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ORIGINAL ARTICLE

Landscape evolution and phylogeography of *Micrablepharus atticolus* (Squamata, Gymnophthalmidae), an endemic lizard of the Brazilian Cerrado

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ABSTRACT

Aim Our aims were to investigate the spatial genetic structure of *Micrablepharus atticolus* and to assess the relative importance of differentiation in plateaus versus depressions, in areas of historical stability versus instability, and in central versus peripheral regions.

Location The Brazilian Cerrado.

Methods We compared the elevational range of *M. atticolus* with that of its sister species, *Micrablepharus maximiliani*, to investigate their putative elevational segregation. We identified past (6, 21 and 130 ka) and current variables associated with the distribution of *M. atticolus*. Based on cytochrome *b* sequences, we compared genetic diversity indexes and neutrality statistics between plateau/depression, stable/unstable and core/periphery populations. We identified geographically homogeneous and maximally differentiated groups of populations and tested the association between genetic and geographical distances. Finally, we traced elevational range on the phylogeny and tested for a significant phylogenetic signal associated with elevation.

Results We found no elevational segregation between *M. atticolus* and *M. maximiliani*. There is high genetic diversity and structuring among populations, with the primary differentiation occurring between north-eastern and south-western Cerrado localities. We recognized three main groups of populations that roughly correspond to the southern, central-northern and north-western portions of the Cerrado, which diverged between 3.5 and 1.5 Ma. Genetic diversity indices indicated no differences between plateaus and depressions or stable and unstable areas, but samples from peripheral isolates in south-western Amazonia exhibited low haplotype and nucleotide diversity and signs of population expansion.

Main conclusions The diversification of *M. atticolus* in the Cerrado was primarily affected by events in the late Neogene. We found no support for the plateau/depression and stability/instability hypotheses, but we did find support for the core/periphery hypothesis. The spatial patterns seemingly resulted from a combination of shifting environmental conditions during climatic cycles, with repeated colonizations of plateaus and depressions, isolation by distance, and divergence in and recolonization of peripheral isolates within Amazonia.

Keywords
Biogeography, climatic cycles, core–periphery, genetic diversity, Neotropical region, population expansion, refugia, savannas, South America, stability.

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INTRODUCTION

The origins of high Neotropical biodiversity have fascinated biogeographers since Wallace (1876). Most work, however, has focused on forest habitats, as open habitats were originally thought of as secondary, species-poor formations (Bond & Parr, 2010). More recently, a growing number of studies have rapidly altered this picture, revealing the biotic singularity of South American open landscapes (reviewed in Werneck, 2011). Nevertheless, knowledge of the spatial patterns of genetic diversity and the mechanisms driving such patterns in the Neotropical region is still biased towards forest habitats. For instance, only a few phylogeographical studies have been conducted in the South American Cerrado (e.g. Collevatti *et al.*, 2009; Prado *et al.*, 2012; Werneck *et al.*, 2012a).

The Cerrado is the largest Neotropical savanna, with high levels of diversity and endemism, especially among plants and squamates (Oliveira & Marquis, 2002). It is highly threatened and considered one of the world's biodiversity hotspots (Myers *et al.*, 2000). Colli (2005) presented a biogeographical scenario for the diversification of the Cerrado herpetofauna, which indicated that most lineage diversification took place during the Palaeogene and Neogene. The compartmentalization of the Cerrado landscape between plateaus (above 500 m) and depressions (below 500 m), which may have occurred *c.* 2–3 Ma, is presumably one of the most important vicariance events in the region (Nogueira *et al.*, 2011; Werneck, 2011), a hypothesis that is hereafter called the plateau/depression hypothesis. Plateaus are separated by depressions holding the main rivers of the Cerrado, namely the Araguaia, Tocantins, São Francisco, Paraguai and Paraná rivers. Depressions are much younger than plateaus, were formed by erosive processes during the Quaternary, and harbour seasonally dry forests or gallery forests; conversely, the ancient plateaus are dominated by savannas. Apparently, younger lineages of Cerrado birds (neoendemics) are associated with depressions, whereas older lineages (palaeoendemics) are associated with plateaus (Silva, 1997).

A historically important explanation for Neotropical diversification is the Pleistocene refuge hypothesis (Haffer, 1969). Although this hypothesis has been challenged (e.g. Colli, 2005; Hoorn *et al.*, 2010), there is some evidence to support it. We refer to it hereafter as the stability/instability hypothesis. Populations within refuges are expected to have higher genetic diversity than populations in unstable areas, which should present signatures of recent population expansion (e.g. Carnaval *et al.*, 2009). Werneck *et al.* (2012b) used palaeodistribution modelling to predict areas suitable for the occurrence of the Cerrado in the mid-Holocene (6 ka), Last Glacial Maximum (LGM, 21 ka) and Last Interglacial (LIG, 130 ka). In the mid-Holocene model, the distribution of suitable conditions for the Cerrado was similar to the current distribution, whereas during the LGM, there was a contraction of this distribution, and the largest expansion was observed during the LIG (Werneck *et al.*, 2012b). Thus, although the modelled scenario disagrees with the traditional view of Cerrado expansion during glacial maxima (Ledru, 2002), it points to changes in vegetation distribution, highlighting the importance of testing the stability/instability hypothesis.

Demographic parameters, such as effective population size (N_e) and migration (m), affect the spatial patterns of genetic diversity (Vucetich & Waite, 2003). One well-known pattern is isolation by distance, in which genetic differentiation is proportional to geographical distance simply due to the restricted gene flow between populations located far from each other (Wright, 1943). A more complex model is the core/periphery hypothesis, which predicts lower genetic diversity and higher differentiation in marginal populations based on the assumption of a higher population density in the core, leading to low N_e in peripheral areas and low gene flow caused by isolation (Eckert *et al.*, 2008; Hardie & Hutchings, 2010). Given the large extent of the Cerrado, it is likely that such gradients of genetic diversity occur among its endemics.

The lizard *Micrablepharus atticolus* Rodrigues, 1996 (Gymnophthalmidae) is endemic to the Cerrado, inhabiting open physiognomies and being broadly distributed and locally abundant (Vitt, 1991; Rodrigues, 1996; Vieira *et al.*, 2000). The species is also found in peripheral isolates of the Cerrado within Amazonia (Vitt & Caldwell, 1993; Gainsbury & Colli, 2003). These characteristics make the species an excellent model with which to investigate patterns resulting from the dynamic history of the Cerrado. The congeneric *Micrablepharus maximiliani* (Reinhardt and Lütken, 1861) has a broader distribution, occurring in the Caatinga and the Pantanal as well as the Cerrado. Nogueira (2006) hypothesized that *M. atticolus* and *M. maximiliani* are segregated along an elevational gradient, the former occurring in highlands and the latter in lowlands. However, this hypothesis was based on limited observations of both species in the same locality and not on formal analyses.

Here, we use molecular (cytochrome *b*) and distributional data to test the hypothesis of elevational segregation between the two species of *Micrablepharus* and identify the historical and ecological predictors of the distribution of *M. atticolus*. We conduct exploratory phylogenetic and phylogeographical analyses to assess patterns of genetic variation, which we contrast with elevation, geomorphology, and current and past distribution models of *M. atticolus* to identify the mechanisms that shaped the species' diversification. More specifically, we evaluate the predictions of the core/periphery, plateau/depression and stability/instability hypotheses. We predict that peripheral populations should have lower genetic diversity and signs of population reduction due to reduced gene flow and more harsh/stochastic peripheral environments. Considering that plateaus are older than depressions, we also predict lower genetic diversity and signs of population expansion in the latter due to recent colonizations. Finally, if areas identified as stable based on species distribution models (SDMs) served as refugia during climate cycles, we predict that they harbour higher genetic diversity than

unstable areas and that the latter areas will show signs of population expansion following recent colonizations.

MATERIALS AND METHODS

Spatial distribution

We compiled distribution records of *M. maximiliani* from Nogueira (2006) and those of *M. atticolus* from the literature and major Brazilian collections (see Appendix S1 in Supporting Information). Because elevation values were not normally distributed, we used the nonparametric Wilcoxon test (Quinn & Keough, 2002) to test for elevational segregation between the two species. To distinguish elevational segregation where species distributions overlap from possible bias due to differences in the extents of geographical species distributions, we performed three different tests comparing all records of *M. atticolus* with the following: (1) total records of *M. maximiliani*; (2) records of *M. maximiliani* outside the *M. atticolus* distribution; and (3) records of *M. maximiliani* within the *M. atticolus* distribution.

We built SDMs of *M. atticolus* for the present, mid-Holocene, LGM and LIG with Maxent v. 3.3 (Phillips *et al.*, 2006). To allow comparisons with the models of Werneck *et al.* (2012b), we used the same environmental variables (nine bioclimatic layers and elevation). We obtained current climatic and elevation variables from WorldClim (http://www.worldclim.org) and palaeoclimate data for the LIG from Otto-Bliesner *et al.* (2006) and for the LGM and mid-Holocene from the ECHAM3 atmospheric General Circulation Model (GCM) (DKRZ, 1992). We excluded records from Alta Floresta (Mato Grosso), Nova Monte Verde (Mato Grosso), and Ourilândia do Norte (Pará) from the analyses because they represent Cerrado isolates within a forest matrix (Fig. 1) and would behave as outliers, strongly influencing the SDMs. Thus, we had a total of 108 spatially unique occurrence points, 25% of which were randomly withheld for testing. Using the present-day SDM, Cerrado limits, occurrence records and personal field experience, we delimited the contiguous, contemporary distribution of *M. atticolus*. We considered records from sites close to the boundaries of this contiguous distribution of the Cerrado to be peripheral populations (Fig. 2). To define stable areas (presumptive refugia) for the species, we transformed the four SDMs into presence–absence maps by selecting threshold values for which sensitivity equals specificity, maximizing the agreement between observed and modelled distributions and balancing the costs arising from an incorrect prediction

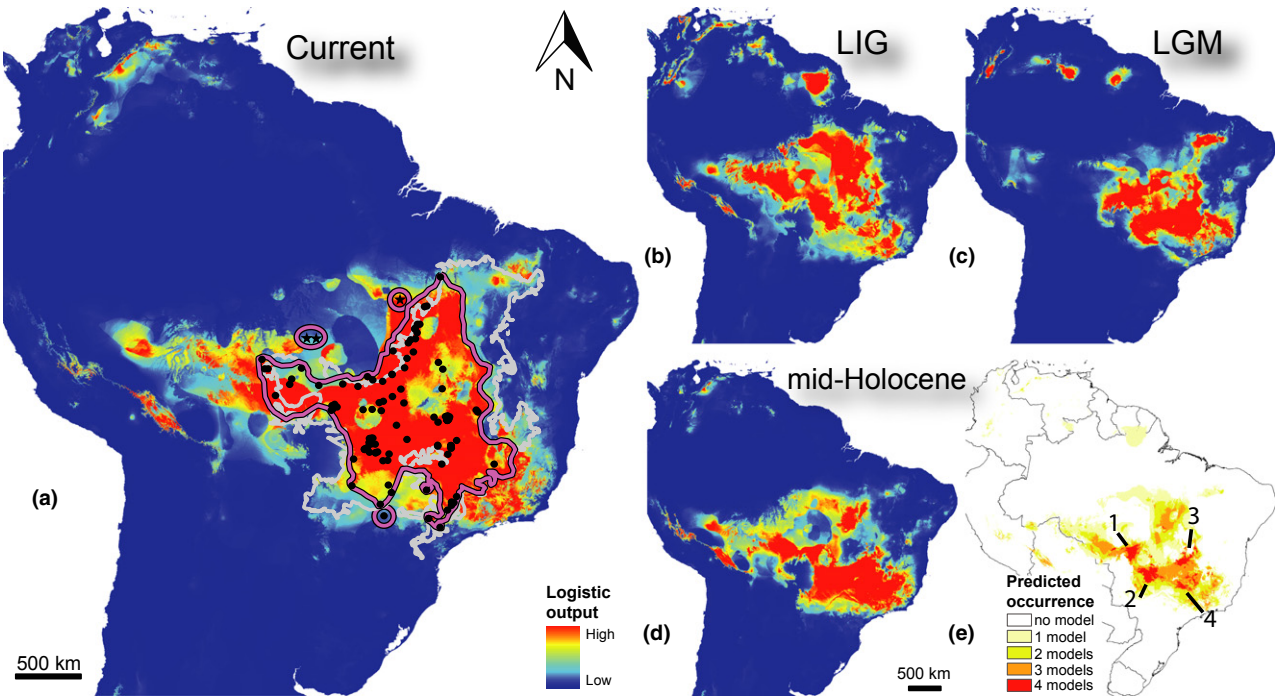

Figure 1 Species distribution models (SDMs) of *Micrablepharus atticolus* from the Brazilian Cerrado under past and current environmental conditions. (a) Occurrence points of *M. atticolus* used in Maxent modelling (black dots) and the current species distribution model (SDM) (gradient colours) and distributional range (pink). Stars indicate the localities excluded from the modelling, namely Alta Floresta (MT), Nova Monte Verde (MT), and Ourilândia do Norte (PA), due to their long distance from the central Cerrado (grey). Pink circles indicate occurrence records isolated from the core distribution of the species. (b–d) SDMs of *M. atticolus* during: (b) the mid-Holocene (6 ka); (c) Last Glacial Maximum (LGM, 21 ka); and (d) Last Interglacial (LIG, 130 ka). (e) Stable and unstable areas for the species as defined by the overlap of the current, mid-Holocene, LGM and LIG SDMs: 1 = Planalto dos Guimarães; 2 = Planalto de Caiapônia; 3 = Planalto Central; 4 = Planalto da Canastra.

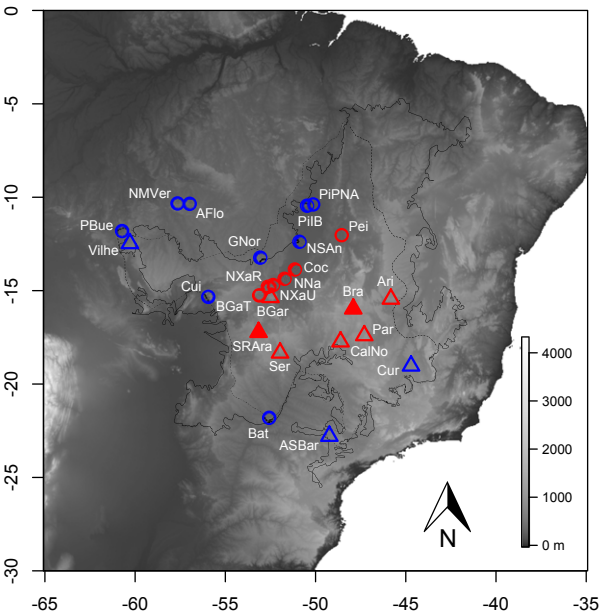

Figure 2 Distribution of Brazilian *Micrablepharus atticolus* samples used in the molecular analyses. The solid line represents the limits of the current Cerrado distribution, and the dashed line represents the limits of the current, contiguous distribution of *M. atticolus*. Red: core areas; blue: peripheral areas. Circle: depression; triangle: plateau. Solid symbols: stable areas (presumptive refugia); empty symbols: unstable areas. AFlo, Alta Floresta; Ari, Arinos; ASBar, Águas de Santa Bárbara; Bat, Bataguassu; BGar, Barra do Garças; BGaT, Barra do Garças (PCH Toricoejo); Bra, Brasília; CalNo, Caldas Novas; Coc, Cocalinho; Cui, Cuiabá; Cur, Curvelo; GNor, Gaúcha do Norte; NMVer, Nova Monte Verde; NNa, Nova Nazaré; NSAn, Novo Santo Antônio; NXaR, Nova Xavantina (Rancho Ponte de Pedra); NXaU, Nova Xavantina (UNEMAT); Par, Paracatu; PBue, Pimenta Bueno; Pei, Peixe; PiIB, Pium (Ilha do Bananal); PiPNA, Pium (Parque Nacional do Araguaia); Ser, Serranópolis; SRAra, Santa Rita do Araguaia; Vilhe, Vilhena.

against the benefits gained from a correct prediction (Manel *et al.*, 2001; Pearson *et al.*, 2006). We considered areas where the four presence–absence maps overlapped to be stable (Fig. 2).

To assess the relative importance of historical and ecological predictors of the contemporary distribution of *M. atticolus*, we used a generalized linear model (GLM) with a logit link function (Quinn & Keough, 2002). Ecological predictors consisted of the environmental variables used in the Maxent modelling, and the historical predictors consisted of the potential distribution of the Cerrado during the mid-Holocene, LGM and LIG as continuous variables, as well as stable (refugia) areas defined by the overlap of binary Cerrado models (Werneck *et al.*, 2012b). We randomly sampled 500 points inside and 500 points outside the distribution of *M. atticolus* and extracted values for each predictor using the ArcGIS Multiple Raster-Value Extractor extension (Pérez, 2007), using presence–absence points as the dependent variable in the model. We manually selected predictors in a forward, stepwise manner, using the Akaike information criterion (AIC) and chi-square tests to evaluate their significance (Tabachnick & Fidell, 2007). We assessed predictor importance via model averaging with the package MuMIn 1.0 for R (Burnham & Anderson, 2002). We conducted all GIS-based analyses in ArcGIS 9.3 (ESRI) and all statistical analyses in R 2.13.0 (R Development Core Team, 2011).

Molecular data

We used 116 tissue samples of *M. atticolus* from 30 localities (Appendix S1). We extracted total DNA from samples of liver, muscle, tail, or toes with a DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, CA, USA). We amplified a cytochrome *b* (cyt *b*) fragment of nearly 800 bp by PCR, using primers CB1-5 and CB3-3 as described by Palumbi (1996), and obtained sequences from Macrogen Inc (Seoul, Korea). We edited the sequences in the Staden Package (Staden *et al.*, 2003a,b) installed through eBioX 1.5.1 (Lagercrantz, 2008). As expected for protein-coding mitochondrial genes, there were no gaps; therefore, we aligned the sequences by eye. We recognize the limitations of single-locus phylogeography, as well as the challenges of assessing genealogical concordance in multi-locus studies (Brito & Edwards, 2009); therefore, some of our conclusions must be regarded as preliminary.

To assess levels of genetic diversity, we calculated the number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (H_d), and nucleotide diversity (π) with DnaSP 5.10.01 (Librado & Rozas, 2009). As we had 13 incomplete sequences and DnaSP excludes sites with missing values, we used a total of 661 bp. We considered each locality as a population, except for five localities from the Distrito Federal and two localities from Pium, which we pooled due to their close proximity. We report genetic diversity and population expansion indices for well-sampled localities [14 to 16 samples: Brasília, Nova Xavantina (Rancho Ponte de Pedra), Pimenta Bueno, Pium and Vilhena] and for plateau/depression, core/periphery and stable/unstable areas.

To determine the significance of differences in genetic diversity between plateau/depression, core/periphery and stable/unstable areas, we used a rarefaction analysis (Sanders, 1968) of h with EcoSim (Gotelli & Entsminger, 2001) based on 1000 randomized samples using the following options: independent sampling, species richness index and abundance level as in the sample with the lowest number of individuals. Next, we used a Z-test (Zar, 2010) for hypothesis testing. To detect genetic signals of population expansion, we calculated F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) statistics and tested their departures from neutrality based on 10,000 coalescent simulations with DnaSP. To assess levels of gene flow among localities, we calculated F_{ST} (Hudson *et al.*, 1992) with DnaSP, excluding samples represented by a single individual.

To determine whether non-random patterns of genetic diversity exist over the landscape, we first used a Mantel test

(Quinn & Keough, 2002) to evaluate the association between genetic distance and geographical distance matrices. We extracted geographical distances between populations using the ArcGIS extension Hawth's Tools (Beyer, 2004). We obtained mean genetic distances between sampling points with MEGA 5.05 (Tamura *et al.*, 2011) with pairwise deletion of missing sites and using a maximum composite likelihood model with a gamma parameter equal to 1.31, according to the model selected by jModelTest 0.1.1 (Posada, 2008). We conducted the Mantel test using package APE 2.7.2 for R (Paradis, 2006) with 10,000 permutations. Next, to define groups of populations that are geographically homogeneous and maximally differentiated from each other, we performed a spatial analysis of molecular variance (SAMOVA) with SAMOVA 1.0 (Dupanloup *et al.*, 2002) with 100 simulated annealing processes and pairwise distances between haplotypes. As we had no a priori hypotheses concerning the best number of populations, we examined results from 2 to 20 groups and based our decision on the proportion of total variation due to between-group differentiation (F_{CT}). We plotted F_{CT} values as a function of the number of populations and looked for the point representing an inflection on the curve.

To reveal general patterns of geographical structure, we conducted a spatial analysis of principal components (sPCA, Jombart *et al.*, 2008, 2009) with the package ADEGENET 1.3-2 for R (Jombart, 2008; Jombart & Ahmed, 2011), using 999 Monte Carlo permutations to identify and test the significance of global and local structures. Based on the results of these tests and on a scree plot, we determined which components should be retained for interpretation, searching for an abrupt decrease in eigenvalues (Jombart *et al.*, 2008).

We represented haplotype relationships by a median-joining network using Network 4.6.0 (http://www.fluxus-engineering.com/, Bandelt *et al.*, 1999). To avoid information loss, we performed two analyses: one including only complete sequences (DS1, n = 103, 715 bp) and the other including all sequences, excluding sites with missing occurrences (DS2, n = 116, 661 bp). We used the default epsilon and weighting parameters and cleared networks with maximum parsimony post-processing to eliminate unnecessary median vectors (Polzin & Daneshmand, 2003). We excluded a sample from Sapezal from the analysis because of its long distance from other *M. atticolus* sequences (see Results).

To assess the historical relationships between unique haplotypes, we conducted a Bayesian phylogenetic analysis with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), including all samples and sites, with *Vanzosaura rubricauda* and *M. maximiliani* as outgroups (cyt *b* sequences supplied by F.P. Werneck, Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil). We used jModelTest to select the best-fit nucleotide substitution model. Because a transitional model with a proportion of invariant sites and gamma-distributed rate variation (TIM2 + I+G) was selected, we changed the default settings for the structure of model parameters in MrBayes to *nst* = 6 and *rates* = *invgamma*. We fixed no priors and estimated the model parameters during the analyses. We performed two independent runs for 5×10^6 generations, with chain sampling every 100th generation, producing a total of 50,001 trees. We maintained all other settings at the default settings so that each run used one cold and three hot chains in a Markov chain Monte Carlo model composition (MC3) approach. We determined stationarity by visual inspection of results and calculated a 50% majority-rule consensus, discarding the first 200 generated trees (20,000 generations). We considered branches with posterior probability values equal to or greater than 0.95 as well-supported.

We estimated divergence times among lineages with BEAST 1.6.1 (Drummond & Rambaut, 2007). As no gymnophthalmid fossils are known, we used a standard 2% million^{-1} years divergence rate for lizard mitochondrial DNA, with a 1% standard deviation and a relaxed molecular clock (Thorpe *et al.*, 2005). We used a GTR+G+I model and priors estimated by the jModelTest analysis and ran a Markov chain Monte Carlo (MCMC) analysis with 3×10^7 generations, sampled every 1000 generations and repeated three times to avoid local optima. We used Tracer 1.5 (Rambaut & Drummond, 2007) to analyse the BEAST output and define burn-in for tree construction. We calculated the 50% majority-rule consensus, excluding the first 5000 generated trees.

To investigate the effects of distribution among plateaus versus depressions on the genetic structure of *M. atticolus*, we traced elevation on the Bayesian phylogenetic tree. We traced elevation both as a binary (above and below 500 m) and as a continuous characteristic, using maximum likelihood and maximum parsimony, respectively, for ancestral state estimation with Mesquite 2.74 (Maddison & Maddison, 2010). In addition, we also tested for a significant phylogenetic signal in elevation using the K statistic (Blomberg *et al.*, 2003) and a randomization test with 1000 simulations using the package PHYTOOLS (Revell, 2012) for R (R Development Core Team, 2011).

RESULTS

Spatial distribution

We compiled 116 unique geographical records for *M. atticolus* and 51 for *M. maximiliani*. The two species co-occurred in only 18 of the 167 localities where at least one of them was recorded, much less than expected by chance (binomial test, $P < 0.001$). Records of *M. atticolus* showed a mean elevation occurrence of 577.6 ± 309.1 m. There was a significant difference in elevational occurrence between the two species when considering the total range of *M. maximiliani* (*M. maximiliani*: 389.0 ± 324.4 m; W_{165} = 4082.5, $P < 0.001$) or only the records of *M. maximiliani* outside the *M. atticolus* range (*M. maximiliani*: 254.2 ± 258.2 m; W_{138} = 2265.5, $P < 0.001$). However, when considering only the area where their ranges overlap, there was no segregation along the elevational gradient (*M. maximiliani*: 508.8 ± 333.5 m;

W_{141} = 1817; P = 0.196). Overall, *M. atticolus* occurs at higher elevations than its congener, but low-elevation records of *M. maximiliani* are present outside of the *M. atticolus* distribution (Cerrado), i.e. there is no elevational segregation where their ranges overlap. Despite occurring at similar elevations where their ranges overlap, the two species tend to not co-occur in the same localities.

The compiled records of *M. atticolus* are primarily within the Cerrado boundaries, with some records from open enclaves within forest formations, particularly in the Amazon Forest (Fig. 1a). The receiver operating characteristic (ROC) curve presented a high area under the curve value (AUC = 0.96), indicating that the SDM is better than a random prediction. This conclusion was confirmed by the correct assignment of test data under the model, which performed significantly better than the null hypothesis. The SDM of *M. atticolus* is highly coincident with the Cerrado, except for the low probability in the north-east and the over-prediction towards south-eastern and north-western Cerrado (Fig. 1a). The palaeomodels suggested noticeable changes in suitable area over the last 130 ka (Fig. 1). During the LIG, suitable areas were concentrated north-west of the current distribution, in the current range of the Amazon Forest. The smallest predicted range was observed in the LGM, when the SDM predicted two major disjunct areas, one in south-east and another in the north-west Cerrado, divided by the Tocantins and Araguaia Basins. During the mid-Holocene, there was a lower probability of the occurrence of the species in northern Cerrado compared with the current prediction. The stable areas defined by the overlap of presence–absence maps are primarily located in high-elevation regions of the current distribution in the Guimarães Plateau, Caiapônia Plateau, Central Plateau and Canastra Plateau (Fig. 1e).

The best GLM included the following predictors, in increasing order of importance: LIG, mid-Holocene, precipitation of the driest month, precipitation of the wettest quarter, temperature seasonality, precipitation seasonality and isothermality. Importance indexes, coefficients, and the significance of predictors are presented in Table 1.

Table 1 Results of the logistic regression relating the distribution of the Brazilian Cerrado endemic lizard *Micrablepharus atticolus* to environmental and historical variables. The importance of variables was assessed using model averaging.

Variable	Coefficient	z	P	Importance
Last Interglacial (LIG, 130 ka)	2.01	8.58	< 0.001	1.00
Holocene (6 ka)	2.19	10.06	< 0.001	1.00
Precipitation of driest month (BIO14)	−4.51	−4.48	< 0.001	1.00
Precipitation of wettest quarter (BIO16)	1.54	4.82	< 0.001	1.00
Temperature seasonality (BIO4)	1.76	3.52	< 0.001	0.98
Precipitation seasonality (BIO15)	−1.33	−2.70	0.007	0.88
Isothermality (BIO3)	0.91	2.04	0.042	0.71
Intercept	−1.27	−4.45	< 0.001	NA

NA, not applicable.

Molecular data

The cyt *b* gene is highly diverse in populations of *M. atticolus*, with many unique haplotypes and F_{ST} values that indicate high inter-population structuring (see Appendix S1). The rarefaction analyses indicated no difference in h between depressions and plateaus (z = 0.981, P = 0.327), smaller h values in stable areas (z = –6.327, P < 0.001), and smaller h values in peripheral areas (z = –4.373, P < 0.001). Therefore, the predictions of low genetic diversity in populations inhabiting peripheral areas, depressions and areas of instability were partially corroborated (Table 2). In addition, samples from Pimenta Bueno had low haplotype and nucleotide diversities. These samples came from peripheral isolates of the Cerrado in low-elevation, unstable areas (Fig. 2). Most predictions of population decline in peripheral areas or population expansion in depressions or unstable areas were not corroborated (Table 2), with the exception of Vilhena, which contains peripheral isolates of the Cerrado in high-elevation (Parecis Plateau) unstable areas (Fig. 2). Contrary to our expectations, we found signs of population expansion in Brasília (a plateau, stable, core area) (Fig. 2).

The Mantel test indicated a significant association between genetic and geographical distances (Z = 82.8, P = 0.024). The SAMOVA indicated that, from three groups on, the proportion of total variation explained by between-group differences (F_{CT}) increased steadily with the number of groups; therefore, we adopted three groups as the general structure for describing the variation of the species (F_{CT} = 0.45, P < 0.001). These groups roughly correspond to the southern (S), north-western (NW), and central-northern (CN) portions of the Cerrado (Fig. 3a). Tests implemented in the sPCA analysis indicated the presence of a significant global structure [max(t) = 0.110, P = 0.002] and a lack of local structure [max(t) = 0.048, P = 0.992], meaning that neighbouring study sites had a tendency to be similar. Based on the eigenvectors, only the first component of global structure was chosen for interpretation, which revealed a differentiation between localities from the south-west and north-east (Fig. 3b). Median-joining networks produced with DS1 and DS2 were similar to each other and presented a highly structured pattern, as expected from the F_{ST} values, corroborating the SAMOVA and sPCA results (see Appendix S2). There was no haplotype sharing between sampled locations except for Pium (Ilha do Bananal), Pium (Parque Nacional do Araguaia), and Novo Santo Antônio (Fig. 2).

In general, the Bayesian phylogenetic analysis presented well-supported clades, and both runs achieved the same topology (Fig. 4), which was highly congruent with haplotype networks (Appendix S2). A single sample of *M. atticolus* from Sapezal, Mato Grosso, clustered with *M. maximiliani*,

Table 2 Genetic diversity parameters and neutrality tests to assess population size changes for samples of *Micrablepharus atticolus* from the Brazilian Cerrado. Samples were classified to test the predictions of the core/periphery, plateau/depression and stable/unstable hypotheses. Parameters and tests for well-sampled localities are also indicated.

Sample	n	S	h	H_d	π	F_S	$P(F_S)$	R_2	$P(R_2)$
Depression	67	132	34	0.950	0.042	0.418	0.624	0.102	0.583
Plateau	48	101	29	0.956	0.037	−0.845	0.428	0.116	0.669
Stable	18	50	6	0.732	0.016	5.997	0.986	0.106	0.183
Unstable	97	154	57	0.974	0.042	−7.326	0.089	0.087	0.456
Core	59	131	34	0.958	0.036	−1.653	0.355	0.086	0.314
Periphery	56	116	29	0.942	0.034	−0.011	0.555	0.094	0.411
Brasília (plateau, stable, core)	16	4	5	0.667	0.001	−1.977	*0.024	0.108	*0.020
Nova Xavantina (depression, unstable, core) (Rancho Ponte de Pedra)	14	17	6	0.747	0.008	1.716	0.800	0.148	0.532
Pimenta Bueno (depression, unstable, periphery)	15	1	2	0.343	0.001	0.597	0.602	0.171	0.410
Pium (depression, unstable, periphery)	14	13	8	0.901	0.005	−1.416	0.196	0.120	0.177
Vilhena (plateau, unstable, periphery)	14	9	9	0.923	0.003	−4.892	0.001	0.093	0.005

n, number of sequences; S, number of polymorphic (segregating) sites; h, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity, F_S, Fu's (1997) F_S statistic; R_2, Ramos-Onsins & Rozas' (2002) R_2 statistic.

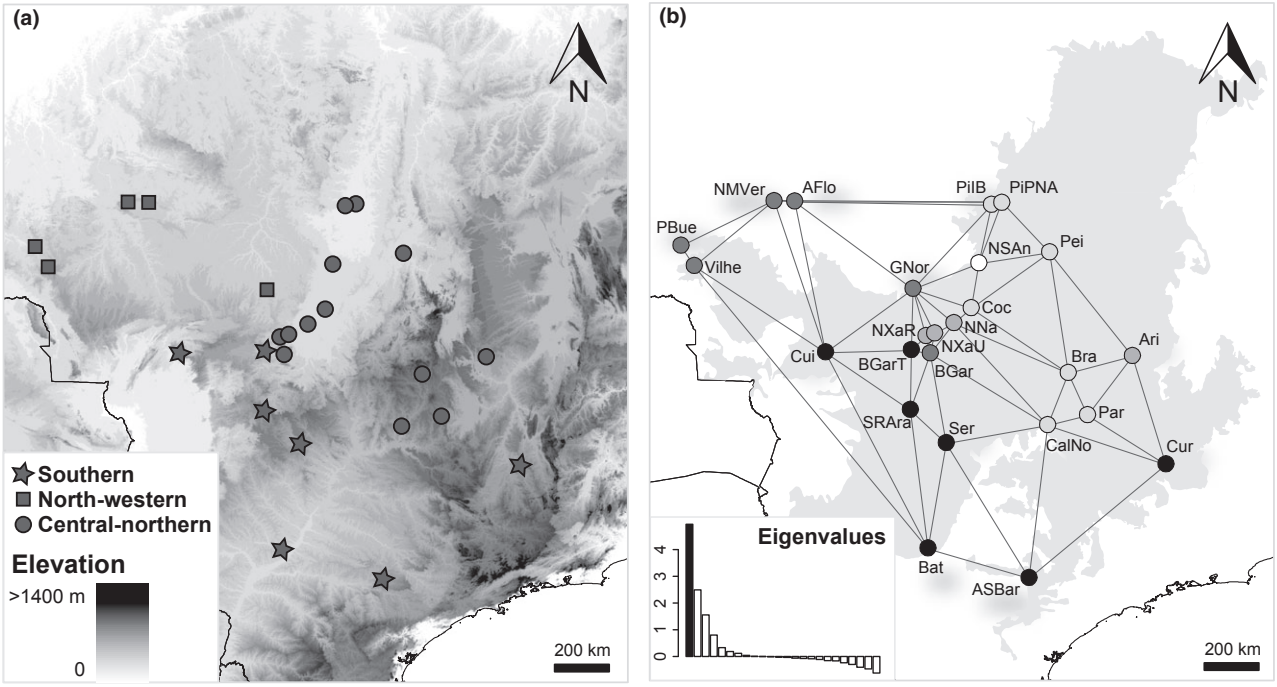

Figure 3 (a) Groups of *Micrablepharus atticolus* samples from the Brazilian Cerrado defined by the spatial analysis of molecular variance (SAMOVA) analysis on the elevational background. (b) Spatial analysis of principal components (sPCA) for cyt *b* sequences of *M. atticolus* from the Brazilian Cerrado (shown in grey). Sample localities (circles) are connected by a Delaunay triangulation, used to define the spatial weightings. Scores on the first principal component are represented by a greyscale gradient, indicating a differentiation of south-west from north-east samples. On the inset scree plot, positive eigenvalues correspond to global structure, whereas negative eigenvalues correspond to local structure; the black bar indicates the eigenvalue represented on the map. AFlo, Alta Floresta; Ari, Arinos; ASBar, Águas de Santa Bárbara; Bat, Bataguassu; BGar, Barra do Garças; BGaT, Barra do Garças (PCH Toricoejo); Bra, Brasília; CalNo, Caldas Novas; Coc, Cocalinho; Cui, Cuiabá; Cur, Curvelo; GNor, Gaúcha do Norte; NMVer, Nova Monte Verde; NNa, Nova Nazaré; NSAn, Novo Santo Antônio; NXaR, Nova Xavantina (Rancho Ponte de Pedra); NXaU, Nova Xavantina (UNEMAT); Par, Paracatu; PBue, Pimenta Bueno; Pei, Peixe; PiIB, Pium (Ilha do Bananal); PiPNA, Pium (Parque Nacional do Araguaia); Ser, Serranópolis; SRAra, Santa Rita do Araguaia; Vilhe, Vilhena.

suggesting a possible identification error or a case of hybridization. Because we did not have access to the voucher specimen, we chose to exclude this sample from further analyses. Except for this case, all other *M. atticolus* samples formed a monophyletic group. Haplotypes from Barra do Garças, Mato Grosso (this and all other localities mentioned hereafter are

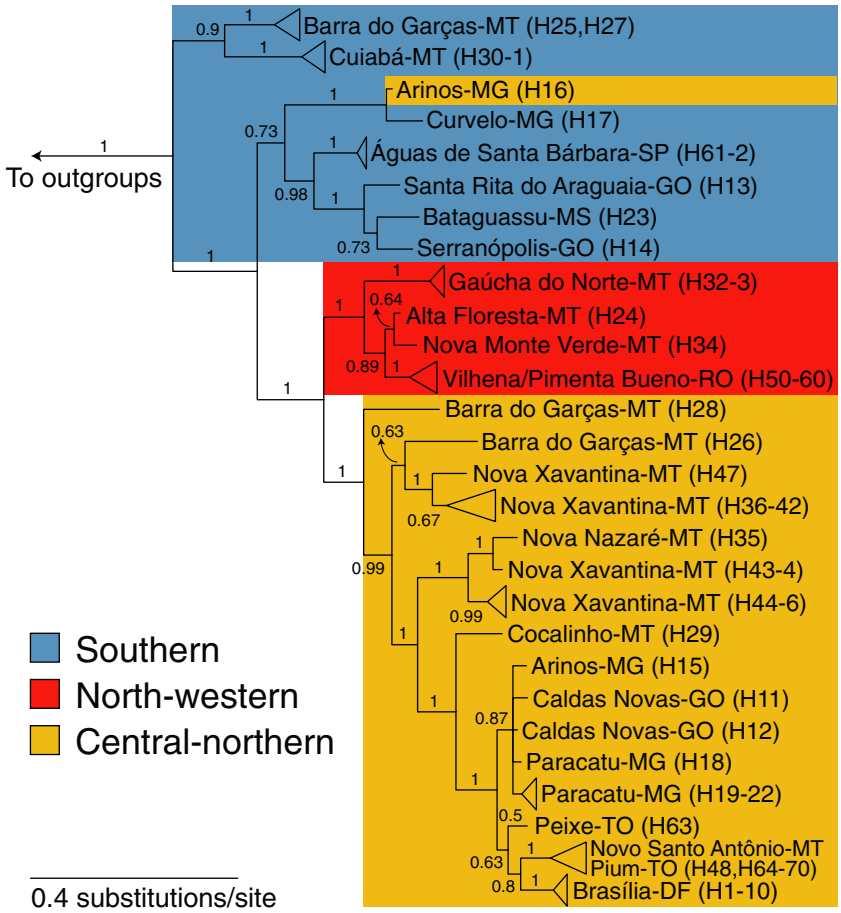

Figure 4 Bayesian phylogenetic tree for *Micrablepharus atticolus* from the Brazilian Cerrado based on a cytochrome *b* fragment. *Vanzosaura rubricauda* and *Micrablepharus maximiliani* samples were used as outgroups. Numbers indicate the posterior probability for branches. Terminal names are localities and haplotype numbers. Colours correspond to the groups defined by the spatial analysis of molecular variance (SAMOVA) (see Fig. 3a). DF, Distrito Federal; GO, Goiás; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso, RO, Rondônia; SP, São Paulo; TO, Tocantins.

depicted in Fig. 2), were distantly placed on the tree. One of these individuals grouped with the haplotypes from Nova Xavantina (Rancho Ponte de Pedra), whereas the other two formed a basal group with the samples from Cuiabá, separated from all other *M. atticolus* samples. This constituted the first deep split within the species. A second split separates the southern Cerrado samples (Arinos, Curvelo, Águas de Santa Bárbara, Santa Rita do Araguaia, Bataguassu and Serranópolis) from the remaining sequences. Subsequently, there is a split between haplotypes from north-western and central-northern Cerrado. Within the north-western clade, samples from Rondônia formed a monophyletic group, which is closely related to Alta Floresta and Nova Monte Verde. This is a group from Cerrado isolates within Amazonia and is closely related to the haplotypes from Gaúcha do Norte, at the Cerrado–Amazonia transition (Fig. 2). The central-northern clade contains samples from the Distrito Federal (Brasília), Goiás (Caldas Novas), Mato Grosso (Barra do Garças, Cocalinho, Nova Xavantina, Nova Nazaré, Novo Santo Antônio), Minas Gerais (Arinos and Paracatu), and Tocantins (Peixe and Pium).

All three BEAST runs recovered the same topology (Fig. 5). This topology was very similar to the MrBayes tree except for the position of Peixe (all localities depicted in Fig. 2), which grouped with Paracatu and Caldas Novas (BEAST) instead of Brasília and Pium (MrBayes, but with a low support value). Divergence between the two species of *Micrablepharus* was estimated to have occurred *c.* 6.5–11 Ma. The first divergence within *M. atticolus* took place *c.* 2.3–3.5 Ma, at the end of the Pliocene and onset of the Pleistocene. The second split, separating the southern haplotypes, occurred *c.* 1.8–2.8 Ma. The north-western samples, including the Cerrado isolate haplotypes, diverged from the other samples *c.* 1.3–2.2 Ma.

Tracing elevation as either a binary (Fig. 6a) or a continuous (Fig. 6b) characteristic revealed a complex history of *M. atticolus* occupation across the topographical landscape. In the binary model, most ancestral proportional likelihoods for assignment to a particular state were inconclusive in the basal nodes. The distribution of sampling localities across the elevational landscape is given in Fig. 2. There was no significant phylogenetic elevation signal ($K = 0.063$, $P = 0.169$).

DISCUSSION

Since its original description, *M. atticolus* has been regarded as strongly associated with the Cerrado formation in Brazil (Rodrigues, 1996; Vieira *et al.*, 2000; Colli *et al.*, 2002). Our compilation of distribution records corroborates the view that the species is in fact a Cerrado endemic. The best

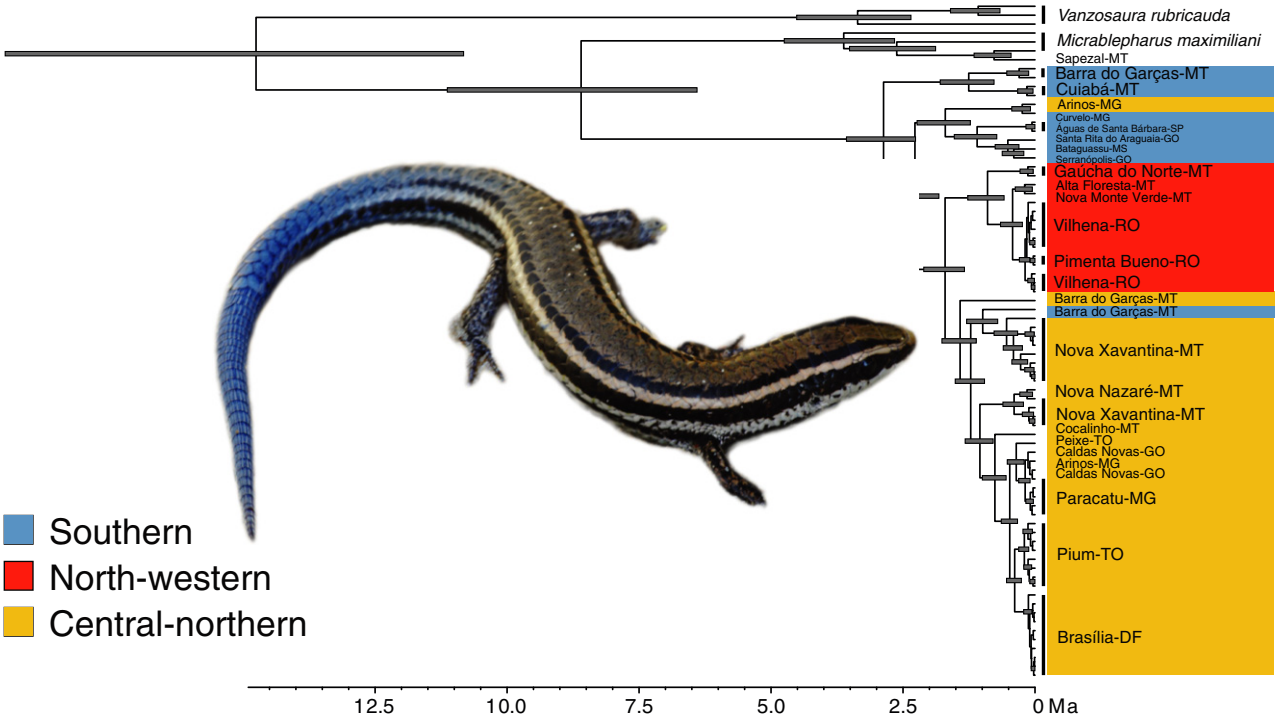

Figure 5 Divergence time estimation for *Micrablepharus atticolus* based on a relaxed molecular clock using BEAST. Boxes indicate the 95% confidence interval of the divergence time. Colours correspond to the groups defined by the spatial analysis of molecular variance (SAMOVA) (see Fig. 3a).

predictors of the current distribution of *M. atticolus* consist of ecological predictors, which highlight the markedly seasonal climate of the Cerrado, and historical predictors, which reflect the ancient distributions of the biome (Werneck *et al.*, 2012b).

The lack of elevational segregation between *M. atticolus* and *M. maximiliani* suggests that the diversification of the genus was not influenced by topography, contrary to the pattern suggested for other Cerrado taxa (Silva, 1997; Colli, 2005; Werneck, 2011). Nevertheless, it is possible that *M. atticolus* and *M. maximiliani* segregate ecologically, due either to ecological interactions or to differences in physiological requirements. Nogueira (2006) proposed a pattern of local segregation between the two species of *Micrablepharus*, claiming that records where both species occur in sympatry are rare. Our results corroborate this view, indicating that localities where the two species co-occur are in fact very rare. *Micrablepharus maximiliani* is seemingly more abundant in cerrado *sensu stricto* (Vitt *et al.*, 2007; Nogueira *et al.*, 2009), a savanna physiognomy with moderate vegetation coverage, whereas *M. atticolus* is found primarily in more open areas (Vieira *et al.*, 2000; Gainsbury & Colli, 2003). A fine-scale gradient analysis in areas where the two species co-occur, along with detailed ecophysiological data, should help to identify the mechanisms that limit their coexistence.

We found high levels of genetic diversity among populations of *M. atticolus* and a significant relationship between genetic and geographical distances. This pattern can result from isolation by distance but also from vicariance or barriers to gene flow (Manel *et al.*, 2003). Identifying the relative roles of these factors in the spatial patterns of genetic variation is not a simple task (Meirmans, 2012), especially considering the limitations of using a single gene. Nevertheless, in view of the large spatial scale of our study, the high heterogeneity of the Cerrado landscape (Silva *et al.*, 2006; Nogueira *et al.*, 2009), and the known barriers to gene flow (e.g. the hundreds of kilometres of rain forest separating peripheral isolates from the core of the Cerrado), it is clear that vicariance played a significant role. The sPCA revealed a marked differentiation between north-eastern and south-western Cerrado populations (Fig. 3b), which generally agrees with the results from the SAMOVA (Fig. 3a), networks (Appendix S2), and phylogenetic analyses (Figs 4 & 5). Groups defined by the SAMOVA are coincident with major clades except for the southern Cerrado group, which is paraphyletic and composed of the two most basal clades in the phylogeny (Figs 3a & 4). However, both basal clades have low support values, showing unclear relationships among their haplotypes. This group corresponds to the least-sampled region in this study; thus, the use of more localities and more individuals per locality from southern Cerrado is warranted. Nevertheless, the frog *Hypsiboas albopunctatus*, also broadly distributed in the Cerrado, has a basal clade in Chapada dos Guimarães, a south-east clade, and a central Cerrado clade, closely resembling our findings for *M. atticolus*, including divergence dates (Prado *et al.*, 2012).

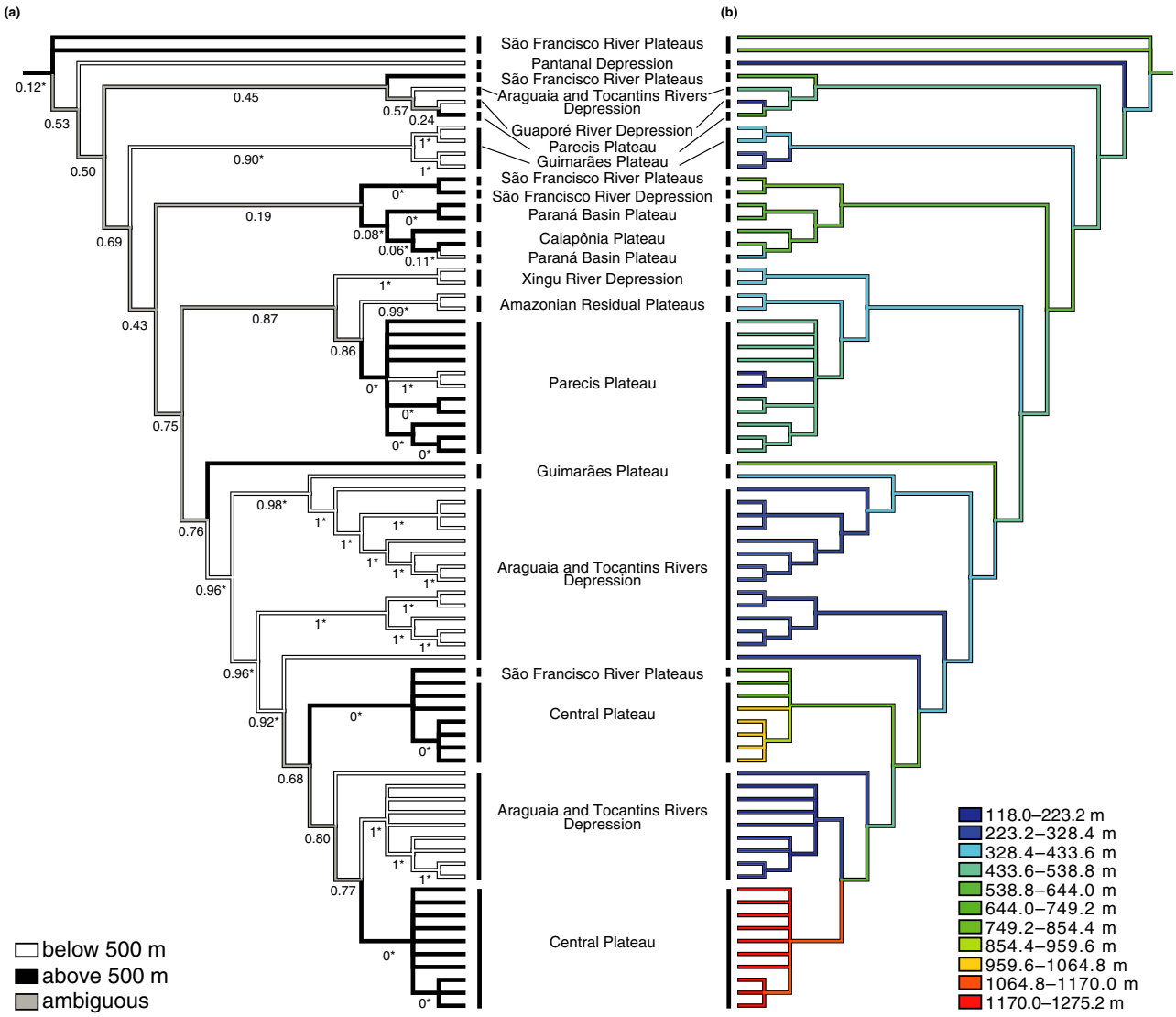

Figure 6 Tracing of the elevation of locality records upon a Bayesian phylogenetic tree of Brazilian *Micrablepharus atticolus* haplotypes. (a) Elevation coded as a binary character, representing plateaus (> 500 m) or depressions (< 500 m), with ancestral state reconstruction based on maximum likelihood. Values indicate the proportional likelihood of ancestral occurrence on depressions. Asterisks indicate a significant difference between likelihoods for occurrence on depressions or plateaus. (b) Elevation coded as a continuous character, with ancestral state reconstruction based on maximum parsimony.

The more ancient divergence times of *M. atticolus* coincide with the final uplift of the Central Brazilian Plateau during the late Neogene (Silva, 1997). However, the phylogenetic tree is not congruent with the pattern hypothesized by Werneck (2011), i.e. older lineages with higher genetic diversity on plateaus, with signs of recent population expansion in lower and younger areas (depressions). We also found no association between genetic diversity and elevation and no significant phylogenetic elevation signal. Therefore, it appears that *M. atticolus* repeatedly colonized both plateaus and depressions and that geographical distance played a more prominent role than elevation in population differentiation.

The SDMs of *M. atticolus* in the mid-Holocene, LGM and LIG differed from the models constructed for the Cerrado by Werneck *et al.* (2012b). Areas with suitable conditions for the species shift between periods along a north-west/south-east diagonal. Stable areas are also different from the Cerrado palaeomodels, with four main stable areas coincident with high elevations. Nevertheless, the palaeomodels for the Cerrado and *M. atticolus* agree that suitable areas were more broadly distributed in the LIG than in the LGM, challenging views that favour the expansion of open formations during drier and colder periods (Ledru, 2002; Mayle & Beerling, 2004; Mayle *et al.*, 2004). Contrary to our predictions, we found lower genetic diversity in stable areas (refugia) and no deviations from neutrality in either stable (refugia) or unstable areas. This result could stem from difficulties regarding the inference of stable areas (Collevatti *et al.*, 2013) or from the ability of populations to withstand or adapt to shifting environmental conditions during climatic fluctuations

(Hoffmann & Sgrò, 2011; Moritz *et al.*, 2012). Regardless of this, most divergence times between haplotypes from different localities are far more ancient than the time scale covered by the palaeomodels, with most populations having already differentiated by the LIG and possibly having persisted even during periods of unfavourable climatic conditions. A recent phylogeographical study on the *Rhinella crucifer* group of toads from the Brazilian Atlantic Forest also did not reveal congruence between genetic patterns and predicted stable areas during the last 130 ka (Thomé *et al.*, 2010).

Molecular dating indicated that lineages from Cerrado peripheral isolates were already differentiated at least 500 ka. This estimation is much older than the presumed savanna expansions towards the Amazonian border *c.* 3–9 ka (Freitas *et al.*, 2001) and during the LIG, as suggested by our palaeomodels. Apparently, several savanna expansions and retractions in south-western Amazonia occurred during the Neogene and Quaternary. Our molecular dating supports the presence of open formations in the contact zone between north-western Cerrado and south-western Amazonia around the middle Pleistocene. Peripheral areas as a whole, and Pimenta Bueno in particular (Fig. 2), had the lowest haplotype and nucleotide diversities among all the large sampled populations, which suggests the effects of drift following isolation. Pimenta Bueno is more distant from central Cerrado than Vilhena (Fig. 2) and exhibits low lizard diversity (Gainsbury & Colli, 2003), which is consistent with our genetic data. Furthermore, we found genetic signs of population expansion in Vilhena, which suggests recolonization of Cerrado peripheral isolates.

CONCLUSIONS

Our results revealed a complex history of diversification of *M. atticolus* in Cerrado, with events that took place in the late Neogene playing a prominent role in this diversification. In contrast to previous studies, we found no support for the plateau/depression hypothesis, as repeated occupations of both plateaus and depressions were identified. We also found no support for the stability/instability hypothesis, with no evidence of higher genetic diversity in reconstructed Cerrado refugia, even though we could not entirely reject the hypothesis. Finally, our results partially support the core/periphery hypothesis, indicating that the formation of peripheral isolates of the Cerrado in south-western Amazonia during climatic cycles led both to a loss of genetic diversity in isolated populations and to the recolonization of Cerrado enclaves, with genetic signs of population expansion. Overall, the plateau/depression, stability/instability and core/periphery hypotheses are oversimplifications of the complex and dynamic history of the Cerrado and cannot fully explain the diversification of its biota. The spatial patterns of genetic structure have seemingly resulted from a combination of shifting environmental conditions during climatic cycles; repeated colonizations of plateaus and depressions, isolation by distance, and divergence in and recolonization of peripheral isolates within Amazonia. In addition to considering these processes in tandem, future studies should also benefit from the use of multiple loci and comparative phylogeographical analyses of several organisms with different habitat requirements.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Locality records, samples, and cyt *b* haplotypes and statistics.

Appendix S2 Networks of cytochrome *b* haplotypes based only on complete sequences and based on all sequences, excluding sites with missing occurrences.

BIOSKETCHES

Marcella Gonçalves Santos's interests include the ecology, evolution, phylogeography and biogeography of South American herpetofauna. This work is the result of her MSc dissertation and is part of the efforts of the research group led by **Guarino R. Colli** to uncover patterns and processes associated with the diversification of the herpetofauna of South American open biomes, especially the Cerrado.

Author contributions: M.G.S, C.N., L.G.G. and G.R.C. conceived the ideas; M.G.S. collected the data; M.G.S. and L.G.G. analysed the data; M.G.S. led the writing of the manuscript, and C.N., L.G.G. and G.R.C. reviewed and improved the manuscript.

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