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Biome variation, not distance between populations, explains morphological variability in the orchid bee *Eulaema nigrita* (Hymenoptera, Apidae, Euglossini)

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Abstract – The sampling of orchid bees across different biomes provides an excellent opportunity to analyze morphological variability in this group. We examined the morphological variation of populations of *Eulaema nigrita* in the context of the determination of geographic origin. It also evaluated population variability of this group in different landscapes from two important biomes, Atlantic Forest, Brazilian Savanna, and the transition zone between them. These analyses were achieved by documenting the pattern of morphological differentiation using geometric morphometric analysis of the wings of 256 individuals, sampled from nine locations, from four different physiognomies. Results indicated that individuals from the Atlantic Forest physiognomies are larger than the ones from the Savanna. The clusterization of individuals was based on landscape features of the sampling areas. The results indicate little or even a complete absence of population structure. Overall, site-specific population differentiation is not strongly supported by wing traits, reinforcing the statement consistent with the broader pattern that orchid bees have a high migratory capacity. However, the subtle differentiation may indicate the existence of locally adapted traits kept by female philopatry or phenotypic plasticity triggered by environmental factors yet to be studied.

Euglossini / geometric morphometrics / Brazilian Savanna / Atlantic Forest

1. INTRODUCTION

Bees are the most frequent flower visitors in forests (Neff and Simpson 1993) and provide

crucial pollination services in a wide number of agricultural and natural ecosystems (Klein et al. 2007; Brown and Paxton 2009; Willmer 2011). In the Neotropical region, the Euglossini tribe, commonly known as orchid bees, stands out as particularly important pollinators (Tonhasca et al. 2003). Studies have shown that both male and female orchid bees pollinate a wide number of plant species while foraging for nectar, pollen, and resins (see Ramirez Arriaga and Martinez Hernandez 1998; Ramirez et al. 2002; Ospina-

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Torres et al. 2015; Ramírez 2019). Specifically, 10% of Neotropical Orchidaceae, around 700 orchid species, is pollinated by male euglossine bees (Ramírez et al. 2002). Euglossine bees are abundant in low- or mid-elevation forests, comprising up to 25% of species in bee communities (Roubik and Hanson 2004). Many euglossine species have a strong association with forested areas and show the highest community richness in areas where precipitation exceeds 2000 mm/year (Dressler 1982).

Euglossine bees have recently attracted interest from the academic field, especially in the last two decades, with an increasing number of studies designed to catalog and recognize the diversity of this group throughout the Atlantic Forest (e.g., Rebêlo and Garófalo 1997; Sofia et al. 2004; Ramalho et al. 2009; Nemésio 2009; Rocha-Filho and Garófalo 2013, 2014; Giangarelli et al. 2015; Costa and Franco 2017; Machado et al. 2018), with additional studies on the population dynamics and genetic diversity of some species (e.g., Cerântola et al. 2011; Freiria et al. 2012; Rocha-Filho et al. 2013; Boff et al. 2014; Penha et al. 2015; Grassi-Sella et al. 2018). Among the approximately 206 known species of orchid bees, one of the most studied is *Eulaema nigrita* Lepelletier, which has a relatively broad geographical distribution (Moure 1950) and tends to inhabit open areas (Peruquetti et al. 1999). This species presents excellent flight and dispersal abilities (Ackerman 1983; Raw 1989) and is an extremely generalist species, with respect to floral diet (Dressler 1982; Ramalho et al. 2009; Silva et al. 2012). *El. nigrita* is relatively abundant in several ecosystems (Rebêlo et al. 2003), among them the Atlantic Forest (e.g., Aguiar and Gaglianone 2012; Cordeiro et al. 2013; Rocha-Filho and Garófalo 2014; Machado et al. 2018) and Brazilian Savannas (e.g., Ramalho et al. 2009; Faria and Silveira 2011; Silveira et al. 2015). Due to its occurrence as a dominant species in open and urban areas, it is considered to be an indicator species of disturbed areas (Morato et al. 1992; Peruquetti et al. 1999; Nemésio and Silveira 2007).

In the last 20 years, geometric morphometrics of wings has become an important tool for the identification and evaluation of insect diversity,

especially in bees (e.g., Francisco et al. 2008; Franco 2011; Franco 2016). This method describes shape by using the Cartesian coordinates of landmarks plotted on an image of a morphological structure and allows the researcher to reconstruct the shape of the studied structure. Because this approach employs a comprehensive statistical analysis to extract spatial information from morphological structures, it is more quantitative and accurate than traditional morphometrics (Bookstein 1998; Mitteroecker and Gunz 2009; Franco 2008, 2012). Geometric morphometric analysis of wings has proved to be a practical methodology for the discrimination of cryptic species (Francisco et al. 2008; Hurtado-Burillo et al. 2016) and geographic ecotypes (Franco 2011; Bonatti et al. 2014; Grassi-Sella et al. 2018) and for the identification of species (Franco 2009, 2012) and subspecies (Oleksa and Tofilski 2014; Silva et al. 2015). Its efficacy as a sensitive tool for differentiating among groups in these ways stems from wing shape being relatively conserved among individuals of the same species (Debat et al. 2003; De Souza et al. 2015). In this study, we used morphometric tools to investigate the population structure of *El. nigrita* sampled from nine different locations in the Atlantic Forest and Brazilian Savannas, in order to investigate species morphological responses to changes along with the tropical landscapes.

2. MATERIAL AND METHODS

2.1. Sampling

We used male individuals of *El. nigrita*, which were collected in Atlantic Forest remnants and Brazilian Savannas in the states of São Paulo (SP) and Mato Grosso do Sul (MS) (Table S1 and S2, Supporting information; Figure 1). Within the Atlantic Forest, we chose to consider different physiognomies. For Atlantic Rain forest, we collected bees in Parque Estadual Turístico do Alto Ribeira (PETAR - 24° 27' 36" S, 48° 36' 0" W) and in Ubatuba (UB - 22° 27' 0" S, 52° 10' 0" W). For Atlantic semi-deciduous forest, samples were collected in Estação Ecológica de Paulo de Faria (PF - 19° 57' 35" S, 49° 31' 46" W),

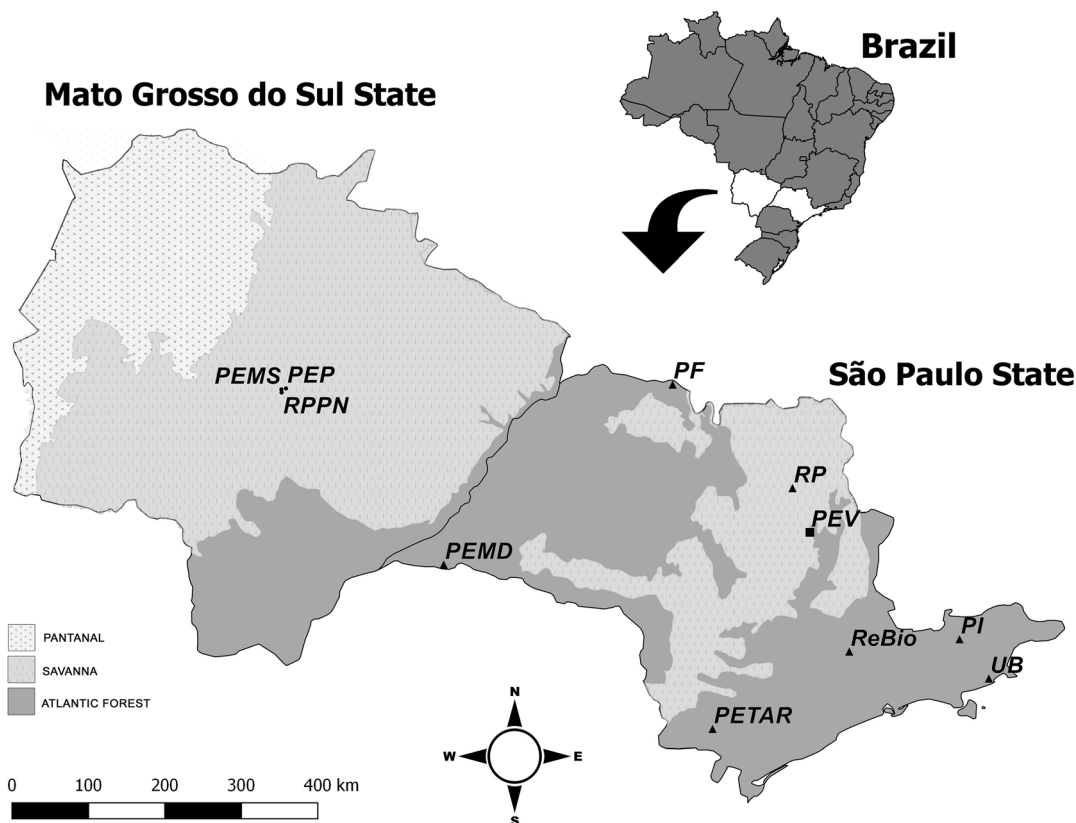


Figure 1. Sampling locations of the *Eulaema nigrita* in Atlantic Forest remnants and Brazilian Savannas in the states of São Paulo (SP) and Mato Grosso do Sul (MS). Estação Ecológica de Paulo de Faria (PF); Reserva Biológica da Serra do Japi (ReBio); Parque Estadual de Vassununga (PEV); Ubatuba (UB); Pindamonhangaba (PI); Parque Estadual Morro do Diabo (PEMD); Parque Estadual Turístico do Alto Ribeira (PETAR); Ribeirão Preto (RP); Campo Grande (CG). Black dots—samples collected in biome Atlantic Forest; black triangles—samples collected in biome Brazilian Savannas; black squares—samples collected in transition area Atlantic Forest and Brazilian Savanna in Parque Estadual de Vassununga (PEV).

Pindamonhangaba (PI - 22° 55' 50" S, 45° 27' 22" W), Parque Estadual Morro do Diabo (PEMD - 22° 27' 0" S, 52° 10' 0" W), Reserva Biológica da Serra do Japi (ReBio - 23° 13' 51" S, 46° 56' 06" W), Ribeirão Preto (RP): Mata de Santa Tereza (21° 12' 54" S, 47° 50' 36" W), and Campus USP (21° 09' 31" S, 47° 51' 55" W). Parque Estadual de Vassununga (PEV - 21° 43' 30" S, 47° 34' 60" W) is placed in a transition area between the Atlantic Forest and Brazilian Savannas. We also sampled bees in three urban forest remnants in Brazilian Savannas in Campo Grande (CG) – MS: Parque Estadual do Prosa (PEP - 20° 27' 09" S, 54° 33' 38" W), Parque Estadual Matas do

Segredo (PEMS - 20° 23' 51" S, 54° 35' 21" W), and Reserva Permanente do Patrimônio Natural da UFMS (RPPN - 20° 30' 30" S, 54° 36' 58" W).

Bees were collected from December 1997 to November 1998 and from January to December 1999 in PF; from April 2011 to February 2013 in ReBio and PEV; April 2014 to March 2015 in UB and PI; May 2014 to April 2015 in PEMD and PETAR; during July 2014 in RP – Mata de Santa Tereza and during August 2017 in RP – Campus USP. In Campo Grande (CG) – MS, males were sampled during November 2016 in PEP and PEMS and during March 2017 in RPPN. The sampling method was based on Rebêlo and

Garófalo (1991), with minor modifications. In brief, we used baits with scents prepared from cotton wool wrapped in gauze and tied with a string; they were fixed to tree branches at a height of 1.5 m above the ground and at a distance of 5–10 m apart. Bees were collected with entomological nets on sunny days from 8:00 to 15:00 h. For more details concerning the sampling methods, see Costa and Franco (2017).

2.2. Morphometric analyses

Geometric morphometrics To analyze patterns of wing venation, the right forewings of 256 *El. nigrita* males were mounted between a microscope slide and coverslip and photographed with a digital camera connected to a stereomicroscope. A tps file was built from the images using the software tpsUtil version 1.70 (Rohlf 2015) to prepare a dataset of the Cartesian coordinates of the plotted landmarks. Eighteen landmarks (Figure 2) were plotted on each wing using tpsDig2 version 2.26 (Rohlf 2015). Landmarks were chosen according to previous studies on males of *Euglossini* (Francoy et al. 2012; Quezada-Euán et al. 2015).

Data analysis Geometric morphometric analyses were conducted using the software MorphoJ version 1.06d (Klingenberg 2011), whereby they were Procrustes aligned to identify the points of shape variation. We first produced a Procrustes fit to eliminate variation caused by differences in size, position, and orientation of the wings, and the residuals of this regression were used as “size free” variables. These data were used as input in a principal component analysis (PCA), canonical variant analysis (CVA), and discriminant function analysis (DFA). Bees were grouped according to their biomes and physiognomies. A leave-one-out cross-validation test was performed to assess the accuracy of the data. We also calculated the Mahalanobis square distances between the centroids of the groups’ distribution and used it to construct a dendrogram of morphological proximity based on the neighbor-joining algorithm using MEGA 7.0 (Kumar et al. 2016). We ran a Mantel test using statistical package R version 3.3.3 (R Development Core Team 2017) on the

Mahalanobis square distances between the centroids of the groups and the geographic distances among the sampling locations (measured by Google Earth 7.3.0.3832/2017). We also employed the Mantel test on the Procrustes distances and the geographic distances between sites. We also used the wing centroid size as a proxy for body size (Zelditch et al. 2004; Outomuro and Johansson 2011) to assess whether bees from different populations differed in size (Rattanawanee et al. 2017). The centroid size was compared among biomes and physiognomies. This data was first tested for its normality and, as the distribution did not fit a normal distribution, they were compared using ANOVA on Ranks, on software Sigmatat version 3.5. The pair-to-pair comparison was made using Dunn’s test, with $\alpha = 0.05$

3. RESULTS

Statistical differences were found in the mean shape of the wing of *El. nigrita* among the biomes (Table 1) and also among physiognomies (Table S3, Supporting information). The first 18 factors of these measures explained 90.98% of the total data variability, with the first two PCs explaining 29.54% of the total data variability (PC1 explained 16.17% and PC2 explained 13.38%; Figure S1, Supporting information). The two first canonical variate functions were significant for the discrimination of bees from different areas ($p < 0.0001$; Figure 3). The scatterplot (Figure 3) shows a subtle separation among areas, although some overlapping can be observed. Based on the first CVA axes (Figure 3), we found two groups, one placed in the left-hand quadrant, Atlantic Forest, and another group in the right-hand quadrant with Brazilian Savannas. Bees from the transition area overlap with individuals from the other two areas. This result is confirmed when bees were grouped according to their physiognomies (Figure 4), in which two groups were found, where one group is placed in the left-hand quadrant, semi-deciduous Forest and rain Forest (physiognomies from Atlantic Forest), and the other group in the right-hand quadrant with Brazilian Savannas; the transition area overlaps with other areas. The discriminant analysis

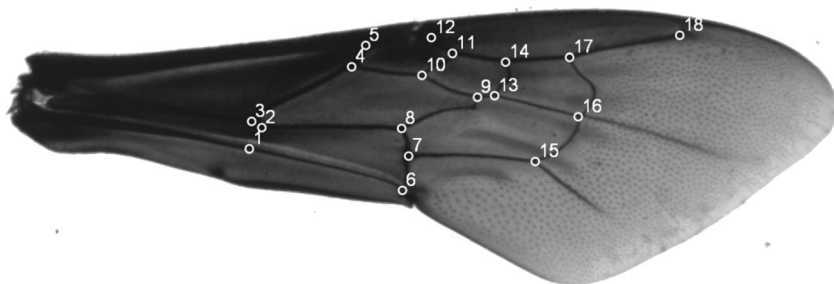


Figure 2. Forewing of *Eulaema nigrita* males, with 18 landmarks used in the geometric morphometric analysis.

showed 91.37% and 89.18% accuracy, for biomes (Table S4, Supporting information) and physiognomies (Table S5, Supporting information) respectively. Assignment of the specimens to geographical areas correctly classified approximately 68% of the total analyzed bees, which were grouped according to their biomes and physiognomies (cross-validation test showed 68.91% and 67.67% accuracy, for biomes and physiognomies respectively) (Tables S4, S5, Supporting information).

The two dendrograms of morphological proximity presented a similar topology (Figure 5), indicating similar relationships among populations. Figure 5a shows the transition area close to Atlantic Forest and Savanna in the external branch. A similar topology appeared when we analyzed morphological proximity about physiognomies (Figure 5b), rainforest, and semi-deciduous forest are placed together in one branch, close to the transition area, and Savanna remained outside

the other physiognomies. The Mantel test correlating the geographic distances between fragments and the Mahalanobis squared distance and Procrustes distance between groups presented non-significant results ($r = 0.368$, p values = 0.067 and $r = 0.379$, p values = 0.078, respectively).

The analysis based on the centroid size indicated that there were statistically significant differences among bees from different physiognomies ($p = 0.000084$) and among biomes ($p = 0.00041$). Bees from Brazilian Savanna were smaller than the ones from the transition area and also from the other physiognomies and biomes (Figure 6a, b, respectively). No statistically significant differences were found among the other groups in both analyses, biomes, and physiognomies.

4. DISCUSSION

Area and distance among different landscapes are the main factors used to explain species

Table I. Mahalanobis squared distances (below diagonal) and Procrustes distances (above diagonal) between mean wing shapes of the *Eulaema nigrita* from different Brazilian biomes. Respective p values, given in brackets, were obtained from permutation tests (10,000 permutation runs). *Transition area Atlantic Forest and Brazilian Savanna in Parque Estadual de Vassununga (PEV)

	Transition area*	Savannas	Atlantic Forest
Transition area*		0.012 (0.0002)	0.009 (0.0014)
Savannas	2.439 (< 0.0001)		0.013 (< 0.0001)
Atlantic Forest	1.649 (0.0234)	2.352 (< 0.0001)	

diversity (McCoy and Mushinsky 1994) and population dynamics (Alder 1994; Aldaz 1994). Among the tools used to evaluate population dynamics, geometric morphometrics is especially promising in studies of insects due to its efficiency and low cost to benefit ratio. In orchid bees and other bees, geometric morphometric analysis of landmark Cartesian coordinates is a useful approach in studies of macro- and micro-evolution, being able to distinguish both species and populations. For example, geometric morphometrics of wings was used to identify species of *Euglossa* genus (Francoy et al. 2012). It was similarly employed to evaluate population structuring associated with landscape and habitat isolation of two species in Atlantic Forest, *Euglossa annectans* and *Euglossa truncata* (Grassi-Sella et al. 2018). The geometry of wing shape is less subject to environmental influence and more informative, in evolutionary terms (Dujardin 2008; Jirakanjanakit et al. 2008) than analysis of wing size (traditional morphometrics). Although there is a genetic basis to size variation, it can be labile and influenced by environmental features, e.g., temperature (Gibert et al. 2004; Polak et al. 2004) or relative humidity (Morales-Vargas et al. 2010) or food availability and/or quality (Quezada-Euán et al. 2011). Since there is a relation between wing size and body size in bees (Bullock 1999), our centroid size analysis indicated a variation in the size of individuals from different physiognomies and biomes. It is known that the pollen offer throughout the year can influence the body size of *El. nigrita* individuals (Peruquetti 2003). Brazilian Savanna biome presents a seasonal variation in plant activities, with an increasing pollen availability during the wet season (Batalha and Martins 2004; Leite et al. 2006). It may reflect directly on the smaller final mean size of males throughout the year. The same observation was made when comparing human-disturbed environments to undisturbed ones (Pinto et al. 2015).

However, despite the differences in size, which can be due to the above-explained factors, this study found an overall wing shape similarity among *El. nigrita* populations, and a lack of population differentiation using wing shape data. Even though the sampling areas

are up to 1000 km apart, discriminant analysis for each of the nine *El. nigrita* geographical populations (Figure S2, Supporting information) indicated slight dissimilarities between all populations, although the scores of Mahalanobis and Procrustes distances were not correlated with geographical distance. This result is corroborated by the Mantel test, which showed no significant correlation between the morphological and geographical distances. Consequently, if there is micro-differentiation among *El. nigrita* populations, geographic distance seems to be an unimportant component that could explain the intra-specific morphometric variability. At the site sampling (or population) level, the morphospace of the first two canonical variables showed that there was no clear-cut separation between the groups.

Previous studies have shown that Euglossini bees have a high capacity to fly long distances (Janzen 1971; Kroodsmá 1975; Ackerman et al. 1982), which is one of the major characteristics of this tribe and is a plausible explanation for the absence of population structure and the similarity of these bee populations. Raw (1969) reported *El. nigrita* as a high dispersal species. Euglossine males fly long distances to search for nectar, but also for the collection of floral fragrances, which can play the role in sexual selection (Janzen 1971; Kroodsmá 1975; Ackerman et al. 1982; Roubik and Hanson 2004).

Our results agree with previous observations that orchid bees have a weak population structure (Grassi-Sella et al. 2018). Similar to this previous study, our sampling sites are surrounded by urban areas and large monocultures, which could be a barrier for the dispersal of the individuals. Nonetheless, we observed high similarity among all groups. As demonstrated by Dick et al. (2004), long-distance dispersal can contribute to the maintenance of gene flow, although other studies indicate that the flight range among orchid bees can vary considerably (Wikelski et al. 2010; Pokorny et al. 2015). Analysis of mitochondrial and microsatellite polymorphisms of some *El. nigrita* populations of our study show that there is no detectable population structuring and high genetic diversity, with continuous

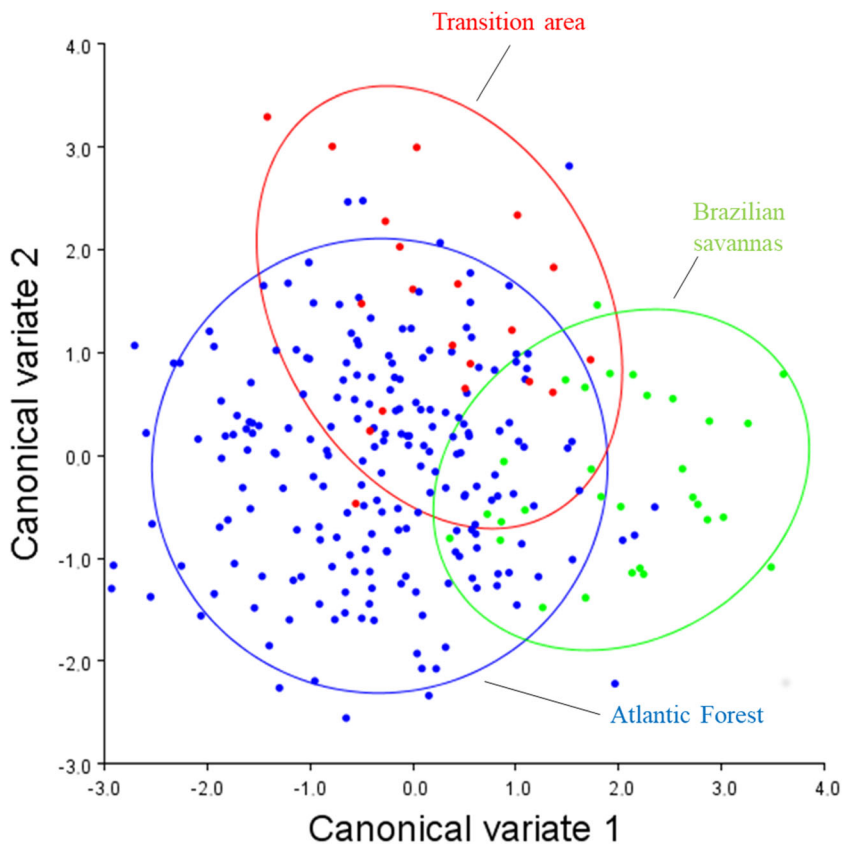


Figure 3. Two-dimensional ordination of *Eulaema nigrita* bees from Atlantic Forest remnants and Brazilian Savannas in the states of São Paulo (SP) and Mato Grosso do Sul (MS) based on canonical variate analysis (CVA). Transition area Atlantic Forest and Brazilian Savanna in Parque Estadual de Vassununga (PEV). Scatterplot of individual scores from the canonical variant analysis of landmarks when bees were grouped according to their biomes; the first two canonical variate functions explained 76.26% and 23.74% of the between-group variance, respectively.

gene flow occurring naturally among them (unpublished results). This corroborates other studies that have shown that euglossine bees exhibit high genetic diversity and low population structure (Souza et al. 2010; Cerântola et al. 2011; Zimmermann et al. 2011; Suni and Brosi 2012; Rocha-Filho et al. 2013). Dick et al. (2004) observed identical mitochondrial DNA haplotypes in cross-Andean populations of *El. nigrita*. Another study found no significant genetic differentiation between samples of *El. nigrita* from island and mainland, as well as *Eulaema cingulata* and *Euglossa cordata* (Rocha-Filho et al. 2013), corroborating the idea that the distance between the areas did not constitute a barrier for these species.

However, a slight eco-region pattern in wing size and shape variation was detected when populations were pooled. It is worth mentioning that our sampling sites are located within the Atlantic forest fragments, Brazilian Savanna areas, and a transition area between these biomes. Both areas are among the top 25 global biodiversity hotspots due to the high number of endemic species, many of which live under the constant threat of decline (Myers et al. 2000). When we analyzed the dendrograms of morphological similarity, built based on both the Mahalanobis and Procrustes distances (Figure S3, Supporting information), we observe the same pattern, with aggragation following environmental characteristics. This

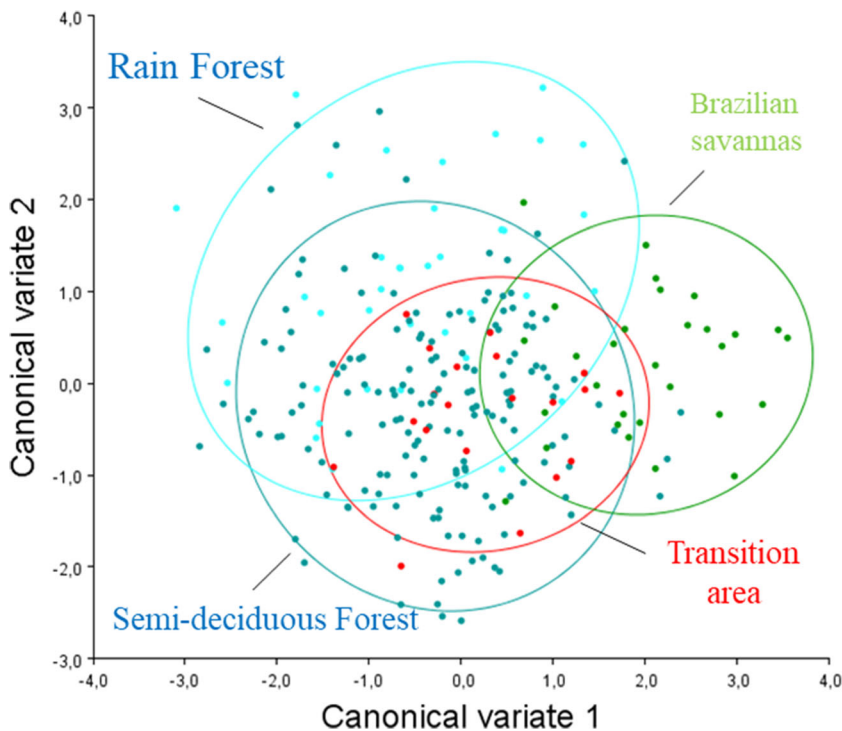


Figure 4. Two-dimensional ordination of *Eulaema nigrita* bees from Atlantic Forest remnants and Brazilian Savannas in the states of São Paulo (SP) and Mato Grosso do Sul (MS) based on canonical variate analysis (CVA). Transition area Atlantic Forest and Brazilian Savanna in Parque Estadual de Vassununga (PEV). Scatterplot of individual scores from the canonical variate analysis of landmarks when bees were grouped according to their physiognomies; the first two canonical variate functions explained 58.33% and 23.93% of the between-group variance, respectively.

tendency of grouping by landscape features may be due to phenotypic plasticity, a common feature found in insects (Whitman and

Agrawal 2009) or to the existence of locally adapted traits that are kept due to the philopatric behavior of Euglossini females

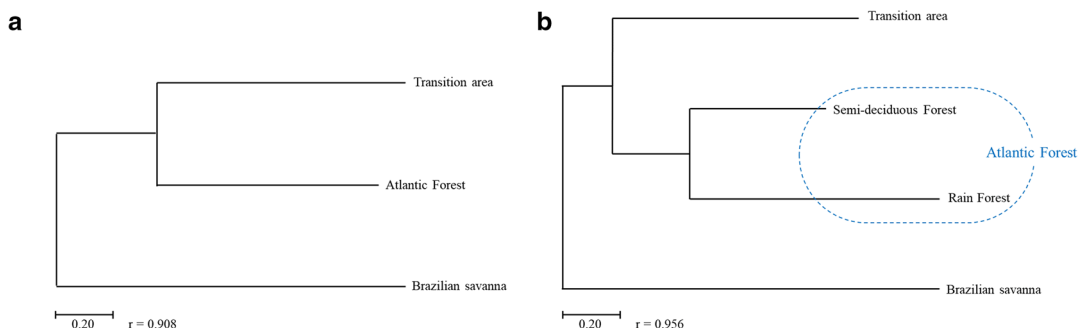


Figure 5. Neighbor-joining dendrogram of *Eulaema nigrita* bees constructed based on the Mahalanobis squared distances between the centroids. Transition area Atlantic Forest and Brazilian Savanna in Parque Estadual de Vassununga (PEV). **a** Neighbor-joining dendrogram of *E. nigrita* when were grouped according to their biomes. **b** Neighbor-joining dendrogram of *E. nigrita* when bees were grouped according to their physiognomies.

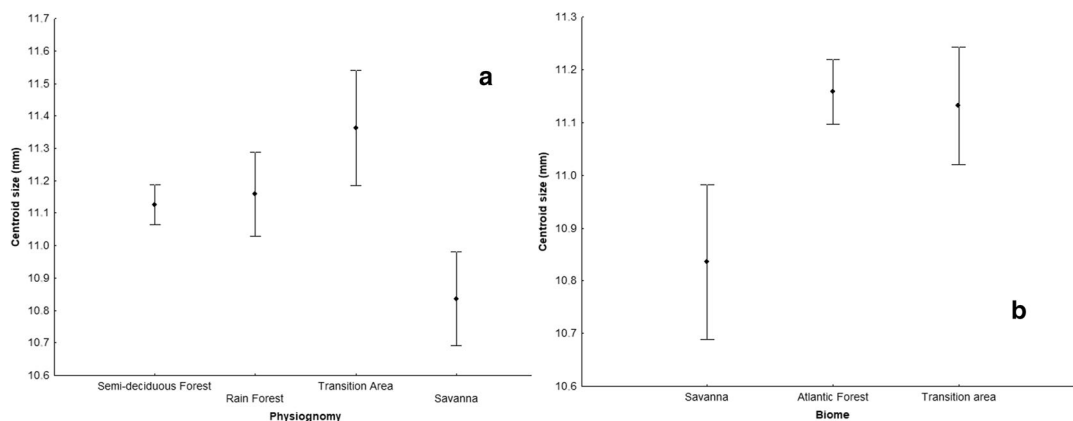


Figure 6. Mean size and standard deviation of the centroid size of *Eulaema nigrita* wings from different **a** physiognomies and **b** biomes.

(Garófalo 1992; López-Urbe et al. 2008). Similar results are observed in other orchid bee species (Grassi-Sella et al. 2018), and these results and others studies (Janzen 1971; Kroodsmá 1975; Ackerman et al. 1982) support the hypothesis that males of some euglossine species are able to fly between distant locations, through biomes, likely covering distances that are greater than the closest points between two areas.

For *El. nigrita*, samples from the transition area overlap with samples from different areas, especially for physiognomies like Atlantic semi-deciduous forest and part of Brazilian Savanna. This result corroborates the assertion that the Euglossini fauna from Savannas is a subset of forest species (Anjos-Silva 2008; Faria and Silveira 2011; Martins et al. 2018) given that the specimens from our study and others were captured in the gallery forests of the Brazilian Savannas. According to Martins et al. (2018), the gallery forests of the Brazilian Savannas could constitute an essential route for dispersion of orchid species. Besides, high discrimination rates for biomes and physiognomies were found, but in the cross-validation test, the rate of correct classification was somewhat lower, whose results were very similar to those found in the literature for different populations of other orchid bees species (Grassi-Sella et al. 2018), indicating high dispersion rates for some species of this tribe.

In summary, morphological distances among populations and morphological diversity of *El. nigrita* do not seem to be affected by geographical linear distances; however, the effect of environment on wing traits needs further evaluation to elucidate patterns of environmental gradients acting upon *El. nigrita* across its distribution. It is already known that *El. nigrita* individuals sampled in the same place in different seasons (rainy and dry) and in different years (15 years span) do not present significant shape differences (unpublished results) but further studies are needed to clarify the effects of different environmental characteristics over these morphometric features. Finally, the results of our study expand knowledge of the euglossine population structure, information useful to guide conservation strategies for this group.

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AUTHORS' CONTRIBUTIONS

CPC: data acquisition, data analysis, and wrote the paper. TMF: supervisor and wrote the paper. CCASM, RPD, WMSS, and CAG: data acquisition. All authors read and approved the final manuscript. Funding information

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La variabilité morphologique de l'abeille à orchidée *Eulaema nigrita* (Hymenoptera, Apidae, Euglossini) peut s'expliquer par la variation du biome, mais pas par la distance entre les populations.

Euglossini / morphométrie géométrique / savane brésilienne / forêt atlantique.

Morphologische Variabilität bei der Prachtbiene *Eulaema nigrita* (Hymenoptera, Apidae, Euglossini) kann durch Variation des Bioms erklärt werden, aber nicht durch die Distanz zwischen Populationen.

Euglossini / geometrische Morphometrie / Brasilianische Savanne / Atlantischer Wald.

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