Geometric morphometrics of the forewing shape and size discriminate *Plebeia* species (Hymenoptera: Apidae) nesting in different substrates

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Abstract. Historically, studies evaluating morphological diversity in stingless bees (Hymenoptera, Apidae: Meliponini) by geometric morphometrics have been used to successfully discriminate taxa and/or populations. Moreover, the use of geometric morphometrics to evaluate phylogenetic morphological variation among stingless bee species has received less attention. Here, we used geometric morphometrics to assess taxonomic discrimination and putative phylogenetic signals for six diapausing stingless bee species (*Plebeia*) occurring in southern Brazil. In all, 12 landmarks were captured from forewings of *P. droryana*, *P. saiqui*, *P. emerina*, *P. remota*, *P. nigriceps* and *P. wittmanni*. Our data show that the centroid size of the forewings reliably discriminated, for example, between *P. droryana* and *P. emerina* from *P. saiqui*. Moreover, this trait does not have a significant phylogenetic signal. In turn, we found that the overall accuracy in discriminating between the six *Plebeia* species according to forewing shape was 84%, while the confusion matrix achieved 71%. Interestingly, our discriminant analysis separated *Plebeia* species nesting in tree cavities from those nesting under granitic rocks. The latter group has second cubital (landmarks = 5, 6, 7), first medial (landmarks = 2, 3, 8) and first submarginal cells (landmarks = 3, 4, 9, 10) that are larger than those of species nesting in trees. The forewing shape showed a strong phylogenetic signal, therefore suggesting that its variation may be due to an evolutionary history shared between *Plebeia* species studied here rather than to environmental features. This work sheds light on the value of forewing size and shape attributes in discriminating *Plebeia* species within same genus. We suggest that landmarks separating different taxonomic groups could be incorporated into dichotomous keys to help in identifying clades of complex resolution.

Introduction

In recent decades, most studies investigating the morphological variation of insects have incorporated geometric morphometrics (GM) using Cartesian geometric coordinates rather than linear measurements (Tatsuta *et al.*, 2018). Thus, GM has been an

important analysis tool in evaluating the morphological diversity in insects. As such, changes in the shape and size of their wings, genitals, mandibles and other structures have helped scientists to answer questions on relevant evolutionary, phylogenetic and ecological issues (Tatsuta *et al.*, 2018). For example, insect wings are two-dimensional and their intersections can be considered as homologous landmarks; thus, a comparative analysis may be utilized (Bookstein, 1991; Klingenberg *et al.*, 2002; Žikić *et al.*, 2017). Many insect orders have already been subjected to GM, but Diptera, Coleoptera and Hymenoptera are the three that have studied in the greatest detail (Tatsuta *et al.*, 2018).

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Among Hymenoptera, the Anthophila clade (bees) is the taxon that has been subjected most thoroughly to GM analyses (Aytekin *et al.*, 2007; Tofilski, 2008; Francoy *et al.*, 2009; Owen, 2012; Barour & Baylac, 2016; Falamarzi *et al.*, 2016). Although a wide range of bee taxa have been studied this way (Aytekin *et al.*, 2007; Tofilski, 2008; Francoy *et al.*, 2009; Owen, 2012; Falamarzi *et al.*, 2016), the stingless bees (Apidae: Meliponini) have received special attention from bee researchers (Francoy *et al.*, 2009; Combey *et al.*, 2013; Vijayakumar & Jayaraj, 2013). Most approaches have involved the use of GM as a taxonomic or ecological tool to discriminate populations, lineages, species and genera of stingless bees (Francisco *et al.*, 2008; Nunes *et al.*, 2012; Bonatti *et al.*, 2014; Lima *et al.*, 2016; Sousa *et al.*, 2016; Galaschi-Teixeira *et al.*, 2018).

Geometric morphometrics has proved to be a successful tool in evaluating the taxonomic and population diversity of stingless bees and other taxa (Francoy *et al.*, 2009; Combey *et al.*, 2013; Vijayakumar & Jayaraj, 2013; Rattanawannee *et al.*, 2015; Halcroft *et al.*, 2016; Lima *et al.*, 2016). However, there are no studies evaluating whether both size and shape of wings in different stingless bees are correlated with phylogenetic relationships. If there is no character displacement, it is expected that homologous traits of closely related species will tend to be more similar to each other than those of more distantly related species. Hence, if wing size and shape in stingless bee species follow this pattern, there should be a strong phylogenetic signal in these morphological attributes. Phylogenetic signal has been used in GM to investigate whether the size and shape of morphological structures of organisms have evolved due to a shared evolutionary history or whether they have evolved due to environmental features (Sidlauskas, 2008; Klingenberg & Gidaszewski, 2010; Monteiro, 2013).

In the present study, we employ both GM and phylogenetic comparative methods to analyse the morphological variation in forewing (FW) size and shape of closely related species of *Plebeia* Schwarz. To date, taxonomic and population approaches have been applied to investigate the morphological diversity of some *Plebeia* species based on GM of FW shapes (Francisco *et al.*, 2008; Francoy *et al.*, 2009). However, there are no studies incorporating both approaches to this genus or to other stingless bee taxa. Therefore, we were interested in evaluating whether closely related species of *Plebeia* could be accurately discriminated according to the size and shape of their FWs. Furthermore, we assess the power of phylogenetic signal on these traits throughout the phylogeny of these species, i.e. whether the observed variation in FW size and shape owes more to a shared evolutionary history than to environmental features.

As a whole, *Plebeia* is a polyphyletic taxon that is closely related to the genera *Friesella* Moure and *Lestrimelitta* Friese (Costa *et al.*, 2003; Rasmussen & Cameron, 2010). The *Plebeia* phylogeny has been revised and widely investigated by means of molecular analysis (Werneck, 2016). The genus *Plebeia* is largely distributed in the Neotropical region and comprises 40 species (Camargo & Pedro, 2013). In Brazil, *Plebeia* is represented by 19 species (Pedro, 2014), and it is believed that many more species remain to be described and/or identified (Silveira *et al.*, 2002; Werneck, 2016). *Plebeia* species exhibit a distinctive behaviour in southern Brazil, known as reproductive diapause, where mother queens periodically and gradually decrease and cease egg-laying during the colder months (Santos *et al.*, 2014, 2015). Finally, the taxonomic identification of *Plebeia* individuals is problematic because it is based on the presence or absence of yellowish markings in their frons, which is a weak trait to discriminate different stingless bee species.

Material and methods

At least eight *Plebeia* species are known to exist in the state of Rio Grande do Sul (Brazil) (SEMA, 2014). Of these, there were six species in our scientific collection (Museu de Ciências e Tecnologia, MCT) at the Pontifical Catholic University of Rio Grande do Sul (PUCRS), with enough specimens with available FWs for us to carry out our study. These were *P. droryana* (Friese), *P. emerina* (Friese), *P. nigriceps* (Friese), *P. remota* (Holmberg), *P. saiqui* (Friese) and *P. wittmanni* Moure & Camargo (Table 1; Fig. S1). We selected 30 individuals per species (Table 1), totalling 180 specimens. Outliers were detected using the 'plotOutliers' function in the package GEOMORPH (Adams *et al.*, 2017); we did not include these individuals in later analyses. As far as possible, we strove to include specimens from different years (from 1984 to 2018) and/or from distinct localities (29 municipalities; Fig. S2; Table S1) to avoid pseudoreplication.

Morphometric analysis

We removed the right FW from each specimen using tweezers and placed them temporarily between slides and cover slips, naming them according to voucher number. Then, the FWs were photographed with a digital camera coupled to a high-end stereo microscope (Leica DMC 2900, Singapore). Eleven of the landmarks (LMs) sampled (homologous anatomical points) were type I LMs (sensu Bookstein, 1991), and one (LM 12, maximum curvature of veins) was a type III LM. They were manually digitized twice independently by different persons using the software TPSDIG2 (Rohlf, 2005) (Fig. 1). We used R v.3.5.1 (Ihaka & Gentleman, 1996; R Core Team, 2018) for subsequent statistical analyses.

The TPS file encompassing the FW coordinates of *Plebeia* specimens was used to generate a generalized Procrustes analysis (GPA) using the 'gpagen' function in GEOMORPH (Adams *et al.*, 2017). Generalized Procrustes analysis involves transformations and superimpositions in the data matrix from individuals, including translation, rotation and isotropic rescheduling, providing an alignment that allows for comparison of the optimized shape from specimens in which the mean generates a consensus matrix (Adams *et al.*, 2017). Specifically, GPA translates all specimens to an origin, rescales them to a centroid size (CS) and optimally rotates them by means of least squares up to the coordinates of corresponding points, aligning as closely as possible resulting in a shape represented in a curved space related to Kendall morphospace (Kendall, 1984; Adams *et al.*, 2017). The

Table 1. *Plebeia* species (eight) searched in stingless bee apiaries or museums of scientific institutions in Rio Grande do Sul (southern Brazil) and those species (six) analysed in the present study.

Species	Bees evaluated	Nesting preference
Plebeia catamarcensis (Holmberg, 1903)	NA	–
Plebeia droryana (Friese, 1900)	30	Tree cavities
Plebeia emerina (Friese, 1900)	30	Tree cavities
Plebeia meridionalis (Ducke, 1916)	NA	–
Plebeia nigriceps (Friese, 1901)	30	Granitic rocks
Plebeia remota (Holmberg, 1903)	30	Tree cavities
Plebeia saiqui (Holmberg, 1903)	30	Tree cavities
Plebeia wittmanni Moure & Camargo, 1989	30	Granitic rocks

NA, not applicable, i.e. the number of individuals was insufficient for our purposes (≤ 10).
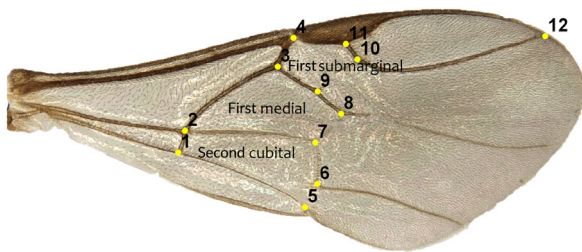

Fig. 1. Landmarks ($N = 12$) on the forewing of *Plebeia droryana*. [Colour figure can be viewed at wileyonlinelibrary.com].

CS is based on the square root of the sum of squared distances between the centre of the object and its landmarks extracted from Procrustes coordinates (Bookstein, 1991).

Regression modelling

To remove the influence of size on a shape that could distort the differentiation of *Plebeia* species, a regression was necessary to analyse the effect of allometry. Such a covariation measure refers to the size-related changes of morphological traits and remains an essential concept in the study of evolution and development (Klingenberg, 2016). Therefore, the patterns of shape covariation with size for a set of superimposed coordinates were tested using the function 'procD.allometry' (Adams *et al.*, 2017). In this function, the FW shape was set as the response variable, and the CS (log-transformed) was used as a predictor variable; *Plebeia* species were assigned as the grouping variable. The allometry test was permuted 9999 times to randomize the residuals for significance testing.

Centroid size is at least theoretically independent of shape, but the shape can be predicted for any CS if there is allometry (Viscosi & Cardini, 2011; Klingenberg, 2016). A residual shape, which is the deviation from the prediction, remains. The residual part of the shape does not covary with the CS or actual size (Klingenberg, 2011). Therefore, as this analysis was significant, our later analyses were performed with allometry-free shapes.

Centroid size in Plebeia *species*

The difference in CS between *Plebeia* species was tested using the function 'procD.lm' (Adams *et al.*, 2017). After that, we performed a pairwise test using the 'permudist' function (p.adjust.method = 'holm') in the MORPHO package (Schlager, 2017). This function compares the distance between two group means with the distances obtained by random assignment (here, 9999 permutations) of observations to these groups (Schlager, 2017).

Discriminant analysis and MANOVA

We performed a discriminant analysis to evaluate how well *Plebeia* species could be discriminated according to FW shapes. This was done using the 'lda' function (method = 'mle') in the MASS package (Venables & Ripley, 2002). We performed cross-validation (leave-one-out) to assess the overall accuracy of the data and to estimate the error rates (Viscosi & Cardini, 2011) after 9999 permutations. We performed a Procrustes multivariate ANOVA using the 'procD.lm' function (Adams *et al.*, 2017) to assess whether *Plebeia* species could be differentiated according to patterns in FW shapes.

Phylogenetic signal and morphospace

As species share an evolutionary history, we expect that closely related *Plebeia* species retain some phenotypic similarities due to their shared ancestry. Thus, to evaluate whether the CS and FW shapes of *Plebeia* species contain any phylogenetic signal, we adapted the phylogenetic tree of *Plebeia* species from Werneck (2016). The tree file (Nexus) was transformed into a phylo file using the package APE (Paradis *et al.*, 2004) and subsequently into an ultrametric tree. For the analysis, we employed the consensus matrix of GPA to test for congruence between the size or shape of FW of *Plebeia* species and their evolutionary history following Klingenberg & Gidaszewski (2010).

We then used the function 'physignal' (9999 permutations) in GEOMORPH (Adams *et al.*, 2017), setting the CS and FW shapes as response variables and the *Plebeia* phylogeny as a predictor variable. The physignal function was used because it estimates the degree of phylogenetic signal from a set of Procrustes-aligned specimens present in CS or shape data for a given phylogeny. As a result, the *K*-statistic evaluates the degree of phylogenetic signal in a dataset compared with what is expected under a Brownian motion model of evolution. For geometric morphometric data, the approach is a mathematical generalization of the kappa statistic (Blomberg *et al.*, 2003) which is appropriate for highly multivariate data (Adams, 2014). Significance testing is conducted by permuting the morphometric data among the tips of the phylogeny. The phylogenetic signal of FW shapes was corrected for allometry-free shapes after removing the size effects as described earlier.

The phylogenetic signal of CS was projected onto a phylogenetic tree of *Plebeia* species using the function 'contMap' in PHYTOOLS (Revell, 2012). The phylogenetic signal

Table 2. Results of Procrustes regression for shape−size covariation (allometry) on forewings of *Plebeia* species.

Homogeneity of slopes test

	ResDf	RSS	SS	MS	Rsq	*F*	*Z*	Pr(>F)
Common allometry	173	0.133						
Group allometries	168	0.125	0.007	0.001	0.040	2.118	5.645	**1.00E−04**
Total	179	0.194						

Type I (Sequential) - sums of squares and cross-products
Randomized residual permutation (9999) procedure used

	Df	SS	MS	Rsq	*F*	*Z*	Pr(>F)
Log(size)	1	0.003	0.003	0.018	4.529	3.340	**1.00E−04**
Species	5	0.056	0.011	0.293	14.721	11.852	**1.00E−04**
Residuals	173	0.133	0.000	0.688			
Total	179	0.194					

Bold values mean statistical significances lower than 0.001.
ResDf, Residual degrees of freedom; RSS, residual sum of squares; SS, sums of squares; MS, mean squares; Rsq, R-squared (the coefficient of determination); F, the F-values; Z, the Z-values or standard score, i.e. standard deviations from their means. Positive values when raw score is above the mean; Pr(>|F|): probability to find F-values by chance; Df, degrees of freedom.

of FW shapes was projected onto a *Plebeia* phylogenetic tree with a multivariate morphospace (phylomorphospace) using the allometry-free shapes through the function 'plotGMPhyloMorphoSpace' (Adams *et al.*, 2017). This function generates a plot from the principal dimensions of tangent space for the set of Procrustes-aligned specimens (Adams *et al.*, 2017). Thus, it allows for visualization of the differences in FW shapes of *Plebeia* species in a phylogenetic context as a phylogenetic principal components analysis (Revell, 2009). In other words, phenotypic evolution can be visualized in phylomorphospace, where the extant taxa and the phylogeny are projected into the morphological trait space and visualized along the first two axes of this space using principal components analysis (Sidlauskas, 2008). Thereby, the resulting phylomorphospace illustrates both the magnitude and the direction of morphometric change inferred along each branch (Sidlauskas, 2008).

Shape means versus target Plebeia *species*

We performed a canonical variate analysis using the function 'CVA' in MORPHO (Schlager, 2017), where the CVA scores were used to plot the shape mean of FW based on the consensus matrix from GPA against the mean of each *Plebeia* species. For this, we used the function 'plotRefToTarget' (Adams *et al.*, 2017). Then, the differences among FW shapes were magnified five times to facilitate biological diagnostics, even though such a procedure must be interpreted with caution (Viscosi & Cardini, 2011).

Results

Allometry and centroid size

We found that FW shape of *Plebeia* species is significantly correlated with CS (Table 2). Therefore, all subsequent analyses were performed with allometry-free shapes. Overall, the

CS is different between *Plebeia* species (Procrustes ANOVA, $F_{5,174} = 30.52$, $P < 0.001$). We found that the CS of FWs was more similar between *P. droryana* and *P. emerina* than among *P. nigriceps*, *P. remota* and *P. wittmanni* (Fig. 2; Table 3). Additionally, there was no difference between the CS of *P. remota* and *P. saiqui* (Fig. 2; Table 3).

Discriminating Plebeia *species according to forewing shapes*

The discriminant analysis had an overall accuracy of 84%. As such, we have evidence that *Plebeia* species differ in the shape of their FW (Procrustes MANOVA, $F_{5,175} = 15.38$, $P < 0.001$). The main discrimination, however, was found between *Plebeia* species nesting in granitic rocks (red scales = *P. nigriceps* + *P. wittmanni*), which were separated from species nesting mainly in tree cavities (Fig. 3; Fig. S3). Thus, when shape differences between a reference (LD minimum, red line) were plotted against a target (LD maximum, black line and shadow), e.g. to LD1 (70.46%), we could see that the second cubital (LM = 5, 6, 7), first medial (LM = 2, 3, 8) and first submarginal (LM = 3, 4, 9, 10) cells are larger in the morphocluster composed of *Plebeia* species nesting in granitic rocks versus those nesting into trees (Fig. 3, lower subplot).

On the other hand, our confusion matrix (cross-validation) obtained 71% accuracy in properly identifying individuals to their correct *Plebeia* species. For example, *P. saiqui* had the highest discrimination power (83%), whereas *P. droryana* had the lowest accuracy (46%), sometimes being erroneously ascribed to several other species as *P. emerina, P. remota* and *P. saiqui* (Table 4).

Phylogenetic signal of forewing traits and mean shapes

We did not find a phylogenetic signal for the CS of FW of *Plebeia* species ($K = 0.32$, $P = 0.84$). This suggests that this trait
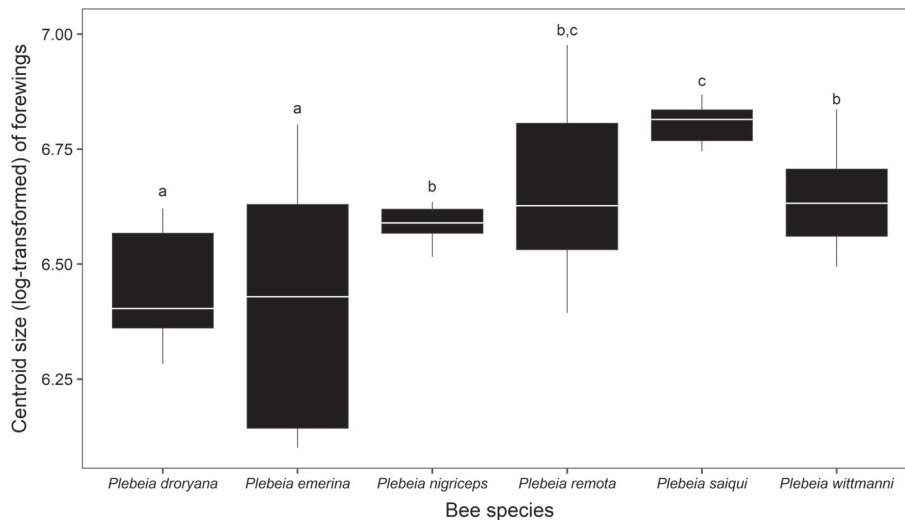
Fig. 2. Comparison between centroid sizes (log) of forewings of six diapausing stingless bee *Plebeia* species. Box, first and third quartiles; whiskers, minimum and maximum ranges of variation; median (white line), second quartile. Different letters represent significant differences (see Material & Methods for details).

Table 3. Permutation test for group differences [log(size) ~ species]. Here, we compare the distance between pairs of *Plebeia* species with the distances obtained by random assignment of observations to these taxa.

	Plebeia droryana	*Plebeia emerina*	*Plebeia nigriceps*	*Plebeia remota*	*Plebeia saiqui*	*Plebeia wittmanni*
Plebeia droryana		19.90	96.93	165.27	250.42	116.21
Plebeia emerina	1.000		113.84	182.18	267.32	133.11
Plebeia nigriceps	**0.031**	**0.006**		68.34	153.48	19.27
Plebeia remota	**0.001**	**0.001**	0.191		85.14	49.06
Plebeia saiqui	**0.001**	**0.001**	**0.001**	0.078		134.20
Plebeia wittmanni	**0.004**	**0.002**	1.000	0.488	**0.001**	

Upper diagonal, distances; lower diagonal, *P*-values (bold for significant values).
Bold values mean statistical significances lower than 0.05.

does not follow a Brownian model and, therefore, that the average size of centroid may have been acquired by convergence (Fig. 4). On the other hand, we found a strong phylogenetic signal for FW shape of *Plebeia* species ($K = 0.83$, $P = 0.02$; Fig. 5). This may be taken as evidence of the common evolutionary history of this trait (Fig. 5). In the phylomorphospace plot, the closely related species of *Plebeia*, such as *P. droryana*, and *P. emerina*, and *P. saiqui*, were found near one another (Fig. 5). Similarly, *P. nigriceps* and *P. wittmanni* are grouped together, whereas *P. remota* was isolated from all other species. This indicates that a strong magnitude and direction of morphometric change for FW shape emanated from a central morphological point [origin (0, 0), hypothesized ancestors].

Overall, the Procrustes-fitted shapes after canonical variance analysis of each *Plebeia* species compared with the mean shape demonstrated that *P. nigriceps* and *P. wittmanni* possess visually major differences with respect to other species mainly related to LMs 3, 4 and 8 (Fig. 6). Additionally, *P. remota* shows a great displacement of landmark 3 that is dislocated forward landmark 4 in contrast to the shape mean (Fig. 6). On the other hand, *P. droryana*, *P. saiqui* and *P.*

emerina show an opposite displacement, i.e. LM 4 was dislocated forward of LM 3 as compared with the shape mean (Fig. 6).

Discussion

We found that the difference in FW shape of six *Plebeia* species may be associated with allometry, i.e. the former trait covaries with augmentation in CS. Therefore, it is necessary to use only the shape residuals (i.e. allometry-free shapes) for evaluating morphological variation in the FW of *Plebeia* species. Furthermore, by incorporating GM with phylogenetic comparative methods, we were able to identify a significant phylogenetic signal on this trait, demonstrating that such an approach is important to finding evolutionary patterns.

Overall, our data demonstrate that there is variation in FW size as well as in shape of *Plebeia* species. Therefore, the morphological attributes analysed here may be used to successfully discriminate between these stingless bees in southern Brazil. For example, *P. droryana* may be erroneously ascribed to four other *Plebeia* species. However, when their FW shape is compared
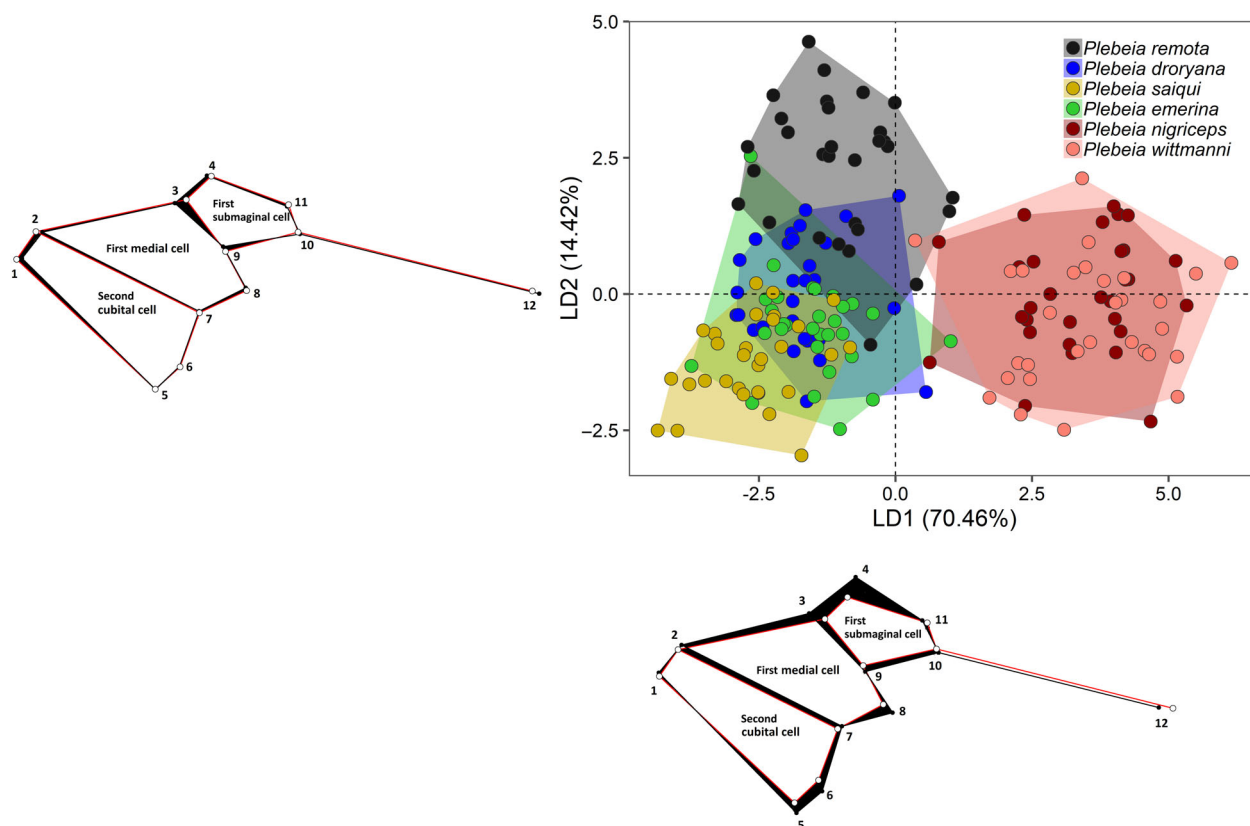
Fig. 3. Ordination of six diapausing stingless bee species, *Plebeia* spp., based on discriminant analysis. Polygons coloured red represent *P. nigricipes* and *P. wittmanni*, bees that nest in granitic rocks (or similar); other colours represent *P. droryana*, *P. emerina*, *P. remota* and *P. saiqui*, bees that nest in tree cavities (or similar). Lower and right subplots show LD1 minimum (red) and maximum (black), and LD2 minimum (red) and maximum, respectively. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 4. Confusion matrix (overall accuracy = 71%).

Actual	Predicted Plebeia droryana	Plebeia emerina	Plebeia nigriceps	Plebeia remota	Plebeia saiqui	Plebeia wittmanni
Plebeia droryana	**0.46**	0.23	0.00	0.06	0.23	0.00
Plebeia emerina	0.16	**0.60**	0.00	0.03	0.16	0.03
Plebeia nigriceps	0.03	0.03	**0.80**	0.00	0.00	0.13
Plebeia remota	0.10	0.10	0.00	**0.80**	0.00	0.00
Plebeia saiqui	0.10	0.06	0.00	0.00	**0.83**	0.00
Plebeia wittmanni	0.00	0.00	0.16	0.03	0.00	**0.80**

against the shape mean (red contour in Fig. 6), we see a great similarity with *P. emerina*. Moreover, LMs 5 and 8 (black points) from the FW of *P. droryana* slightly displace inwards of the second cubital and first medial cells, respectively, reducing its area and providing reliable attributes to discriminate both species pairs.

We also found that when the CS of FWs (not only their shape) of *Plebeia* species differs depending on the studied taxa, it can be useful as an additional attribute to help discriminate between distinctive taxa, as suggested by Aytekin *et al.* (2007). This procedure has also been successfully employed to differentiate stingless bee populations elsewhere (Francoy *et al.*, 2009, 2016;

Combey *et al.*, 2013; Halcroft *et al.*, 2016; Galaschi-Teixeira *et al.*, 2018). For example, although *P. droryana* can be mistaken for four other *Plebeia* species when the FW shape is not analysed in detail (cross-validation), the use of CS reduced this number to only one species (*P. emerina*). Furthermore, CS may corroborate those findings observed in the confusion matrix. For example, sister species such as *P. nigriceps* and *P. wittmanni* had a similar misclassification error rate of *c.* 80% after the cross-validation test. Both species were erroneously ascribed to each other and their CS was not significantly different.
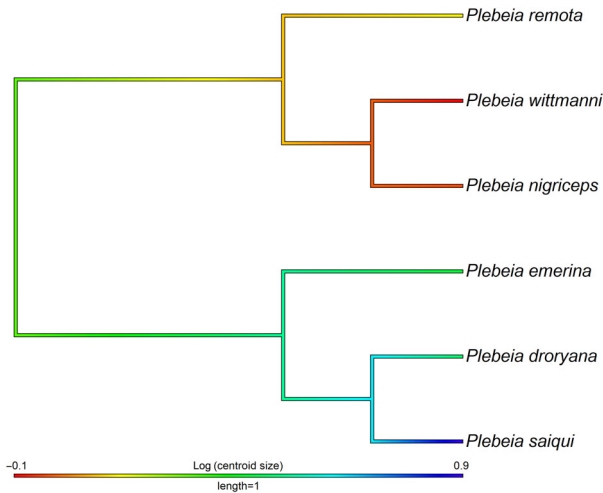
Fig. 4. Phylogenetic tree recovering the topology of six species of *Plebeia* (adapted from Werneck, 2016) mapped as continuous traits with ancestral states reconstructed by maximum likelihood estimation (graded colour). This plot is mainly intended to visualize the centroid size of forewings of *Plebeia* (red, low value; blue, high value), not to quantify the ancestral states. [Colour figure can be viewed at wileyonlinelibrary.com].

As the six *Plebeia* species evaluated in this study are phylogenetically closely related to each other, this may have contributed to masking the power of discrimination among these taxa. Hence, the addition of more LMs captured from forewings and/or hindwings could reduce the misclassification error rate found in this study. Here, we extracted 12 landmarks from the

FW of six *Plebeia* species. Nevertheless, historically, entomologists have obtained seven to 20 LMs from bee wings, depending of bold venation and its capacity to clearly discern as much as possible the homologous anatomical marks (Aytekin *et al.*, 2007; Combey *et al.*, 2013; Nunes *et al.*, 2013; Falamarzi *et al.*, 2016; Francoy *et al.*, 2016; Prado-Silva *et al.*, 2018). Further, other insect structures, such as genitalia, mandibles and head, are adequate attributes but are rarely used in GM for evaluating morphological variation in bees (Tatsuta *et al.*, 2018).

Interestingly, our discriminating analysis based on FW shape separated *Plebeia* species nesting in rocks or similar substrates (*P. nigriceps*, *P. wittmanni*) (Wittmann, 1989) from those living in tree cavities (*P. droryana*, *P. saiqui*, *P. emerina*, *P. remota*) (Michener, 2007). It is known that wing venation might affect bee flight in some way as wing flexibility results in different aerodynamic forces and affects the ability to take off (Mountcastle & Combes, 2013). Nevertheless, at this time, we cannot assert whether this is due to the common evolutionary history between *P. nigriceps* and *P. wittmanni* or, alternatively, if other ecological features, such as the ability to load food or nest material (for details see Polidori *et al.*, 2013), are applying any selective pressure to FW shapes of *Plebeia* species nesting in rocks.

We did not find any phylogenetic signal for wing CS, but we did find a strong phylogenetic signal for FW shape of *Plebeia* species visualized through the phylomorphospace. The phylomorphospace is a useful approach because it enables us to map the history of a clade's morphological diversification and to infer the magnitude and direction of shape change along branches of a given phylogeny (Sidlauskas, 2008; Klingenberg & Gidaszewski, 2010; Monteiro, 2013). As such, if a phylogenetic signal in wing shape is absent or weak, then closely
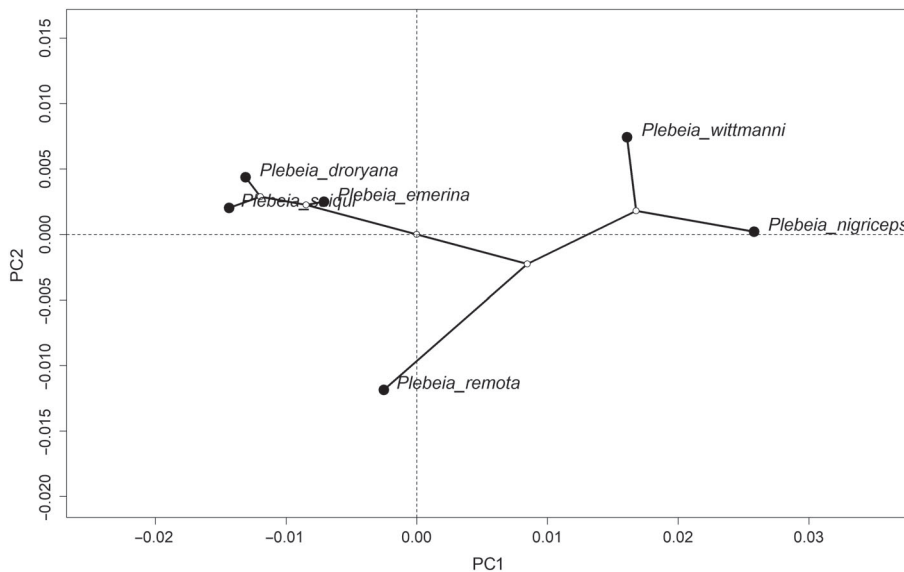

Fig. 5. Phylogeny of *Plebeia* adapted from Werneck (2016) depicted on the phylomorphospace of the consensus matrix of a generalized Procrustes analysis (GPA). Such a representation allows us to project the phylogeny into shape space in order to view evolutionary shape changes of forewings of *Plebeia* species obtained from the phylogenetic signal analysis. Here, the first two principal components of variation are shown.
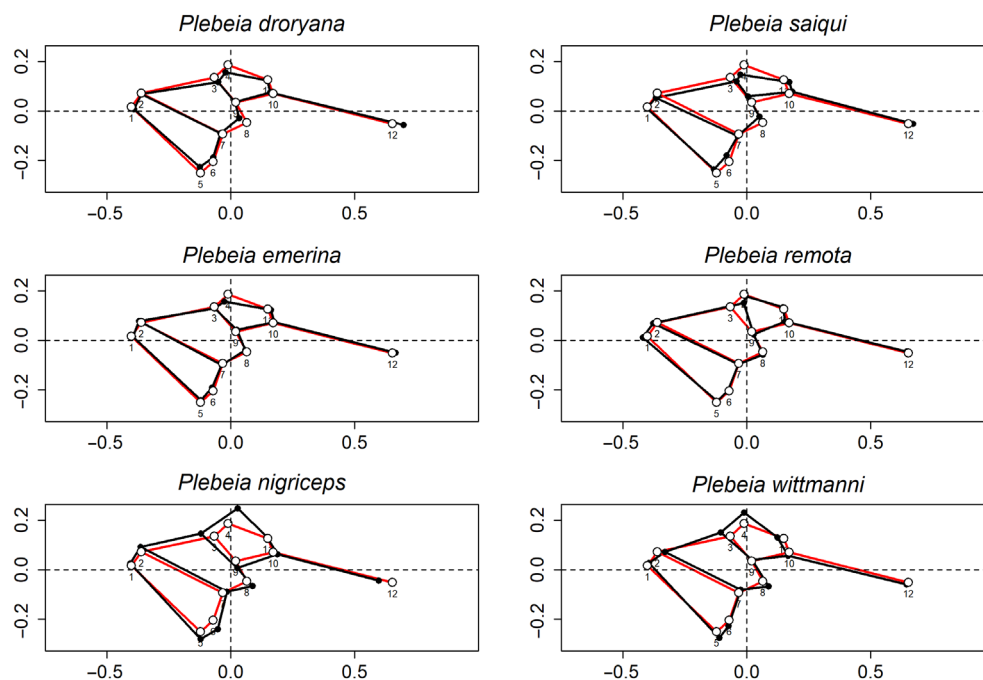
Fig. 6. All Procrustes-fitted shapes. The shapes are based on landmarks digitized onto interpretative drawings of the *Plebeia* specimens and must be interpreted with caution as shape differences have been magnified by a factor of five to aid in the description of shape differences and biological interpretation. Here, polygons coloured red represent shape mean of FW based on the consensus matrix from GPA against the mean (polygons coloured black) of each *Plebeia* species. [Colour figure can be viewed at wileyonlinelibrary.com].

related species tend to be far from one another in morphometric space (Sidlauskas, 2008; Klingenberg & Gidaszewski, 2010; Monteiro, 2013). On the other hand, if the phylogenetic signal is strong, it suggests that FW shapes of sister-species like *P. nigriceps* and *P. wittmanni* may be due to the evolutionary history shared between them rather than to environmental factors.

Conclusions

In summary, the use of phylogenetic comparative methods in geometric morphometric analysis was successful in helping us to understand how morphological variation in FW size and shape between closely related species of stingless bees (in this study, *Plebeia*) has evolved. As such, we can assume that both size and shape of FWs possess significant differences and power to discriminate between *Plebeia* species occurring in southern Brazil. Our main finding here is that second cubital (LM = 5, 6, 7), first medial (LM = 2,3,8) and first submarginal (LM = 3, 4, 9, 10) cells are larger in *Plebeia* species nesting in granitic rocks than in those nesting in trees, data that could be incorporated into new taxonomic keys. Obviously, our analysis can be improved if, in future studies, we add more specimens (perhaps freshly sampled in flowers or nests) and more *Plebeia* species inhabiting other localities in Brazil.

Finally, insect wings are one of the best morphological structures in GM analysis, because they are two-dimensional.

However, we suggest that other morphological attributes could be incorporated into similar analyses to improve accuracy in the discrimination of phylogenetically related species of stingless bees.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. *Plebeia* species evaluated in this study.

Fig. S2. Localities from state of Rio Grande do Sul, southern Brazil, based on geographical coordinates of municipalities where the specimens of the six species of *Plebeia* were sampled (yellow points). Note that the relative size of points indicates the proportion of *Plebeia* individuals sampled (details in Table S1).

Fig. S3. Morphological distance. Dendrogram using Mahalanobis distance between Plebeia species after canonical variate analysis. This clustering analysis did not properly recover the phylogeny suggested by Werneck (2016) for *Plebeia* species evaluated in this study. In fact, geometric morphometric analysis does not seem to accurately reconstruct the phylogeny of organisms (Rohlf, 1998; Klingenberg and Gidaszewski 2010, *but see* Zelditch et al. 1995, 1998). However, it may be useful for us to understand how shape

is evolving throughout the evolutionary history of distinct clades (Rohlf, 1998; Klingenberg & Gidaszewski, 2010) as depicted by phylomorphospace in the main text.

Table S1. Data on *Plebeia* species analysed in this study.

Acknowledgements

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