



Effects of Pleistocene climate changes on species ranges and evolutionary processes in the Neotropical Atlantic Forest

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Received 15 March 2016; revised 1 May 2016; accepted for publication 2 May 2016

The effects of global glaciations on the distribution of organisms is an essential element of many diversification models. However, the empirical evidence supporting this idea is mixed, in particular with respect to explaining tropical forest evolution. In the present study, we evaluated the impacts of range shifts associated with Pleistocene global glacial cycles on the evolution of tropical forests. In particular, we tested the predictions: (1) that population genetic structure increases with fragmentation variation between the present and the Last Glacial Maximum (LGM) and also (2) with geographical range instability; and (3) that genetic diversity increases with range stability and (4) decreases with fragmentation variation between periods. To address our predictions, we studied population genetic structures and modelled present and past distributions of 15 Atlantic Forest (AF) endemic birds. Afterwards, we evaluated the relationship of population genetic parameters with metrics of species range shifts between the present and the LGM. We found that geographical ranges of AF birds changed in concert with Pleistocene glacial cycles but, unexpectedly, our findings suggest the novel idea that ranges during glacial maxima were slightly larger on average, as well as equally fragmented and displaced from the interglacial ranges. Our findings suggest that range shifts over the late Pleistocene impacted on the diversification of forest organisms, although they did not show that those range shifts had a strong effect. We found that a combination of fragmentation variation across time, small current range size, and range stability increased population genetic structure. However, neither fragmentation, nor range stability affected genetic diversity. Our study showed that evolutionary responses to range shifts across AF birds have a high variance, which could explain the mixed support given by single-species studies to the action of Pleistocene range shifts on population evolution. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 856–872.

KEYWORDS: Atlantic Forest – birds – niche modelling – Pleistocene glaciations – population genetic structure.

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INTRODUCTION

The effects of global glacial cycles on the distribution of organisms is an essential element of many diversification models, in particular with respect to explaining tropical forest evolution (Moritz *et al.*, 2000;

Birmingham, Dick & Moritz, 2005). For example, according to the forest refugia hypothesis (Haffer, 1969; Haffer & Prance, 2001), global glaciations created cycles of retraction and expansion of forests. During retraction phases, forests were fragmented and reduced to islands (i.e. forest refugia systems), where populations diverged in allopatry (vicariance). Then, during the subsequent expansion phase, divergent populations extended their ranges until they reached populations that diverged in other refugia. A recent criticism of the refugia model considers that shifts in forest species composition, instead of fragmentation, have been the main impact of global glaciations in the Neotropics (Colinvaux & De Oliveira, 2001; Bush & Metcalfe, 2012). Species composition shifts could also impact biological diversification, without the need of replacement of biomes. For example, dry forests could act as gene flow barriers for rainforest organisms. Consequently, shifting from humid to dry forest could promote isolation and population divergence of rainforest organisms. In another model, the gradients model, dissimilar selection regimes across forest types are important diversification drivers (Endler, 1977; Smith, 1997; Jump *et al.*, 2008). Because glacial cycles modify forest distribution and composition (Bush & Metcalfe, 2012), they are also able to alter selection regimes and gene flow patterns, impacting on diversification. A last model considering the range shifts important for evolution is the vanishing refuge hypothesis (Vanzolini & Williams, 1981; Damasceno *et al.*, 2014), which explains diversification by combining forest retractions and composition shifts associated with climate changes, vicariance, and adaptation to new forest types.

The aforementioned diversification models share predictions. For example, contact areas (i.e. suture zones) between recently diverged populations should be geographically congruent among species (Hewitt, 2000; Moritz *et al.*, 2000; Costa & Leite, 2012; Cabanne *et al.*, 2013). According to the refugia model, this is expected because forest retraction creates isolated refugia where populations diverge, and also adjacent regions where divergent populations converge after forest expansion. Contact areas should be geographically congruent across species because the process impacts most forest taxa in a similar manner. Congruent contact regions are also expected according to the gradients model, where different taxa should evolve in adjacent but different forest types, and contact regions may be congruent in forest ecotones. We consider that another prediction shared by these models is a positive correlation between population genetic structure (e.g. F_{ST}) and the range shifts of organisms across global glacial cycles. This is because organism range shifts would

have modified the genetic connectivity among populations, contributing to divergence.

Finally, if global glacial cycles have driven diversification, this should be reflected by the genetic diversity patterns. In particular, we expected a positive relationship between species range stability and their genetic diversity (Moritz *et al.*, 2000; Carnaval *et al.*, 2009) because strong range shifts might have diminished effective population sizes, and also because genetic diversity is proportional to effective size (Hedrick, 2011). In this context, because effective population size could be proportional to species range size, a positive link between genetic diversity and species range size would also be expected; but see Bazin, Glemin & Galtier (2006).

The Atlantic forest (AF) of Brazil, north-eastern Argentina, and eastern Paraguay is amongst the richest and most endangered rainforests in the world (Galindo-Leal & Câmara, 2003; Costa & Leite, 2012) (Fig. 1). This is a suitable biome for studying geographical range shifts linked to glacial cycles and any evolutionary effects because palynological studies (Behling & Negrelle, 2001; Behling, 2002; Ledru *et al.*, 2005), as well as early models of forest palaeodistribution (Carnaval & Moritz, 2008; Carnaval *et al.*, 2009), suggest that it was severely affected by climate changes during the Pleistocene. These studies propose that, during the maximum of glaciations, the southern AF was strongly fragmented by the advance of grasslands and savannah-like vegetation, whereas the central AF was relatively stable, and the northern AF expanded. More recently, Carnaval *et al.* (2014) presented a new palaeomodel for the AF that is different from previous models (Carnaval & Moritz, 2008) because it does not indicate dramatic forest retractions linked to glaciations in the southern portion of the biome.

There is mixed evidence suggesting that Pleistocene glaciations, as well as the associated forests range shifts, have driven the evolution of AF organisms. Support for significant evolutionary impacts of glaciations comes from several phylogeographical studies and a wide spectrum of taxa, such as forest birds (Cabanne *et al.*, 2008; D'Horta *et al.*, 2011; Maldonado-Coelho, 2012; Raposo Do Amaral *et al.*, 2013), frogs (Carnaval *et al.*, 2009, 2014), and bees (Batalha-Filho *et al.*, 2010). However, this idea has been recently challenged by an equivalent number of phylogeographical studies, also based on a wide range of taxa, such as frogs (Thomé *et al.*, 2010, 2012, 2014; Amaro *et al.*, 2012; Carnaval *et al.*, 2014), birds (Batalha-Filho, Cabanne & Miyaki, 2012; Cabanne *et al.*, 2013) planarians (Alvarez-Presas *et al.*, 2011; Álvarez-Presas *et al.*, 2014), and small mammals (Leite *et al.*, 2016). Moreover, some of the later studies have proposed that the AF was

not dramatically fragmented during glaciations, or even that forest dynamism was not important for evolution (Thomé *et al.*, 2014; Leite *et al.*, 2016). Overall, it is still unclear whether this lack of congruence across studies is the consequence of a high variance of the evolutionary and demographic response of species to range shifts, as suggested by Smith *et al.* (2014), or whether it is a result of glaciations and range shifts not having a strong impact on the population evolution of AF organisms.

Niche modelling allows us to explore and compare distributions of organisms across different time periods (Hijmans *et al.*, 2005; Peterson *et al.*, 2011), helping us to understand how range shifts affected populations and species evolution. For example, studying species ranges during the present (Holocene) and during the Last Glacial Maximum (LGM; 18 000–21 000 years BP) may help assess the

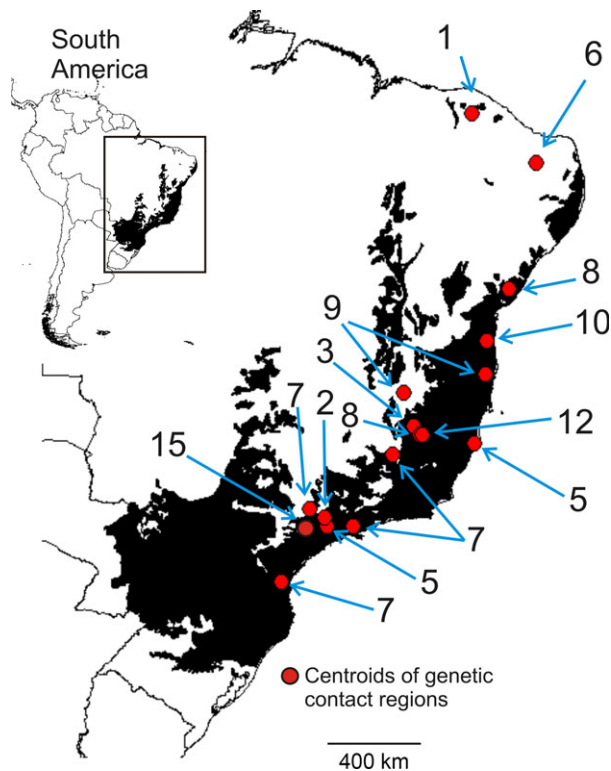


Figure 1. Atlantic Forest distribution and centroids of contact regions between recently diverged populations as described by BAPS (i.e. clusters). The inset represents the study area. Numbers represent taxa of each contact region (Table 1): 1, *Sclerurus scansor cearensis*; 2, *Sclerurus scansor scansor*; 3, *Synallaxis ruficapilla*; 5, *Xiphorhynchus fuscus*; 6, *Xiphorhynchus atlanticus*; 7, *Conopophaga lineata*; 8, *Conopophaga melanops*; 9, *Thamnophilus ambiguus*; 10, *Myrmoderus loricata*; 12, *Pyriglena leucoptera*; 13, *Schiffornis virescens*; 14, *Tachyphonus coronatus*; 15, *Myiothlypis leucoblephara*.

direction and magnitude of range shifts that occurred repetitively during the last Pleistocene glacial cycles (e.g. last 500 000 years). Even though the LGM may be too recent to explain intraspecific evolution, it represents one end of the climatic variation that occurred throughout the late Pleistocene (i.e. a peak of a glaciation). Therefore, because at least the last five glaciations have been cyclic, with a similar duration and intensity (Anderson, Goudie & Parker, 2007; Bush & Metcalfe, 2012), species ranges at the LGM are expected to be a good representation of scenarios during previous glaciations, and a similar rationale could be applied to the present (i.e. Holocene) and previous interglacial periods. The present study investigated current species distributions and those at the LGM and, for the first time, obtained models of range shifts between periods, aiming to better understand the effects of Pleistocene climate alterations on population evolution.

The objective of our research was to evaluate the evolutionary impacts of Pleistocene climate changes on forest organisms. In particular, according to the hypothesis that Pleistocene species range shifts have driven population evolution we predicted (Fig. 2A): (1) that population genetic structure increases with fragmentation variation between the present and the LGM, as well as (2) with geographical range instability; and also (3) that genetic diversity is positively related to range stability and (4) decreases with fragmentation variation between periods. In addition, (5) we expected that genetic diversity increased with present range size. Two additional questions were considered: (1) what kind of geographical range shifts experienced forest taxa between the LGM and present? (2) Are contact areas between genetically differentiated clusters distributed randomly? To address our predictions and questions, we studied population genetic structures and modelled present and past distributions of a sample of AF endemic birds. Subsequently, we evaluated the relationship of population genetic parameters with metrics of species range shifts between the present and the LGM. We found that a combination of fragmentation variation, small range size, and range stability increased the genetic structure, and also that range size and altitudinal distribution affected genetic diversity.

MATERIAL AND METHODS

STUDIED TAXA

We studied 15 AF passerines (Tables 1, 2). We selected target taxa according to the criteria: (1) being forest dependent and restricted to the AF biome (Stotz *et al.*, 1996) and (2) having a mitochondrial DNA data set obtained from samples collected

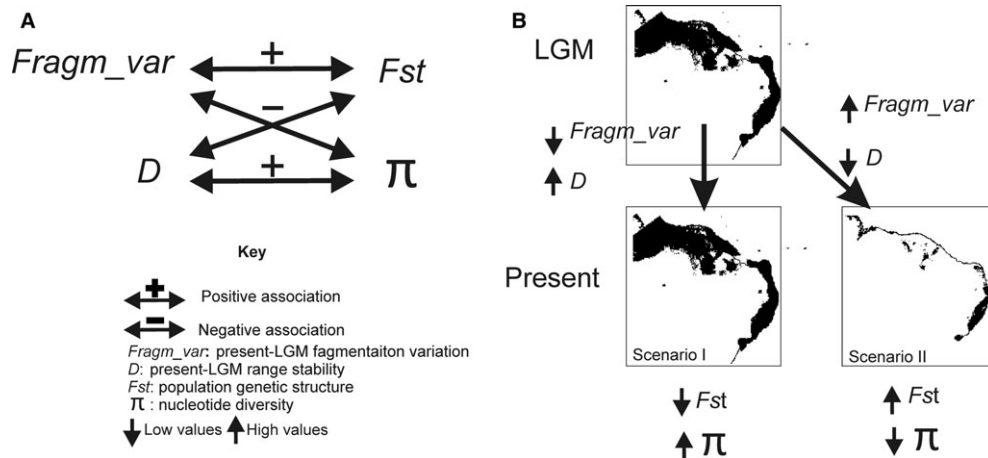


Figure 2. Predictions of the working hypothesis that range shifts between the Last Glacial Maximum (LGM) and the present impacted population evolution. A, tested predictions in terms of fragmentation variation between the LGM and the present (*Fragm_var*), range stability (*D*), population genetic structure (F_{ST}) and on genetic diversity (π). B, two examples of scenarios and predicted behaviour of range shift (*Fragm_var* and *D*) and population genetic parameters (F_{ST} and π).

through most of their geographical distribution. Finally, all taxa are abundant with well-known geographical distributions, which helped to evaluate niche models obtained with MAXENT (see below).

SPECIES RANGE MODELS

We modelled species ranges, instead of the biome range (Carnaval *et al.*, 2014), because the distribution of stable populations would depend on each species, according to its habitat requirements (Gómez & Lunt, 2006; Porto, Carnaval & da Rocha, 2013; López-Urbe *et al.*, 2014). Georeferenced occurrence localities of the 15 target species were gathered from data from our own field work, from different museum collections, from ORNIS (www.ornisnet.org), and from XENO Canto (www.xeno-canto.org). The locations of the points of occurrence were verified using Google Earth 7.0 (www.google.com/earth), and possible mislabelled coordinates were excluded. The study area (AF) is strongly impacted by deforestation (Galindo-Leal & Câmara, 2003; Costa & Leite, 2012) and, because deforestation might cause a sampling bias toward regions with preserved forest, we took special care to incorporate historic records that would represent the pre-deforestation species range. In this case, we only sampled historic records (e.g. > 100 years) that were geographically congruent with other forest species records, which would indicate existence of past forest. To obtain an even geographical distribution of records, only samples separated at least by 20 km of each other were used.

To obtain species distribution models, we used the maximum entropy algorithm, as implemented in MAXENT, version 3.3.3 (Phillips, Anderson & Schapire, 2006). This presence-background modelling technique has performed well in comparisons with other such techniques (Elith *et al.*, 2006; Hernández *et al.*, 2006; Phillips & Dudík, 2008; Wisz *et al.*, 2008). To estimate the distribution range of each species, the bioclimatic variables used for the modelling approach were chosen from the 19 layers available in the WorldClim dataset (Global Climate Data; www.worldclim.org) (Hijmans *et al.*, 2005). We rejected correlated environmental variables (Peterson *et al.*, 2011) and selected relevant variables following a rationale of permutation importance > 5%. The bioclimate layers were used with a resolution of 2.5' arc-min, and delimited by the following rectangle (study area): north-eastern corner $x = -30.5325$ and $y = 1.2614$ and south-western corner $x = -61.1071$ and $y = -36.1285$. We selected a study area that does not adjust strictly to the AF distribution because some of the target species are marginally distributed in gallery forests of the Cerrado and Chaco (e.g. *Xiphorhynchus fuscus* and *Myiothlypis leucoblephara*), and also because we wanted to evaluate the dislocation of ranges between periods (Present and LGM). General conditions for analyses were: randomtestpoints: 25; replicates: 10; replicate-type: subsample; maximumiterations: 5000. For small data sets (< 50 records) we used a 10-fold cross validation approach, which consisted of randomly splitting the occurrence data into 10 equal size groups (folds) and creating models by 10 replicates

Table 1. Analyses of MAXENT range models of a sample of Atlantic Forest endemic birds

Taxa	N*	Average AUC	Max. altitude (km)	Range _{Present} [†] (pixels)	Range _{LGM} [‡] (pixels)	Range _{LGM} /Range _{Present}	Fragm _{Present} [§]	Fragm _{LGM} [¶]	Fragm _{Var}	D**
1 <i>Sclerurus scansor cearensis</i>	44	0.99	0.7	472	9330	19.77	6.3559	1.6845	4.6714	0.4631
2 <i>Sclerurus scansor scansor</i>	71	0.926	1.5	42 314	80 787	1.91	0.1418	0.0955	0.0463	0.5165
3 <i>Synallaxis ruficapilla</i>	74	0.911	1.8	44 631	62 022	1.39	0.0672	0.0841	-0.0169	0.5832
4 <i>Automolus leucophthalmus</i>	86	0.914	0.9	42 844	74 328	1.74	0.1867	0.1274	0.0594	0.4336
5 <i>Xiphorhynchus fuscus</i>	90	0.863	1.5	58 245	104 097	1.79	0.1717	0.0934	0.0783	0.6472
6 <i>Xiphorhynchus atlanticus</i>	46	0.987	0.7	1969	10 621	5.40	3.5551	0.5088	3.0463	0.4087
7 <i>Conopophaga lineata</i>	105	0.88	2.4	37 617	132 436	3.52	0.3988	0.1232	0.2756	0.5538
8 <i>Conopophaga melanops</i>	47	0.974	0.8	17 384	37 181	2.14	0.4027	0.1763	0.2264	0.4969
9 <i>Thamnophilus ambiguus</i>	41	0.977	0.4	7040	12 465	1.78	1.1364	0.4586	0.6778	0.3552
10 <i>Myrmoderus loricata</i>	39	0.94	1.2	10 467	43 740	4.18	1.2420	0.4388	0.8032	0.6016
11 <i>Myrmoderus squamosa</i>	58	0.98	1	6210	23 092	3.72	0.4831	0.4097	0.0734	0.4944
12 <i>Pyriglena leucoptera</i>	72	0.894	1.8	49 825	10 2551	2.06	0.2007	0.1338	0.0669	0.6132
13 <i>Schiffornis virescens</i>	119	0.907	1.5	46 748	75 469	1.61	0.2781	0.2059	0.0722	0.3989
14 <i>Tachyphonus coronatus</i>	77	0.917	1	41 171	57 778	1.40	0.1214	0.2152	-0.0938	0.5381
15 <i>Myiothlypis leucoblephara</i>	92	0.95	2	69 639	75 831	1.09	0.0574	0.1315	-0.0741	0.5889

*Number of independent records used for the niche models.

†Present range.

‡Range at Last Glacial Maximum (LGM).

§Fragmentation index for present.

¶Fragmentation index for LGM.

**Stability between the present and the LGM (for further details, see Material and methods).

Table 2. Genetic analysis of a sample of Atlantic Forest endemic birds

Taxa	mtDNA gene	N^\dagger	π^\ddagger	Number of populations (BAPS) [§]	F_{ST}	Data origin
1 <i>Sclerurus scansor cearensis</i>	cytB	17	0.0027	2	0.87**	D'Horta <i>et al.</i> (2011)
2 <i>Sclerurus scansor scansor</i>	cytB	29	0.0052	2	0.38**	D'Horta <i>et al.</i> (2011)
3 <i>Synallaxis ruficapilla</i>	cytB	11	0.0109	2	0.67*	Batalha-Filho <i>et al.</i> (2013)
4 <i>Automulus leucophthalmus</i>	cytB	21	0.0026	1	0	D'Horta (2009)
5 <i>Xiphorhynchus fuscus</i>	cytB	27	0.0095	3	0.76**	Cabanne <i>et al.</i> (2008)
6 <i>Xiphorhynchus atlanticus</i>	cytB	10	0.0011	2	0.68**	Cabanne <i>et al.</i> (2008)
7 <i>Conopophaga lineata</i>	CR	173	0.0461	4	0.79**	Dantas <i>et al.</i> (2014)
8 <i>Conopophaga melanops</i>	CR	30	0.0200	3	0.83**	Present study (Genbank accession numbers KX352275–KX352304)
9 <i>Thamnophilus ambiguus</i>	cytB	21	0.0055	3	0.68**	Lacerda, Marini & Santos (2007)
10 <i>Myrmoderus loricata</i>	ND2	47	0.0032	2	0.48**	Raposo Do Amaral <i>et al.</i> (2013)
11 <i>Myrmoderus squamosa</i>	ND2	40	0.0007	1	0	Raposo Do Amaral <i>et al.</i> (2013)
12 <i>Pyriglena leucoptera</i>	ND2	48	0.0045	2	0.29**	Maldonado-Coelho (2012)
13 <i>Schiffornis virescens</i>	cytB	26	0.0018	2 [¶]	0	Cabanne <i>et al.</i> (2013)
14 <i>Tachyphonus coronatus</i>	cytB	50	0.0051	2 [¶]	0	Present study (Genbank accession numbers KX352305–KX352354)
15 <i>Myiothlypis leucoblephara</i>	cytB	54	0.0051	2	0.28**	Batalha-Filho <i>et al.</i> (2012)

* $P < 0.05$, ** $P < 0.01$.

[†]Number of sequences used in the genetic analyses.

[‡]Nucleotide diversity based on uncorrected pairwise distances.

[§]Number of populations described by BAPS.

[¶]Clusters described by BAPS overlapped geographically; thus, we assumed $F_{ST} = 0$.

and leaving out each fold in turn (Phillips *et al.*, 2006). To determine whether the model's discrimination capacity is better than random chance, current models were validated by accessing the area under the receiver operating characteristic curve: the AUC. The AUC ranges from 0.5 (random accuracy) to a maximum value of 1.0 (perfect discrimination).

We projected models into two palaeoclimate scenarios simulating the LGM period (21 000 years BP): CCSM3 (Community Climate System Model; <http://www.cesm.ucar.edu>), and MIROC (Model of Interdisciplinary Research on Climate; <http://www.ccsr.utokyo.ac.jp/kyosei/hasumi/MIROC/tech-repo.pdf>). To convert continuous models into binary maps (presence/absence), we evaluated two thresholds: minimum training presence and equal training sensitivity and specificity (ETSS). We report only the results obtained with the ETSS threshold because: (1) the distribution models using these two thresholds did not differ notably and (2) the ETSS produced models that adjusted better to the current known distribution of species (less commission error), according to our personal experience with the studied species and according to published maps (Ridgely & Tudor, 2009). MAXENT infiles and results in ascii files are provided in the Supporting information (Data S3).

GENETIC ANALYSIS

We studied mitochondrial DNA (mtDNA) sequences to evaluate population genetic structure and genetic diversity. Markers used were: cytochrome B (cytbB), control region (CR) or NADH dehydrogenase 2 (ND2), depending on the species (Table 2). We analyzed sequences that were specifically collected for the present study (i.e. of *Conopophaga melanops* and *Tachyphonus coronatus*), as well as public sequences available from Genbank (Table 2). We also used cytochrome oxidase I sequences obtained from Genbank to control for the lack of independence introduced by the evolutionary relationship among taxa, where we estimated a matrix of pairwise genetic distances (TNei + G, $\alpha = 0.55$) to be used in the statistical analyses. For laboratory procedures and tissue sample details, see the Supporting information, Data S2.

We employed the Bayesian clustering algorithm implemented in BAPS, version 6.0 (Corander *et al.*, 2008) to estimate the most likely number of genetically differentiated populations for each species. We surveyed for the probability of a different number of genetic clusters, ranging from $K = 1$ to 20. We fixed $K_{\max} = 20$ and each search was performed under the models of *mixture analysis* and *spatial clustering of individuals*. In those cases when two or more genetic

clusters with a geographical coherence (i.e. no geographically overlapping or randomly scattered clusters) were detected, we estimated population genetic structure (F_{ST}) using the analysis of molecular variance approach, with ARLEQUIN, version 5.1 (Excoffier & Lischer, 2010). If clusters described by BAPS overlapped geographically or were randomly scattered, we considered an $F_{ST} = 0$. F_{ST} values were calculated using noncorrected pairwise differences and P -values were obtained after 1000 permutations. We used the 'mapfile' output files from BAPS to map contact regions between divergent populations. The 'mapfile' consists of a GIS shapefile that describes the geographical distribution of genetic clusters. Nucleotide diversity values (π) were estimated in ARLEQUIN using noncorrected pairwise differences. For species with more than 50 sequences available, π was estimated using a subset of 30 randomly selected sequences.

Finally, to test for geographical congruence amongst cluster contact areas identified by BAPS, we investigated whether geographical centroids of contact areas were randomly distributed with the test average nearest neighbor implemented in ARCGIS, version 10.1 (Environmental Research Institute). The later test considered, as the study region, the minimum area enclosing the mentioned centroids. When different BAPS clusters did not co-occur or did not come into contact, we considered, as centroids, the midpoint of the shortest geographical distance between samples of the two considered clusters.

GEOGRAPHICAL RANGE AND GENETIC STATISTICAL ANALYSIS

For each target taxon, we recorded the following metrics to be used in posterior analyses: fragmentation index for the present and for the LGM ($Fragm_{Present}$ and $Fragm_{LGM}$); difference of fragmentation between the present and the LGM ($Fragm_{var} = Fragm_{Present} - Fragm_{LGM}$); range stability between the present and the LGM (D); maximum altitude of occurrence at present ($Max. altitude$); and present and LGM range sizes ($Range_{Present}$ and $Range_{LGM}$). Each variable is explained below.

The fragmentation index ($Fragm$) for each period was estimated using the formula $Fragm = (\#Fragms / Range) 1000$; where $\#Fragms$ was the number of fragments of at least 1000 km², isolated by at least one pixel (approximately 4.8 km) and $Range$ was the total range in pixels. We estimated $\#Fragms$ in ARCGIS, version 10.1 using the World Mercator (EPSG: 3395) coordinate system, converting rasters to vectorial files and estimating fragment areas. We only considered fragments equal or larger than 1000 km²

because our preliminary analysis indicated it to be a size that maximizes $Fragm_{var}$. A fragment separation of 4.8 km is sufficient to isolate most AF forest passerines (Zurita *et al.*, 2012).

The overall range stability between periods (D) consisted of the metric Schoener's D (Schoener, 1968) estimated between the present and the LGM ranges of each taxa in ENMTools (Warren, Glor & Turelli, 2010). Briefly, D varies from 0 to 1, being 0 when fully divergent models are compared, and 1 when models are equal. To include, in the estimation of D , those portions of present and LGM models where sea level changed between periods, we converted sea level regions of all rasters (background, pixel value -9999) into areas with the lowest probability of occurrence (pixel value 0). A preliminary analysis of our data set indicated that D and $Fragm_{var}$ did not co-vary (Spearman nonparametric correlation test, $P = 0.49$), which confirmed that each variable was sensitive to different aspects of range shifts.

$Range_{LGM}$ and $Range_{Present}$ size was estimated as the number of presence pixels at LGM and present, respectively.

For *Max. altitude*, we used the highest limit of occurrence of each species at the Serra dos Órgãos mountain range, south-eastern Brazil. We used this region as a reference because abundance variation across altitude is relatively well known there (Stotz *et al.*, 1996; Mallet-Rodrigues, Parrini & Pacheco, 2007; Mallet-Rodrigues *et al.*, 2010; G. S. Cabanne, unpubl. data) and also because it is where most of our target taxa occur. In this respect, we obtained maximum altitudes from Mallet-Rodrigues *et al.* (2010) and split taxa into those from lowlands ($Max. altitude < 1500$ m) and from highlands (≥ 1500 m). We split the sample into lowlands and highlands to obtain an objective and repetitive criterion for identifying taxa that could be cold-adapted. Typically, cold-adapted taxa would be tagged according to the condition of association with southern AF (Carnaval *et al.*, 2014), although we consider that our target taxa do not fit well a northern/southern definition. Nevertheless, it is worth noting that 100% of the target taxa from highlands ($N = 7$) (Table 1) have range limits in southern AF, and thus both criteria of *Max. altitude* and of association with southern AF are in agreement. For species that do not occur at Serra dos Órgãos (e.g. *Sclerurus scansor cearensis*, *Xiphorhynchus atlanticus*, and *Myrmoderus squamosa*), we used maximum altitudes across their entire distribution (G. S. Cabanne, unpubl. data).

We used the Fisher randomization test (Fisher, 1935) implemented in RUNDOM PRO, version 3.14 (Jadwiszczack, 2009) to compare metrics of the species distributions. The stable range area, or refugium

area, was estimated by the formula $(Range_{LGM} \cap Range_{Present})/Range_{Present}$.

We studied the relationship of the genetic constitution of taxa (F_{ST} and π) with metrics of range shifts between the present and the LGM (*Fragm_var* and π). Briefly, we used the nonparametric procedure of Smouse, Long & Sokal (1986) (see also Manly, 1991), also known as the multiple Mantel test (Legendre & Legendre, 2012), to obtain partial correlation coefficients between matrices of F_{ST} and π with matrices of the metrics: (1) *Fragm_var*; (2) D ; (3) $Range_{Present}$; and (4) *Max. altitude*. To obtain a single global test for F_{ST} and π , we averaged parameters measured from each LGM climate model (e.g. *Fragm_var* of CCSM and MIROC). The partial correlation analyses were performed using pairwise distances instead of raw data, aiming to keep the validity of the procedure (Legendre & Legendre, 2012). Thus, we first converted each metric into a matrix of pairwise distances (pairwise differences between values of each taxa). Therefore, statistically tested predictions were stated in terms of the association between matrices of pairwise differences, which is another way of expressing the relationships shown in Figure 2A.

All partial correlation analyses were performed on standardized values (Quinn & Keough, 2002) in FSTAT, version 2.9.3.2 (Goudet, 2002), with 20 000 replicates. The final model estimation used backward variable selection, based on the stepwise elimination of nonsignificant variables with lesser contribution according to the overall sum of squares. To control for the lack of independence introduced by evolutionary relationships among taxa, we used a matrix of pairwise genetic distances (*Evol. distance*) estimated with COI sequences (see Genetic analysis). In addition, because the genetic data set consisted of different mtDNA markers (Table 2), which could differ slightly in variation levels, we used an indicator matrix constructed with a dummy variable (Quinn & Keough, 2002) to specify when different genes were involved.

RESULTS

NICHE MODELS AND RANGE SHIFTS

We modelled the present and LGM distribution of each taxon (Fig. 3 and Table 1). Models presented a good performance because most average AUC values exceeded 0.9 (Peterson *et al.*, 2011). For further details (i.e. MAXENT files, distribution of records, AUC values, BIOCLIM variables, and probability of occurrences), see Supporting information (Data S1, S3). The models suggest that most range shifts between the LGM and the present consisted of

displacements, coupled with small shrinkages or no absolute range size change (Table 1). We found that only 65% of present distributions overlapped with LGM ranges (refugia areas, mean proportion of stable regions = 0.65, interval: 0.57–0.72; $N = 15$). Also, the mean of the ratio $Range_{LGM}/Range_{Present}$ across taxa deviated from one (Fisher's test $P < 0.01$), indicating that LGM distributions were on average 2.6 times larger than present distributions (mean $Range_{LGM}/Range_{Present} = 2.62$, interval: 1.09–19.77; $N = 15$). Regarding fragmentation levels, no significant difference was found between present and the LGM fragmentations (mean $Fragm_{LGM}/Fragm_{Present}$ across taxa = 0.75, Fisher test $P = 0.13$; $N = 15$).

We explored the relationship between the range shift metrics and maximum altitude, and found that D , *Fragm_var* and $Range_{LGM}/Range_{Present}$ were significantly correlated with maximum altitude variation (partial correlation $r_{D-Max. alt} = 0.659$, $P < 0.0001$, $r^2 = 43.5$; partial correlation $r_{Fragm_var-Max. alt} = -0.4817$, $P < 0.0001$, $r^2 = 23.2$; partial correlation $r_{Range_{LGM}/Range_{Present} - Max.alt} = -0.33$, $P < 0.0001$, $r^2 = 10.9$). We controlled for an evolutionary relationship in the previous tests, although neither partial correlation with *Evol. distance* was significant ($P > 0.05$). Scatterplots indicate that lowland birds had higher $Range_{LGM}/Range_{Present}$ and higher *Fragm_var*, as well as smaller stability (D) between periods (Fig. 4).

ANALYSIS OF CONTACT AREAS

The number of genetically differentiated populations (Clusters) for each target taxon varied from 1 to 4 (Table 2). The contact areas between clusters denoted by BAPS ($N = 17$) (Fig. 1) were randomly distributed (nearest neighbour ratio = 1.05, $P = 0.7$, $Z = 0.38$).

ANALYSIS OF POPULATION GENETIC STRUCTURE AND GENETIC DIVERSITY

The metric F_{ST} varied from 0 to 0.87, and π varied from 0.0007 to 0.0461 (Table 2). Pairwise F_{ST} variation was positively correlated with fragmentation variation between the LGM and the present, and with range stability variation; furthermore, it was negatively correlated with present range size variation (Table 3). Altogether, these three significant factors explained approximately 31% of the total variation in pairwise differences of F_{ST} . Scatterplots indicated that F_{ST} increased with higher fragmentation difference between the present and the LGM, and with intermediate to high values of D . By contrast, F_{ST} decreased with larger present ranges (Fig. 5A, B, C).



Figure 3. Binary models of distribution of 15 Atlantic forest birds. Distributions were projected for the present and for two circulation models of the Last Glacial Maximum (21 kyr BP). Insets represent study areas for widely distribution taxa and for taxa restricted to northern Atlantic forest.

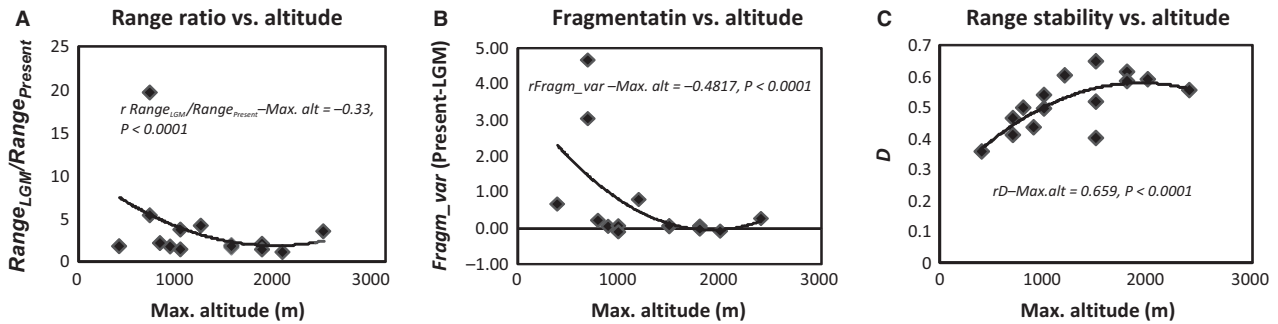


Figure 4. A, scatterplot linking the ratio of ranges between the Last Glacial Maximum (LGM) and the present ($Range_{LGM}/Range_{Present}$) with maximum altitude of occurrence. B, scatterplot linking fragmentation variation between the present and the LGM ($Fragm_var$) with maximum altitude. C, scatterplot linking range stability between the present and the LGM (D) with maximum altitude. Partial correlation coefficients between matrices of pairwise difference metrics are also shown (for further details, see Material and methods). The tendency lines of scatterplots were obtained with a polynomial function of second order.

Genetic diversity variation was positively correlated with maximum altitude and present range size (Table 3). Effect size of both factors is low because taken together only explain 35.43% of the variation of π across taxa. Scatterplots indicate that lowland taxa had the smallest π , and that taxa with intermediate present size ranges showed the highest π (Fig. 5D, E).

DISCUSSION

We have studied the evolutionary impacts of Pleistocene climate changes on forest organisms. Our first finding, in contrast to what is proposed in literature (Behling, 2002; Behling *et al.*, 2007; Carnaval & Moritz, 2008), suggest that ranges of the studied taxa during the LGM were on average larger and equally fragmented compared to the present. Second, we confirmed that range shifts are positively linked to intraspecific genetic differentiation, although only with low effect (Table 3). Third, we observed that genetic diversity was only correlated with altitudinal distribution and present range size. Lastly, our study shows that evolutionary response across AF birds to historical range shifts have a high variance. This finding could explain the lack of a clear pattern for the evolutionary impacts of Pleistocene range shifts on the AF, in particular when single-species studies are considered.

CONGRUENCE BETWEEN NICHE MODELS AND PHYLOGEOGRAPHICAL PATTERNS

Our niche models presented good performance and are in agreement with previous phylogeographical studies of the target taxa. For example, studies on

M. leucoblephara and *Shiffornis virescens* found shallow population genetic structure and demographic stability during the LGM, suggesting low or inexistent late Pleistocene range shifts for both species (Batalha-Filho *et al.*, 2012; Cabanne *et al.*, 2013). Our niche models are compatible with these results because both species showed very small range shifts between the LGM and the present (Table 1; see also Supporting Information, Data S1). Also, previous studies on *Sclerurus scansor cearensis* and *X. atlanticus* found strong population genetic structures and genetic evidence of demographic instability in certain geographical regions (Cabanne *et al.*, 2008; D'Horta *et al.*, 2011), which matched our modelled range shifts (Table 1). Moreover, *S. s. cearensis* presented the strongest range shift of all our studied taxa, with its LGM range being 19.7 times larger than in the present. Lastly, Maldonado-Coelho (2012) found different demographic histories across populations of *Pyrrhuloxia leucoptera*, with stability in the central region of the AF and instability in the southern portion of the biome. These findings are in agreement with our models (Fig. 3; see also Supporting information, Data S1), which suggest that, during the LGM, *P. leucoptera* expanded toward central and northern AF, coupled with shrinkages across southern populations, although without an important absolute shift of the species total range ($Range_{LGM}/Range_{Present} = 2$).

WHAT KIND OF RANGE SHIFTS OCCURRED BETWEEN THE LGM AND THE PRESENT?

Our niche models suggest that LGM ranges of AF birds were slightly larger than in the present, as well as equally fragmented and displaced from the interglacial distributions (Fig. 3). This finding was

Table 3. Partial correlation analyses between population genetic structure (F_{ST}) and nucleotide diversity (π) with metrics of range shifts between the Last Glacial Maximum and the present

Dependent factor	Independent factor	Partial correlation*	Percentage of variance explained
F_{ST}	<i>Max. altitude</i>	n.s.	
	<i>mtDNA gene</i>	n.s.	
	<i>Fragm_var</i>	0.316 ($P < 0.001$)	9.98
	<i>D</i>	0.34 ($P < 0.001$)	11.56
	<i>Range_{Present}</i>	-0.306208 ($P < 0.001$)	9.37
	<i>Evol. distance</i>	n.s.	
	<i>All variables</i>		30.91
π	<i>Max. altitude</i>	0.514 ($P < 0.001$)	26.31
	<i>mtDNA gene</i>	n.s.	
	<i>Fragm_var</i>	n.s.	
	<i>D</i>	n.s.	
	<i>Range_{Present}</i>	-0.315 ($P < 0.001$)	10.12
	<i>Evol. distance</i>	n.s.	
	<i>All variables</i>		36.43

Analyses are based on pairwise difference matrices of each metric (see Material and methods).

*Significance limit was $P = 0.025$ because of the test of F_{ST} and π shared data.

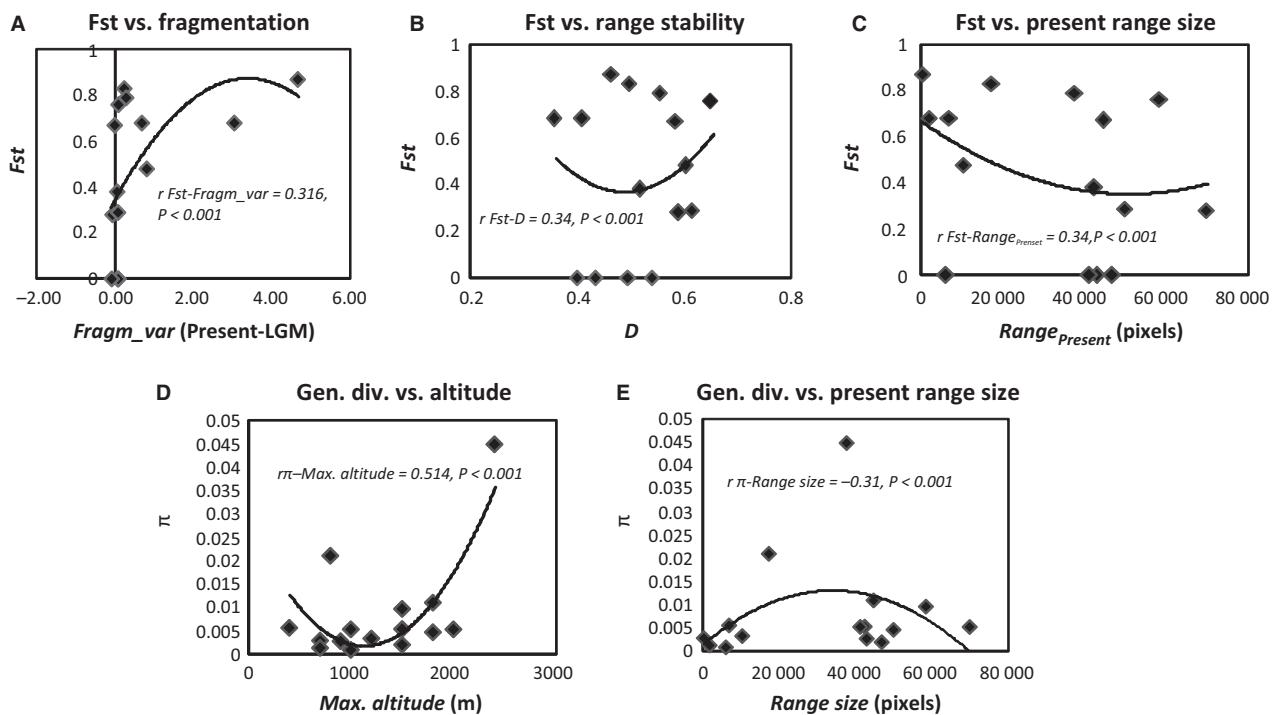


Figure 5. Scatterplots linking population genetic parameters of Atlantic Forest birds with range shift metrics. A, population genetic structure (F_{ST}) vs. fragmentation variation (*Fragm_var*) between the present and the Last Glacial Maximum (LGM). B, F_{ST} vs. range stability (*D*) between the present and the LGM. C, F_{ST} vs. present range size. D, nucleotide diversity (π) vs. maximum altitude. E, π vs. *D*. Partial correlation coefficients between pairwise difference matrices of metrics are also shown (for further details, see Material and methods). The tendency lines of scatterplots were obtained with a polynomial function of second order.

unexpected and in disagreement with previous studies proposing that AF organisms had smaller ranges during the LGM (Behling, 2002; Behling *et al.*, 2007;

Carnaval & Moritz, 2008). However, the aforementioned notion of smaller ranges during the LGM came from only comparing refugia areas with

present areas, and not from comparing absolute areas. Indeed, in our dataset, refugia areas were smaller than present ranges (i.e. only 65% of present distributions are refugia). However, we argue that, for evolutionary studies, it is advantageous to evaluate shifts in total ranges because these shifts will reflect absolute demographic events of the whole species (e.g. bottlenecks), instead of only evaluating refugia areas. In summary, current distributions of AF organisms are not fully stable, in accordance with previous studies (Carnaval & Moritz, 2008), although, at the community level, it appears that absolute range sizes did not change dramatically between the LGM and the present. Moreover, in some species, LGM ranges were larger than in the present (Table 1).

Our results suggesting that some AF bird ranges were larger at the LGM than in the present could be explained by taking into account events that could have expanded forest past distributions. In this case, according to the models of Figure 3 taken together, our results could be explained by range expansions into regions currently covered by the savanna Cerrado and the dry forest Caatinga, as well as by retraction of sea level during the maximum of glaciations and the likely consequent forest expansion offshore of the present coastline. For example, the species *Conopophaga lineata* showed a strong expansion during the LGM into central and north AF, as well as expansion into regions of the present-day Cerrado (Fig. 3). In another example, *C. melanops* mostly expanded into the coastal lowlands and into areas of the present day Cerrado and Caatinga. Our explanation is in accordance with palynological and climatological studies indicating expansions during glaciations of humid forest into the Cerrado and Caatinga, coupled with some forest retraction in southern AF regions (Ledru, Salgado-Labouriau & Lorscheitter, 1998; Behling, 2002; Ledru, 2002; Cheng *et al.*, 2013; Ledru *et al.*, 2016). Moreover, our results are also in agreement with studies indicating a retraction of sea levels during glaciations (Rabineau *et al.*, 2006; Anderson *et al.*, 2007; Prentice, Harrison & Bartlein, 2011; Leite *et al.*, 2016). However, it is not clear which process would be proportionally more important to explain, at the community level, the observed range dynamism (Raposo Do Amaral *et al.*, 2016). According to our models (Fig. 3), range expansions into continental areas, instead of into coastal lowlands, appear to explain a higher proportion of range shifts between the LGM and the present.

A lack of strong range shifts between the LGM and the present, or even larger LGM ranges, was also observed in other niche models of tropical and temperate forest organisms (Amaro *et al.*, 2012;

Manthey, Klicka & Spellman, 2012, 2014; Walstrom, Klicka & Spellman, 2012; Gür, 2013; Leite *et al.*, 2016). However, the present study is the first to suggest that some AF birds expanded their ranges during the maximum of glaciations. Moreover, a lack of strong range shifts between the LGM and the present is also compatible with other phylogeographical studies of AF taxa (Thomé *et al.*, 2010, 2014; Amaro *et al.*, 2012), as well as with recent whole-biome distribution models (Carnaval *et al.*, 2014; Sobral-Souza, Lima-Ribeiro & Solferini, 2015; Leite *et al.*, 2016).

Carnaval *et al.* (2014) suggest that AF cold-associated taxa (mainly southern biome taxa) are currently in their contraction climatic phase. If the later hypothesis is correct, then, in the present, these taxa should have smaller ranges and larger fragmentation levels than in the past. AF cold-associated taxa could be comparable to highland taxa of our sample (*Max. altitude* > 1500 m) because the later taxa are associated with colder climates. Our results indicate that taxa with different maximum altitudes presented different range stability, different ratios between the LGM and present ranges, and different fragmentation variation between periods (Fig. 4). However, lowland taxa (instead of highland taxa) appeared to be in a contraction phase because they tend to have larger LGM ranges ($Range_{LGM}/Range_{Present} > 1$) and higher levels of present fragmentation ($Fragm_{Present} - Fragg_{LGM} > 0$) (Fig. 4A, B). Moreover, lowland taxa tend to have the least stable ranges (Fig. 4C), which is compatible with the impact of sea level changes noted previously. Therefore, our results on range shifts vs. altitude do not indicate that cold-associated taxa are in their contracted phase.

Perhaps our sampled highland taxa did not suffer strong range shifts because they were free to move their distributions as a result of their ranges being more centred in the continent, instead of being restricted to the coastal mountain ranges as in the case of most lowland taxa. A larger comparative study across the whole altitudinal gradient of the AF would clarify the mechanism underlying the previous results.

ARE CONTACT REGIONS RANDOMLY DISTRIBUTED?

Even though contact areas appeared to be clustered (Fig. 1), they did not depart statistically from a random distribution. Even though other studies indicate that contact zones are geographically congruent in the AF (De Mello Martins, 2011; Costa & Leite, 2012; Cabanne *et al.*, 2013), to our knowledge, this is the first study to have performed a formal statistical test of this hypothesis.

This result does not cause us to reject the hypothesis that range shifts affected the evolution of AF taxa; however it does rule out the hypothesis that events that triggered these changes (e.g. forest range dynamics) were sufficiently strong to affect all organisms in the same way. It is more likely that each species responded to forest shifts differently, according to their ecological requirements, dispersal capacity, population age, etc., which is in agreement with findings in other tropical forests (Smith *et al.*, 2014). This result also strengthens the observation that a refugia hypothesis based on whole biome models is not always a good representation of individual species refugia (Porto *et al.*, 2013).

WHICH FACTORS AFFECTED POPULATION GENETIC STRUCTURE?

The results confirmed predictions of the working hypothesis suggesting that range shifts drive population evolution (Fig. 2A and Table 3). We have found that population genetic structure variation was positively correlated with both fragmentation variation and range stability. In addition, range size was negatively correlated with population genetic structure. The effect of these later factors taken together is small, explaining approximately 31% of the total variance at most. This low effect size to explain F_{ST} variation suggests that factors other than range shifts linked to the LGM affected the evolution of populations, such as different range shifts related to previous glaciations, landscape geological evolution, and bioclimatic barriers persistent across the late Pleistocene (Ledru *et al.*, 2016).

The positive relationship observed between F_{ST} and D was unexpected. The highest F_{ST} raw values were associated with medium raw values of D (Fig. 5B), which translated into the positive partial correlation between pairwise F_{ST} and D variation (Table 3). Even though unpredicted, this is a likely pattern because the lowest D values (closer to 0) would be related to the strongest range shifts that could have erased any genetic structure (e.g. by driving local extinctions and allowing colonization from neighbour regions). At the same time, the highest D values imply lesser range shifts, allowing high gene flow rates and population effective sizes that could preclude population divergence.

Population genetic structure variation across species was negatively correlated with current range size variation (Fig. 5C). A likely explanation for this pattern may be that taxa with larger ranges could have a higher dispersal capacity, which would preclude a strong population divergence. In this case, a positive correlation between range size and dispersal capacity is predicted. An alternative explanation

for the relationship observed between genetic structure and range size could consider that the distribution of intraspecific lineages is restricted by the climate heterogeneity found in larger ranges, as suggested by Carnaval *et al.* (2014). In accordance with the previous hypothesis, larger ranges spanning a wide variety of climates would drive a lower number of lineages that are capable of coping with this heterogeneity, diminishing population genetic structure, and diversity. Another alternative explanation for the link between genetic structure and range size variation could be that reduced ranges may represent smaller population effective sizes, with genetic drift becoming a more intense factor contributing to higher F_{ST} values.

Our results are also in accordance with other aspects of the study of Carnaval *et al.* (2014). Carnaval *et al.* (2014) evaluated the relationship between the metric phylogeographical endemism (PE) and forest stability in a sample of AF vertebrates (90% amphibians). PE has higher scores in geographical regions where high levels of intraspecific genetic variation are restricted to smaller areas. Here, we employed F_{ST} , which is expected to be positively correlated with PE (A. C. Carnaval, unpubl. data). Carnaval *et al.* (2014) found a negative relationship between PE and range size of the climate space occupied by the lineage, and a positive relationship between PE and forest stability, which is a good match with our findings.

However, our study is not in full agreement with Carnaval *et al.* (2014) in that F_{ST} was not correlated with maximum altitudes. The study by Carnaval *et al.* (2014) suggests that AF species would react to forest shifts differently depending on whether they are associated with a warm or cold climate. For example, cold-associated taxa may be less sensitive to forest fragmentation than other taxa, and therefore their population structures should not be linked to forest shifts. As noted previously, our highland taxa are associated with colder climates, whereas lowland taxa are associated with a warmer climate. Thus, if AF species react to forest shifts differently depending on the climate with which they are associated, a significant relationship is predicted between maximum altitude and F_{ST} . However, our analysis did not confirm this prediction (Table 3). A larger sampling across the whole altitudinal range of the AF will allow clarification of the later results.

WHAT FACTORS AFFECTED GENETIC DIVERSITY?

By contrast to our expectations, we found no association between historical changes of geographical ranges and genetic diversity. Only altitudinal

distribution and differences in present range size across taxa explained genetic diversity variation (Fig. 5D, E and Table 3).

We observed the smallest genetic diversity in lowland taxa (Fig. 5D). A likely explanation for this pattern is that these organisms have been impacted more by long-term range shifts than by highland taxa. Strong range shifts could have determined lower population effective sizes and therefore lower genetic diversities. This later idea is supported by lowland taxa showing smaller ranges in the present than during the LGM, as well as higher fragmentation levels than during the LGM, whereas highland taxa showed more even levels of both fragmentation and absolute ranges between periods (Fig. 4A, B). Moreover, the previous results are reflected in the fact that lowland taxa presented the least stable ranges (Fig. 4C). However, because these range shifts across maximum altitude were not correlated with genetic diversity (Table 3), we argue that factors other than the addressed range shift should have impacted on genetic diversity.

We confirmed our expectation of a positive correlation between genetic diversity and present range size. The highest diversity values were associated with medium size ranges (Fig. 5E). This result is in agreement with the aforementioned hypothesis that larger ranges spanning a wide variety of climates would drive a lower number of lineages, which represents a scenario predicting that taxa with larger ranges will show lower genetic diversities than taxa with smaller ranges. Future studies should focus on obtaining further evidence supporting the observed relationship between genetic diversity and altitude.

CONCLUSIONS

Our findings provide evidence that the Pleistocene range shifts affected the diversification of forest organisms, although with a low effect. A combination of fragmentation variation across time, small current range size and range stability increased population genetic structure. Our study showed that evolutionary response to range shifts across AF birds have a high variance, which could explain the mixed support given by single-species studies to the action of past range shifts on population evolution. Also, our findings suggest the novel idea that ranges of AF species during the LGM were slightly larger, equally fragmented and geographically displaced from their interglacial ranges.

Overall, range stability with the addition of intermediate fragmentation shifts across time, and not stability alone as stated earlier (i.e. Carnaval &

Moritz, 2008), seem to affect intraspecific evolution. This finding is in agreement with the existence of species that occur in stable habitats but show a strong phylogeographical structure associated with dynamics and powerful gene flow barriers, such as many forest birds from the Amazon basin (e.g. Ribas *et al.*, 2012; Sousa-Neves, Aleixo & Sequeira, 2013). In the AF, persistent bioclimatic barriers instead of intense forest fragmentation associated with glaciations may be related to the evolution of strong population genetic structures.

ACKNOWLEDGEMENTS

The present study was funded by the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), and the Agencia Nacional de Promoción Científica y Tecnológica (Argentina). This study was co-funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (Brazil, BIOTA 2013/50297-0), National Science Foundation (USA, DOB 1343578), and National Aeronautics and Space Administration (USA), and part of it was also performed at the Research Center on Biodiversity and Computing (BioComp) of the Universidade de São Paulo (USP), supported by the USP Provost's Office for Research. We are thankful to the curators of the bird collections of the Field Museum of Natural History, as well as the Laboratório de Biodiversidade e Evolução Molecular, ICB, Universidade Federal de Minas Gerais, Brazil, for allowing access to their collections. We also thank to Dan Zornizer and three anonymous reviewers for their comments that helped to improve the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

- Data S1.** MAXENT analysis: modelled maps for each taxa and final BIOCLIM variables.
Data S2. Samples and procedures for the genetic data collection.
Data S3. MAXENT analysis: infiles and MAXENT outputs for all taxa (ascii files).