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Phylogeography of an Atlantic forest passerine reveals demographic stability through the last glacial maximum

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ABSTRACT

In this study we analyzed the phylogeographic pattern and historical demography of an endemic Atlantic forest (AF) bird, *Basileuterus leucoblepharus*, and test the influence of the last glacial maximum (LGM) on its population effective size using coalescent simulations. We address two main questions: (i) Does *B. leucoblepharus* present population genetic structure congruent with the patterns observed for other AF organisms? (ii) How did the LGM affect the effective population size of *B. leucoblepharus*? We sequenced 914 bp of the mitochondrial gene cytochrome b and 512 bp of the nuclear intron 5 of beta-fibrinogen of 62 individuals from 15 localities along the AF. Both molecular markers revealed no genetic structure in *B. leucoblepharus*. Neutrality tests based on both loci showed significant demographic expansion. The extended Bayesian skyline plot showed that the species seems to have experienced demographic expansion starting around 300,000 years ago, during the late Pleistocene. This date does not coincide with the LGM and the dynamics of population size showed stability during the LGM. To further test the effect of the LGM on this species, we simulated seven demographic scenarios to explore whether populations suffered specific bottlenecks. The scenarios most congruent with our data were population stability during the LGM with bottlenecks older than this period. This is the first example of an AF organism that does not show phylogeographic breaks caused by vicariant events associated to climate change and geotectonic activities in the Quaternary. Differential ecological, environmental tolerances and habitat requirements are possibly influencing the different evolutionary histories of these organisms. Our results show that the history of organism diversification in this megadiverse Neotropical forest is complex.

1. Introduction

The Atlantic forest (AF) is among the top five biodiversity hotspots in the world and these areas hold more than 60% of all terrestrial species on the planet (Myers et al., 2000; Galindo-Leal and Câmara, 2005). It is distributed along the coastal regions of eastern Brazil to eastern Paraguay and northeastern Argentina (Galindo-Leal and Câmara, 2005) (Fig. 1). Also, the AF now holds only between 11.4% and 16% of its original forest cover (Ribeiro et al., 2009).

The high species diversity in the AF may be a consequence of various evolutionary processes. Some of them possibly occurred in the Quaternary, especially in the late Pleistocene (Whitmore and Prance, 1987). During this period the planet went through many glacial and interglacial cycles, when continental glaciers advanced and retreated and forests suffered cycles of reduction, fragmentation and expansion (Sant'Anna Neto and Nery, 2005). Palynological data analyses show that the last glacial maximum in the AF occurred between 18 and 48 thousand years ago, with open areas (grasslands) in southeastern Brazil that extended 750 km northward, from latitude 28°/27°S to at least 20°S (Behling and Lichte, 1997; Behling, 2002). The final expansion of forested areas to its current distribution occurred only at the end of the Holocene (Behling, 2002; Behling and Pillar, 2007).

One of the most discussed hypotheses on the origin of the Neotropical biodiversity during the Pleistocene is the theory of forest refugia (Haffer, 1969; Vanzolini and Williams, 1970; Brown and Ab'Saber, 1979). According to this theory, refugia are islands of humid forest isolated by open vegetation, such us grasslands or dry forest. These rainforests would shrink during glacial maxima (forming refugia) and expand during warmer periods (interglacial cycles), while areas of open vegetation would behave the other

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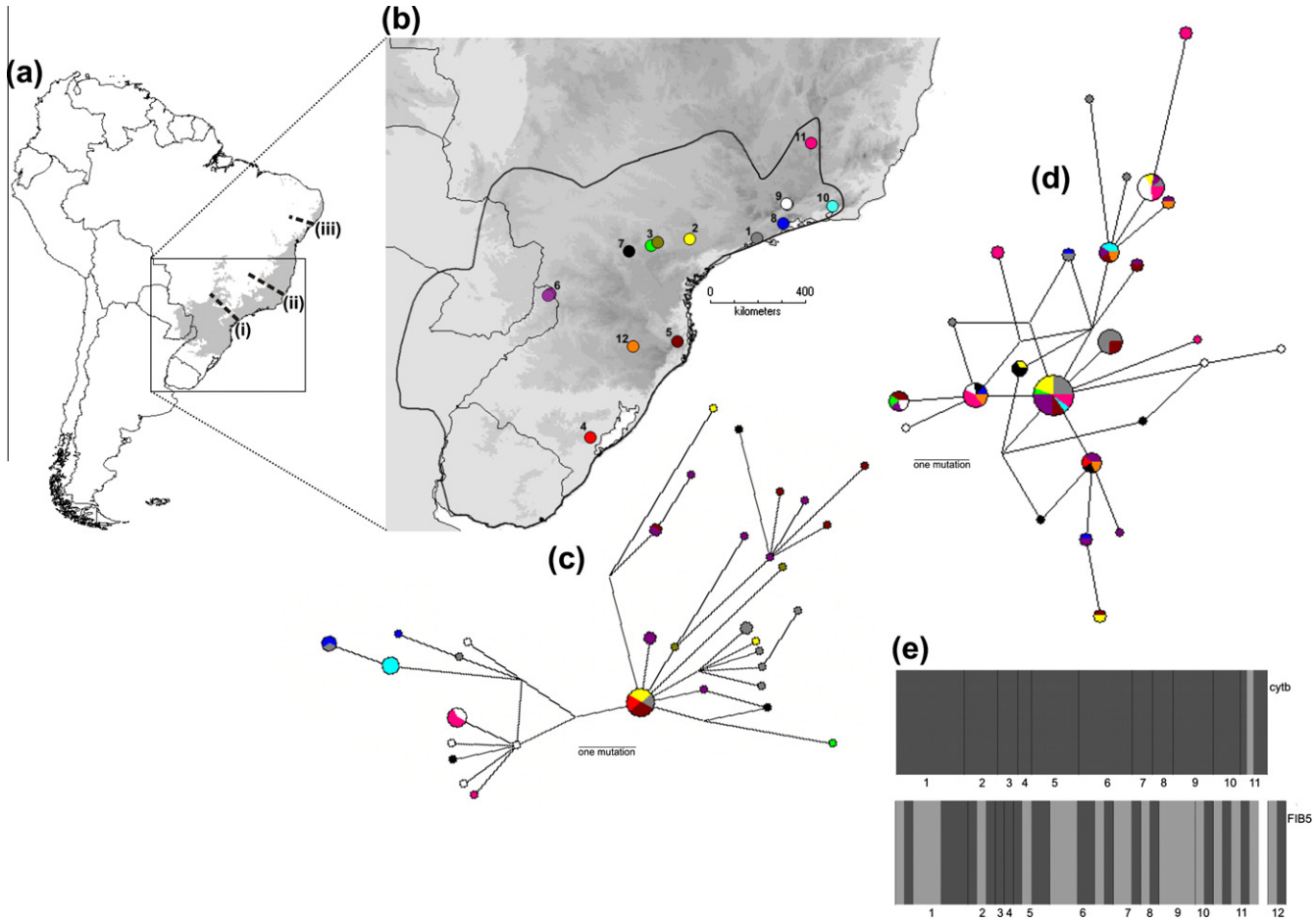

Fig. 1. (a) Main phylogeographic breaks observed in Atlantic forest organisms (see Section 4). Gray area represents the limits of the Atlantic forest and dotted lines the phylogeographic breaks. Break i observed by: Grazziotin et al. (2006), Cabanne et al. (2007, 2008, 2011), Carnaval et al. (2009), Martins et al. (2009), Batalha-Filho et al. (2010), Thomé et al. (2010), and d'Horta et al. (2011). Break ii observed by: Cabanne et al. (2007, 2008), Carnaval et al. (2009), Ribeiro et al. (2010), Thomé et al. (2010), and d'Horta et al. (2011). Break iii observed by: Cabanne et al. (2008) and Carnaval et al. (2009). (b) Sampling sites of *B. leucoblepharus*. The black continuous line shows the approximate limit of the species distribution based on Ridgely and Tudor (1996). The circles indicate the sites and their colors are represented in the haplotype networks. Sampling site numbers represent operational localities used in AMOVA and BAPS analyses. The different gray tones in the map represent elevation (the darker the higher are the altitudes). (c) Haplotype network based on 914 bp of cytb. (d) Haplotype network based on 512 bp of FIB5. Colors in networks correspond to colors in the map. (e) Bayesian analysis of population structure (BAPS) of *B. leucoblepharus*. Clustering based on 914 bp of cytb, and 512 bp of FIB5. Colors indicate the best numbers of clusters for each marker. Numbers represents the operational localities according to the map.

way round. Thus, the geographic distribution of species that inhabit rainforests should have accompanied these cycles of contraction and expansion of forests during the Pleistocene. If these populations were isolated for enough time to accumulate differences between them, even if they subsequently got in contact again after the expansion of forests, no gene flow would occur. However, geological and palaeoclimatic studies questioned the existence of Pleistocene refugia (Colinvaux et al., 2000; Bush and de Oliveira, 2006).

Another possible driver of diversification is rivers as barriers to gene flow for terrestrial animals. This hypothesis was initially proposed by Alfred Russell Wallace (1852), and recent studies showed that rivers played an important role for diversification in Amazonia (Ribas et al., 2012), but not in the AF (Maldonado-Coelho, 2012). Another hypothesis is the ecological gradient theory (Smith et al., 1997, 2001) that suggests that diversification can occur in parapatry, i.e., species or lineage differentiation may occur due to differential selective pressures acting on different and adjacent habitats. This hypothesis was evaluated and supported by Cabanne et al. (2011) in the intra-specific diversification of a bird that inhabits the AF and gallery forest of Caatinga, Cerrado, and Chaco. Finally, geotectonic activity during the Quaternary (including movement of geological faults in Brazil; Saadi et al., 2002) may have originated gene flow barriers that triggered diversification of some AF organisms (Batalha-Filho et al., 2010; Thomé et al., 2010).

Recent phylogeographic and paleoclimatic modeling studies do not reject the Pleistocene refuge theory as possible explanation of intra-specific diversification of Neotropical species (Cabanne et al., 2007, 2008; Carnaval and Moritz, 2008; Solomon et al., 2008; Carnaval et al., 2009; d'Horta et al., 2011; Martins, 2011; Maldonado-Coelho, 2012). Phylogeographic studies focused on organisms that occur in the AF are still scarce (Grazziotin et al., 2006; Cabanne et al., 2007, 2008; Carnaval et al., 2009; Fitzpatrick et al., 2009; Batalha-Filho et al., 2010; Thomé et al., 2010; Cabanne et al., 2011; d'Horta et al., 2011) compared to its high species diversity. These phylogeographic studies revealed three main phylogeographic breaks shared among taxa (Fig. 1a), but there is no clear temporal congruence between estimated dates of divergence of these populations or lineages (see Section 4 for more details).

In the present study we performed a phylogeographic analysis to study the historical demography of the white-rimmed warbler, *Basileuterus leucoblepharus*, an endemic bird of the AF (Passeriformes, Parulidae). We based the study on nuclear and mitochondrial

DNA sequences for testing the effect of the last glacial maximum on its demographic history.

This species is distributed from the states of Minas Gerais and Rio de Janeiro to Rio Grande do Sul in Brazil to Paraguay, Uruguay and the northeastern and eastern Argentina (Fig. 1b; Ridgely and Tudor, 1996; Sick, 1997). It can be fairly common and easily identified by its characteristic song. It occurs from uplands in its northern distribution (e.g. between 800 and 2200 m in latitude 22°) to sea level in the southern part of its distribution. *B. leucoblepharus* lives inside moist and shaded forests (Sick, 1997), but also occurs in secondary and mixed forests in agroecosystems (i.e. *Eucalyptus* sp. and *Araucaria angustifolia* plantations), as well as gallery forests within the humid Chaco, and Argentinean and Uruguayan Pampas (Stotz et al., 1996).

In this study we addressed two main questions: (i) Does *B. leucoblepharus* present population genetic structure concordant with patterns observed for other AF organisms (Fig. 1a), such as birds (Cabanne et al., 2007, 2008, 2011; d'Horta et al., 2011; Maldonado-Coelho, 2012), other vertebrates (Grazziotin et al., 2006; Carnaval et al., 2009; Fitzpatrick et al., 2009; Martins et al., 2009; Thomé et al., 2010), invertebrates (Batalha-Filho et al., 2010) and plants (Ribeiro et al., 2010)?; and (ii) How did the last maximum glacial affect the effective population size of *B. leucoblepharus*?

2. Material and methods

2.1. Sampling and molecular analyses methods

Samples (muscle and blood) of 62 specimens from 15 localities in Brazil and Argentina were collected between 2000 and 2009 (Table 1, Fig. 1b). Samples are deposited in three collections in Brazil: Laboratório de Genética e Evolução Molecular de Aves (Instituto de Biociências, Universidade de São Paulo); Laboratório de Biodiversidade e Evolução Molecular (Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais); and Departamento de Genética (Universidade Federal do Rio Grande do Sul). Specimens' vouchers of all muscle samples are deposited at Museu de Zoologia da Universidade de São Paulo (MZUSP). The identification codes of samples, specimens' vouchers and GenBank accession numbers are presented in Table 1.

Total DNA was extracted following Bruford et al. (1992). The mitochondrial gene cytochrome b (cytb) was amplified with primers L-14841 and H-16065 (Lougheed et al., 2000) and the nuclear beta-fibrinogen intron 5 (FIB5) with primers FIB5 and FIB6 (Marini and Hackett, 2002). PCR (25 μL) contained template DNA (50 ng), 1X of *Taq* buffer (GE Healthcare), dNTPs (0.32 μM), 0.5 μM of each primer and 0.5 U of *Taq* polymerase (GE Healthcare). PCR conditions were: an initial denaturation step at 94 °C for 3 min and 30 s; followed by 35 cycles at 94 °C for 35 s, 56 °C (cytb) or 51–56 °C (FIB5) for 40 s and 72 °C for 1 min; plus a final extension step at 72 °C for 9 min.

Amplicons were purified using polyethylene glycol 20% (PEG) precipitation (Sambrook et al., 1989). This DNA was then directly sequenced in both directions with the same amplification primers using Big Dye terminator 3.0 cycle sequencing kit (Applied Biosystems) following the manufacturer's protocol. Sequences were analyzed in an automated sequencer ABI PRISM 3100 (Applied Biosystems).

2.2. Sequence editing, haplotype reconstruction and recombination test

The electropherograms were edited and assembled in contigs using the software package Phred, Phrap, Consed (Ewing et al., 1998; Ewing and Green, 1998; Gordon et al., 1998). Sequences were aligned using the CLUSTAL W method (Higgins et al., 1994) in MEGA4 (Tamura et al., 2007). All alignments were inspected and corrected visually.

Initially, we codified double peaks present in both strands in the FIB5 sequence electropherograms as ambiguous sites according to the IUPAC code. We did not find any indel heterozygote. The gametic phase of heterozygote individuals was resolved using the algorithm PHASE (Stephens et al., 2001) with the default settings in DnaSP 5 (Librado and Rozas, 2009) and 0.7 as the minimum probability. Individuals with lower probabilities were removed from further analyses. We used PHI test in SPLITSTREE4 (Bruen et al., 2006; Huson and Bryant, 2006) to check for recombination in the FIB5 sequences. This test was used due to its power to distinguish recombination events from homoplasies (Bruen et al., 2006).

2.3. Population structure tests

Median-joining networks (Bandelt et al., 1999) were obtained using NETWORK 4.5.1.0 (www.fluxus-engineering.com) in order to study the relationships between haplotypes and their geographic distribution. To check the level of population genetic structure among localities we performed an analysis of molecular variance (AMOVA, Excoffier et al., 1992) with two hierarchical levels for each gene separately using ARLEQUIN 3.11 (Excoffier et al., 2005). We also calculated an AMOVA with three hierarchical levels for the cytb. For the third level of this analysis, we separated in two groups those localities south and north of the phylogeographic break i (Fig. 1a). In order to verify if there is correlation between geographic and genetic (pair wise Φ_{ST}) distances we used Mantel test (Sokal and Rolf, 1995) using ARLEQUIN 3.11. Significances of these analyses were obtained with 1000 permutations.

In addition, we used BAPS 5.3 (Bayesian Analysis of Population Structure; Corander and Marttinen, 2006; Corander et al., 2008) to access the number of populations without prior information of the sampling location for mitochondrial and nuclear datasets. Due to the difference in ploidy, BAPS analysis was performed for each gene separately. Firstly, we ran the analysis assuming mixture model to determine the most probable number of populations (k) given the data. After that the resulting mixture clustering was used for an admixture analysis with 500 reference individuals and repeated the admixture analysis 500 times per individual. The analyses were repeated five times to check for convergence between different runs.

2.4. Demographic analyses

The nucleotide diversity per site (π) and number of haplotypes (l) were calculated in DnaSP 5. In order to test if there is any evidence of demographic expansion, the neutrality test indexes Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) and the population size change test R_2 (Ramos-Onsins and Rozas, 2002) were calculated for each gene using DnaSP 5. The significances of these tests were obtained based on 1000 coalescent simulations. In order to identify the putative direction of the demographic expansion (north to south, or vice versa) we plotted the nucleotide diversity per site (π) of each locality against its latitude in degrees, and we performed a linear regression. It is expected that higher values of π is associated with a larger and more stable historical population size (Spellman and Klicka, 2006). Thus, localities with higher values of π could be associated to past refugia.

In addition, to analyze population size dynamics through time for both loci combined, we used the Extended Bayesian Skyline Plot method (EBSP; Heled and Drummond, 2008) implemented in BEAST 1.6.1 (Drummond and Rambaut, 2007). Two independent EBSP runs were obtained using the following parameters: an initial

Table 1
Samples of *B. leucoblepharus*: localities sampled, geographic coordinates, sample size (*N*) per locality, sample code, voucher and GenBank accession numbers for each marker.

Locality	Coordinates	*N*	Sample code	Voucher[d]	GenBank cytb	GenBank FIB5
1. Nova Lima – MG, BRA	19°58′S; 43°49′W	8	B0627[a]	–	–	JX488594
			B0628[a]	–	–	JX488595
			B0629[a]	–	JX488543	JX488596
			B0630[a]	–	JX488544	JX488597
			B0631[a]	–	JX488545	JX488598
			B0705[a]	–	JX488546	–
			B1279[a]	–	–	JX488599
			B1281[a]	–	–	JX488600
2. Itamonte – MG, BRA	22°21′S; 44°46′W	2	12017[b]	MZUSP82630	JX488535	JX488586
			12020[b]	MZUSP82629	JX488536	JX488587
3. Resende – RJ, BRA	22°23′S; 44°45′W	4	12014[b]	MZUSP82617	JX488533	JX488584
			12015[b]	MZUSP82627	JX488534	JX488585
			12023[b]	MZUSP82628	JX488537	JX488588
			12024[b]	MZUSP82616	JX488538	JX488589
4. Parque Nacional Serra dos Órgãos – RJ, BRA	22°26′S; 43°00′W	4	13000[b]	MZUSP82588	JX488539	JX488590
			13006[b]	MZUSP82589	JX488540	JX488591
			13008[b]	MZUSP82587	JX488541	JX488592
			13019[b]	MZUSP82586	JX488542	JX488593
5. Parque Estadual Morro Grande – SP, BRA	23°41′S; 45°51′W	10	779[b]	–	JX488492	JX488547
			820[b]	–	JX488493	JX488548
			825[b]	–	JX488494	JX488549
			828[b]	–	JX488495	JX488550
			830[b]	–	JX488499	JX488554
			834[b]	–	JX488497	JX488552
			835[b]	–	JX488498	JX488553
			852[b]	–	JX488496	JX488551
			853[b]	–	JX488500	JX488555
			854[b]	–	JX488501	JX488556
6. Buri – SP, BRA	23°42′S; 48°34′W	5	1240[b]	MZUSP76135	JX488502	JX488557
			1251[b]	MZUSP75604	JX488503	JX488558
			1254[b]	–	JX488504	JX488559
			2962[b]	–	JX488505	JX488570
			2966[b]	–	JX488506	JX488571
7. Cunha – SP, BRA	23°07′S; 44°56′W	3	12003[b]	MZUSP 81387	JX488530	JX488581
			12006[b]	MZUSP82632	JX488531	JX488582
			12007[b]	MZUSP82631	JX488532	JX488583
8. Ortigueira – PR, BRA	24°12′S; 50°55′W	3	11407[b]	MZUSP75679	JX488527	JX488578
			11408[b]	–	JX488528	JX488579
			11412[b]	–	JX488529	JX488580
9. Pinhalão – PR, BRA	23°58′S; 50°03′W	1	1296[b]	MZUSP75641	JX488507	JX488560
10. Wenceslau Braz – PR, BRA	23°51′S; 49°48′W	2	1384[b]	–	JX488508	–
			1388[b]	–	JX488509	JX488561
11. Campo Belo do Sul – SC, BRA	27°53S; 50°45′W	3	DA29326[c]	–	–	JX488601
			DA29328[c]	–	–	JX488602
			DA29522[c]	–	–	JX488603
12. Rancho Queimado – SC, BRA	27°40′S; 49°01′W	7	2150[b]	–	JX488512	JX488565
			2153[b]	–	JX488513	–
			2160[b]	–	JX488514	JX488566
			2161[b]	–	JX488515	JX488567
			2164[b]	–	JX488516	JX488568
			2165[b]	–	JX488517	JX488569
			2196[b]	MZUSP82783	JX488518	–
13. Arroio do Padre – RS, BRA	31°22′S; 52°23′W	2	2135[b]	–	JX488510	JX488563
			2138[b]	–	JX488511	JX488564
14. Parque Uruguaí, Uruzú, Misiones, ARG	25°51′S; 55°10′W	1	1409[b]	–	JX488519	JX488562
15. Paraje Maria Soledad, Misiones, ARG	25°51′S; 53°59′W	7	10345[b]	–	JX488520	JX488572
			10358[b]	–	JX488521	JX488573
			10364[b]	–	JX488522	–
			10371[b]	–	JX488523	JX488574
			10383[b]	–	JX488524	JX488575
			10385[b]	–	JX488525	JX488576
			10387[b]	–	JX488526	JX488577

BRA – Brasil; ARG – Argentina.
Tissue collections:
[a] Laboratório de Biodiversidade e Evolução Molecular, Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais.
[b] Laboratório de Genética e Evolução Molecular de Aves (LGEMA), Instituto de Biociências, Universidade de São Paulo.
[c] Departamento de Genética, Universidade Federal do Rio Grande do Sul.
[d] Samples with museum voucher correspond to muscle samples, the remaining ones are blood samples. Specimens are deposited in Museu de Zoologia da Universidade de São Paulo (MZUSP).

UPGMA tree, linear model, 50 million steps, parameters sampled every 10,000 steps and a burn-in of 10%. The hLRT test did not reject the molecular clock hypothesis for both loci. The best fit substitution model for each partition (loci) was determined by Modeltest 3.7 (Posada and Krandall, 1998). We used 1.05% (*s.d.* 0.05%) per lineage per million years as the mutation rate for cytb (Weir and Schluter, 2008) under a normal prior distribution. Based on the probabilities obtained, BEAST estimated the mutation rate for FIB5 under a default lognormal prior distribution. To check the convergence of parameters between runs and analysis performance (ESS values > 200) we used TRACER 1.5 (http://beast.bio.ed.ac.uk/Tracer).

2.5. Coalescent simulations

In order to evaluate the demographic history we simulated scenarios in BAYESSC, a modified version of SERIAL SIMCOAL (Anderson et al., 2005; Chan et al., 2006), and evaluated the goodness of fit of the observed data to the simulations. The general procedure followed Richards et al. (2007). We also followed the methodology suggested by Voight et al. (2005) that considers several summary statistics and results on a single posterior probability for each model. This procedure has been widely applied in recent studies (i.e.: Belle et al., 2006; Fabre et al., 2009; Ghirotto et al., 2010; Cabanne et al., 2011; Fischer et al., 2011; Brace et al., 2012). The rationale of this procedure consists in testing models by simulating genealogies under specific demographic scenarios and then evaluating the fit of the empirical data to the simulated data (Knowles, 2009). If the empirical (observed) data fits well to the simulated data, this given demographic scenario would receive a high posterior probability and therefore, be supported. Alternatively, if the observed data does not fit well into the distribution of the simulated data, the posterior probability would be low and the specific scenario would not be supported.

The models that were tested differed in the timing and number of bottlenecks (Fig. 2, models A to E). Also, as genetic signals of expansion may be found in a demographically stable island system (see Nielsen and Beaumont, 2009; Peter et al., 2010), we also simulated two island models that originated before the Pleistocene (Fig. 2, models F and G). This date was estimated based on the divergence of *B. leucoblepharus* and its sister species *B. flaveolus* (Lovette et al., 2010). For the island models we grouped sampling localities by geographic proximity into five populations as follows: pop 1 – localities 1–4; pop 2 – locality 5; pop 3 – localities 6–10; pop 4 – localities 11–13; pop 5 – localities 14 and 15. Grouping by geographic proximity was the simplest way to recreate a plausible island model. Other configurations of island models could have been evaluated, but they would be more complex. Complex models should be avoided in this kind of procedure because they would get a higher posterior probability but without being plausi-

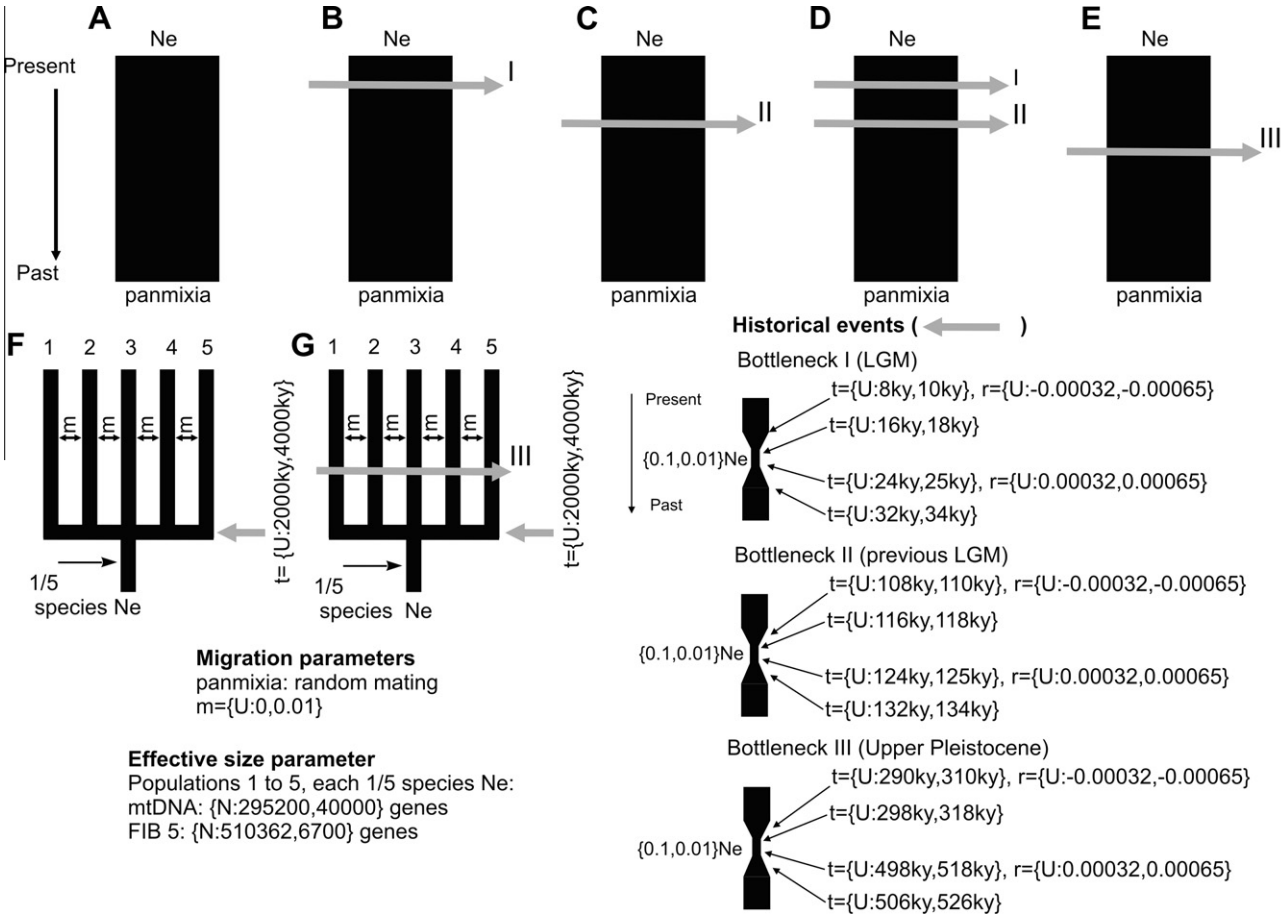

Fig. 2. Parameters of the historical demographic models of *B. leucoblepharus* evaluated by coalescent simulations in BAYESSC. For all models we simulated populations that suffered two types of historical events: bottleneck (I–III) and population fragmentation. The prior distribution of time of events (t), migration (m) and of the demographic growth rate (r), are specified. Time is expressed in thousand years (ky). Uniform prior distributions are denoted with an *U*. Normal prior distributions are denoted by and *N*, the mean value and standard distribution (SD). Intensities of bottlenecks are indicated by the proportion of the current effective size (*Ne*) that persisted during the bottleneck. Time is expressed in kilo-years (ky). LGM: last glacial maximum.

Table 2
Summary statistics for cytochrome b (cytb) and beta-fibrinogen intron 5 (FIB5) of *B. leucoblepharus*.

Locus	*N*	π	*l*	*D*	F_s	R_2
cytb	55	0.00562	34	-1.7072^{*}	-27.1696^{***}	0.0494^{**}
FIB5	86	0.00536	25	-1.3053^{ns}	-14.1737^{***}	0.0554^{*}

N: sample size; π: nucleotide diversity per site; *l*: number of haplotypes; *D*: Tajima's test; F_s: Fu's test; R_2: Ramos-Onsins and Rozas' test; ^{ns} not-significant.
* $0.10 > p > 0.05$.
** $p < 0.05$.
*** $p < 0.01$.

ble (Nielsen and Beaumont, 2009). Specifically, Nielsen and Beaumont (2009) suggested to use simple models as a baseline against which to compare more complex models.

We simulated bottlenecks that reduced the population effective size (*Ne*) to 1–10% of the current size. We estimated *Ne* from theta ($\Theta = 2\mu Ne$) that was obtained assuming the F84 model of sequence evolution (Felsenstein and Churchill, 1996), and empirical base frequencies and transition/transversion ratios with a Markov chain Monte Carlo (default settings) in LAMARC 2.1.2b (Kuhner, 2006). We assumed that *Ne* from each present population and from ancestral populations in models F and G (Fig. 2) was 1/5 of the species' *Ne*. Effective number of genes was introduced in BAYESSC infiles as presenting a normal distribution with a mean equal to the maximum likelihood estimation of *Ne* and standard deviation estimated from Θ confidence interval. For mtDNA we used a mutation rate as a uniform interval 1.05×10^{-8}–9.14×10^{-7} changes/site/generation (Weir and Schluter, 2008). The transition bias was 0.85, the mutation rate gamma distribution was 0.84 and the number of mutation categories was six. For FIB5, the mutation rate was obtained from the EBSP analysis in BEAST; the uniform rate interval was 3.86×10^{-9}–6.99×10^{-10} changes/site/generation. The transition bias was 0.7, the mutation rate gamma distribution was 0.77 and the number of mutation categories was six. We assumed a generation time of 1 year as Cabanne et al. (2008).

For each model we ran 1000 simulations per marker (mtDNA and FIB5). Then, for each simulated data we estimated three summary statistics [nucleotide diversity – π, Tajima's *D* (Tajima, 1989), and number of haplotypes – *h*] to obtain null distributions against which we tested the observed data (Hickerson et al., 2006). For evaluating the goodness of fit of the observed data to the simulated one, we followed Voight et al. (2005) and used the two-tailed empirical likelihood *pi* of each summary statistics *i*;

$$pi = 1 - [2 \times (|0.5 - p|)] \quad (1)$$

being *p* the proportion of simulated values equal or higher than the observed summary statistic. When the observed statistic fell outside the simulated distribution we attributed $pi = 0.00001$, as in Cabanne et al. (2011). Then, we combined the three *pi* values by

$$C_{obs} = -2\sum_{i=1}^{k} \ln p_i \quad (2)$$

and obtained its significance. The significance was assessed by comparing C_{obs} against a null distribution of *C* obtained following Voight et al. (2005) and Fabre et al. (2009). For each simulated dataset, each summary statistic was compared with the other values representing the empirical distribution of the statistic from simulation. Specifically, we treated the value of each summary statistic as the observed value and calculated with Eq. (1) its p_{sim}-value relative to the remaining 999 simulated data. Then, the null distribution of *C* was obtained by combining with Eq. (2) p_{sim}-values across summary statistics and the significance of C_{obs} was obtained as in step 1 (Eq. (1)). This procedure generated two-tailed combined *p*-values associated to each marker. Finally, the overall *P*-value for the model was obtained by combining *p*-values of both markers by the parametric Fisher's method in METAP (Whitlock, 2005), which is authored by Ge (available at http://compute1.lsrc.duke.edu/softwares/MetaP/metap.php). We rejected models when $P \leqslant 0.05$.

3. Results

3.1. Diversity and genetic structure

We obtained 914 bp of cytb ($N = 55$) and 512 bp of FIB5 ($N = 114$ sampled chromosomes). No indels were observed in cytb, but FIB5

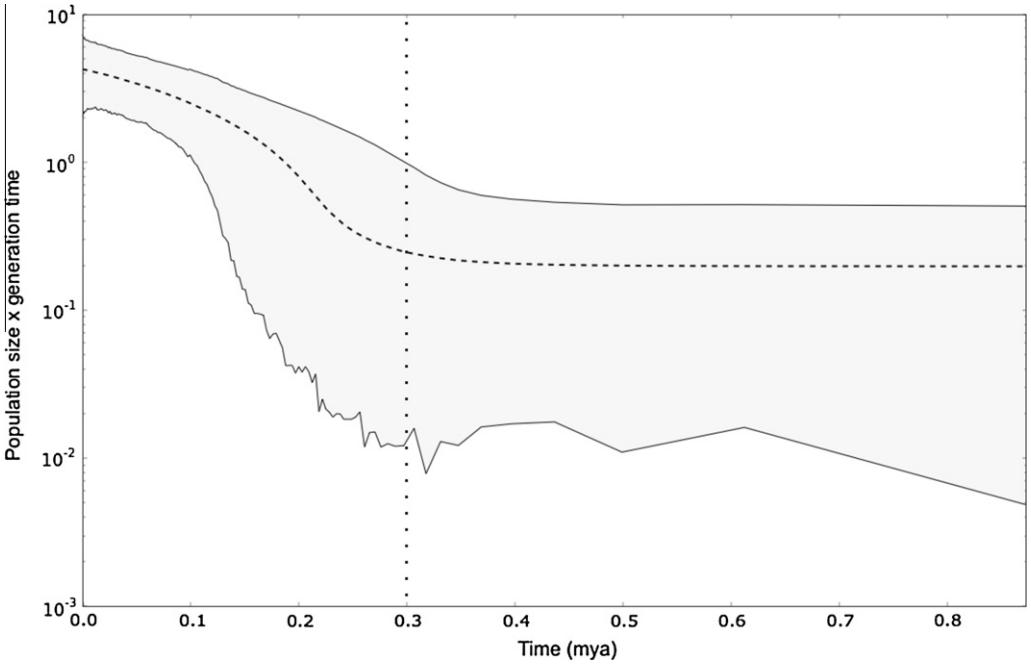

Fig. 3. Extended Bayesian Skyline Plot (EBSP) result. The dotted horizontal line shows the median estimate of the EBSP and the gray line shows the upper and lower 95% highest posterior density limits. The dotted vertical line illustrates the approximate time when demographic expansion of *B. leucoblepharus* started. The *Y*-axis is in ln scale.

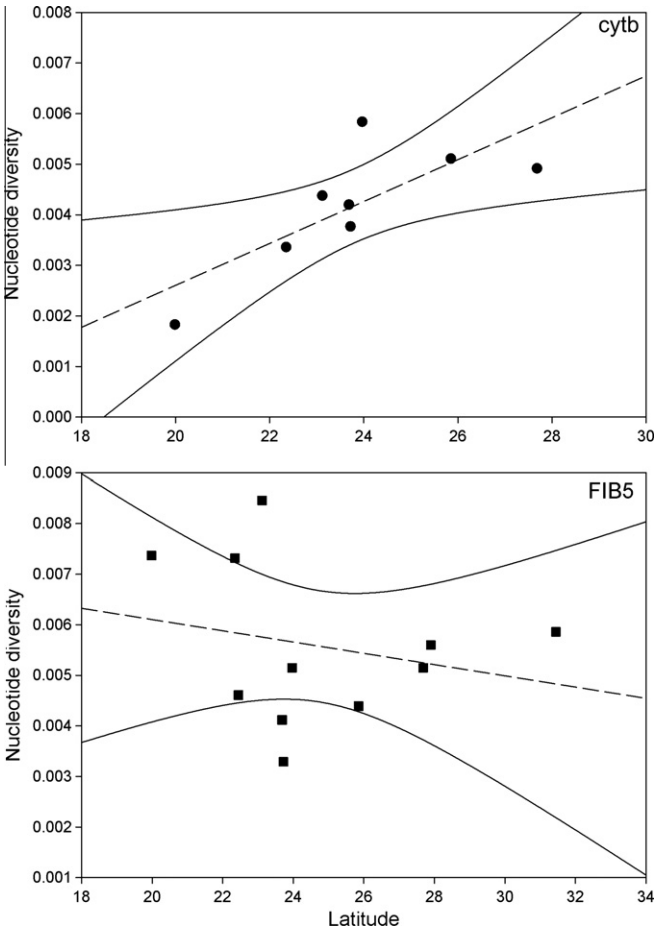

Fig. 4. Correlation between nucleotide diversity (π) of mitochondrial (cytb) and nuclear (FIB5) genes, and degrees of latitude of localities. Dashed lines represent the fitted linear model and the solid lines represent 95% of confidence intervals calculated for the regression line.

had an indel of 6 bp in ten samples from various localities. There were 40 polymorphic sites in cytb and 41 in FIB5 after excluding indels and ambiguous sites. No unexpected stop codons or ambiguous peaks in the electropherograms were found in cytb sequences, suggesting that they were of mitochondrial origin. The haplotype reconstruction by PHASE resolved a total of 43 samples (86 sampled chromosomes) with $p > 0.7$, and the remaining 11 specimens with low PHASE probability were removed from further analyses. The PHI test ($p = 0.09$) excluded the hypothesis of recombination in FIB5. The summary statistics for both loci are shown in Table 2.

We used different strategies to identify population genetic structure. Haplotype networks based on each of the two marker sequences showed absence of genetic structure. The most common and internal haplotypes were found in geographically distant locations (Fig. 1). AMOVA indicated that the highest percentage of genetic variation was observed within localities, 69.8% ($p < 0.00001$) for cytb and 92.3% ($p = 0.02053$) for FIB5, indicating that the genetic structure among localities is low (mtDNA) or absent (FIB5). When we applied the three hierarchical level AMOVA we did not find strong genetic structure between the hypothetical groups (variation among groups = 18.23%; variation among localities within groups = 17.53%; variation within localities = 64.23%). Mantel test indicated no correlation between genetic and geographic distances [$r_{cytb} = 0.2567$ ($p = 0.107$); $r_{FIB5} = 0.1108$ ($p = 0.201$)], also suggesting absence of isolation by distance between localities. BAPS analysis indicated presence of two and three populations for mitochondrial and nuclear genes, respectively (Fig. 1e). Notwithstanding, these clusters were not spatially coherent for either markers and therefore confirmed absence of population structure.

3.2. Historical demography

All neutrality test values were significant (except Tajima's *D* for FIB5), indicating sign of demographic expansion in both loci (Table 2). According to Ramos-Onsins and Rozas (2002), F_s and R_2 tests are more robust in detecting events of demographic expansion, and F_s is more suitable for larger samples while R_2, for smaller samples.

The EBSP indicated that *B. leucoblepharus* experienced a demographic expansion starting about 300,000 years ago (Fig. 3), possibly associated to climate changes during the late Pleistocene. The demographic expansion event dated here is older than the last glacial maximum (LGM), when paleopalinological records indicate that open areas (grasslands) were present in the AF between 48,000 and 18,000 years ago (Behling and Lichte, 1997; Behling, 2002). Thus, we performed coalescent simulations to test the effect of the LGM on the effective population size of *B. leucoblepharus*.

The relationship between π and latitude indicated a possible demographic expansion from south to north for cytb ($R^2 = 31.36\%$, $p = 0.029$), but for FIB5 there is no correlation between latitude and π ($R^2 = 5.21\%$, $p = 0.4998$) (Fig. 4).

3.3. Coalescent simulations

We simulated mtDNA and FIB5 sequences under seven different demographic scenarios to explore whether populations suffered specific bottlenecks, as expected according to the history of the AF, or alternatively, followed a panmictic or finite island model. The simulated models are (Fig. 2): (A) a panmictic stable population;

Table 3
Values of likelihood for nucleotide diversity (π), Tajima's *D*, and number of haplotyes (*l*) and overall likelihood for models of demographic scenarios for *B. leucoblepharus* based on mtDNA and FIB5 datasets.

	Demographic model	mtDNA				FIB5				Overall *P*-value
		$p\pi$	*pD*	*pl*	Combined *p*-values	$p\pi$	*pD*	*pl*	Combined *p*-values	
A	Panmixia, stability	0.00001	0.016	0.87	0.00001	0.006	0.088	0.868	0.076	0.00001146
B	Panmixia, bottleneck LGM	0.364	0.136	0.00001	0.00001	0.45	0.074	0.132	0.254	0.00003526
C	Panmixia, bottleneck 120 ky ago	0.304	0.294	0.342	0.61	0.384	0.264	0.26	0.584	0.7239*
D	Panmixia, bottlenecks LGM and 120 ky ago	0.68	0.236	0.00001	0.006	0.594	0.18	0.054	0.32	0.0139
E	Panmixia, bottleneck 300–500 ky ago**	0.614	0.6918	0.755	0.3483	0.42	0.4225	0.3159	0.8305	0.6481*
F	Stable finite island, origin 2–4 m.y.a. ago, gene flow 0–0.01	0.004	0.024	0.284	0.010	0.07	0.114	0.418	0.212	0.0152
G	Model F, bottleneck 300–500 ky ago	0.928	0.994	0.258	0.474	0.00001	0.106	0.052	0.00001	0.00006284

* Scenarios not rejected.
** Date in accordance with observed results from EBSP analysis.

(B) a panmictic population that suffered a bottleneck during the LGM; (C) a panmictic population that suffered a bottleneck during the glaciation previous to the LGM; (D) a panmictic population that suffered bottlenecks during the last two glacial maxima; (E) a panmictic population that suffered a bottleneck during the upper Pleistocene (300,000–500,000 years ago), according to the results of EBSP (Fig. 3); (F) a stable finite island system connected by gene flow and that originated before the Pleistocene (2–4 m.y.a. ago); (G) model F but including a bottleneck at the upper Pleistocene.

We initially estimated the effective number of genes to be used in simulations. Species' *Ne* for mtDNA was 1,476,000 (SE 200,000) genes and for FIB5 2,551,810 (SE 33,700) genes. We used one fifth of the total species' *Ne* as the effective size of each of the five populations used in simulated models: 295,200 genes for mtDNA and 510,362 for FIB5.

Five models were rejected (overall $P < 0.05$; Table 3), namely, a single panmictic and stable population (model A), a bottleneck during the LGM (B), two consecutive bottlenecks during the last two glaciations (D), and island systems (F and G). The last result shows that our data does not support the possibility that the observed expansion signal could be explained by an island model.

There were two models that were not rejected. The simplest scenario suggests that the observed data could have been produced after a bottleneck that occurred at the maximum glaciation before the last one (model C, ~120,000 years ago). The other scenario suggests a bottleneck at mid Pleistocene (model E, 300,000–500,000 years ago).

4. Discussion

4.1. Phylogeographic structure and demography of B. leucoblepharus

The results of the haplotype networks, AMOVA, and comparison between genetic and geographic distances based on both mitochondrial and nuclear loci indicate absence of a strong population genetic structure in *Basileuterus leucoblepharus* (Fig. 1). Nevertheless, BAPS analysis indicated the presence of clusters for both loci, but these groups did not present any geographic structure (Fig. 1e). However, Bayesian approaches implemented in BAPS and Structure (Pritchard et al., 2000) are very sensitive to low levels of genetic differentiation (i.e. F_{ST} around 0.03–0.05; Latch et al., 2006), thus, when F_{ST} values are very low the number of incorrect assignments is high (Latch et al., 2006). In sum, even though AMOVA also indicated that 30% of the genetic variation of cytb is allocated between localities, no clear geographic structure was observed. Yet, the three hierarchical level AMOVA showed that just 18% of variation is allocated between the hypothetical groups. Thus, our data indicated absence of genetic structure in *B. leucoblepharus*.

Interestingly, the phylogeographic pattern we found in *B. leucoblepharus* is not congruent with previous phylogeographic studies of other AF organisms (Fig. 1a) that show recurrent geographic discontinuities possibly caused by vicariant events associated to climate changes or geotectonic activities in the Quaternary (Grazziotin et al., 2006; Cabanne et al., 2007, 2008; Carnaval et al., 2009; Martins et al., 2009; Batalha-Filho et al., 2010; Ribeiro et al., 2010; Thomé et al., 2010; d'Horta et al., 2011).

The absence of a strong differentiation among localities and the significant signal of demographic expansion (Table 2 and Fig. 4) suggest that *B. leucoblepharus* suffered a bottleneck followed by demographic expansion. This expansion occurred approximately in mid Pleistocene, as suggested by the EBSP, and is likely related to climate changes that occurred locally in that period. This date estimate is not congruent with the end of the LGM, when the glaciers started to retreat between 20,000 and 14,000 years ago (Sant'Anna Neto and Nery, 2005; Anderson et al., 2007). Therefore, our results suggest that *B. leucoblepharus* maintained its population size relatively stable during the LGM in the AF, contrarily to what would be expected according to the Pleistocene forest refuge model.

The particular pattern exhibited by *B. leucoblepharus*, in comparison to other organisms from the AF (Grazziotin et al., 2006; Cabanne et al., 2007, 2008; Carnaval et al., 2009; Martins et al., 2009; Batalha-Filho et al., 2010; Ribeiro et al., 2010; Thomé et al., 2010; d'Horta et al., 2011; Cabanne et al., 2011; Maldonado-Coelho, 2012), might be associated to different habitat requirements presented by these species. In addition, the phylogeographic barriers observed for some endemic taxa from AF (Fig. 1a) may have acted as selective ecological filters (see Toon et al., 2010), i.e., only species with higher tolerance to habitat fragmentation were able to maintain gene flow or disperse across these barriers. *B. leucoblepharus* is relatively tolerant to habitat fragmentation (Stotz et al., 1996), and thus, barriers that affected several other AF forest birds (references as above) were probably not strong enough to preclude gene flow in *B. leucoblepharus*. This would explain the absence of phylogeographic structure in this species.

According to Moritz et al. (2000), the location, size and existence of forest refugia during the glacial maximum are dependent on the ecological and environmental tolerances of each species. Thus, *B. leucoblepharus* seems to have been able to maintain a stable population through the late Pleistocene, even during peaks of glaciations when forests fragmented and the climate was temperate (Behling, 2002). Contrarily to other AF birds that present a strong population genetic structure and historical demography affected by late Pleistocene glaciations, such as *Sclerurus scansor* (d'Horta et al., 2011) and *Xiphorhynchus fuscus* (Cabanne et al., 2007, 2008), the current southeastern distribution limit of *B. leucoblepharus* reaches temperate regions with discontinuous forest; namely the riparian forest from humid Chaco and pampas grasslands in eastern Argentina and Uruguay. Also, in the core of the AF *B. leucoblepharus* is found in montane forests where winters are temperate to cool (personal observation by HBF, Stotz et al., 1996), and in forest fragments within the Cerrado (Stotz et al., 1996). Thus, *B. leucoblepharus* seems to have been more tolerant to the climatic changes of the late Pleistocene than *S. scansor* and *X. fuscus*.

We rejected the presence of a bottleneck and demographic expansion at the LGM, and apparently the ecological flexibility of *B. leucoblepharus* might have been determinant for this response. However, it is not clear why this bird seems not to have been affected by the last glaciation, but affected by the previous glaciation, as suggested by the observed data (Fig. 3) and the simulations (Fig. 2). The two last glaciation maxima seem to have been similar in duration and intensity of cooling (Petit et al., 1999; Anderson et al., 2007), therefore, the effect on forests and their organisms should have been similar. Knowledge on the detailed paleogeographic and paleo-vegetation histories of the AF during the mid Pleistocene is almost inexistent. At the global scale this period coincides with the Mid-Brunhes event, a transition period from relative cool interglacial and small amplitude of glacial/interglacial temperature variation to warmer interglacials with larger amplitude of temperature variation (Anderson et al., 2007). However, the conditions in the AF and how organisms were affected during this period are unknown. Anyway, even though simulations did not favor a single date for bottlenecks, they suggest that there was a strong one. More phylogeographic studies are needed to identify the nature of the demographic expansion suffered by *B. leucoblepharus*.

4.2. Phylogeographic patterns in the Atlantic forest: a new pattern revealed by Basileuterus leucoblepharus?

In general, phylogeography studies of AF organisms show three main recurrent breaks (Fig. 1a): (i) one break in the state of São

Paulo, close to the valleys of the rivers Paraíba do Sul, Tietê and Ribeira do Iguape (snake, birds, frogs, bats, and toad; Grazziotin et al., 2006; Cabanne et al., 2007, 2008, 2011; Carnaval et al., 2009; Martins et al., 2009; Batalha-Filho et al., 2010; Thomé et al., 2010; d'Horta et al., 2011); (ii) one break in the state of Minas Gerais, close to the valleys of the rivers Jequitinhonha and Doce (bird and frogs; Cabanne et al., 2007, 2008; Carnaval et al., 2009; d'Horta et al., 2011); and (iii) a break in northeastern Brazil, close to the mouth of the São Francisco river (bird and frogs; Cabanne et al., 2008; Carnaval et al., 2009).

Regarding phylogeographic breaks i and ii, the dates of the origins of the river valleys are older than the estimated intraspecific divergence dates, and therefore the geologic origin of the valleys could not have been the primary cause of lineage divergence. Phylogeographic studies of AF birds (Cabanne et al., 2007, 2008; d'Horta et al., 2011), bats (Pavan et al., 2011) and frogs (Carnaval et al., 2009) could not reject that lineage divergences were associated with habitat fragmentation due to the glacial cycles in the Pleistocene. These studies were also congruent with a model of Pleistocene forest refugia in the AF based on paleoclimatic modeling of the LGM (Carnaval and Moritz, 2008). However, it is interesting to note that a snake (Grazziotin et al., 2006) also presents a phylogeographic break in this same region (break i, Fig. 1a). Even though these barriers show congruence in space, the estimated dates were not congruent (e.g. for break i: 3.87 m.y.a. for the snake and 0.39 m.y.a. for a bird). Moreover, two other studies with bees (Batalha-Filho et al., 2010) and toads (Thomé et al., 2010) suggest that some of these phylogeographic breaks also coincide with putative barriers associated to neotectonic activities.

The ecological niche modeling of the AF during the LGM by Carnaval and Moritz (2008) indicated that the forested areas in the southern portion of this biome were more fragmented than forests at the northern portion. Some recent studies have confirmed this hypothesis (Cabanne et al., 2007, 2008; Carnaval et al., 2009; Martins et al., 2009; d'Horta et al., 2011; Martins, 2011). However, our results were not congruent with the Carnaval and Moritz (2008) hypothesis because we did not find a strong genetic structure, a bottleneck followed by a strong signal of demographic expansion at the LGM, and a tendency of expansion from north to south (Figs. 1 and 3). Moreover, the comparison of nucleotide diversity of the mtDNA versus latitude showed that the south holds higher levels of genetic diversity (Fig. 4). This result disagrees with the latitudinal gradient hypothesis, which states that higher diversity levels should be found in lower latitudes (Miller et al., 2010; d'Horta et al., 2011). Furthermore, our simulations rejected the scenarios where the effects of LGM were tested (Table 3). Thomé et al. (2010) observed toad lineages that survived in the southern portion of the AF, and this is also not congruent with the Carnaval and Moritz (2008) hypothesis.

Both traditional and model-based phylogeographic approaches allowed us to infer about the evolutionary history of *B. leucoblepharus* and the diversification dynamics within the AF. To our knowledge, the present work revealed the first example of an AF organism (*B. leucoblepharus*) without a strong population genetic structure and whose population size did not change during the LGM. Thus, our results show that more AF organisms should be investigated to help in the reconstruction of the evolutionary history of this biome. As more taxa are studied, new scenarios shall be revealed. Costa (2003) suggested that speciation in the Neotropics could not be explained by any single model of vicariance or climatic change. Thus, it is possible that no general pattern for organisms' diversification in the AF will arise, but a complex range of scenarios shall be described.

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