

Identification of cryptic species and morphotypes in male *Euglossa*: morphometric analysis of forewings (Hymenoptera: Euglossini)

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Received 3 February 2015 – Revised 10 April 2015 – Accepted 17 April 2015

Abstract – Males of sibling orchid bees *Euglossa viridissima* and *Euglossa dilemma* are morphologically cryptic, except for the number and shape of mandibular teeth. An alternative morph of *E. viridissima* has a third tooth similar to males of *E. dilemma*. We used this model system to evaluate the potential of wing morphometrics for the resolution of these groups. We found differences in the size characters of forewings of *E. viridissima* and *E. dilemma* albeit with substantial overlapping amongst them. However, geometric morphometrics of forewing vein intersections separated both species and, to a lesser extent, morphotypes. A discriminant analysis of the shape of the radial cell showed separation between all three groups, too, albeit with higher misclassification between *E. viridissima* and *E. dilemma*. We show that sibling cryptic species and morphotypes can be identified by geometric morphometrics, supporting its application with other methods as powerful aids to infrageneric taxonomy in bees.

orchid bee / cryptic species / morphotypes / geometric morphometrics / forewing

1. INTRODUCTION

Euglossines, commonly called orchid bees (Hymenoptera, Apidae, Euglossini) are neotropical, iridescent colored insects, well known for the males' behavior of collecting volatiles from orchids and other sources (Dressler 1982). *Euglossa* Letreille is the largest genus of orchid bees with over 100 described species (Roubik and Hanson 2004).

Electronic supplementary material The online version of this article (doi:10.1007/s13592-015-0369-7) contains supplementary material, which is available to authorized users.

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Manuscript editor: Klaus Hartfelder

The taxonomy of Euglossini is mostly based on the analysis of male traits (Roubik and Hanson 2004). Detailed morphological revisions of male Euglossini have resulted in the discovery of several sibling and cryptic species (Oliveira and Nemésio 2003; Ramírez 2006; Rasmussen and Skov 2006; Nemésio and Bembé 2008; Hinojosa-Díaz et al. 2011, 2012; Nemésio and Engel 2012). These types of revisions can make use of the additional information obtained from morphometric multivariate methods and molecular plus biochemical tools (Eltz et al. 2011; Pokorny et al. 2014).

Insect wing morphometrics has been shown to be a quick and inexpensive method that provides reliable resolution on the specific and infra-specific levels of various taxa (Ruttner 1988; Haas and Tolley 1998; Klingenberg et al. 2001;

Dujardin et al. 2003). Recently, geometric morphometric approaches have shown higher statistical power compared to traditional morphometrics for species separation (Bookstein 1991; Zelditch et al. 2004; Francoy et al. 2011; Zinetti et al. 2013). By eliminating the effect of size, statistical comparison of landmarks used in geometric morphometrics can reveal differences of actual shapes or forms with greater power of discrimination (Mitteroecker and Gunz 2009). In bees, geometric morphometrics of the forewing has been capable of discriminating operational taxonomic units (Aytekin et al. 2007; Francoy et al. 2008, 2011; Kandemir et al. 2011). Importantly, such identification seems to conform to those based on molecular markers (Francoy et al. 2012; Oleksa and Tofilski 2015). However, in bees, the use of geometric morphometrics for the discrimination of cryptic species has remained uninvestigated, in spite of its promising use in other insect taxa (Baylac et al. 2003; Pizzo et al. 2006; Gurgel-Goncalves et al. 2011; Muñoz-Muñoz et al. 2011; Mitrovski—Bogdanović et al. 2013). Overall, the application of geometric morphometrics to assess intraspecific morphological differentiation and intra-sexual plasticity is still a largely unexplored field in bees (Danforth and Desjardins 1999).

In this respect, male orchid bees of sibling *Euglossa viridissima* Friese and *Euglossa dilemma* Bembé and Eltz represent a unique model for evaluating geometric morphometric methods in the identification of cryptic species and morphotypes altogether. The *E. viridissima*–*E. dilemma* species pair exists in sympatry in the Tehuantepec Isthmus and the Yucatan Peninsula of Mexico (Eltz et al. 2011). Morphometric methods based on measures of body size have failed to separate sibling *E. viridissima* and *E. dilemma* males which remain cryptic for several characters traditionally used in bee morphometrics (Ramirez-Pech 2009). So far, males of both species can only be separated phenotypically by their number and shape of mandibular teeth. Males of *E. viridissima* are commonly bidentate (2D, >90 % of individuals) but occasionally tridentate (3D) with the middle dentition near the distal one, whereas *E. dilemma* are always tridentate with the middle dentition placed

centrally (Eltz et al. 2011). Females of both species are superficially identical and can only be separated by molecular markers (Eltz et al. 2011; May-Itzá et al. 2014). Male 2D and 3D *E. viridissima* are chemically and genetically indistinguishable (Eltz et al. 2011; Pokorný et al. 2014; Ramírez et al., unpublished data). Moreover, both male types emerge from clutches of the same mother (T. Eltz and Quezada-Euán, unpublished data), confirming that they are in fact individuals of the same species.

In this study, we evaluated the power of wing morphometrics for identifying cryptic species and species morphotypes in bees using as a model the *E. viridissima*–*E. dilemma* species complex. Both traditional morphometrics based on wing size (traditional morphometrics) and geometric morphometrics of shape were included in the study.

2. MATERIALS AND METHODS

2.1. Material collection

Males of *E. viridissima* and *E. dilemma* were collected in 13 localities across the Yucatan Peninsula during 2010 using synthetic benzyl benzoate (BB), 1,8-cineole (C), eugenol (E), methyl salicylate (MS), p-dimethoxybenzene (PDMB), and methyl cinnamate (TMC) as baiting substances (Pokorný et al. 2013). Individuals were kept in ethanol and stored at -20°C awaiting further analyses. Males were separated into *E. dilemma* and *E. viridissima* and the two morphs in the latter in accordance to the number of mandibular teeth and their position. For all statistical analyses, the three a priori groups consist of 44 2D and 23 3D specimens of *E. viridissima*, and 41 of *E. dilemma* were identified.

2.2. Preparation of forewings

From each specimen, the right forewing was dissected from the base of the radial vein with the help of forceps and mounted between glass slides to keep them flat. Wings were photographed using an OpticFilm 7200 scanner (Plustek) for diapositives at 3600 dpi. We used 28×48 mm, 1.55-mm thickness microscope slides to sandwich wings, which fitted the slide scanner.

2.3. Analyses of size

For the analyses of size, we used distances and angles formed by vein intersections. We first recorded 18 landmarks of the intersections of the veins on the right forewing (Figure 1). Cartesian coordinates of points on the photograph of the forewing of each individual male were registered using the software tpsDig2 version 2.12 (Rohlf 2008). After this, all configurations were aligned using Procrustes superimposition by means of the software CoordGen6 (Sheets 2006).

Ten inter-landmark distances and seven angles between vein intersections were then calculated using the program TMorphGen (Sheets 2006) generating a set of “traditional” morphometric measurements.

A general measure of forewing size for each specimen was also obtained using the values of the centroid size for the distances between vein intersections calculated by CoordGen6. The distances and angles formed by forewing venation landmarks used in the analyses are shown in Table I.

The mean values for each distance between vein intersections, centroids, and angles were compared between the three male types by means of ANOVA. Multiple comparisons were conducted by means of a Duncan’s range test. After this, we could identify which characters showed differences between groups. In a further step, we used the forewing traits that showed significant differences between groups in a multivariate approach by means of a principal components analysis (PCA).

Three principal components were obtained with their associated eigenvalues. These were used to calculate the percentage variation explained by each component. Scores for each male for each component were calculated using the coefficients assigned to each individual variable. To test for overall size differences between individuals of the three groups, the resulting scores were compared by means of ANOVA. Comparisons between

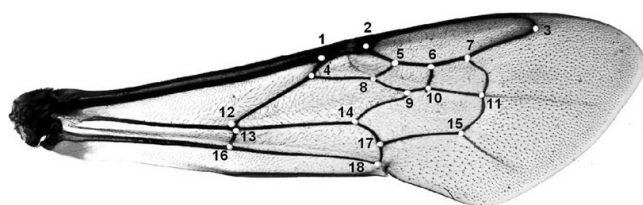


Figure 1. Landmarks of forewing vein intersections used in the analyses of size and shape of males of *Euglossa dilemma* and *Euglossa viridissima* morphs.

Table I. Distances and angles formed by venation landmarks used in the analyses of forewing size and shape.

Distances between vein intersections	Landmark 1	Landmark 2	Landmark 3 (angle intersect)
1	13	14	–
2	1	13	–
3	11	15	–
4	12	16	–
5	8	16	–
6	10	12	–
7	3	9	–
8	7	18	–
9	3	5	–
10	6	18	–
Angles			
1	7	15	16
2	6	7	18
3	12	14	15
4	4	8	5
5	8	11	12
6	10	13	1
7	2	3	9

male types were conducted by means of Duncan’s multiple range tests. The PC scores were plotted on a bidimensional scale using PC1 and PC2 which represent the largest amount of variation in the data.

2.4. Analyses of shape

The same 18 landmarks (vein intersections), as in Figure 1 were used for the analysis of the shape of forewing venation pattern. Additionally, we also analyzed the shape of the radial cell in 28 males of each, 2D *E. viridissima* and *E. dilemma*, plus ten males of 3D *E. viridissima*. We analyzed the radial cell because it has been shown to accurately separate races of *Apis mellifera* (Francoy et al. 2006). For the analysis of radial cell shape, we used semilandmarks (points not anatomically defined on the structure) as it has a curvilinear shape (Figure 2). To define the contour of the radial cell, we used 18 semilandmarks produced between landmarks 1 and 13 in the radial cell (Figure 1) using the option “comb” of the program MakeFan6 (Sheets 2006).

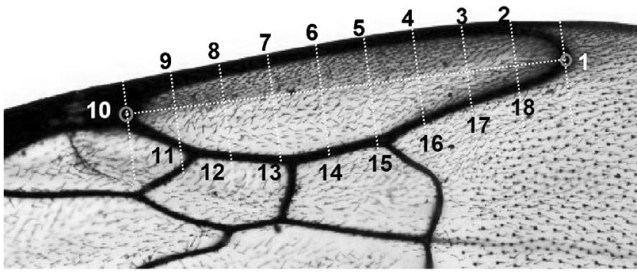


Figure 2. Semilandmarks used in the analyses of shape of radial cell of males of *Euglossa dilemma* and *Euglossa viridissima* morphs. The numbers 1 and 10 indicate real landmarks from which equidistant points were defined around the cell contour.

The landmarks and semilandmarks were submitted to canonical variates analyses to evaluate the relative power of discrimination of the pattern of forewing venation and radial cell shapes, respectively. The analyses were based on partial warp scores on the Procrustes superimposed images using the CVAgen software (Sheets 2006). We calculated the number of significant CVA axes at $P=0.05$ and, afterwards, computed the canonical variates scores of individual males. Additionally, we used Mahalanobis distances to assign all the specimens to one of the three groups loaded. The CVA scores for each individual of the three types of male were compared by means of ANOVA. We performed jackknifed assignments of the specimens in order to test the accuracy of the CVA scores in establishing differences between male types. Finally, the CVA scores were plotted on a bidimensional scale against the Canonical variates 1 and 2 to detect group differences in shape.

3. RESULTS

3.1. Analyses of size

The comparison of size between species and morphs was based on the vein intersection distances, centroids, and angles of the forewing (traditional morphometric measures) at univariate and multivariate levels. The results of ANOVA to compare male types at the univariate level are presented in Supplementary Table 1.

The statistics of PCA using distances between forewing vein intersections are presented in Supplementary Tables 2–4.

The Kaiser's measure of sampling adequacy was 0.874 which indicated that the correlation

matrix was adequate for PCA. The first two components included 84 % of the total variation (76.7 % for PC1 and 7.4 % for PC2, respectively). PC1 was positively correlated with the 13 distances between vein intersection of the forewing (Supplementary Table 1) probably indicating that PC1 is largely an overall size measure. The mean score values for PC1 were significantly different between the three male types ($F=36.05$; $df=2$, 107; $P=0.001$; Table II). The results of Duncan's multiple range test showed that the mean of the PC1 scores of 2D and 3D *E. viridissima* males were not significantly different from each other (0.531 and 0.428, respectively) but that they differed from those of *E. dilemma* (−0.810). This means that distances between forewing intersections are smaller in males of *E. dilemma* compared with both morphotypes of *E. viridissima*. For both PC2 and PC3, there were no significant differences between the scores of the three groups ($F=2.7$; $df=2$, 107; $P=0.07$ and $F=0.37$; $df=2$, 107; $P=0.69$, respectively; Table II).

The individual scores were plotted against PC1 and PC2 (Figure 3). The distribution of both morphotypes of *E. viridissima* is evident towards the right of PC1 compared with *E. dilemma* confirming the smaller size of these individuals for these characters. However, in spite of statistical differences of size between *E. viridissima* and *E. dilemma* along PC1, some individuals overlapped, especially the 3D morph with the latter. Nevertheless, individuals of both *E. viridissima* morphotypes substantially overlapped along this component (Figure 3). Similarly, overlapping of both species and morphotypes was evident along PC2. This result is consistent with the ANOVA results, indicating a difference in the means relative to within group variance but does indicate substantial overlap of the individuals along these variables.

3.2. Analyses of shape

The results of the analyses of shape of the pattern of forewing venation by MANOVA indicated statistical differences amongst the three male types (Wilk's $\lambda=0.001$; $P<0.001$). Two canonical variates showed significant differences between groups (axis 1 $\lambda=0.551$, $\text{chisq}=61.97$,

Table II. Comparison of mean of scores for males of *Euglossa dilemma* and *Euglossa viridissima* morphs for each of the three principal components derived from forewing distances between vein intersections.

	Male type			F value	Prob. F
	<i>E. viridissima</i> 2D	<i>E. viridissima</i> 3D	<i>E. dilemma</i>		
PC 1	0.531a (0.117)	0.428a (0.162)	-0.810b (0.121)	36.05	0.001
PC 2	0.147 (0.148)	0.215 (0.205)	-0.279 (0.153)	2.70	0.072
PC 3	0.045 (0.151)	0.096 (0.209)	-0.103 (0.157)	0.37	0.692

Standard deviations are presented in brackets

$df=6$, $P=0.001$ and axis 2 $\lambda = 0.865$, $chisq=15.03$, $df=2$, $P=0.001$). Since there were three groups of specimens in the study, there could be a maximum of two statistically significant axes, as occurred here.

The ANOVA of CVA scores revealed significant differences for CV1 ($F=118.39$; $df=2$, 107; $P=0.001$; Table III) between the means of *E. dilemma* (20.42) and the two morphs of *E. viridissima* but not between the latter two (-9.35 for 3D and -14.13 for 2D ones). Significant differences were also found for CV2 scores ($F=25.64$; $df=2$, 107; $P=0.001$). The means of *E. viridissima* 3D morph (20.21) differed from the 2D morph and *E. dilemma* which were not significantly different amongst them (-8.12 and -14.39, respectively) (Table III).

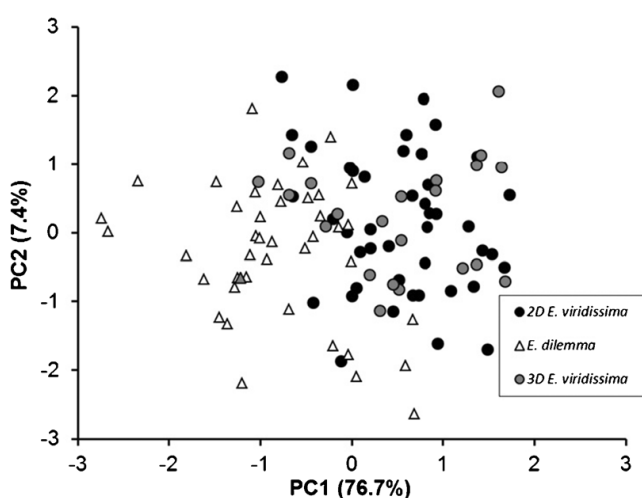


Figure 3. Plot of individual scores of males of *Euglossa dilemma* and *Euglossa viridissima* morphs against principal components 1 (PC1) and 2 (PC2) from the analysis of distances between forewing vein intersections. The percentage of variation explained by each component is presented in brackets.

A jackknifed assignment test showed that 96 % of the *E. dilemma* specimens were correctly assigned to their group, while the rest were incorrectly assigned to *E. viridissima* 3D (4 %). In the case of *E. viridissima* 2D and 3D, 4 % were incorrectly assigned to *E. dilemma*, respectively. More incorrect assignments occurred between 2D and 3D *E. viridissima*. In the case of 2D males, 34 % were incorrectly assigned to the 3D morphotype and in the case of the latter, 43 % were incorrectly assigned to the 2D morphotype. The plot of the distribution of the scores against canonical variates 1 and 2 is presented in Figure 4. A clear separation between *E. dilemma* and the two *E. viridissima* morphs is evident against CV1 compared with a higher overlap between the latter two. Overlapping of the three groups is also evident along CV2.

Statistical differences amongst the three male types was also evident with the analysis of shape of the radial cell (MANOVA Wilk's $\lambda=0.001$; $P<0.001$). Two canonical variates showed significant differences between groups (axis 1 $\lambda=0.4407$; $chisq=50.801$; $df=6$; $P=0.001$ and axis 2 $\lambda = 0.718$; $chisq=20.468$; $df=2$; $P=0.001$).

The ANOVA of CVA scores revealed significant differences for CV1 ($F=19.46$; $df=2$, 65; $P=0.001$; Table III). The mean of the *E. viridissima* 2D morph (0.336) was different from both, the 3D morph (-0.230) and *E. dilemma* (-0.243) that were not different between each other. Significant differences were also found for CV2 scores ($F=14.53$; $df=2$, 107; $P=0.001$). The means of the *E. viridissima* 3D morph (-0.628) differed from the 2D morph and *E. dilemma* which were not significantly different

Table III. Comparison of means of CVA scores of males of *Euglossa dilemma* and *Euglossa viridissima* morphs for landmarks of forewing venation and semilandmarks of radial cell.

Forewing venation landmarks	Male type			F value	Prob. F
	<i>E. viridissima</i> 2D	<i>E. viridissima</i> 3D	<i>E. dilemma</i>		
CV1	-14.13a (1.65)	-9.35a (2.41)	20.42b (1.22)	118.39	<0.001
CV2	-8.12a (3.22)	20.21b (2.32)	-14.39a (2.47)	25.64	<0.001
Radial cell semilandmarks					
CV1	0.336a (0.07)	-0.230b (0.11)	-0.243b (0.07)	19.46	<0.001
CV2	-0.005a (0.08)	-0.628b (0.13)	0.215a (0.08)	14.53	<0.001

Standard deviations are presented in brackets

amongst them (-0.005 and 0.215 ± 2.4 , respectively).

A jackknifed assignment test showed that 67.8 % of the *E. dilemma* specimens were correctly assigned to their group, 11 % to *E. viridissima* 3D and 21.4 % to *E. viridissima* 2D. In the case of *E. viridissima* 2D and 3D, 11 and 10 % were incorrectly assigned to *E. dilemma*, respectively. In the case of *E. viridissima* 2D males, 11 % was incorrectly assigned to the 3D morphotype and in the case of the latter, 0 % was incorrectly assigned to the 2D morphotype. The plot of the distribution of the scores against canonical variates 1 and 2 is presented in Figure 5. A complete overlap along CV1 exists between *E. dilemma* and 3D *E. viridissima*. However, a separation is observed between

E. dilemma and 2D *E. viridissima* along the same CV1. Moreover, a degree of separation is observed between the two *E. viridissima* morphotypes along both CV1 and CV2.

4. DISCUSSION

The additional morphometric information used in our study reinforces the identity of *E. viridissima* and *E. dilemma* as separate species (Eltz et al. 2011). Moreover, characters of size and shape of the forewing were capable of separating both morphotypes within *E. viridissima*. However, it was evident that the degree of separation and its associated significance depended on the type of forewing character used.

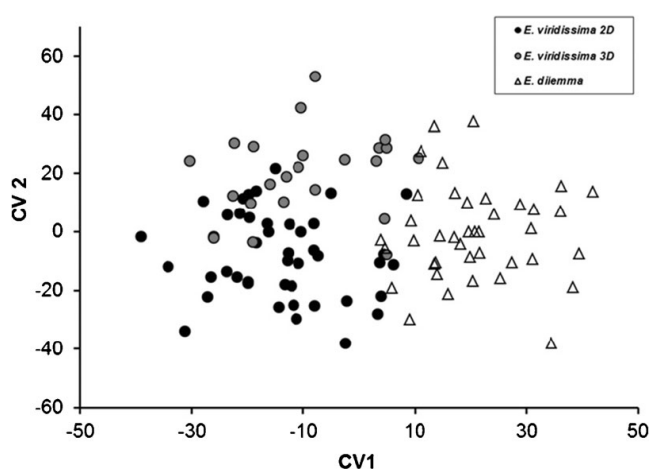


Figure 4. Plot of individual scores of males *Euglossa dilemma* and *Euglossa viridissima* morphs against canonical variates 1 (CV1) and 2 (CV2) from the analysis of landmark pattern of forewing venation.

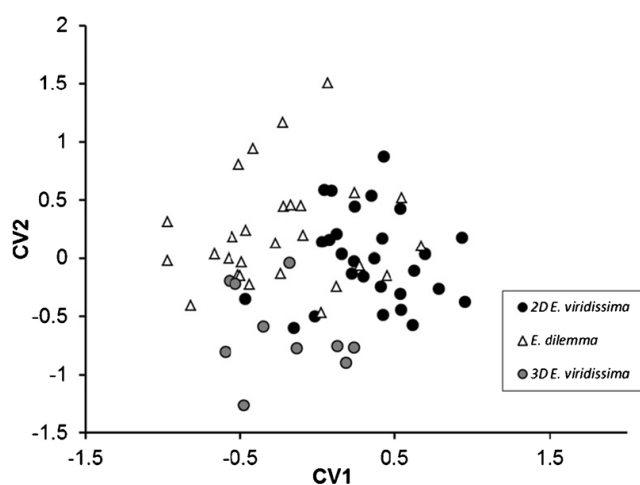


Figure 5. Plot of individual scores of males of *Euglossa dilemma* and *Euglossa viridissima* morphs against canonical variates 1 (CV1) and 2 (CV2) from the analysis of semilandmark pattern of the forewing radial cell.

Overall, characters related to forewing size showed differentiation between species but not morphotypes, whereas characters related to the shape of wing cells varied also between morphotypes within species. In a former study, using various anatomical characters, *E. viridissima* and *E. dilemma* showed no differences in an overall estimation of size (Ramirez-Pech 2009; Eltz et al. 2011). However, in this recent approach including a more detailed analysis of forewing vein intersections, both species showed morphological differentiation in this structure. Within the characters that we used to compare size, the angles between vein intersections did not show substantial differences neither between species nor morphotypes. On the other hand, all distances between vein intersections showed significant differences between male species.

In the analyses of shape, the best discriminator between species was the whole pattern on forewing venation that reported the highest level of separation and the lowest percentage of misclassification between species. The semilandmarks associated with the shape of the radial cell showed separation amongst the three groups, albeit with higher misclassification rates between species. These findings suggest that better classification of cryptic species is expected when the shape of the whole pattern of forewing venation is analyzed compared with individual cells.

Our results support to extending the use of forewing geometric morphometrics as a powerful tool for the identification, not only of close bee lineages but of sibling cryptic species and morphotypes within species too. The sensitivity of the method was capable of revealing differences between 2D and 3D *E. viridissima*. These males have, until now, only been identified by their number of mandibular teeth, but here we show that other features in their anatomy are also substantially different. Highly variable DNA markers (Microsatellites, SNPs) as well as chemical characters (perfumes, cuticular hydrocarbons) are clearly different between *E. dilemma* and *E. viridissima* but show no systematic variation amongst 3D and 2D males of the latter, confirming that they are in fact polymorphic individuals of the same species (Eltz et al. 2011;

Pokorny et al. 2014; Ramirez et al. unpublished data).

Polymorphism is widely present in insects. It can be produced by alternative genotypes or may be the genotype response to variation in the environment (Fordyce 2006). To date, only a few studies have documented in detail the extent of intra-sexual plasticity in the social Hymenoptera (ants, bees, and wasps) and they have been mostly conducted on females (but see Danforth and Desjardins 1999). The physiological basis of male wing polymorphism in bees, thus, is not clearly understood. In other insect taxa, male wing polymorphism can result from variation in both genetic and environmental factors (Zera 2004). Ultimately, hormonal differences, particularly in the titer of juvenile hormone during the last juvenile instars might explain the development of different morphs (Zera 2004). It has also been shown that the development of wings involves numerous genes that act from egg to adulthood, so stresses that act at different times could alter their shape in specific ways (Hoffmann et al. 2005). Similarly, the observed wing shape differences between 2D and 3D *E. viridissima* males could be the result of stressful conditions during their development, although both morphs seem to persist across time and space (Eltz and Quezada-Euán unpublished data) possibly indicating little effect of environmental constraints (see Simmons et al. 2000).

Our results on forewing morphology of 2D and 3D *E. viridissima* males indicate that polymorphism apparently extends to traits of their anatomy other than dentition. Several selective mechanisms may explain the maintenance of polymorphisms within a species. Disruptive selection and heterozygote advantage are likely to play important roles but the most common explanation is frequency-dependent selection (Ajuria-Ibarra and Reader 2013). It would be daring to suggest a mechanism for the maintenance of 2D and 3D males in *E. viridissima*. It is therefore important to understand the mechanism(s) that preserve both morphotypes to help explain how genetic diversity is maintained within these populations and the evolutionary biology of orchid bees.

Due to their particular mode of sexual attraction, seemingly based on non-visual mating signals (chemical display by males; Eltz et al. 2005),

species of euglossines could have diverged without substantial changes in morphology and meristic characters (Eltz et al. 2008), thus remaining cryptic under traditional alpha taxonomy (Bickford et al. 2007). In this respect, geometric morphometrics data can be of much help in the taxonomic revision of euglossines.

ACKNOWLEDGMENTS

We would like to thank projects Conacyt 103341 “Identificación de especies crípticas e indicadores de diversidad genética en abejas nativas de México” and DAAD-Proalmex B330/336/11 “Divergencia en linajes de *Euglossa* (Hymenoptera: Euglossini) en relación con la disponibilidad de fragancias a lo largo de una clina ecológica en la Península de Yucatán” for the financial support.

Identification d’espèces cryptiques et morphotypes chez des mâles d’*Euglossa*: analyse morphométrique des ailes antérieures (Hymenoptera: Euglossini)

abeille à orchidée / morphométrie géométrique / aile / espèce jumelle

Identifizierung kryptischer Arten und von Morphospezies in *Euglossa* -Männchen: Morphometrische Analyse der Vorderflügel (Hymenoptera: Euglossini)

Prachtbienen / kryptische Arten / Morphotypen / geometrische Morphometrie / Vorderflügel

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