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Coastal-inland divergence and postglacial expansion in the populations of the orchid bee *Euglossa annectans*

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ABSTRACT

Biogeographic patterns in the southernmost portion of the Atlantic Forest (AF) are still elusive for a high number of endemic species where the main phylogeographic hypothesis in AF (e.g., CM model or AF expansion) fails to predict refuges during the Last Glacial Maximum (LGM). *Euglossa annectans* is an orchid bee species endemic to AF, distributed throughout the longitudinal edges of the southern portions of this biome. Combining ecological niche modelling and genetic analysis, we investigated whether the Pleistocene climate fluctuations played some role in the population structure of this species. We analysed 640 bp of the *Cytb* gene and eight microsatellite loci from males sampled from twelve localities along *E. annectans* distribution. Microsatellite markers indicated two main groups with a disjunction between inland and coastal areas. Although mitochondrial data showed no spatial structure, we found signals of a bottleneck followed by population expansion after the LGM. Pleistocene climatic models revealed a fragmented scenario with suitable areas concentrated at the coastal continental shelf and the northern-west inland areas, both in line with the genetic nuclear markers' structure and synchronic with the mitochondrial bottleneck at the LGM. The climatic instability along the Pleistocene has played a central role in determining the phylogeographic and demographic patterns of the *E. annectans*, resulting in a genetic structure reported for the first time in this area.

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Introduction

The Atlantic Rainforest (AF) domain is a complex combination of vegetal physiognomies, largely determined by a wide range of latitudinal (from 3°S to 31°S), longitudinal, (35°W to 60°W), and altitudinal (up to 2892 meters, "Pico da Bandeira" mountain) components, and therefore a variety of pluviometric levels and climatic conditions (Ribeiro et al., 2009). Such variation has shaped a matrix of different forest types throughout the AF history in which the Ombrophilous Dense Forest (DF), the Mixed Ombrophilous Forest (MF), and the Semideciduous Seasonal Forest (SF) correspond to the dominant forest types (Oliveira-Filho & Fontes, 2000). These forests compose distinct endemic zones for different organisms (Tabarelli et al., 2010), including orchid bees (Apidae: Euglossini) (Ramírez et al., 2010) represented by around 50 species in AF, from which

almost 50% are endemic to this biome (Nemésio, 2009).

Hypotheses based on the Pleistocene climatic variations have become some of the most accepted theories for explain the high rates of diversification in the AF (Carnaval & Moritz, 2008; Porto et al., 2013). In particular, the model of Carnaval and Moritz (2008, hereafter "CM Model") presents predictions often corroborated by population-genetic and phylogeographic data (Batalha-Filho & Miyaki, 2016; Frantine-Silva et al., 2017). According to the CM model, refuges at the northern portion of the AF distribution have constituted zones of climatic stability through the Last Glacial Maximum (LGM). However, the common refuges attributed to the CM model do not cover stable areas at the southernmost portions of AF as those occupied by *Euglossa* (*Glossura*) *annectans* Dressler 1982. On the other hand, other studies have indicated more complex scenarios for different species through the Late Pleistocene at AF

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WFS and SHS conceived the initial idea; WFS and DCG conducted the lab, field work and collected the data with additional material from GDC; WFS and SHS analysed the data; and WFS led the writing with assistance from all the authors.

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(Porto et al., 2013; Thomé et al., 2010, 2014), leading to the proposition of different hypotheses such as the existence of geographical barriers (Thomé et al., 2014), neotectonism (Batalha-Filho et al., 2010), and even one also based on climatic instability but predicting expansion of the AF toward the continental shelf due to the marine regression at LGM periods instead AF retraction to refuges (Leite et al., 2016a).

Orchid bees are especially useful models for studying the Quaternary climatic variations (Frantine-Silva et al., 2017; López-Urbe et al., 2014), mainly due to their intrinsic relationship with forested habitats (Giangarelli, Aguiar, et al. 2015), especially the wet forest (>2000 mm/year) wherein communities' richness is particularly high (Dressler, 1982). These bees are specialists in the collection of chemical compounds that males store in their hind tibia to latter exposure in courtship display (Dressler, 1982; Eltz et al., 2005), increasing the pressure of sexual selection and the possibility of speciation (Brand et al., 2020). Independent studies have pointed out that the Quaternary climatic fluctuations have shaped the diversification of the maternal lineages in orchid bees (Frantine-Silva et al., 2017; López-Urbe et al., 2014). However, the majority of AF phylogeographic studies have focused on wide latitudinal distributions, wherein the dense forest dominates the landscape, and the AF refuges are often corroborated (Carnaval & Moritz, 2008; Frantine-Silva et al., 2017). Thus, the influence of Pleistocene climatic fluctuations on the AF longitudinal component still elusive. In such scenario, *E. annectans* became a good model since it is endemic to AF and widely distributed throughout the inland semideciduous forest (Faria & Melo, 2007) with records in dense forest at the AF coast (Faria & Melo, 2007; Giangarelli, Aguiar, et al., 2015; Pinto et al., 2019), covering the southern and southwestern edges of the AF. The species pollinates 24 Angiospermae families (Cortopassi-Laurino et al., 2009), nesting alone or with other females (Garófalo et al., 1998), reactivating nests through successive generations with records for as long as five years (Cortopassi-Laurino et al., 2008). *Euglossa annectans* is prone to a high diploid male production (around 10%; Frantine-Silva, Gaglianone, et al., 2021; Giangarelli, Freiria, et al., 2015) compared to the other orchid bees (Giangarelli, Freiria, et al., 2015), highlighting the importance of the studies covering its genetic diversity and structure.

The present study aimed to evaluate the influence of the Quaternary climatic variations on the genetic diversity and structure of *E. annectans* throughout its distribution as proxy of its phylogeographic history. To this end, ecological niche models are compared with genetic structuring patterns from mitochondrial

and nuclear markers, assessing the chronological alignment of climatic variations with the demographic and structuring patterns of *E. annectans* in the last 130 Ka as well as the concordance with the current main hypothesis for phylogeographic patterns in the Atlantic Forest.

Materials and methods

Study areas and bee sampling

Twelve remnants of Seasonal Semideciduous Forest and Ombrophilous Dense Forest from the southern portion of the Atlantic Forest were sampled for *E. annectans* males, covering an area of ~280,000 km², including most of the known species distribution (Figure 1(A); Table 1). Orchid bees are solitary bees occasionally observed in nature (Dressler, 1982). Since orchid bee males are attracted by volatile chemical compounds which they use later in species-specific bouquets for sexual display (Eltz et al. 2005; Zimmermann et al. 2006), samplings were conducted using scent baits as follows: benzyl benzoate, benzyl acetate, beta-ionone, eucalyptol, eugenol, methyl butyrate, methyl cinnamate, methyl salicylate, and vanillin as in (Giangarelli, Aguiar, et al. 2015). We used aerial insect nets and traps (as in Ramalho et al., 2009) with the same essences between the years 2009 and 2016. Representative individuals of the samplings were deposited in the Zoological Museum of the State University of Londrina (MZUEL).

Molecular procedures

Total DNA was extracted with a phenol-chloroform protocol (Penha et al., 2015) from the thoracic musculature or the right hind leg of bees. Amplification and sequencing of the *Cytochrome b* (*Cytb*) mitochondrial gene were performed according to Frantine-Silva et al. (2017), using a combination of primers drawn from the sequences of *Euglossa iopoe-cila* Dressler 1982 (*Eiop-F1*:3'-CGAGGTCGCAAACATCT-5') with the primer CB-N-11367 (Crozier et al., 1991), annealing at 48 °C. PCR fragments were sequenced in an ABI-PRISM 3500-XL automatic sequencer (Applied Biosystems, Foster City, CA, USA). Nuclear information was assessed through eight microsatellite loci described for *E. annectans*: Ann2, Ann3, Ann4, Ann6, Ann8, Ann24, Ann37, and Ann41 (Paxton et al., 2009), following the same annealing temperatures originally described for the respective loci and the protocol described by Penha et al. (2015). Samples were genotyped in an ABI3500-XL automatic sequencer (Applied Biosystems, Foster City, CA, USA).

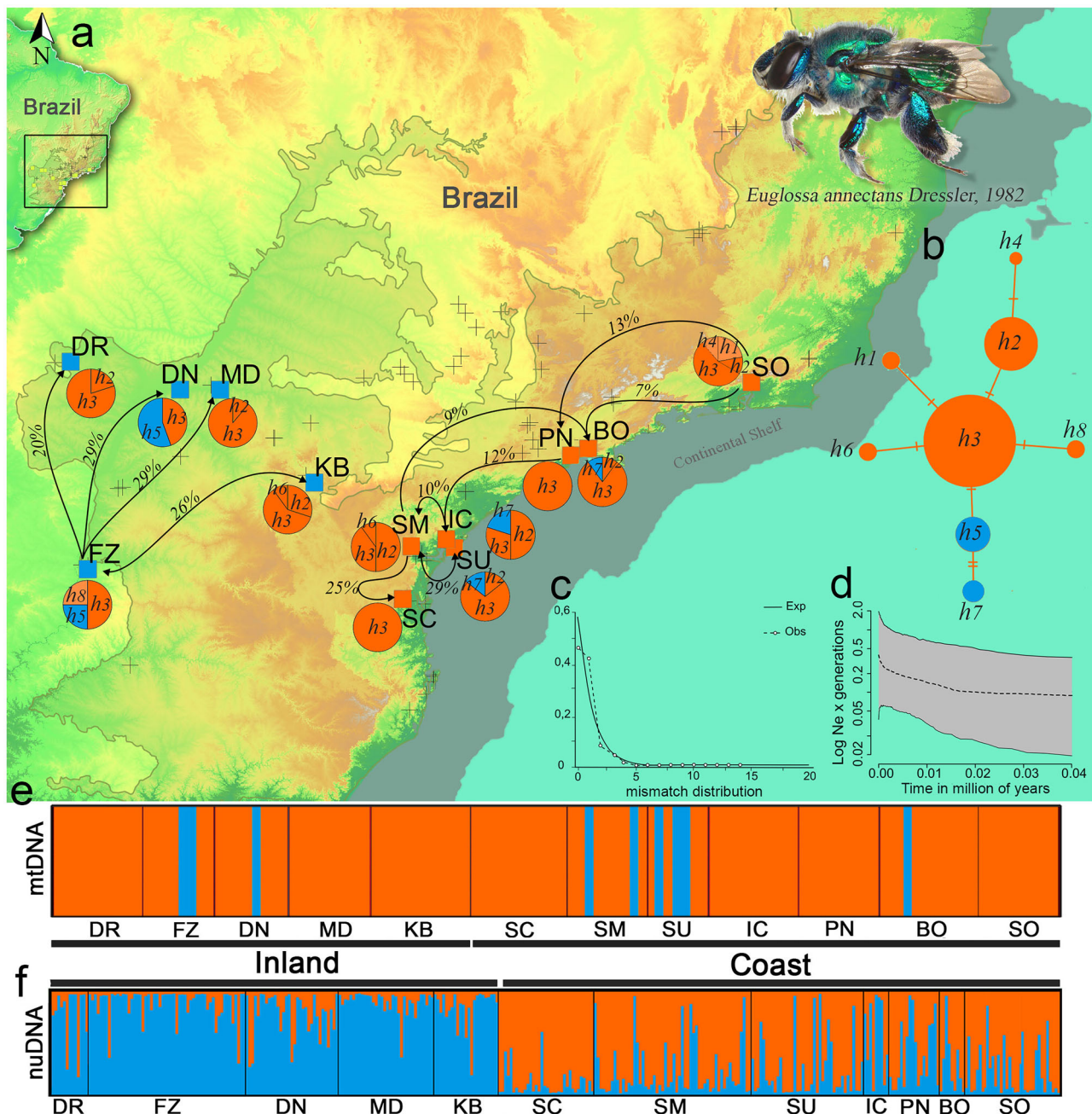


Figure 1. Geographic distribution and genetic structure of *Euglossa annectans* through southern Atlantic Forest (highlighted background area): (A) Light-grey crosses pinpoint the known records of *E. annectans* used to the niche models; pie charts represent the haplotype frequencies at each sampling site coloured as indicated by the small squares; black arrows and percentages indicate the estimated relative number of migrants between sites according to the Bayesian assignment test (only values >5%); (B) haplotype median joining network, coloured as the genetic clusters; (C) mismatch distribution chart, solid line represent the frequency of the expected pairwise differences under a population expansion scenario, and the dotted lines represent the frequency of observed differences; (D) Bayesian Extended Skyline Plot, dotted lines shows the Log of the estimated effective population size (*N_e*) through time in millions of years and solid lines display the 95% confidence interval; (E) Bayesian genetic structuring bar chart for the most likely *k*-cluster grouping (*K*=2) based on the mitochondrial *Cytb* gene sequences and; (F) microsatellite data (sites: DR-Dourados, FZ-Foz do Iguaçu, DN-Diamante do Norte, MD-Teodoro Sampaio, KB-Telêmaco Borba, SC-Joinville, SM-Salto Morato, SU-Superaui, IC-Cananéia, PN-Mogi das Cruzes, BO-Salesópolis, SO-Teresópolis).

Analysis of molecular data

Mitochondrial DNA

DNA SP 5.0 (Librado & Rozas, 2009) was used to identify the haplotypes, calculate nucleotide and haplotype diversity, mismatch distribution, and the neutrality tests of Tajima's *D* (Tajima, 1989), Fu's *F_s*

(Fu, 1997), and the *R₂* statistic (Ramos-Onsins & Rozas, 2002). Haplotype median-joining networks were inferred using POPART v1.7 (Leigh & Bryant, 2015). The effective population size fluctuations through time was estimated by the "Extended Bayesian Skyline Plot" (EBS) method as

Table 1. Sampling sites and number males of *Euglossa annectans* captured in the present study.

ID	Municipality	Locality	FT	Latitude	Longitude	N
SO	Teresópolis	PARNA da Serra dos Órgãos	DF	−22.493	−43.0733	40
BO	Salesópolis	Estação Biológica de Boraceia	DF	−23.631	−45.8697	9
PN	Mogi das Cruzes	Parque das Neblinas	DF	−23.748	−46.1633	22
SU	Superagui	Superagui National Park	DF	−25.331	−48.1558	60
SM	Salto Morato	RPPN Salto Morato	DF	−25.182	−48.2978	46
IC	Cananéia	Ilha do Cardoso State Park	DF	−25.305	−48.8967	11
SC	Joinville	Pirabeiraba	DF	−26.207	−49.0431	35
KB	Telêmaco Borba	RPPN Fazenda Monte Alegre	SF	−24.212	−50.5572	26
MD	Teodoro Sampaio	State Park Morro do Diabo	SF	−22.621	−52.1717	40
DN	Diamante do Norte	ESEC Caiuá	SF	−22.618	−52.8564	35
FZ	Foz do Iguaçu	Iguaçu National Park	SF	−25.695	−54.4367	60
DR	Dourados	APP Serra da Picadinha	SF	−22.142	−54.7317	14

The areas code (ID), location and forest types (FT), as well as latitude and longitude are shown RPPN – Privy Reserve of Nature Protection; APP – Permanent Protection Area; forest types are described in the text DF – Ombrophilous Dense Forest; SF – Semideciduous Seasonal Forest.

implemented in BEAST 2.3.2 (Drummond & Rambaut, 2007) following the parameters for runs and dating described in Frantini-Silva et al. (2017) with HKY + I + G model indicated by the JMODELTEST 1.1 (Posada, 2008). The spatial structure for mtDNA data was accessed by Bayesian inference through BAPS v6.2 (Corander et al., 2008), using the spatial grouping option with K ranging from 1 to 15. Isolation by distance (IBD) was tested using the Mantel test (1000 permutations) in GENALEX (Peakall & Smouse, 2006), also used to estimate pairwise genetic structure indices and AMOVA framework considering two groups, as estimated by the nuclear markers with 1000 permutations.

Nuclear DNA: microsatellites

Genotyped data was visualized and checked for alleles out of the range using the GENE MARKER v2.2.0. GENALEX was used to calculate the mean number of alleles in each population (\bar{N}_A), mean number of effective alleles (\bar{N}_E), number of private alleles (N_P), and the mean expected heterozygosity (\bar{H}_e), G_{ST} , G'_{ST} (Hedrick, 2005), and $Dest$ (Jost, 2008). Different approaches were used to access the genetic structuring among the several samples. Bayesian analysis via STRUCTURE (Pritchard et al., 2000) was employed to estimate the most likely number of groups (K) as follows: K -groups ranging from 1 to 15; 20 interactions in each K ; MCMC with 1,000,000 steps; burning of 10%. The statistical significance of inferred groups was tested through the AMOVA framework in GENALEX. The linearized values of G_{ST} were used to investigate possible IBD patterns between the samples through the Mantel test with 1000 permutations. Finally, BAYESASS 3.0 (Wilson & Rannala, 2003) was used to estimate the migration rates and its directions between pairs of localities through a Bayesian approach. Four independent runs were performed with 1,000,000 generations of MCMC, sampling parameters every 1000 generations with a burning of 100,000 generations, with other parameters in default.

Ecological niche modelling

Ecological niche models (ENM) for the current time were constructed and projected to the middle Holocene (MID; 6 Ka), Last Glacial Maximum (LGM; 21 Ka), and last interglacial period (LIG; 130 Ka) using MAXENT v3.3.3k (Phillips et al., 2006) based on 71 georeferenced records (Figure 1(A); Appendix S1). A set of 11 out of 19 WorldClim variables was selected via PCA, comprising the 95% variation (in order of contribution: BIO10, BIO5, BIO14, BIO17, BIO1, BIO9, BIO19, BIO3, BIO12, BIO15, and BIO4), excluding the variables BIO8 and BIO11 due to the high correlation with the variables BIO10 and BIO9 ($r^2 > 0.8$, $p < 0.001$), respectively. Models were evaluated according to the area under the curve (AUC), the Akaike information criterion corrected for small sample sizes (AICc), Bayesian information criterion (BIC) and the log of probability of the models ($LogL$) through ENMTOOLS (Warren et al., 2021). For MID and LGM, three General Circulation Models (GCM) were used, including the CCSM4, MPI-ESM, and the MIROC-ESM (see worldclim.org). Variables were downloaded at 2.5 arcminutes and projected to WGS84 30 arcseconds using QGISv2.14 (Quantum GIS Team, 2016). Layers were cropped between latitudes -5 and -35 and longitudes -73 and -34 . Models were generated using MAXENT's default parameters, cross-validation at 25%, 10 replications for each model and a threshold of equal sensitivity and specificity, creating a consensus when there were more than one GCMs available.

Results

Euglossa annectans: diversity, phylogeography and demography

The sequences of the *Cytb* gene from 112 males resulted in 640 bp and eight different haplotypes (h) (Table 2). We observed a relatively low overall haplotypic diversity ($Hd = 0.520$), but an important variation among the sampled localities caused by fixed haplotypes in PN, SC, and MD. The highest Hd and

Table 2. Genetic diversity indexes and neutrality tests for 640 bp from the *Cyt b* gene of *Euglossa annectans*.

ID	Cytochrome b gene								Microsatellites						
	N	S	h	Hd	π (%)	Fs	D	R ₂	N	N _A	sd	N _E	sd	N _P	He
SO	9	3	4	0.69	0.15	-1.03	-0.35	0.16	34	8.5	1.3	5.2	0.7	2	0.78
PN	10	0	1	0.00	0.00	0.00	0.00	0.00	9	4.1	0.4	3.0	0.4	1	0.64
BO	10	2	3	0.37	0.06	-1.16*	-1.40*	0.20	18	5.5	0.9	3.9	0.5	0	0.73
IC	10	2	3	0.66	0.11	-0.04	0.22	0.20	9	5.3	0.5	4.3	0.5	0	0.78
SU	7	4	4	0.81	0.17	0.26	-1.43	0.26	40	7.1	0.7	3.7	0.3	2	0.72
SM	10	4	3	0.68	0.25	1.48	0.56	0.20	56	8.0	0.7	3.9	0.6	1	0.70
SC	10	0	1	0.00	0.00	0.00	0.00	0.00	34	7.1	0.9	4.7	0.8	5	0.74
KB	10	2	3	0.60	0.10	-0.27	-0.18	0.19	23	5.8	0.5	3.5	0.5	0	0.67
MD	9	1	1	0.00	0.03	-0.26	-1.08	0.31	34	7.9	1.2	5.1	0.9	1	0.77
DN	9	1	4	0.75	0.08	1.01	1.40	0.27	33	7.4	1.1	4.7	0.8	3	0.75
FZ	8	2	5	0.85	0.13	-0.07	0.41	0.21	56	9.9	1.6	5.3	1.0	1	0.76
DR	10	1	2	0.35	0.05	0.41	0.01	0.17	13	4.6	0.4	3.2	0.4	0	0.65
Total	112	7	8	0.52	0.11	-3.38	-1.08	0.05*	359	13.7	5.6	4.2	0.2	-	0.73

N – number of specimens analysed; S – segregating sites; h – number of haplotypes; Hd – haplotypic diversity; π – nucleotide diversity; Fs – Fu's neutrality test; D – Tajima's D test; R₂ – Ramos-Onsín and Rozas neutrality test; N_A – average number of alleles; N_E – effective number of alleles; N_P – number of private alleles; He – expected heterozygosity. * $p \leq 0.05$.

nucleotide diversity (π) were respectively observed in FZ ($Hd=0.857$) and SM ($\pi=0.253\%$). The haplotypic distribution shows a homogeneous pattern influenced by the most common haplotypes *h*3 (60.6%), *h*2 (20.2%), and *h*5 (8.5%), with no indication of spatial structuring (Figure 1(A)). A star-like network (Figure 1(B)), typically related to scenarios of population expansion after genetic bottlenecks or founder effects (Ramírez-Soriano et al., 2008), was observed and corroborated by the R_2 statistics (Table 2) and the mismatch distribution (Figure 1(C); Appendix S2). The EBSP demonstrates a fourfold increment in the effective population size from 15 Ka, indicating a post-glacial population expansion (Figure 1(D)). BAPS inference of the genetic structure did not find any defined spatial structure (Figure 1(E)), neither AMOVA simulations (Appendix S3). Comparisons between pairs of samples are shown in Appendix S4.

For the microsatellite markers, 398 males of *E. annectans* were genotyped, but 39 had one or more heterozygous loci and were not included in the analysis. Then, 359 haploid males presented 110 alleles, an average of 12.87 alleles per locus (8–20 alleles). On average, the data showed 0.17% (s.d. 0.42%) of missing data, exclusively in the loci Ann03 and Ann04, respectively 1.11% and 0.28% (Appendix S7). Sixteen private alleles were identified in eight out of 12 localities, but only six of them had a frequency higher than 5% in three sites (BO, SC, and DN). The *He* presented low variation between the localities, ranging from 0.64 to 0.78 (Table 2). STRUCTURE has identified two sets of samples: a coastal (SO, BO, PN, IC, SU, SM, and SC) and an inland group (KB, MD, DN, FZ, and DR) (Figure 1(F)), corroborated by AMOVA ($\Phi_{CT} = 0.0476$; p -value = 0.002). Besides, an important component of the local structure was identified in the comparisons of samples within groups ($\Phi_{SC} = 0.078$) as well as the comparisons between all samples ($\Phi_{ST} = 0.122$; Appendix S3).

Comparisons between pairs of samples are also shown in Appendix S4.

Two main migratory routes were identified through the Bayesian inference between the sampled localities, both concentrated within the respective groups. Among the samples from the "inland" group, migration rates between 20.2% and 28.2% were identified from the FZ to other sampling sites (DR, DN, MD, and KB), with significant migration rates from KB to FZ (26.2%). In the "coastal" group, gene flow rates varied from 3% (SM-IC) to 29% (SM-SU) (Figure 1(A); Appendix S5). Although a trend of isolation by distance was observed for the G_{ST} (Appendix S6) the correlations were not significant.

Niche models and paleodistribution

All models generated for *E. annectans* presented high AUC values, ranging from 0.976 to 0.986. The models show a significant change in the *E. annectans* distribution from the LIG to the present days, with stable areas only in the coastal region and an important variation in the inland portion of the distribution (Figure 2). The suitable areas of the present (Figure 2(a)) shifted from the southern portion of the Atlantic Forest (between 29S–19S and 40W–55W) of a similar, but a more fragmented scenario, in the MID (Figure 2(b)). LGM model (Figure 2(c)) presented the same trend of fragmentation between suitable areas in the coast and inland, creating instability zones between the suitable areas at the continental shelf and inland areas of *E. annectans* paleodistribution. The LIG model (Figure 2(d)) shows a deeper separation between the coastal and inland zones of distribution of *E. annectans*, but with only two large areas.

Discussion

Although biogeographic patterns across the Atlantic Forest (AF) have been continuously assessed, most

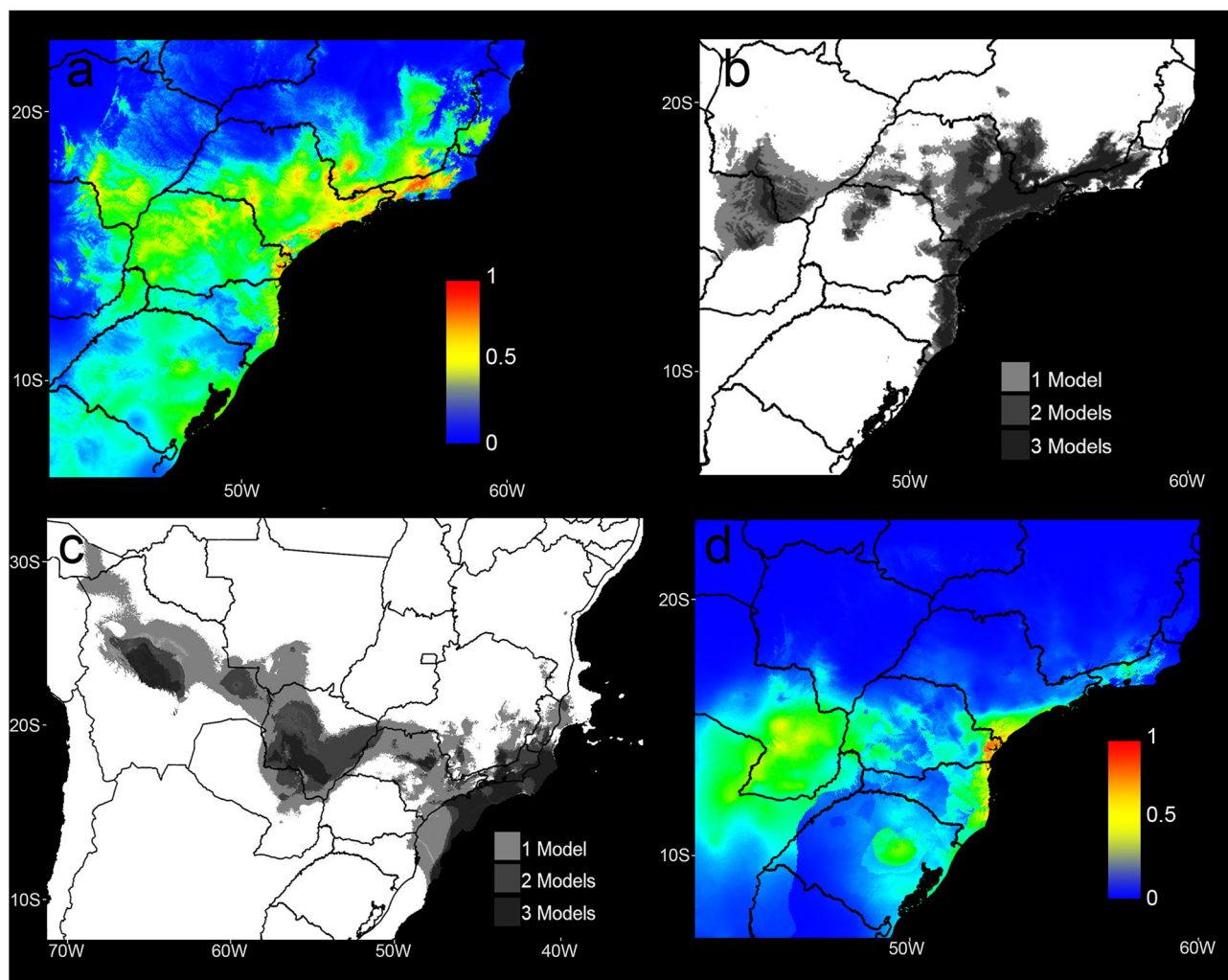


Figure 2. Ecological niche modelling of *Euglossa annectans* for the past 130 Ka: (a) Model of *E. annectans* for current time; (b) consensus for three GCM for the mid-Holocene (6 Ka); (c) last glacial maximum (LGM; 21 Ka), consensus of three GCM; (d) model for the last interglacial period (LIG; 130 Ka). Warmer colours indicate higher suitability for species' occurrence, as indicated in the legend.

of the assumed models embrace conditions usually found along latitudinal gradients (Batalha-Filho et al., 2012; Carnaval & Moritz, 2008; Leite et al., 2016a; Porto et al., 2013). Nonetheless, the drivers of the phylogeographic structure on the AF longitudinal gradients are still elusive for several endemic species. To our knowledge, the present study is the first to investigate the influence of Quaternary climate fluctuations on the phylogeography of a bee species distributed throughout the AF southern and western limits in an approach combining ecological niche models and different molecular markers. The results corroborate the hypothesis that the Late Pleistocene climatic fluctuations played a central role in the demographic history of *E. annectans*. However, it also revealed an inland-costal structuring pattern reported for the first time in the southern AF. The hypothesis that climatic fluctuations had a strong influence on the demography and genetic structure of this species is supported by at least three results: i) the reduction and disjunction of the suitable areas through the last glacial maximum supported by the

observed genetic structure; ii) demographic patterns chronologically aligned with climate fluctuations; (iii) absence of significant geographical barriers other than those influenced by climatic variations. Barriers, such as valleys in river basins or neotectonic events as documented for snakes (Grazziotin et al., 2006), amphibians (Thomé et al., 2014), planarians (Álvarez-Presas et al., 2014), or even bees (Batalha-Filho et al., 2010), can be discarded in the case of *E. annectans* due to several reasons, including: the recent chronology of the demographic events; the high congruence of spatial patterns with the predictions of niche models for microsatellite markers; or even for the high dispersion capacity of orchid bees (Pokorny et al., 2015).

Climatic instability predicts the patterns of structuring and demography

In general, paleoclimatic projections predicted the response of both mtDNA and nuDNA markers. These models indicate a progressive reduction in the

suitable areas for *E. annectans* toward the LGM, leading to the disjunction between populations in coastal and inland areas. Although similar patterns have also been observed in paleoclimatic models for other AF endemic organisms (Batalha-Filho & Miyaki, 2016; Porto et al., 2013; Thomé et al., 2010), herein this inland-coast pattern is supported by the results from the multiple nuclear markers. Moreover, the retraction of suitable areas also predicts the lack of spatial structuring for the mtDNA when we consider the difference of the effective population size (N_e) between the two genomes. In haplodiploid populations, the effective population size of the mtDNA is three times lower than the nuDNA (Zayed, 2004). Therefore, the stochastic effects of the loss of genetic diversity affect more one than the other (Luikart et al., 1998), tending to the loss of the phylogeographic signal in the mtDNA even without a local population extinction (Edwards & Beerli, 2000). Despite having used only a single region in the mtDNA analysis, the *Cytb* gene has been documented as one of the most informative mitochondrial regions in orchid bees (Frantine-Silva et al., 2017) with an evolutionary pattern intrinsically related to all mtDNA due to the physical link between the genes (Hurst & Jiggins, 2005). Besides, the Euglossini tribe has as a sex-asymmetric dispersion (López-Urbe et al., 2014), where males promote the nuclear gene flow (Zimmermann et al., 2011) and females are considered as philopatric organisms, remaining close to the nest of origin (Augusto & Garófalo, 2011), as in the case of *E. annectans* (Boff et al., 2017; Garófalo et al., 1998). Thus, mtDNA would be expected to remain unmixed through time, resulting in either strong isolation by distance pattern (Frantine-Silva et al., 2017; Penha et al., 2015) or a lack of structure under bottleneck scenarios, as herein documented (e.g., Batalha-Filho & Miyaki, 2016). Therefore, it is likely that climatic instability has been driving both genomes to the current measured condition.

The data of *E. annectans* add a new perspective on the events that may have occurred with endemic taxa at the southern portion of the AF. These results gather a set of evidence previously reported only separately, supporting that both demography and genetic structuring of some species may have been affected by the last glacial maximum (LGM). The absence of genetic or geographic structuring is often reported in the AF for organisms with similar distribution of *E. annectans* (Batalha-Filho et al., 2012; Thomé et al., 2014), or the spatial structure is just predicted in the LGM models, but not supported by genetic results (Batalha-Filho & Miyaki, 2016). Herein both, niche models and genetic results, support that LGM has played a central role in current

demography and genetic structure. Although similar demographic signals are reported to another orchid bee the *Euglossa iopoecila*, no similar genetic structure was found (Frantine-Silva et al., 2017). However, *E. iopoecila* is not recorded out of the coastal line, which also reinforces the idiosyncrasy of species endemic to different regions of AF. Overall, the recurrent signs of population expansion in species belonging to different taxa endemic to the southern AF indicate that at least, for part of the fauna, there was a strong influence of climatic fluctuations of the Quaternary, especially the LGM, on the phylogeography and demography of species from southern AF.

The LGM models also point to another important aspect relative to the Euglossini biogeography in the AF, supporting the existence of a connection between the AF and the Amazonian and Andean Rain Forest. Although the molecular data of *E. annectans* do not allow us to direct test this hypothesis, niche models corroborate the central region of the Cerrado as a possible route of gene flow between the AF and southern Amazonia and western Andean, as already reported for birds (Batalha-Filho & Miyaki, 2016; Trujillo-Arias et al., 2017) and other organisms (Ledo & Colli, 2017). This idea is also supported by the presence of stable areas in the central portion of the Brazilian Savanna in the LGM for other Euglossini species, such as *Eulaema cingulata* (López-Urbe et al., 2014). Further studies should specifically address the importance of this connection to orchid bees in the AF.

Reflections of the phylogeographic patterns on the genetic diversity

Regarding genetic diversity, we might observe two different patterns for each of both markers analysed. The microsatellite metrics (N_A , N_E , and H_e) indicate important levels of diversity even in populations with a small sample size (BO, CI, and DR) compared to the other euglossine bees in the AF (Boff et al., 2014; Frantine-Silva, Augusto, et al., 2021; Penha et al., 2015). This pattern is also supported by the Bayesian estimation of the gene flow, which displays an intense spatially structured pattern, in line with the historical separation of the niche clusters. On the other hand, the data from the *Cytb* gene consistently display low values for the equivalent indices in mtDNA such as H_d and π . Such differences might be in part associated with the above-discussed sex-asymmetric dispersion in the orchid bees, as observed to *E. iopoecila* (Penha et al., 2015) and *Eulaema* spp. (López-Urbe et al., 2014). Besides, the difference in genetic diversity between genomes is also partly due to their respective N_e , also discussed above. It is worth noticing that the homogeneity of

the genetic parameters relative to the respective marker also does not follow the expectation of the CM model “*strictu sensu*,” where northernmost areas usually have higher genetic diversity (Carnaval et al., 2009), pointing out that southern AF populations should respond differently to the Late Pleistocene glaciation.

An admixture between CM model and AF hypothesis

The climatic instability and the post-LGM population expansion observed for the *E. annectans* data display a pattern only partially aligned with the refuges of the CM model “*strictu sensu*” (Carnaval & Moritz, 2008). The strict interpretation of this hypothesis predicts that endemic species should have remained or migrate to the refuges at north of the AF during the LGM, resulting at least two consequences: (i) higher levels of genetic diversity would be found at refuges areas; (ii) signs of expansion post-LGM would be observed for populations in southern AF. In agreement with the CM model, we can immediately mention the signals of population expansion post-LMG. On the other hand, the genetic structure of *E. annectans* does not present the expected north-south clustering pattern as other AF organisms (e.g., Frantine-Silva et al., 2017), neither the niche models predict a full-shift of the distribution north toward. Instead, the genetic structure observed has a longitudinal pattern, as predicted in the niche models, and reported by Batalha-Filho and Miyaki (2016). Moreover, only a small portion of the distribution seems to remain geographically as stable as expected to characterize the refuges as the “Bahia refuge” in the CM model. During the LGM, the core of the distribution of *E. annectans* was separated northwest towards inland areas and southeast toward to the continental shelf. Interestingly, this remarkable expansion towards the continental shelf is expected in a second hypothesis, sometimes interpreted as antagonistic to the CM model, the “Atlantic Forest hypothesis” (Leite et al., 2016a), hereinafter “AF hypothesis.” The AF hypothesis states that the exposure of the continental shelf due to marine regression at the LGM provided areas to which the AF expanded instead to retract its domains, and thus population expansion should be identified at the LGM, not after that. Although extension of the AF hypothesis conclusions is debatable (Leite et al., 2016b; Raposo do Amaral et al., 2016), several studies have pointed out suitable areas at the continental shelf in central and southern AF during the LGM (Batalha-Filho & Miyaki, 2016; Thomé et al., 2014). Nevertheless, *E. annectans* and other organisms present signals of post-LGM population

expansion (e.g., Batalha-Filho & Miyaki, 2016). Then, the results herein found and those elsewhere reported indicate a more complex scenario, mixing points from these two apparently antagonistic hypotheses.

Our data highlight the importance of a more comprehensive interpretation of these hypotheses, since distinct species may subject to idiosyncrasies that would shape their evolutionary history. *Euglossa annectans*, for instance, display niche models that indicate an expansion toward the continental shelf in the LGM, as in Leite et al. (2016a); however, such in and out shifts have also pressured the population to a bottleneck, fitting the demographic expectations of the CM model. In other words, the herein data indicate a very dynamic climate scenario during the last 130 Ka in southern portion of AF. Together, the data observed to *E. annectans* and the southern cluster of *E. iopocila* (Frantine-Silva et al., 2017) indicate an environmental instability for orchid bees through the LGM at higher latitudes, which is in line with a general scenario of CM model to the AF. However, considering the number of different niches explored at this portion of the AF, it is important to evaluate species with similar distributions to those of *E. annectans*, assessing whether this pattern will be confirmed as a rule at the southern AF.

Overall, these results display a singular pattern of genetic structuring previously unnoticed for AF organisms. Late Pleistocene climatic fluctuations have been playing a significant role in the definition of this structure, causing a dynamic framework of environmental suitability. These results for *E. annectans* also assemble elements from two different hypotheses regarding the dynamics of refuges throughout the AF Quaternary, but which can be complementary at some points. At this perspective, suitable areas in the higher latitudes of the AF expanded over the continental shelf, but such advance was counterbalanced by the advance of less suitable areas throughout species distribution, leading some populations to fragmentation and genetic bottlenecks. Finally, as climatic variations seem to continue playing a key role in the distribution of *E. annectans*, the measures that prioritize the increase and connection of forested areas between the coast and inland portions of southern AF are of paramount importance to sustain the genetic variability of this species in the future.

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Disclosure statement

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Data availability statement

Data from *Cytb* gene are available in the NCBI GENBANK (MT109653–MT109660) and the microsatellite data is available in Mendeley Data repository under the DOI: 10.17632/7zskzd24p.1.

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