Original article

Integrated landmark and outline-based morphometric methods efficiently distinguish species of *Euglossa* (Hymenoptera, Apidae, Euglossini)

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Abstract – Morphometric methods permit identification of insect species and are an aid for taxonomy. Quantitative wing traits were used to identify male euglossine bees. Landmark- and outline-based methods have been primarily used independently. Here, we combine the two methods using five *Euglossa*. Landmark-based methods correctly classified 84% and outline-based 77%, but an integrated analysis correctly classified 91% of samples. Some species presented significantly high reclassification percentages when only wing cell contour was considered, and correct identification of specimens with damaged wings was also obtained using this methodology.

euglossine bees / species identification / morphometrics / wings / quantitative traits

1. INTRODUCTION

Found only in the Neotropical region, the euglossine bees are an important group of pollinators (Roubik and Hanson 2004; Moure et al. 2007). The males collect odors from a variety of floral and non-floral resources to use as pheromone analogs (Zimmerman et al. 2006). Because they interact with dozens of plant families, not only orchids, are both perennial and abundant, and comprise up to 25% of bee species in communities (Roubik 1989), euglossines play a large role in ecology and conservation. Males are attracted to chemical baits and most current field study is based on this gender. Little is known about the females and their biology (Roubik and Hanson 2004). Currently, one of the largest problems faced by researchers is the lack of taxonomic specialists to identify specimens (Silveira et al. 2006). This situation is slowly abating by development of keys and photographs, potentially used by non-specialists (e.g., Nemesio 2010; Roubik [stri.org/bioinformatics, *Euglossa, Eulaema, Exaerete, Eufriesea*, or Discoverlife.org, Orchid bees of French Guiana] and, furthermore, molecular studies with description of species-specific DNA sequences (Ramírez et al., 2010). In conjunction with such initiatives, user-friendly tools may diminish the work load for taxonomists.

Quantitative traits have long been used for bee taxonomy (Ruttner et al. 1978; Ruttner 1988; Francoy et al. 2006; 2008; 2009; Tofilski 2008; Bloch et al. 2010, among others), and are applied to orchid bees in the present work. Such traits can be analyzed using different morphometric approaches, for example "traditional morphometrics", which consists in applying multivariate statistics analysis to variables, such as length, width, counts, ratios, angles and height (Marcus 1990) and "geometric morphometrics", which capture the geometric form (shape + size) of the analyzed organ using landmarks or outline-based methods (Bookstein 1991; Rohlf and Marcus 1993). Geometric morphometrics is considered a powerful tool in shape analysis and, for several reasons, has been considered superior to traditional morphometrics (for discussion see Bookstein 1991; Rohlf and Marcus 1993; Bookstein 1997; Adams et al. 2004). Furthermore, it is an alternative and useful complement to even refined molecular characterization of species or populations (Smith et al. 2008).

In order to provide accurate bee species identification based on phenotypic traits, recent Neotropical studies serve as an example (Mendes et al. 2007; Francisco et al. 2008; Francoy et al. 2008; 2009; Francoy et al. 2011). A main conclusion from those studies is that wing morphology encompasses sufficient quantitative variability to be used as a marker to discriminate operational taxonomic units. Furthermore, wing morphology is an efficient quantitative marker to discriminate other insect species, as for example, the fly genus *Drosophila* (Moraes et al. 2004; Bubliy et al. 2008).

Despite effective species discrimination from application of landmark- or outline-based methods used independently, the combined results of these two methods have not been investigated. To provide a case study, we had three main objectives. First, we aimed to discriminate five species of the genus *Euglossa* (subgenus *Glossura*, *piliventris* group) by wing morphology using the *partial warps* landmark-based method. Second, we wished to introduce the use of outline-based morphometrics to study phenotypic variation in closely related euglossine bees (Roubik 2004). Third, we attempt to investigate whether combining morphological information from landmark and outline-based methodologies improves discrimination between species. Our ultimate goal was to estimate the correct identification rates of species based only upon wing morphology, which may provide an alternative taxonomic tool for bee species identification.

2. MATERIALS AND METHODS

We used approximately 30 male individuals each of *Euglossa chalybeata*, *Euglossa flammea*, *Euglossa ignita*, *Euglossa imperialis*, and *Euglossa orellana* from Panama and Ecuador. The right forewings were removed with a forceps, placed between microscope slides and photographed with a digital camera attached to a stereomicroscope.

We carried out two different morphometric analyses. The first is based on landmarks variation analysis and the second on outline contour of the wing cells.

For the partial warps analysis, we first built a .tps file from wing images using the software tpsUtil version 1.40 (Rohlf 2008a) and plotted 18 landmarks (Figure 1) in wing vein intersections using tpsDig2 version 2.12 (Rohlf 2008b). The images were then Procrustes aligned (Rohlf and Marcus 1993) and the partial warps and Uniform Components were calculated using tpsRelw version 1.45 (Rohlf 2007). Additionally, we calculated the centroid size of landmarks for each wing. All software used in the analysis is freely available at http://life.bio.sunysb.edu/morph/.

We used our measurements as variables in discriminant analysis (DA) followed by a leave-one-out cross-validation test with the statistical software Statistica (StatSoft, 2001).

For outline analysis, six wing cells (Figure 1) were outlined using image processing software. To quantify shape variability, each cell outline was described by the means of elliptic Fourier descriptors (Kuhl and Giardina 1982). We used 25 harmonics to generate morphological coefficients, used as input in principal components analysis (PCA). The elliptic Fourier descriptors and PCA were performed using the SHAPE package (Iwata and Ukai 2002). The area of contour was used as a size measurement. In order
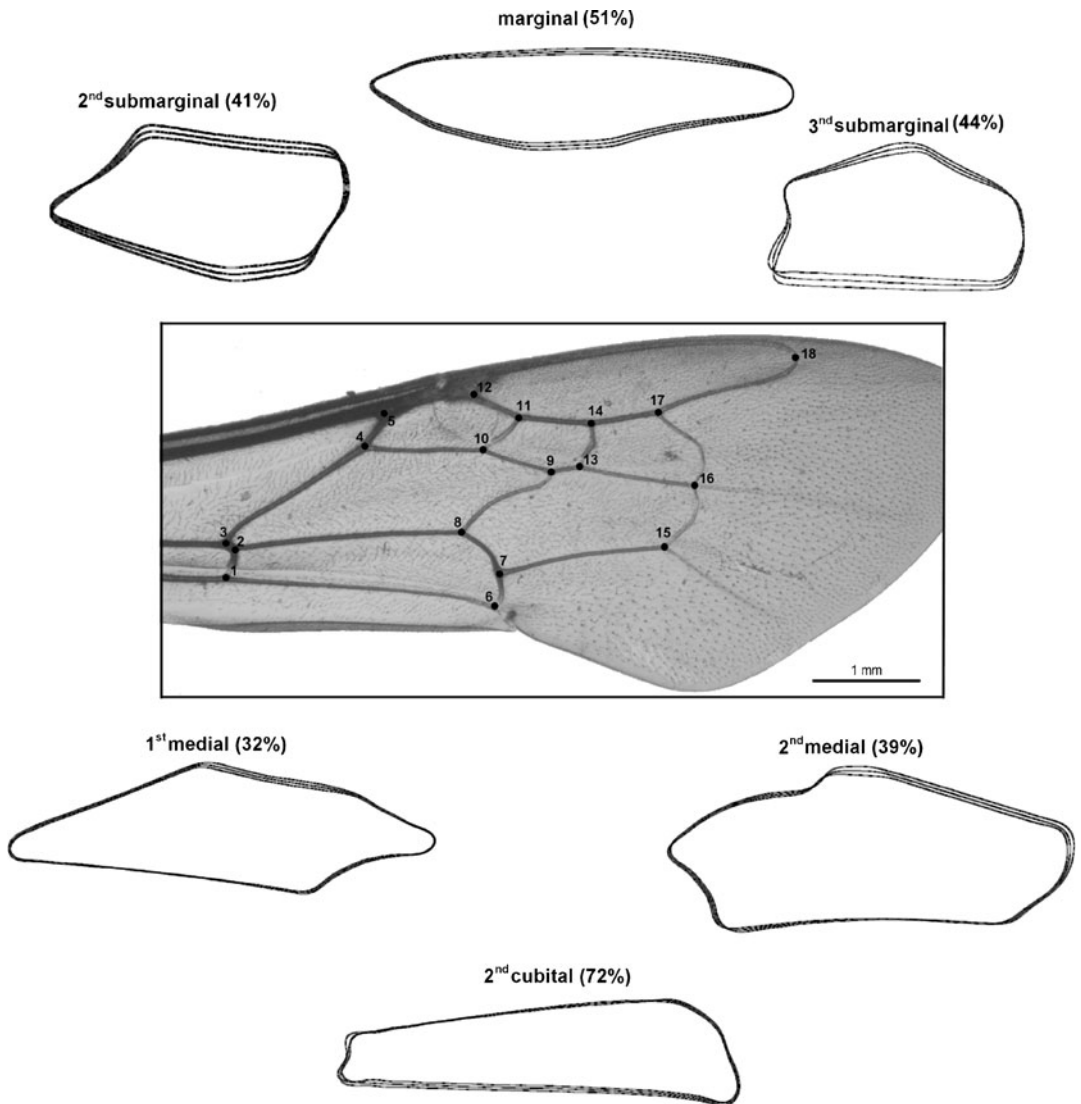
Figure 1. A representative image of the wing of *Euglossa* showing the landmarks scored in each wing (*central figure*). *Above and below* the central image, is showed the overlap of average shapes and standard deviations of the first principal component (PC) of each wing cell, generated through Fourier analysis. The variability percentage, determined by PC, is presented in *parenthesis*.

to quantify morphological variability in each wing cell, we used PCA scores and the area contour as independent variables in a DA, also followed by a leave-one-out cross-validation test.

In addition to independent analysis with both geometric morphometric methods, we performed DA followed by a leave-one-out cross-validation test using the partial warps and centroid size obtained in the landmark-based method and PC generated by contours of second and third submarginal cells, for which we identified higher levels of inter-specific morphological variability.

The efficiency of geometric wing morphometrics for recovering phylogenetic information was determined using the square Mahalanobis distances be-

tween group centroids that was used to construct a dendogram of morphological proximity, compared finally with molecular phylogenetic information (Ramirez et al. 2010).

3. RESULTS

3.1. Landmark-based method

When using centroid size and the 32 partial warps in the DA, 28 out of the 32 partial warps and centroid size contributed significantly ($\alpha=$ 0.05) to group discrimination. Mahalanobis square distances between groups were significantly different from each other (*linear discriminant analysis*, $P=0.001$) and MANOVA indicated statistical differences among all groups (Wilk's $\lambda=0.008$; $P<0.001$). The discriminant analysis placed all five groups separated with some overlap between the confidence ellipses of *E. chalybeata, E. ignita, E. flammea* and *E. orellana. E. imperialis* was the only group completely isolated in the morphometric space from the others (Supplementary Fig. 1).

Linear discriminant functions correctly classified 91% of the individuals, and cross-validation of the data based on DA functions correctly re-assigned 84% of the individuals to their correct original group. The consensus configuration of the landmarks of all five species and the classification functions for the discriminant analysis are in Tables I and II in the Electronic supplementary material (ESM).

3.2. Outline-based method

The variation of harmonic coefficients obtained for each wing cell was reduced to independent PCs, from 7 PCs in the analysis of the second cubital cell, and 11 PCs in the analysis of the first medial cell. In all the cases, the PCs explained more than 90% of the total variation and were further considered as quantitative traits (Table I). The variability found in the first PC of each wing cell, which encloses the major morphological variation percentage, is presented in Figure 1.

We detected significant morphological differentiation in all the wing cells analyzed (Table I). The most informative cells that discriminated species were the marginal, second submarginal and third submarginal cells, respectively (Table I). In order to improve discrimination among the groups, we employed an additional DA based on morphological attributes of the contour of the second and third cells together. We chose these cells because they were among the most informative (Table I) and most inter-specific variability found in them was not related to vein intersection (Figure 1). We did not use the outline of the marginal cell because most of the variation found in this cell overlapped with the variation of the two submarginal cells. Because we outlined the submarginal cells together, inclusion of the marginal cell would cause duplication of information used for DA, as would be the case if we have used the entire set of cell contours.

Analysis of harmonic variation coefficients of the two cells together produced 11 PCs that reflected more than 93% of variability among groups. When using the 11 PCs and cell area, 10 out of the 11 PCs and cell area contributed significantly ($\alpha=0.05$) to discrimination of groups. The MANOVA indicated that all groups were statistically different from each other (Wilk's $\lambda=0.058$; $P<0.0001$) as did Mahalanobis square distances (*linear discriminant analysis*, $P<0.001$). The DA showed greater overlap (ESM Fig. 2) in the confidence ellipsis of the five groups when compared to the landmark-based analysis. The only group that was not greatly superposed was *E. imperialis*, which displayed almost no overlap with the other 4 groups.

The linear discriminant functions classified 84% of the individuals correctly and the cross-validation tests using these linear discriminant functions re-assigned 77% of the individuals to the correct species.

3.3. Combined results of landmark- and outline-based methods

We used the 32 partial warps, centroid size, the 11 PCs generated in the analysis of the second and third submarginal combined cells,

Table I. Sumary of the discriminant analysis for each wing cell of the *Euglossa* species.

Cell	Number of PC (total variation explained)	Wilk's λ (*P* value)	Correct species classification (CV)
Marginal	9 (91.85%)	0.1192 (*P*<0.0001)	74.46 (69,50)
2nd submarginal	10 (92.08%)	0.0914 (*P*<0.0001)	74.67 (70,00)
3rd submarginal	9 (93.43%)	0.1480 (*P*<0.0001)	70.92 (61,70)
1st medial	11 (92.58%)	0.2545 (*P*<0.0001)	60.87 (46,40)
2nd medial	10 (92.25%)	0.1816 (*P*<0.0001)	69.34 (55,50)
2nd cubital	7 (93.02%)	0.1884 (*P*<0.0001)	64.51 (58,90)
2nd + 3rd submarginal	11 (93.51%)	0,0574 (*P*<0.0001)	83,60 (77,10)

PC Principal component; *CV* values of correctly classified individuals in the cross-validation test

and the second and third submarginal cells area in a total of 45 measures. The results are that 32 out of the 45 features contributed significantly (α=0.05) to discrimination among groups. The Mahalanobis square distances between the centroids were statistically different from each other (*P*<0.0001) and the multivariate analysis of the variance indicated that all groups were statistically different (Wilk's λ=0.002; *P*< 0.0001). The discriminant analysis (Figure 2) separated the five groups with little overlap in confidence ellipses of *E. chalybeata* and *E. ignita*, or between the *E. flammea* and *E. orellana*. The graph shows a better separation among the groups than either of the two analyses (Supplementary Figures 1 and 2).

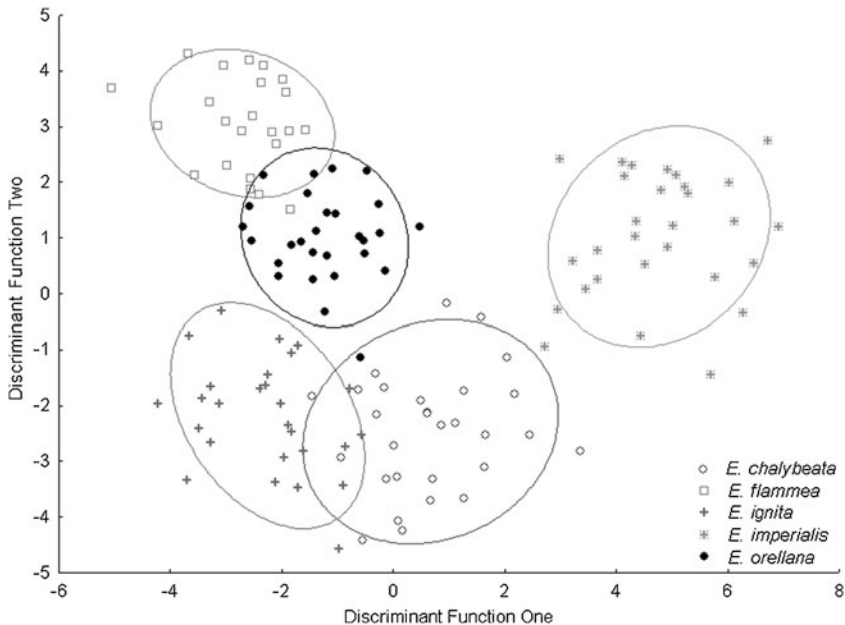The linear discriminant functions correctly classified 97% of individuals and cross-validation

Figure 2. Scatterplot of the combined analysis using wing features extracted using landmark- and outline-based methods from five species of *Euglossa*.

tests of the data using these functions re-assigned 91% of the individuals to the correct species.

We further used the morphological distances between the centroids of the morphological distribution to compare them with phylogenetic hypothesis based on molecular data reported for the same *Euglossa* species (Ramírez et al. 2010). The UPGMA dendogram based on these distances are shown in Figure 3.

4. DISCUSSION

Morphometric methods have been successfully applied for both taxonomic purposes and to appraise biodiversity (Ruttner 1988; Moraes et al. 2004; Schroeder et al., 2006; Bubliy et al. 2008; Francoy et al. 2009; Francoy et al. 2011). Our morphometric analysis indicates marked

Figure 3. a Phylogenetic relationships of the *E. piliventris* species group based on four DNA loci: *cytochrome oxidase* I, *elongation factor* 1-α, *arginine kinase* and *RNA polymerase* II. Support values below the branches indicate parsimony bootstrap while values above the branches correspond the Bayesian posterior probabilities (adapted from Ramírez et al. 2010). b UPGMA dendrogram showing the phenetic relationships among the *Euglossa* species analyzed here according wing morphology. The *scale bar* represents the Mahalanobis distance.

quantitative divergence in male wing morphology of *Euglossa*, in both landmark- and outline-based approaches. Interestingly, some species presented significantly high reclassification percentages when only wing cell contour was considered. These results suggest that wing cell contour can be promising to classify, for example, specimens with damaged wings (it uses only a small part of the wing), while the landmark analysis requires the entire wing to study its venation. Indeed, the features from a single wing cell were used to distinguish subspecies of *Apis mellifera* (Francoy et al. 2006) and the same outline-based method used in this work was successfully applied to identify those subspecies used in apiculture during biblical times, in the Jordan valley (Bloch et al. 2010).

Effectiveness of the landmark-based method has been demonstrated in stingless bees (Francoy et al. 2009) *A. mellifera* (Francoy et al. 2008; Tofilski 2008), *Bombus* (Aytekin et al. 2007) and others (Villemant et al. 2007). Ours is the first study that tests the methodology for euglossines. Another attempt to identify *Euglossa* using other methodologies than traditional approaches was based on allozymes and restriction patterns of mitochondrial genes (López-Uribe and Del Lama, 2007). Using those genetic data, the authors were able to identify some individuals of each species. Our results suggest wing morphometric traits may be more informative.

In order to improve the reclassification rates, we associated in the same matrix the measures obtained by both contour and landmark methodologies to increase the correct classification rates in the cross-validation tests. Other studies have applied these two methodologies to the same specimens but did not combine them (Loy et al. 2000; Kandemir et al. 2009). Based on the data presented here, the integrated approach functions adequately.

The use and importance of quantitative characters for phylogenetic inference is still under debate (Catalano et al. 2010; Klingenberg and Gidaszewski 2010). Considering the species analyzed here, according to a phylogenetic hypothesis based on molecular markers, *E. imperialis* is placed in the most basal lineage
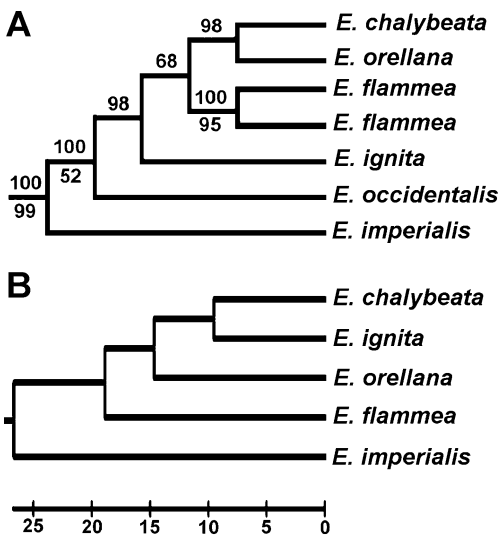
within the *piliventris* species group (Ramírez et al. 2010). Its marginal phylogenetic position is in agreement with the wing quantitative characters analyzed here (Figure 3). This situation suggests that wing morphology could detect a phylogenetic signal in this species group, but comparison of the branch diagrams presented above suggests they did not capture further phylogenetic information. In diagnostic traits, male *E. flammea* and *E. orellana* are extremely similar, while *E. ignita* does not closely resemble any of the other species, nor does *E. imperialis*, particularly with regard to the mid tibial tufts and the sternal cowls (Roubik 2004). In fact, similar approaches in other insect species fail to recover much of the phylogenetic information and only the most basal species was correctly placed in a phylogeny (e.g. Moraes et al. 2004). The greatest incongruence between the trees was the placement of *E. ignita* with *E. chalybeata*, while molecular data, and gross morphology, as mentioned above, do not suggest this arrangement. This incongruence is probably due to different evolutionary rates concerning molecular and morphological data, e.g., selection concerning a particular wing trait.

This study validates use of quantitative wing traits for bee identification. There was significant improvement of classification success when landmark- and outline-based methods were combined. Considering that, the current estimates of biodiversity are likely to be underestimates, especially due to cryptic diversity, the use of precise, quantitative morphology provides an efficient tool to face the lack of taxonomic experts. Furthermore, we were able to verify that, despite utility in recovering taxonomic information, such morphometric information was not informative in phylogenetic constructs for the species studied here.

ACKNOWLEDGMENTS

Distinction efficace entre espèces d'*Euglossa* (Hymenoptera, Apidae, Euglossini) grâce à l'utilisation combinée des méthodes morphométriques par points d'intérêt et par contours.

Euglossine / identification des espèces / morphométrie / aile / caractère quantitatif

Integration von landmarken- und umrissbasierten morphometrischen Methoden kann verschieden Arten von *Euglossa* (Hymenoptera, Apidae, Euglossini) effektiv unterscheiden.

Euglossine Bienen / Identifizierung von Arten / Morphometrie / Flügel / quantitative Merkmale

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