An Acad Bras Cienc (2022) 94(Suppl. 4): e20201206 DOI 10.1590/0001-3765202220201206
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Peripheral isolation and demographic stability are reflected in the genetic diversity of the populations of the Helmeted Manakin: a bird endemic to the gallery forests

THAINARA O. SOUZA, LEILTON W. LUNA, JULIANA ARARIPE, WEBER A. DE G. E SILVA & PÉRICLES S. DO REGO

Abstract: The Cerrado encompasses a complex network of hydrographic basins, which is responsible for the formation and maintenance of the riparian and gallery forests. Alterations in the vegetation resulting from the paleoclimatic changes that occurred during the Pleistocene influenced the current distribution of these humid forests. To understand of the evolutionary dynamics of this landscape on the population structure of the associated organisms, we studied the population genetics of the *Antilophia galeata* (Pipridae), a bird endemic to the gallery forests of the Cerrado. For this, we evaluated the variability of the mitochondrial control region of 71 *A. galeata* specimens from 18 localities distributed throughout the Cerrado. The results indicated the existence of significant substructure among the populations located in the peripheral areas of the Cerrado, in comparison with the populations found in the central portion, reflecting historical changes in the environment. We also found high levels of diversity in all the populations, while the analysis of the demographic history revealed a scenario of stability. Overall, then, these findings indicate that the historical modifications of the gallery forest distribution may have been most accentuated in the peripheral zones, with a greater stability and connectivity persisting in the central portion of the biome.

Key words: *Antilophia galeata*, Cerrado, Late Pleistocene, population structure, paloeclimatic events, South America.

INTRODUCTION

The central Brazilian plateau forms the core area of the Cerrado savanna domain and encompasses the headwaters of a number of important South American hydrographic basins (Oliveira et al. 2019). The hydrographic basins located within the Cerrado are associated with a typical vegetation classified as riparian and gallery forests, which are extremely important for the species richness and composition of the biome (Ribeiro & Marinho-Filho 2005). The five principal basins are those of the Araguaia-Tocantins, Paraná, Paraguay, Parnaíba, and São Francisco rivers (Figure 1). These riparian and gallery forests function as an important repository of the domain's biodiversity (Felfili et al. 2005), acting as "ecological corridors" which interlink and connect the forest elements of the other ecological formations within the region (Aquino et al. 2012). The organisms that inhabit and transit through the Cerrado matrix also depend on these forest environments to feed, reproduce, and nest (Ribeiro et al. 2005).

Paleoenvironmental records of the evolution of the Cerrado landscape indicate that the associated vegetation has had a dynamic history (Ledru 2002, Ledru et al. 2006). The occurrence of

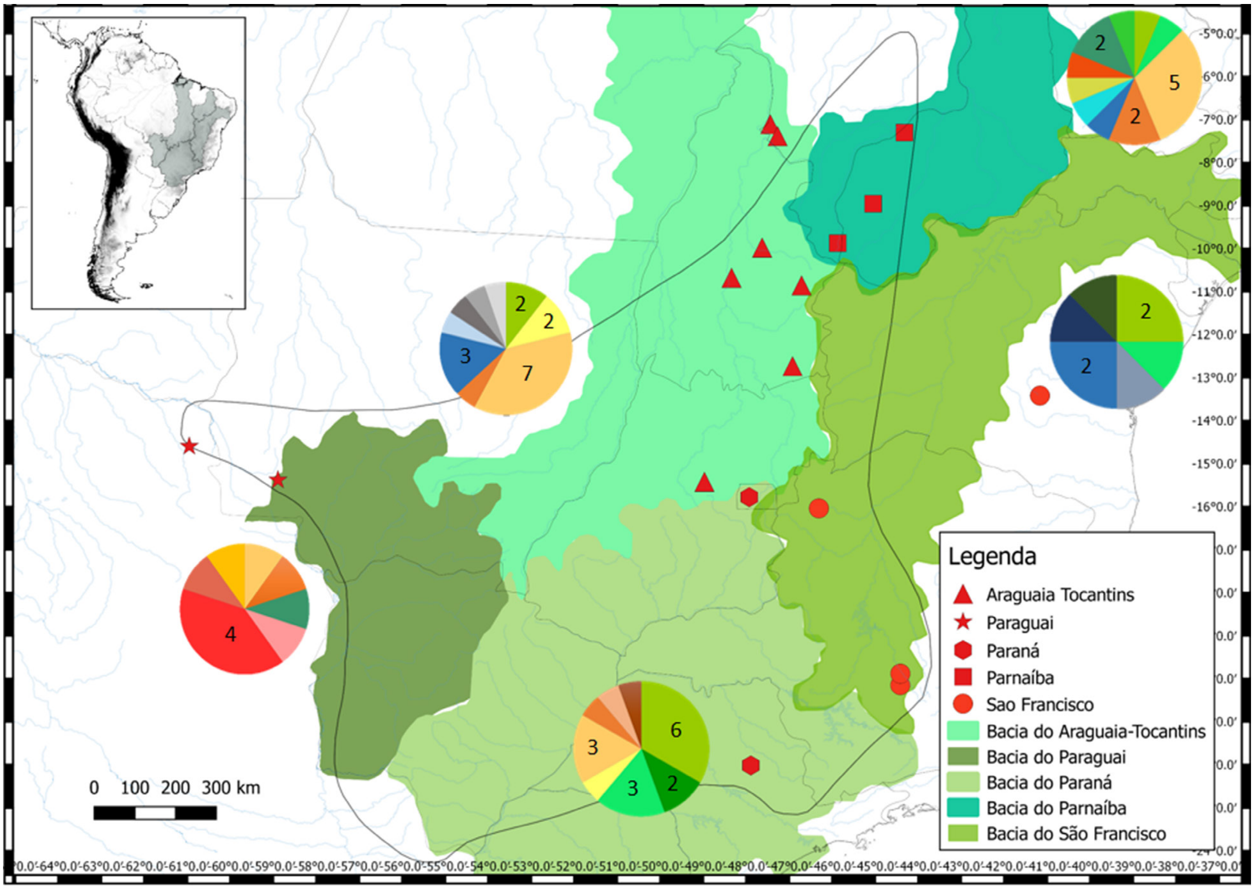

Figure 1. The five principal hydrographic basins of the Cerrado domain. The geographic distribution of *Antilophia galeata* (black line) and the sampling points are differentiated by hydrographic basin. The map of each basin shows the distribution of the haplotypes of Hypervariable Domain I of the mitochondrial Control Region. Each haplotype is represented by a distinct color and the numbers indicate the quantity of specimens that had the respective haplotype.

the forest formations in this domain has been molded by both temporal (over geological and ecological time) and spatial processes (local variation, such as topography, hydrography, and the depth of the water table), in particular, the major climatic pulses of the Quaternary, with long periods of low temperatures (glaciations) alternating with shorter intervals of higher temperatures, that is, the interglacial periods (Salgado-Labouriau 1994, Ribeiro & Walter 1998). The climatic fluctuations that occurred during the Pleistocene were fundamental to the formation of the present-day phytogeographic configuration of the central Brazilian plateau, with the available evidence indicating that the areas currently covered with savanna underwent multiple modifications of their phytophysiognomy and the distribution of different vegetation types (Salgado-Labouriau et al. 1997). However, the changes in climatic conditions that occurred during the Last Glaciation Maximum (LGM) had a more intense effect throughout the tropical region, characterized by a marked decline in both precipitation and temperatures (van der Hammen et al. 1992, Ledru 2002). This cool, dry period, between 20,000 and 18,000 years ago (the Late Pleistocene) resulted in the progressive isolation of humid forests due to the expansion of savannas into areas that are now covered by forest, forming what are known as the Pleistocene refugia (van der Hammen & Hooghiemstra 2000). The available phylogeographic evidence indicates that these processes molded the demographic history and population structure of the species dependent on the forested environments located within the Cerrado, which makes these taxa excellent models for the understanding of the evolutionary processes that formed this environment (Prado et al. 2012, Santos et al. 2014). Studies of plants, reptiles, and birds endemic to the Cerrado indicate that the greatest genetic diversity and the lowest differentiation among populations are found in the central portion of the biome, decreasing toward its edges (Telles et al. 2007, Diniz-Filho et al. 2009, Santos et al. 2014).

One species endemic to the gallery forests of the Cerrado is the helmeted manakin, *Antilophia galeata* (Lichtenstein 1823), which currently has a Least Concern conservation status (BirdLife International 2018). This bird occurs throughout central Brazil, extending to the west as far as the Pantanal, northeastern Paraguay, and Bolivia (Sick 1997, del Hoyo et al. 2004). Leite (2006) found a clear pattern of geographic variation in the morphometric parameters of *A. galeata*, which indicates that the populations restricted to the gallery forests of different hydrographic basins are more or less isolated from each other, leading to intraspecific variation in morphology.

The present-day division of the hydrographic basins of the Cerrado indicates that the biota found in its gallery forests may have also suffered certain limitations of connectivity during the final paleoclimatic alterations of the LGM, which would have triggered population subdivisions in species associated with these forests that are still poorly studied or understood. Given its ample distribution in central Brazil, *A. galeata* is an ideal model for the evaluation of population structure and the distribution of the genetic variability among populations on an ample spatial scale, based on the analysis of a hypervariable mitochondrial region. This analysis elucidated part of the evolutionary history of the species and provided evidence of the probable effects of the most recent climate fluctuations in the region, which has determined so much of the biodiversity of the Neotropics. Based on the analyses, we tested two hypotheses: (i) Events during the Pleistocene (climatic fluctuations) provoked historical shifts in the connectivity of the gallery forests, resulting in the isolation of the *A. galeata* populations, which is reflected in their genetic differentiation, and (ii) the gallery forests formed refugia for the *A. galeata* populations on the central Brazilian plateau during the climate shifts of the Pleistocene, establishing systematic differences in the genetic diversity of the populations found in the principal hydrographic basins.

MATERIALS AND METHODS

Sampling

A total of 71 *A. galeata* specimens were sampled in the present study, collected from 18 localities in the five principal hydrographic basins of the Cerrado biome (of the Araguaia-Tocantins, Paraguay, Paraná, Parnaíba, and São Francisco rivers - Figure 1). Twenty-seven of these samples were obtained in the field between 2015 and 2017 (IBAMA collecting license 48380/2015) at eight points distributed throughout the range of the species in the Cerrado (Supplementary Material - Table SI). These specimens were captured in mist-nets set in gallery forests in the vicinity of river headwaters, with blood samples being collected from each individual and stored in 70% ethanol. The other samples (n=44) represent 10 other Cerrado localities (Table SI) and were obtained on loan from the following institutions: the Federal University of Mato Grosso (n=1), Museum of Natural Science at Louisiana State University (n=9), Zoology

Museum of the University of São Paulo (n=14), the National Museum at the University Federal of Rio de Janeiro (n=4), the Goeldi Museum (MPEG) in Belém (n=3), the University of Brasília (n=9), and the Federal University of São Carlos (n=4). The samples (muscle tissue and/or blood) were conserved in 95% ethanol at -20°C.

Laboratory procedures

The genetic material was extracted using the Wizard® Genomic DNA Purification kit (Promega) following the maker's protocol. The Polymerase Chain Reaction (PCR) was used to amplify Hypervariable Domain I (HDI) of the mitochondrial Control Region, a segment commonly used in studies of population genetics for the evaluation of levels of genetic diversity (Sammler et al. 2012, Jackson et al. 2013, Luna et al. 2017a, Almeida et al. 2019) and the population structure of species (Roukonen & Svist 2002). The Dloop H-739 (Sorenson et al. 1999) and CytB-End (Bensch & Harlid 2000) primers were used to amplify the sequence in a cycle consisting of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds, hybridization at 57°C for 30 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. The PCR products were purified using Polyethylene Glycol 8000 (PEG, 1g/mL) and sequenced in an ABI 3500XL (Applied Biosystems™) automatic sequencer using the BigDye® Terminator v3.1 CycleSequencing kit (Applied Biosystems™).

Population structure analysis

The sequences were aligned in Clustal-W (Thompson et al. 1994) and edited in BIOEDIT (Hall 1999). As previous studies have shown that the hydrographic basins of the Cerrado may be a factor segregating populations dependent on gallery forest, the fixation index (F_{ST}) was calculated to determine the level of genetic differentiation among the *A. galeata* populations occurring in the five principal hydrographic basins of the Brazilian Cerrado (Araguaia-Tocantins, Paraguay, Paraná, Parnaíba, and São Francisco). *A priori*, the F_{ST} index with 1,000 permutations and the Analysis of Molecular Variance (AMOVA) were applied to the populations assumed to be confined to these five hydrographic basins. Both these analyses were run in ARLEQUIN 3.5.2.2 (Excoffier & Lischer 2010), which was also used to apply the Mantel test to evaluate the potential existence of isolation by distance among the populations, based on the correlation between geographic and genetic distances.

The genetic structure was also evaluated using a Bayesian analysis of the potential formation of genetic groups, run in BAPS v 6.0 (Bayesian Analysis of Population Structure - Corander et al. 2003, 2008). This analysis applies a search algorithm that calculates the posterior distribution of the parameters of the model established by the observed data, that is, the haplotypes of the DNA sequences. To determine the most probable number of groups (K), the analysis was based on the assumption that genetic mixing was not spatially hierarchical at an individual level. The probable number of genetic groups was included as a parameter to be estimated, and the best partition (number of groups) was considered to be the arrangement with the greatest probability.

Genetic parameters and demography history

The genetic diversity of the populations was calculated in ARLEQUIN 3.5.2.2 (Excoffier & Lischer 2010), and included the indices of holotype (h) and nucleotide (π) diversity, the number of polymorphic sites, and the number of haplotypes, and their frequency and distribution among the five groups delimited *a priori*. The haplotype network was compiled in Haploviewer (Salzburger et al. 2011) using the Maximum Likelihood algorithm, providing a visual representation of the genealogical relationships among the different haplotypes.

Historical changes in the size of the *A. galeata* population were evaluated using two approaches. The first approach analyzed the deviations from neutral evolution based on Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997), which were applied to determine whether the *A. galeata* populations are in equilibrium, expansion or have passed through a bottleneck (Rogers & Harpending 1992). These tests were also computed in ARLEQUIN 3.5.2.2. The second approach consisted of the evaluation of historical fluctuations in population size estimated by the Bayesian Skyline Plot (BSP), run in BEAST 1.8 (Drummond & Rambaut 2007). This analysis was run over 200×10^8 generations, with the genealogy being sampled every 10,000 generations, with the first 10% of generations being discarded as burn-in. The molecular clock was calibrated based on the intraspecific mutation rate estimated for the HDI by Norman et al. (2014), that is, 0.0348 substitutions per site per million years in a given lineage. A generation time of one year was assumed for the calculation of the effective number of females (*Nef* – Luna et al. 2017a). The Bayesian Skyline Plot (BSP) and Effective Sample Size (ESS) were determined in Tracer 1.6 (Rambaut & Drummond 2007). The HKY model was the best model of nucleotide evolution, based on the Bayesian Information Criterion (BIC), which was used to compile the BSP. The best model of nucleotide evolution was identified in MEGA 7 (Tamura et al. 2013).

RESULTS

Population structure

A total of 355 base pairs (bps) of the Hypervariable Domain I (HDI) of the mitochondrial Control Region were sequenced in the 71 *A. galeata* specimens, with no evidence being found of the presence of indels. A total of 26 polymorphic sites were identified, being used to define 25 haplotypes (Table SII, Figure 2). The GenBank accession numbers of the sequences recorded in the present study are MZ028975 - MZ029045.

The pairwise analysis of the F_{ST} values indicated that the *A. galeata* populations of the São Francisco basin are differentiated significantly from the other population groups (Table I). The population of the Paraguay basin was also differentiated from that of the Paraná basin. No evidence was found of any correlation between geographic and genetic distances (Mantel test: r = 0.0320; p = 0.1768), which indicates that the geographic distance does not influence the genetic differentiation found among the study populations. The AMOVA indicated that most (88.9%) of the observed variability exists within the groups that represent each hydrographic basin (Table II).

The haplotype network reveals that the most common haplotypes are amply distributed in all five hydrographic basins (Table SII, Figure 2). Exclusive haplotypes were identified in each hydrographic basin, although most were rare (Table SII). The BAPS analysis of population structure indicated the presence of three genetic groups (K=3, marginal probability = -328.2651; Supplementary Material - Figure S1) based on the haplotype sequences. This analysis is consistent with the F_{ST} values, with the geographic substructuring of the individuals revealing differences in the frequencies of the three genetic profiles in the populations of the five hydrographic basins, with the populations of the Paraguay and São Francisco basins presenting a higher level of differentiation in their composition in comparison with the other three populations, which are located more centrally.

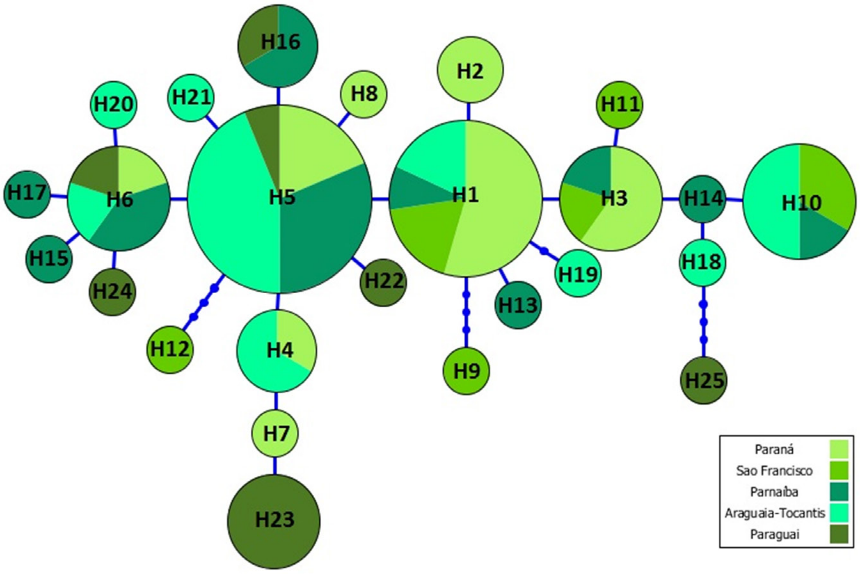

Figure 2. Haplotype network based on the Maximum Likelihood analysis of Hypervariable Domain I of the mitochondrial Control Region of *Antilophia galeata*. The different colors represent each of the five hydrographic basins shown in Figure 1.

Table I. Pairwise fixation indices f (F_{ST}) between the *Antilophia galeata* populations sampled in the gallery forests of the five hydrographic basins of the Cerrado.

	Araguaia-Tocantins	Paraguai	Paraná	Parnaíba	São Francisco
Araguaia-Tocantins	----------				
Paraguai	0,02297	----------			
Paraná	0,03090	0,12433*	----------		
Parnaíba	-0,02113	-0,01264	0,05902	----------	
São Francisco	0,12014*	0,16116*	0,14986*	0,15215*	----------

*$p<0.05$.

Table II. Results of the Analysis of Molecular Variance (AMOVA), considering the groups of *Antilophia galeata* populations present in the gallery forests of the five principal hydrographic basins of the Cerrado domain and the global fixation index (ΦST) values.

	Sum of squares	Components of variability	% of variation
Among groups	6.148	0.12553	10.02
Among populations within groups	2.688	0.01302	1.04
Within groups	73.558	1.11452	88.94
Total	82.394	1.25307	
ΦSC=0.11055 (p=0.17009)			
ΦST=0.11057 (p=0.00587)			
ΦCT=0.10018 (p=0)			

Genetic parameters and demography history

High levels of haplotype and nucleotide diversity were found in the populations of the five hydrographic basins, when analyzed separately (Table III), with the population of the São Francisco basin presenting the highest diversity values (*h* = 0.929; *π* = 0.14011) and the Araguaia-Tocantins population, the lowest values (*h* = 0.848; *π* = 0.08547). High levels of diversity (*h* = 0.910; *π* = 0.09054) were also found for the whole *A. galeata* dataset, without the division by hydrographic basin.

The results of the neutrality tests (*D*) were not significant, in contrast with those of the *Fs* test, which presented significant negative values, which may reflect a degree of deviation from neutrality in all the populations of the different hydrographic basins, except for that of the São Francisco basin (Table III). When the tests are applied to the whole *A. galeata* dataset, without the subdivision by hydrographic basin (Table III), significant negative values were obtained in both cases (*D* = 1.75788, $p < 0.05$; *Fs* = -18.15960, $p < 0.05$), indicating a population in expansion, rather than in equilibrium.

The demographic history defined by the BSP (Figure 3) indicates a scenario of stability in the *A. galeata* populations of the five Cerrado hydrographic basins. Evidence of a pattern of growth followed by stability was found when the whole *A. galeata* dataset was analyzed without considering the basin subdivisions, which is consistent with the results of the previous analyses, presented above.

DISCUSSION

Historical structure of the *Antilophia galeata* population

The variation found in certain morphological features among the *A. galeata* populations indicates that this species may have passed through a period of isolation based on the discontinuities of the gallery forests distributed within the hydrographic basins, which limited gene flow among these populations (Leite 2006). The analysis of the population structure of *A. galeata*, based on the sequences of the mitochondrial Control Region, indicate a pattern of population sub-structuring related to peripheral sampling. Populations located in the more peripheral portions of the Cerrado, that is,

Table III. Summary of genetic diversity and neutrality tests to assess changes in the population size of *Antilophia galeata* in the Cerrado. The samples were grouped to observe the diversity of populations as a single evolutionary unit and also populations within the gallery forests present in the river basins.

	N	NH	*h*	π	D	P (D)	Fs	P (Fs)
Araguaia-Tocantins	19	9	0.848	0.08547	-0.79196	0.22800	-3.05710	0.02800
Paraguai	10	7	0.867	0.10341	-1.38129	0.09900	-2.36149	0.04700
Paraná	18	8	0.856	0.05807	-0.44281	0.35300	-3.56113	0.01200
Parnaíba	16	10	0.900	0.08141	-0.44002	0.34800	-5.44778	0
São Francisco	8	6	0.929	0.14011	-0.70580	0.30100	-1.16498	0.18700
All populations	71	25	0.910	0.09054	-1.75786	0.0090	-18.15960	0

N = number of samples; NH = number of haplotypes; h = haplotype diversity; π = nucleotide diversity; D = Tajima's D; P(D)= probability of Tajima's test; Fs = Fu's Fs; P(Fs)= probability of Fu's test.

in the gallery forests of the Paraguay and São Francisco basins, have a more distinct genetic makeup in comparison with the populations located toward the center of the biome.

Genetic variation in the *A. galeata* populations may reflect past events of environmental change occurring in the Cerrado domain (Silva 1995, 1997, Werneck et al. 2012, Ribeiro et al. 2016). The interaction of global factors, such as glaciations, with regional and local elements, including differences in altitude and geomorphological conditions, have contributed to the final response of the species to the climatic modifications in each region (Sano et al. 2019). Simulations of climatic models for the genus *Antilophia* have revealed the successive expansion and retraction of the gallery forests of the Cerrado during the LGM (Luna et al. 2017b). Under these environmental conditions, it is likely that the gallery forests found in the peripheral portions of the Cerrado domain would have suffered more intense changes in climate which, in turn, provoked a greater degree of differentiation in the genetic profile of the *A. galeata* populations in these areas, in particular, in the outer limits of the distribution of the species.

The palynological data reveal modifications in the distribution and composition of the Cerrado vegetation during the Late Pleistocene

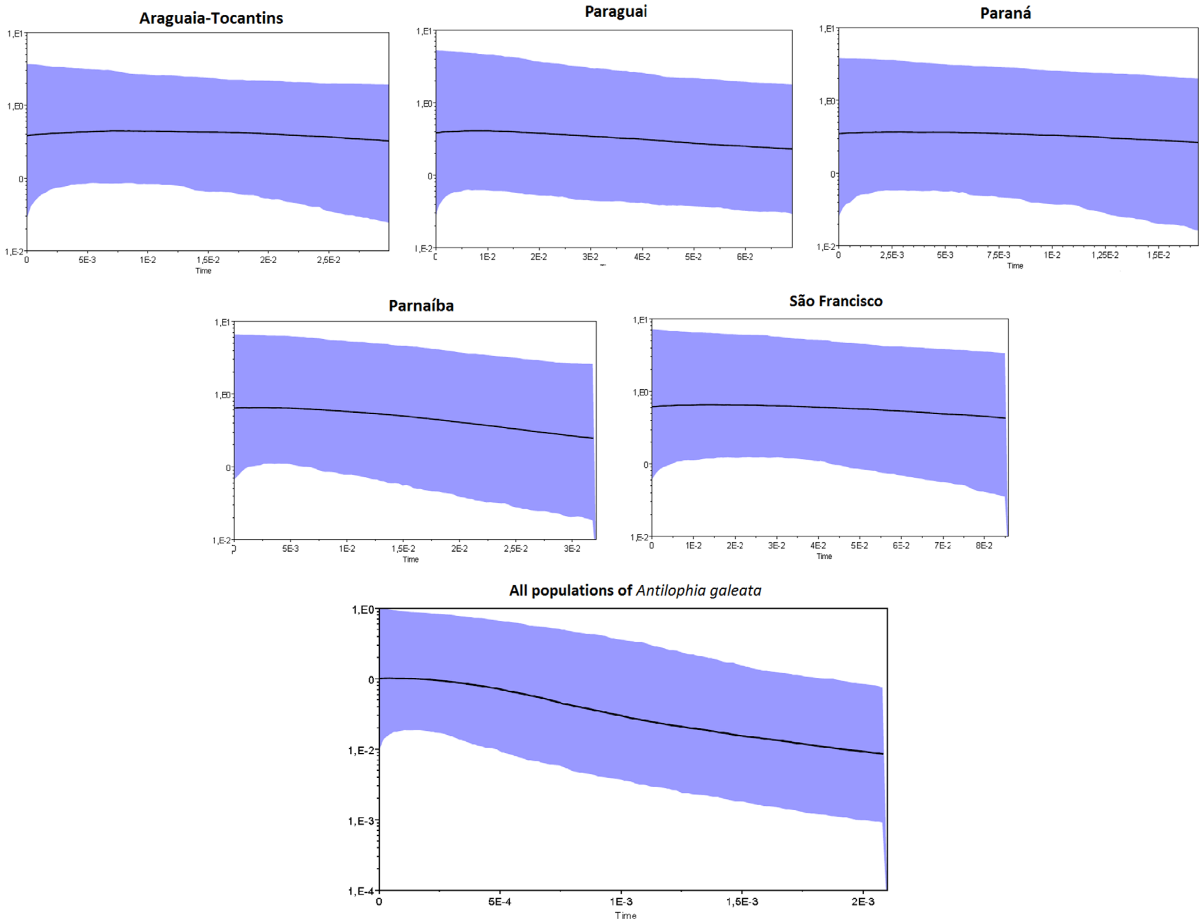

Figure 3. Bayesian Skyline Plot showing the effective size of the *Antilophia galeata* populations found in the five hydrographic basins of the Cerrado and the complete population, considering all the datasets combined. The solid line represents the variation in the effective size of the population over time.

and the Holocene, resulting from paleoclimatic changes (Barberi 2003). These records, together with the geomorphological evidence indicate that a single, large refugium of humid vegetation persisted in the center of the Brazilian Cerrado, in addition to a number of smaller refugia (microrefugia) in the periphery of the domain, located in the southeast, northwest (adjacent to the Amazon biome), and the northeast, in the transition zone with the Caatinga biome during the climatic fluctuations of the Pleistocene, between 20,000 and 13,000 years ago (Ab'Saber 1977, 1983, Bueno et al. 2017). These areas encompass a number of plateaus, such as the central Goiás plateau and the Guimarães plateau, where the surrounding gallery forests may have been little disturbed during the Late Pleistocene, if at all (Ab'Saber 1977, 1983).

The *A. galeata* populations of the gallery forests of the central portion of the Cerrado have relatively similar genetic profiles (Figure 2), with more shared haplotypes, which indicates a greater degree of connectivity and stability (Figure S1, Table SII). However, the portions of the gallery forest that retracted toward the periphery of the domain would have experienced more accentuated changes in their constitution, exerting pressures on the resident forest-dependent species (Ab'Saber 1977, 1983), which would have supported the differentiation of these populations (Leite 2006). This hypothesis of peripheral isolation is supported by the more differentiated genetic profile of the population of the Paraguay basin, and in particular by the genetic differences between the population of the São Francisco basin, in comparison with the other populations of the central Cerrado (Table I). The vegetation of this region, for example, the Central plateau that separates the São Francisco and Tocantins basins, endured profound alterations, and were at least partially replaced by the xeric vegetation that expanded from the region of the Caatinga, while others, such as the Espinhaço plateau, were invaded by the open vegetation that expanded from the cooler and drier areas of South America (Ab'Saber 1983, Bueno et al. 2017).

The unique environmental features that arose in distinct regions of the Brazilian Cerrado during the LGM may have provided preferencial habitats for forest-dependent species, with landscape structure having a fundamental influence on the community of the gallery forests of the Cerrado (Werneck et al. 2012). These distinct ecoregions are formed by well-preserved plateaus and depressions, which are considered to be separate regions, based on the hierarchical analyses of their biophysical attributes (Sano et al. 2019). These well-preserved areas may have served as refugia for *A. galeata* populations during specific periods, when these populations may have been more prone to genetic drift, which would have resulted in their differentiation from the populations inhabiting other regions of the Cerrado.

Genetic diversity and demographic history

The results of the present study indicate that, during the Late Pleistocene, the gallery forests of the Cerrado underwent considerable retractions, resulting in the formation of a major central core, with a number of microrefugia formed by fragments of forest in the peripheral areas of the Cerrado. However, these modifications in the configuration of the forest vegetation did not have a drastic impact on the genetic diversity of *A. galeata*, which remained high in all the populations, even when the diversity is analyzed separately by hydrographic basin (Table III). These significant levels of diversity may be attributed to the persistence of a large and stable population with a long evolutionary history (Grant & Bower 1998), which means that the possible occurrence of discontinuities among the gallery forests did not result in significant changes in the diversity of the *A. galeata* populations confined to the different hydrographic basins.

Ecological studies have shown that *A. galeata* is a highly versatile and adaptive species that is capable of reproducing even in small, isolated, and degraded fragments of forest habitat (Marini et al. 2007, Paniago 2016, Marçal & Lopes 2019, Silva et al. 2017, Tolentino et al. 2018). This may explain why the species was able to maintain such a stable population and preserve its genetic diversity, despite the profound shifts in the configuration of the Cerrado forests over time.

The high levels of diversity found in all the *A. galeata* populations is probably also related to their demographic stability. This hypothesis is supported by the results of the Bayesian Skyline Plot (Figure 3), which indicates that the populations were stable over time. The areas of endemism found within the Cerrado domain are considered to be extremely important for the evolution and maintenance of the fauna of this biome (Werneck 2011). In particular, four principal centers of endemism have been proposed to account for the distribution of the endemic Cerrado birds, resulting from their historical isolation, which has persisted to the present day (Silva 1997, Silva & Bates 2002). Three of these areas are located in the central portion of the Cerrado, on the Central Goiás Plateau, and in the valleys of the Araguaia and Paranã rivers, which are characterized by the presence of gallery forests and enclaves of tropical dry forest (Silva 1997, Silva & Bates 2002).

Two of these important areas of endemism are found in peripheral regions of the Cerrado. One is the Parecis Plateau, in the hydrographic basin of the Paraguay River, while the other is the Espinhaço Plateau, in the São Francisco basin. These areas also represent historically stable tracts of vegetation (King 1956, Braun 1970, Silva 1997, Silva & Bates 2002), which persisted as microrefugia that favored the maintenance of the species' genetic diversity and played an important role in the diversification of the local fauna (Lima-Rezende et al. 2019). This is supported by the differentiation of these areas from the other regions of the Cerrado domain, due to their unique environmental characteristics, which are appropriate for the establishment and persistence of forest-dependent species (Haddad et al. 1988, Rodrigues 1988). These findings should be used to orientate future studies on the distribution patterns of other groups of organisms in these regions.

The genetic parameters described here for *A. galeata* indicate that this species, endemic to the gallery forests of the Cerrado domain, is characterized by the isolation of its peripheral populations, demographic stability, and high genetic variability throughout its distribution. These results indicate that the connectivity of the gallery forests was sustained more extensively in the central portion of the Cerrado during the climatic oscillations of the Quaternary, through "corridors" that maintained the genetic diversity and flux of individuals. This pattern further reinforces the findings of previous studies, which indicate that the dynamic interaction between climate and relief played a fundamental role in the diversification and persistence of the biodiversity of the Cerrado (Santos et al. 2014, Lima-Rezende et al. 2019). However, to test this hypothesis against potential alternative demographic scenarios (e.g., stability between micro refugia with subsequent connection by range expansion), further studies using additional genetic markers (e.g., microsatellites; Souza et al. 2019) coupled with an analysis framework based on the coalescent theory that models different historical demographic change (e.g., ABC; Beaumont et al. 2010) will be necessary.

Acknowledgments

We are grateful to the following institutions for providing samples analyzed in the present study: the bird laboratory at UFMT, the Museum of Natural Science at Louisiana State University, Museu de Zoologia da USP, the Museu Nacional at UFRJ, Museu Paraense Emílio Goeldi, UnB, and UFSC. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) through Universal project 460195/2014-0, Grants 312404/2019-0 to JA, and 311539/2019-0 to PSR, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES process 88881.337398/2019-01 to PSR) and the Universidade Federal do Pará, through the Instituto de Estudos Costeiros and the Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (AQUASIS), together with the Graduate Program in Environmental Biology. LWL was supported by the fellowship of the Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES - 88882.444617/2019-01), through the Doctoral Graduate Program in Zoology, Universidade Federal do Pará (UFPA) and Museu Paraense Emílio Goeldi (MPEG), Belém, Brazil. We thank Stephen Ferrari for the revision of the manuscript.

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SUPPLEMENTARY MATERIAL

Tables SI, SII
Figure S1

How to cite

SOUZA OT, LUNA LW, ARARIPE J, SILVA WAG & REGO PS. 2022. Peripheral isolation and demographic stability are reflected in the genetic diversity of the populations of the Helmeted Manakin: a bird endemic to the gallery forests. An Acad Bras Cienc 94: e20201206. DOI 10.1590/0001-3765202220201206.

Manuscript received on July 31, 2020;
accepted for publication on June 7, 2021

THAINARA O. SOUZA[1]
https://orcid.org/0000-0001-8762-5828

LEILTON W. LUNA[2]
https://orcid.org/0000-0001-7902-6294

JULIANA ARARIPE[1,3]
http://orcid.org/0000-0002-8014-3081

WEBER A. DE G. E SILVA[4]
https://orcid.org/0000-0001-5503-5065

PÉRICLES S. DO REGO[1,3]
http://orcid.org/0000-0002-4537-946X

[1]Universidade Federal do Pará, Instituto de Estudos Costeiros, Laboratório de Genética e Conservação, Rua Leandro Ribeiro s/n, 68600-000 Bragança, PA, Brazil

[2]Universidade Federal do Pará, Departamento de Zoologia, Museu Paraense Emílio Goeldi, Avenida Perimetral, 1901/1907, 66040-170 Belém, PA, Brazil

[3]CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus de Vairão, Rua Padre Armando Quintas 7, 4485-661 Vairão, Portugal

[4]Associação de Pesquisa e Preservação de Ecossistemas Aquáticos, Avenida Pintor João Figueiredo - SESC - Iparana, 61627-250 Caucaia, CE, Brazil

Correspondence to: **Péricles Sena do Rego**
E-mail: periclessena@yahoo.com.br

Author contributions

T.O.S, L.W.L., and P.S.R. designed the study. T.O.S. and L.W.L. collected part of the samples in the field work. T.O.S. performed the laboratory procedures and the computational analysis. T.O.S., L.W.L. and P.S.R. wrote the manuscript with suggestions and revisions of J.A. and W.A.G.S.