5); group II, samples from Santo Antônio do Leverger–MT (#20); group III, samples from the Tocantins–Araguaia depressions (Carolina–MA, Caseara–TO, Palmas–TO) in the Cerrado, from the Cerrado–Caatinga transition (Piripiri–PI), and from the Caatinga–Atlantic Forest transition (Mata de São João–BA); group IV, samples from Mateiros–TO (#3); and group V included samples from 12 localities across the central Cerrado and from Jardim–CE and Crato–CE (#17-18), in the Chapada do Araripe region, a Cerrado enclave in the Caatinga (Fig. 3A). Besides having some geographic correspondence, most groups were not recovered as different species in BPP, except the population from Novo Progresso-PA (#5). Using both algorithms (algorithms 0 and 1, Yang and Rannala 2010), with or without gaps, BPP consistently returned the same result, corroborating the hypothesis that the Novo Progresso–PA sample belongs to a different species. Different BPP runs recovered four species with a posterior probability of 1: the two outgroups (*Vanzosaura rubricauda* and *Acratosaura mentalis*), the Novo Progresso–PA sample, and the last one comprising all remaining *C. modesta* samples as a single species with shallow divergences. The best species tree estimated by BPP (highest posterior probability in all runs) had the same topology recovered by all concatenated phylogenetic analyses. The cytb haplotype network also indicated high divergence between the Novo Progresso–PA sample and the remaining samples of *C. modesta* (Fig. S1).

PHYLOGENETIC RELATIONSHIPS AND DIVERGENCE TIMES WITHIN *Colobosaura modesta*

Our BI and ML concatenated gene tree inference methods recovered similar results. *Colobosaura modesta* was monophyletic in both reconstructions. For brevity, we present only the BI tree (Fig. 3B). Overall, our gene tree inferences indicated shallow divergences and unresolved topologies within *C. modesta*, while the divergence of the Novo Progresso lineage (CHUNB34981; locality #5) is well supported from the remaining populations of *C. modesta* from the Cerrado, Caatinga, and Atlantic Forest (groups II–V) (Fig. 3B). Clades from Chapada do Araripe (#17 and 18), Santo Antônio do Leverger (#20), and in central Brazil (#2 to 4, 6 to 8, 10, 12, 14, 15) are also well supported, but their relationships were poorly resolved (Fig. 3B). The cytb haplotype network also resolves high divergence of the Novo Progresso lineage from the remaining lineages (Fig. S1).

Bayesian Inference implemented in BEAST recovered better resolved relationships within *Colobosaura modesta* than did MRBAYES. The Novo Progresso-PA lineage diverged during the Pliocene, whereas all others diverged during the Pleistocene (Fig. 4). This result was also congruent with the BPP results above and suggests a recent history of colonization and range expansion of *C. modesta* within the dry diagonal.
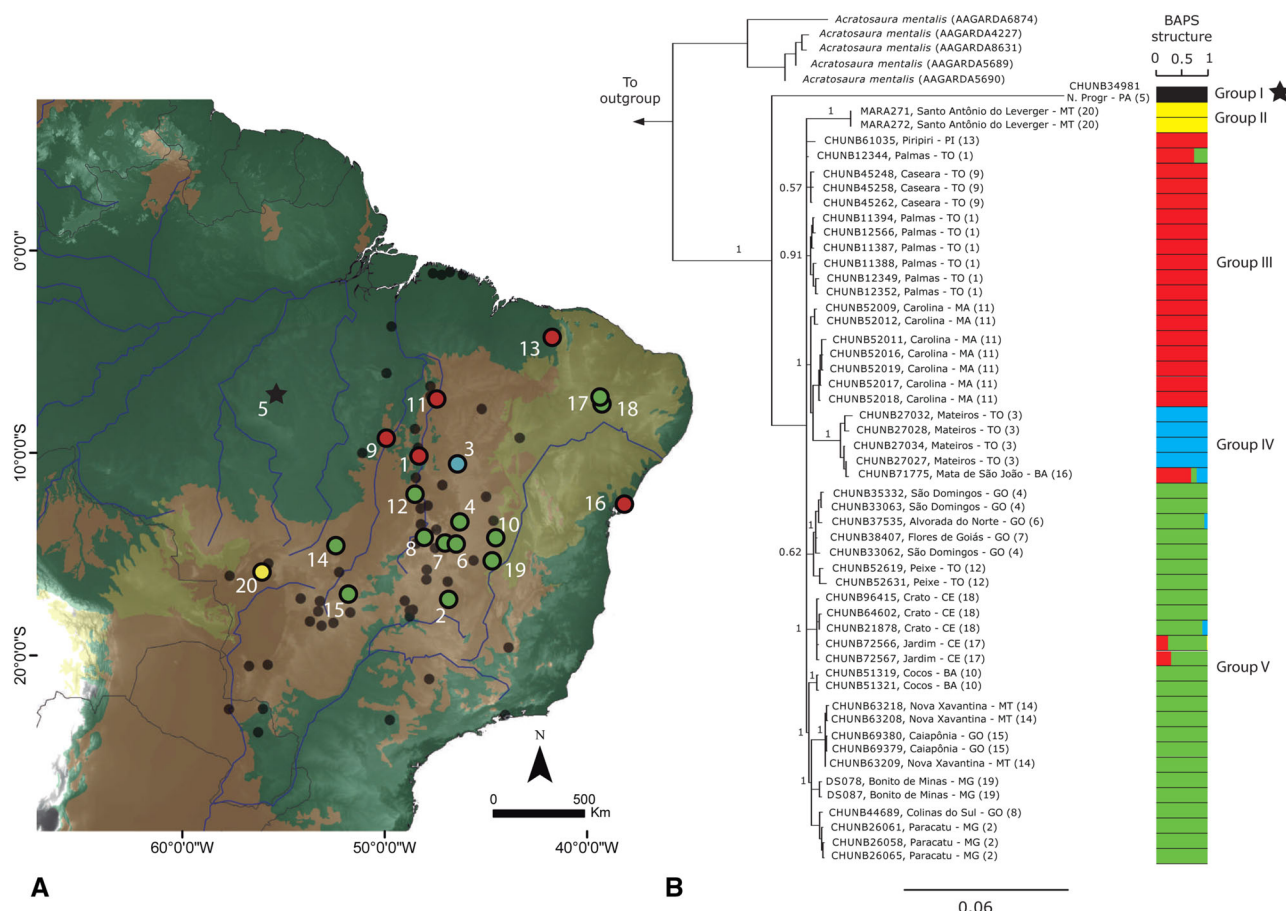
Figure 3. A: Distribution of *Colobosaura modesta* (gray dots) and distribution of the sampled localities of *C. modesta*, with colors based on the groups accessed with BAPS (letter B). On the upper left, a specimen of *Colobosaura modesta* (photo modified from Carlos Cândido). B: Concatenated gene genealogy of *Colobosaura modesta* reconstructed with Bayesian Inference in MrBayes 3.2.5, and genetic structure within *C. modesta* (colored bar) accessed with BAPS 6.0. Each color (five in total) is associated with one different group generated in BAPS. Group one is also represented by a star on the map (letter A). One of the outgroups (*Vanzosaura rubricauda*) was omitted in the figure for visualization purpose. The numbers in the map show different sample sites, also corresponded at the terminals of the phylogenetic tree.

GEOGRAPHIC STRUCTURE AND POPULATION HISTORY OF *Colobosaura modesta*

The Mantel test showed a significant association between genetic structure and geographic distance among sites in *Colobosaura modesta* ($r = 0.41$; $P < 0.001$). However, the partial Mantel test indicated no significant association of genetic distance and altitude resistance, when controlling for the effects of geographic distance ($r = -0.13$; $P = 0.83$). This suggests that the central Brazilian plateaus do not promote genetic breaks within *C. modesta*. The Monmonier's maximum difference algorithm implemented in BARRIER identified three main barriers to gene flow, all at the extremes of the *C. modesta* distribution (Fig. S2). This result provides further evidence that the central Brazilian plateaus did not play an important role in shaping genetic structure within *C. modesta*, suggesting that riparian forests may have acted as an interconnected network, allowing gene flow across the Cerrado

domain, as populations in the core distribution are more similar to each other than to peripheral populations.

Of the 10 SDM algorithms analyzed in BIOMOD2, Random Forest showed the best values of AUC, TSS, accuracy, and bias for our data (Table S4). Projections to the past indicate a *C. modesta* range expansions during the Last Interglacial (LIG), retraction during the LGM, and expansions in current conditions (Fig. 5). Areas with high probability of occurrence of *C. modesta* on montane forests inside Caatinga ("brejos de altitude," e.g., Fig. 1 localities #17 and 18), and on the western part of the distribution (group II, yellow in Fig. 1) were also recovered in the present, LIG and LGM (two of three AOGCMs) projections (Fig. 5). Areas on the restinga of Mata de São João (BA; locality #16 in Fig. 1), in the Atlantic Forest, were not recovered in any projection from the past to the present, suggesting unsuitable conditions for this species through time (Fig. 5). These results
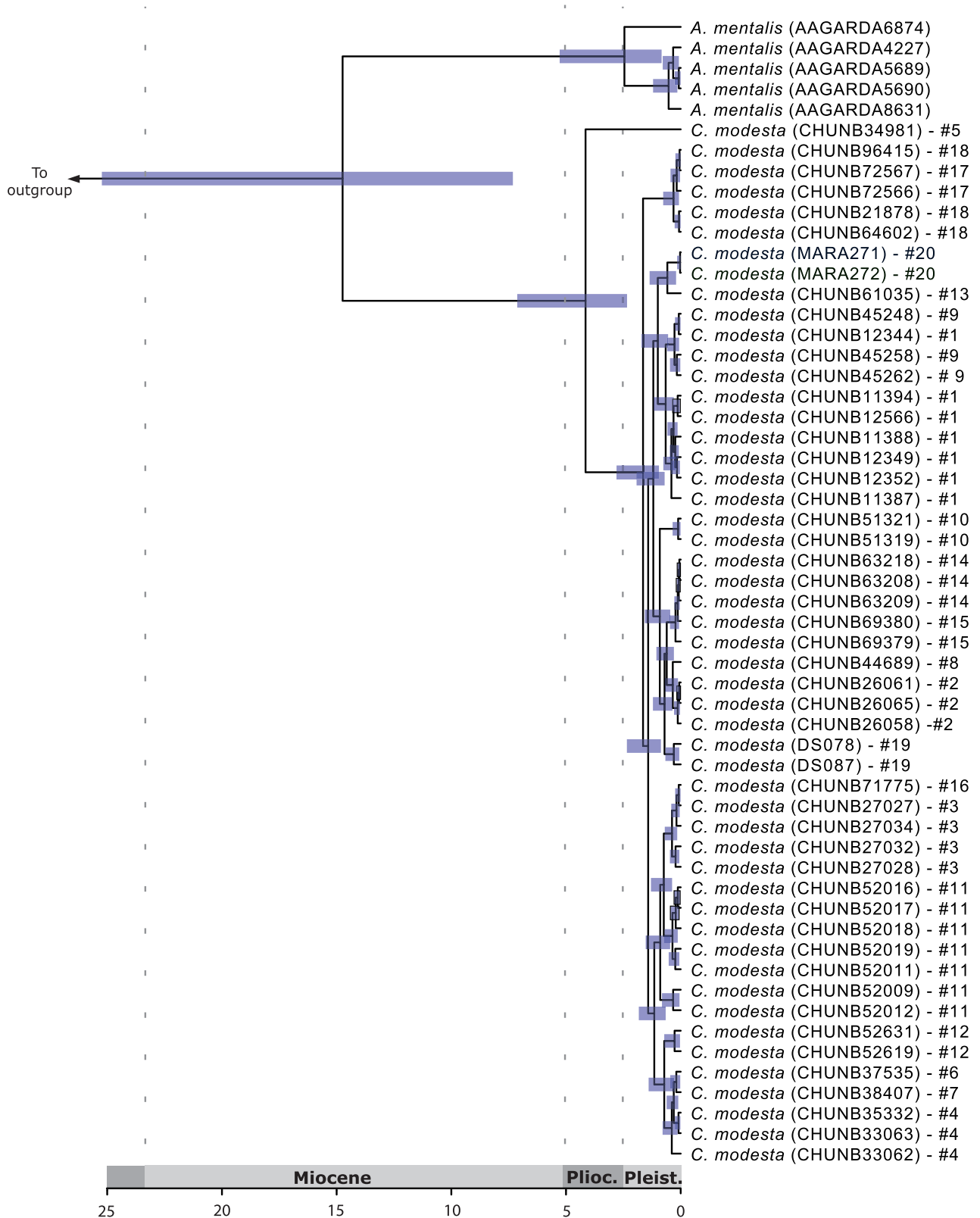
Figure 4. Bayesian phylogenetic reconstruction with divergence times of *Colobosaura modesta* generated in Beast 2.3.5. Horizontal bars represent 95% posterior credibility intervals of the time divergence estimates. Numbers bellow the gray box indicate estimated ages (million years). One of the outgroups (*Vanzosaura rubricauda*) was omitted in the figure for visualization purpose. Numbers at each terminal of the tree are different localities (sample sites) and also correspond to the sample sites in Figures 1 and 3.
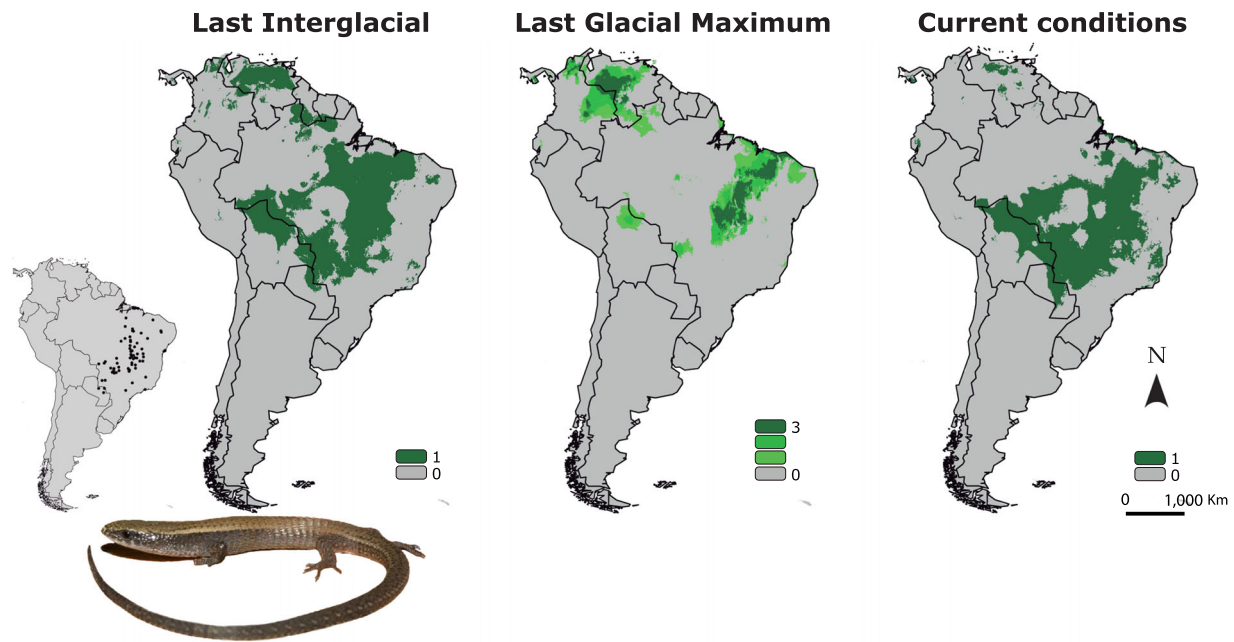
Figure 5. Species Distribution Modelling (SDM) of *Colobosaura modesta* in current conditions and projections to the Last Glacial Maximum (LGM) and Last Interglacial (LIG) using Random Forest. Black dots are occurrence points of *C. modesta* used in SDM. Signs of population expansion were notice mainly in LIG and current projections (photo modified from Carlos Cândido).

suggest the expansion of habitats for *C. modesta* in warm/wet conditions and retraction in cooler conditions, as well as isolation of *C. modesta* into subpopulations with some correspondence to BAPS groups.

The Bayesian Skyline Plots suggested a mid-Pleistocene expansion (Fig. 6A) for group III (red dots in Fig. 3A), while group V populations remained stable (Fig. 6B; green dots in Fig. 3A). These results also showed some congruence with the SDMs, since the projection of the potential LGM distribution of *Colobosaura modesta* is geographically concordant with the distribution of group V. The Approximate Bayesian Computation implemented in DIY-ABC identified Pleistocene population expansions (scenarios 1 and 3 in Fig. 2) as the best scenarios for the demographic history of *C. modesta* (75% explanation together), and a recent LIG population expansion (scenario 3 in Fig. 2) as the most likely among the six competing scenarios (52% explanation; Fig. S2). Scenario 2 accounted for 13% of the explanation (population stability), and the remaining scenarios all had <7% probabilities. All of these results suggest that at least part of *C. modesta* had a recent history of population expansion within the dry diagonal, mainly in Northeastern Brazil, associated with the Pleistocene.

Discussion

PHYLOGEOGRAPHY OF *Colobosaura modesta* AND THE HISTORY OF ITS ASSOCIATED VEGETATION

Pre-Pleistocene geologic (Colli 2005; Hoorn et al. 2010) and Pleistocene climatic changes (Haffer 1969; Vanzolini and Williams 1981) are among the most important hypothesized drivers of Neotropical diversification and, in some cases, both are invoked to explain diversification patterns (Rull 2008; Rull 2011). Werneck (2011) suggested that riparian forests are more related to younger processes in central Brazil and would be characterized by species with weaker genetic structure, lower phylogeographic resolution, and with signatures of population expansion, due to geologically recent erosion in the Cerrado plateaus, and Pleistocene climatic fluctuations effects. Our results support this hypothesis based on demographic and phylogeographic inferences for *Colobosaura modesta*, a typical forest-dwelling organism in the Cerrado, eastern Amazonia, and Caatinga montane forests.

Recent molecular studies suggested that Pleistocene climatic fluctuations were important in shaping the demographic and distributional patterns in northeastern Brazilian frogs (Carnaval and Bates 2007; Thomé et al. 2016; Gehara et al. 2017), squamate reptiles (Zamudio and Greene 1997; Oliveira et al. 2015), and birds (Batalha-Filho et al. 2013). Pleistocene climatic fluctuations allowed the incursion of Amazonian and Atlantic Forest biotas across the Cerrado and Caatinga (Batalha-Filho et al. 2013; Ledo and Colli 2017) and vice versa (Buzatti et al. 2018), an interpretation supported by paleoecological evidence in these same regions (De Oliveira et al. 1999; Pessenda et al. 2004; Pessenda et al. 2010). Our study also corroborates this hypothesis with a forest-dwelling lizard from the dry diagonal, as shown by the phylogeographic, SDM, BSP, and ABC inferences.
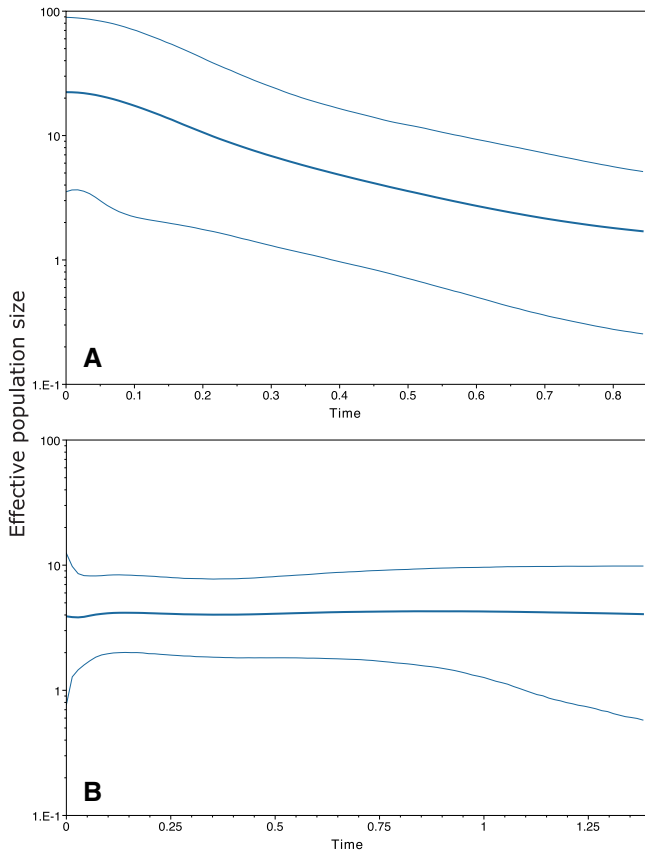
Figure 6. Bayesian skyline plots for subpopulations of *Colobosaura modesta* (A: group III; B: group V) from BAPS, illustrating effective population size (Ne) variation through time. Thicker lines represent the mean population size, and thinner lines represent 95% higher and lower confidence interval of posterior probabilities.

There is evidence that the central Brazilian highlands are important barriers for gene flow for riparian forest-dwelling species (Silva 1996; Valdujo et al. 2013) and for organisms from other vegetation formations (Werneck et al. 2012a; Oliveira et al. 2018). Our partial Mantel test indicated that altitude resistance is not an important variable in explaining genetic diversity and structure within *Colobosaura modesta*; rather, isolation-by-distance played a major role in the genetic structure of the species. Similar results were found in studies of other Cerrado lizards (Santos et al. 2014), frogs (Vasconcellos et al. 2019), spiders (Bartoleti et al. 2018), and palm trees (Melo et al. 2018), suggesting that isolation-by-distance plays an important role in shaping genetic structure of several organismal groups within the Cerrado. Moreover, populations at the periphery of the distribution of *C. modesta* were more genetically differentiated relative to core populations, and these differences were not associated with main river basins in central Brazil. The observed genetic structure of *C. modesta* supports the hypothesis that riparian forests formed interconnected networks promoting intraspecific

gene flow across the Cerrado and northeastern Brazil (Mares and Ernest 1995; Oliveira-Filho and Ratter 1995). In summary, our results indicate that the genetic structure within *C. modesta* may be the result of Pleistocene mesic forest expansions and retractions in Cerrado and Caatinga, isolating peripheral populations in relation to core populations and promoting differences among them.

Our BSP analyses recovered different demographic stories for *Colobosaura modesta* across its distribution; the northeastern population (group III in BAPS) shows a signature of population expansion whereas the central population (group V in BAPS) shows no significant demographic changes through time. These phylogeographic results for the northeastern populations of *C. modesta* are supported by studies of Pleistocene vegetation shifts in northeastern Brazil (De Oliveira et al. 1999; Pessenda et al. 2010). However, results for central populations may have two different causes. For instance, the BSP results for these populations might be an artifact, the "structure effect," due to violation of the panmixia assumption (Heller et al. 2013).

Alternatively, part of the riparian forests in central Brazil might not have experienced significant changes through the Pleistocene. The central and southern Cerrado riparian forests are characterized by different landscapes than the northern and northeastern Cerrado riparian forests (Brasil 1974), and both were subjected to different climatic conditions (Oliveira-Filho and Fontes 2000). The central/southern Cerrado riparian forests are characterized by more pronounced reliefs (embedded in depressions), whereas the northern/northeastern Cerrado riparian forests occur in less pronounced regions of the Brazilian Shield, and with more low-elevation areas toward Amazonia. The more pronounced relief in central Brazil likely provided the necessary conditions for the persistence of riparian forests in the depressions, even during unfavorable periods of the Pleistocene. Further, the South American Convergence Zone brings a lot of precipitation from the Atlantic Ocean and Amazonia to the central and southern Cerrado (Zhou and Lau 1998; Gan et al. 2004; Cheng et al. 2013), as part of the South American Monsoon System. We suggest that this system likely maintained high levels of precipitation in these regions during the Pleistocene (Cheng et al. 2013), thereby favoring the persistence of riparian forests. Palynological studies in the central-southern Cerrado show moister conditions in the LGM than the present day, along with the presence of riparian forests (Salgado-Labouriau 1997; Parizzi et al. 1998; Barberi et al. 2000; Salgado-Laboriau 2005). Moreover, it is also clear that the Atlantic Forest vegetation is better adapted to climatic seasonality than the Amazonian vegetation (Oliveira-Filho and Fontes 2000), allowing the persistence of more Atlantic Forest lineages inside the Cerrado (via riparian forests) than the Amazon.

Paleoecological studies suggest significant Late Pleistocene vegetation shifts in central Brazil, showing wetter/colder

conditions than the present (Salgado-Labouriau 1997; Parizzi et al. 1998; Barberi et al. 2000; Salgado-Laboriau 2005), and wetter conditions in northeastern Brazil (De Oliveira et al. 1999; Auler and Smart 2001; Pessenda et al. 2010). Our study supports the hypothesis that Pleistocene climatic changes also played an important role in the Neotropical region, including the Cerrado domain. Nevertheless, deep divergences of typical plateau taxa (Werneck et al. 2012a; Domingos et al. 2014; Guarnizo et al. 2016) and environmental niche modeling studies (Werneck et al. 2012b) suggest that climatic changes were not consistent across the entire Cerrado or across all taxa. In other words, vegetation shifts were driven by climate changes in the Cerrado during the Pleistocene, but these did not modify the entire Cerrado landscape.

The plateaus contain different edaphic characteristics compared to valleys, so typical savanna elements may have persisted in plateaus (stability areas) even as they receded overall during the unfavorable Pleistocene conditions. Some studies suggest that some western Cerrado regions maintained a stable vegetation during glacial times (Burbridge et al. 2004), and that these regions also contained genetically variable and deeply divergent populations of amphibians (Prado et al. 2012) and reptiles (Santos et al. 2014; Guarnizo et al. 2016). Moreover, large climatic stable regions were also suggested for the central-eastern Cerrado (Werneck et al. 2012b). Currently, riparian forests account for 10–20% of the Cerrado vegetation cover (Ribeiro and Walter 2008) and were probably larger in more favorable past climates (De Oliveira et al. 2020). These more extensive plant communities would have been interconnected along the headwaters of river basins, but they would not dramatically replace the savanna Cerrado vegetation communities. Palynological evidence suggest that even with these riparian forest shifts, the common grassland communities would dominate savanna landscapes, as evidenced by high densities of these species in the pollen record (Salgado-Labouriau et al. 1998). In summary, it is likely that the Cerrado biota is composed of both old and new lineages that were shaped by different phenomena, and they occupy different landscape features and habitats in the biome.

LIMITATIONS OF THE STUDY AND OTHER PHYLOGEOGRAPHICAL HYPOTHESES

The phylogeographic history of *Colobosaura modesta* reflects reduced population structure in the core distribution (central Cerrado and part of northeastern Brazil), and higher differentiation between the core and the peripheral populations (Novo Progresso – PA, Santo Antônio do Leverger – MS, and Mata de São João – BA). This result was also supported by the BSPs and SDMs of *C. modesta*, mainly for group V (Fig. 1, color green). We did not formally test other hypotheses associated with isolation-by-distance and Pleistocene climatic changes, such as the center/periphery

hypothesis (Pironon et al. 2017) or the isolation-by-instability (Vasconcellos et al. 2019); neither of these were included in the original objectives of this study, and would both require different sampling designs to be tested. The center/periphery hypothesis predicts lower differentiation among populations in the core distribution of a species, due to higher population density, and higher differentiation in marginal populations, due to low N_e in peripheral areas and limited gene flow due to isolation (Hardie and Hutchings 2010). "Isolation-by-instability" suggests that the present-day genetic structure of organisms is explained by the effect Pleistocene climate changes. During unfavorable conditions, populations were isolated in refugia and characterized by restricted gene flow and lower genetic diversity. Some intraspecific phylogeographic studies of the dry diagonal biota are consistent with these predictions of Pleistocene climatic changes (Santos et al. 2014; Melo et al. 2018; Vasconcellos et al. 2019); the genetic structure of *C. modesta* might also be explained by these hypotheses, but a wider geographic sampling of the species and additional genetic markers are necessary to test them.

SPECIES BOUNDARIES IN *Colobosaura modesta*

Colobosaura modesta (Reinhardt and Lütken 1862) is currently considered the only valid species within the genus *Colobosaura*. A new species, *C. (Perodactylus) kraepelini* (Werner 1910), was described based on only one young female (SVL = 40 mm) from Puerto Max, Paraguay (Werner 1910), but later synonymized with *C. modesta* (Amaral 1932). Similarly, *C. landii* (Cunha 1977) was described from specimens collected in primary forests of eastern Amazonia (Vila do Curupati, northeastern Pará), in a different habitat from the previous known records of *Colobosaura*. These specimens and other individuals from eastern Pará were later recognized as conspecific with *C. modesta* (Cunha and Nascimento 1982, 1983; Nascimento et al. 1987). These data and the results in this study support the hypothesis *C. modesta* is a single, relatively "young" lineage, but the pronounced divergence of the Serra do Cachimbo (Novo Progresso – PA) *C. modesta* sample from all remaining samples (and results of the BPP analysis), suggest that it be treated as a "candidate species," pending further study. All other populations occupying the remaining distribution of *C. modesta* (eastern Pará, Cerrado riparian forests, montane and submontane forest enclaves in the Caatinga, and restinga habitats in northeastern Bahia) are conspecific on the basis of all available evidence.

Other endemic species of reptiles and amphibians are known from the Novo Progresso region, within the Serra do Cachimbo mountain range, including the lizard *Tropidurus insulanus* (Rodrigues 1987) and *Dendropsophus cachimbo* (Napoli and Caramaschi 1999), indicating the biogeographic importance of this region. As noted above, the collection of more specimens from more localities throughout the region, coupled with additional

data, is essential to test our "candidate species" hypothesis for the Novo Progresso individual. The locality of this specimen occurs in the well-known "Arc of Deforestation" (Aldrich et al. 2012; Ferreira et al. 2014), a region with high diversity and intense habitat loss where most of the Amazonian deforestation is concentrated. Recently a new genus of gymnophthalmid lizard was described for the region (Colli et al. 2015), reflecting our "biodiversity knowledge gap." Given current rates of habitat loss within this "Arc of Deforestation," many species could go extinct before being discovered if conservation efforts are not implemented.

In summary, our results corroborate the hypothesis of a "mixed history" within the Cerrado: older lineages are associated with Cerrado-dwelling organisms, while younger lineages are associated with forest-dwelling organisms. This pattern highlights the importance of riparian forests within the dry diagonal, in the larger biodiversity context of South America. These forests have permitted intraspecific gene flow among regions, thereby maintaining genetic diversity within this lizard. Considering that the Neotropical biodiversity was assembled and became megadiverse through extensive interchange among biomes (Antonelli et al. 2018b), the importance of riparian forests should not be neglected in the larger context of dry diagonal biodiversity conservation. Further, we identify a genetically divergent and geographically isolated *C. modesta* population, referred to here as a "candidate species," that requires further study. This candidate species is apparently restricted to the Serra do Cachimbo region, which, due to its biogeographic singularity and intense deforestation, is in need of urgent conservation.

AUTHOR CONTRIBUTIONS

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DATA ARCHIVING

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