The LINNEAN SOCIETY of London ZOOLOGICAL Journal of the Linnean Society

*Zoological Journal of the Linnean Society*, 2011, **163**, 1064–1076. With 7 figures

# Characterization of the orchid bee *Euglossa viridissima* (Apidae: Euglossini) and a novel cryptic sibling species, by morphological, chemical, and genetic characters

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*Received 7 August 2010; revised 9 February 2011; accepted for publication 18 February 2011*

In orchid bees, males signal their availability as mates by fanning 'perfumes', i.e. blends of volatiles that are collected from environmental sources and stored in hind leg pouches. The chemical composition of such perfumes in males with either two or three mandibular teeth has previously led to the discovery of two sympatric, cryptic lineages within *Euglossa viridissima* Friese on the Yucatan peninsula, Mexico. Here, we combine chemical, morphological, and genetic data for an integrated characterization of the two lineages. The lectotype of *E. viridissima* Friese in the Museum of Natural History in Vienna has two mandibular teeth, and the species name *viridissima* must thus be assigned to the predominantly bidentate lineage, whereas the completely tridentate lineage is described as a novel species, ***Euglossa dilemma* sp. nov.** Bembé & Eltz. Chemical profiling and microsatellite genotyping revealed that *E. viridissima* males can occasionally (~10% of individuals) express a third mandibular tooth, but this tooth is not positioned centrally on the mandible as in *E. dilemma*, but is displaced towards the tip. Thus, males of the two lineages can be unambiguously diagnosed by mandibular characters alone. Based on 889 bp of CO1 sequence data, we confirm that *E. viridissima* and *E. dilemma* constitute a monophyletic group within the genus *Euglossa*. However, CO1 alone failed to separate these two lineages due to the lack of parsimony-informative sites. Both species occur in broad sympatry across Central America, but the orchid bees recently introduced to Florida have three mandibular teeth in males, i.e. belong to *E. dilemma*.


doi: 10.1111/j.1096-3642.2011.00740.x

ADDITIONAL KEYWORDS: Apinae – chemotaxonomy – cryptic diversity – pheromone analogue – reinforcement – reproductive character displacement – sister species – speciation.

## INTRODUCTION

Sibling species can often be distinguished by differences in secondary sexual characters that underlie divergent selection due to their function as mate recognition signals. However, not all taxa communicate reproductive signals visually and are therefore not easily recognized by taxonomists (Bickford *et al*., 2007). In insects, chemical signals are frequently involved in mate location and recognition (Cardé & Baker, 1984; Roelofs, 1995), and chemical dichotomies are sometimes a first clue to differentiate cryptic species (Byers & Struble, 1990). Cryptic sibling

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species have also been discovered in neotropical orchid bees (Euglossini) that use environmental odours for communication (Roubik, 2004; Bembé, 2008; Eltz *et al*., 2008). Males are attracted to volatiles produced by flowers and certain non-floral sources, e.g. fungus-infected wood, tree wounds, and faeces (Vogel, 1966; Dressler, 1982; Roubik & Hanson, 2004). They collect the emitted volatiles and store them in voluminous pockets on their hind tibiae, where complex and species-specific blends of 'perfume' accumulate (Eltz, Roubik & Lunau, 2005; Eltz *et al*., 2007). Later, perfumes are exposed by males during courtship display, for which they perch on woody stems in the forest understorey (Bembé, 2004; Eltz, Sager & Lunau, 2005). Although this has not been demonstrated directly with bioassays, it is believed that perfume signals target females and serve to communicate availability for mating. A function in the context of mate recognition is indirectly supported by the finding that chemical blends are different between species, particularly between closely related species (Zimmermann, Ramírez & Eltz, 2009).

Recently, chemical analysis of male perfumes has revealed cryptic diversity in what was previously regarded as a single species, *Euglossa viridissima* Friese, a medium-sized, metallic green orchid bee species distributed from Mexico to Costa Rica (Eltz *et al*., 2008). Males of all *E. viridissima*-like bees are distinguished from other *Euglossa* by two large patches of hair on the second sternite and the characteristic shape of midtibial hair tufts (Roubik & Hanson, 2004). However, Dressler (1978) had already noted that males with those characters are variable with respect to the number (three or two) of mandibular teeth, with three being more common. Chemical analysis of male perfumes from populations in the Yucatan peninsula revealed that most tridentate individuals contained a set of highly characteristic compounds [2-hydroxy-6-nona-1,3-dienylbenzaldehyde (HNDB) isomers] as major perfume ingredients, whereas those compounds were completely absent in bidentate males (Eltz *et al*., 2008). The distinction in perfume composition was associated with striking differences in olfactory sensitivity revealed by electroantennography towards HNDB isomers, which could explain differential perfume accumulation by males. Finally, allele frequencies of microsatellite loci were different between tridentate and bidentate individuals, suggesting that the two morphotypes belong to two reproductively isolated lineages (Eltz *et al*., 2008). However, the assignment of males based on the number of mandibular teeth alone was not perfect, possibly because males of the bidentate lineage occasionally express a third tooth. In the present paper we further explore morphological, chemical, and genetic variation in this sibling species complex, with the aim to identify morphological characters that allow unambiguous diagnosis of males of the two lineages. Based on such characters, and on a comparison with type material, we describe one of these lineages as representing *Euglossa dilemma* sp. nov. Furthermore, we confirm the monophyly of the sibling group within the framework of a recently published molecular phylogeny of the Euglossini, and provide additional data on geographical ranges.

## MATERIAL AND METHODS

### Baiting and sampling

To obtain specimens and to assess local proportions of bidentate and tridentate males, baiting with commercially available attractants (*p*-dimethoxybenzene, eugenol, and methyl cinnamate) was done at various localities in southern Mexico from October 2006 to July 2009. Males were captured with hand nets, morphotyped using a hand lens, and partly preserved for later chemical, genetic, and/or morphometric analysis. In total, 198 males from the localities Xmatkuil, Chablekal, San Crisanto, and Coba (see Fig. 7 below) were subjected to detailed perfume analysis and microsatellite genotyping. These 198 individuals include 63 that have already been analysed (Eltz *et al*., 2008). A separate set of 245 individuals was used for general morphometrics. These were from Yucatecan localities 1 and 3–13 of Eltz *et al*. (2008). Finally, we compiled data on the geographical range of the two lineages, including data from baiting assays in various localities in southern Mexico as well as from surveys of pinned specimens in entomological collections.

### Chemical analysis of male perfumes

Male perfumes were extracted from hind legs in 0.5 mL hexane. Coupled gas chromatography/mass spectrometry was carried out with a HP 5890 II chromatograph fitted with a DB-5 column (30 m × 0.25 mm Ø × 0.25 μm film thickness) and a HP 5972 MSD. Injection was splitless, and the oven programmed from 60 to 300 °C at 10 °C $min^{-1}$. Extracts of 176 males from the localities (1) Xmatkuil, (2) Chablekal, (3) San Crisanto, and (4) Coba were subjected to detailed perfume analysis as described in Eltz *et al*. (2008). Structure assignment of extracted compounds was carried out by comparison of mass spectra and retention indices with those of authentic reference samples or those given in the literature (Adams, 2001). Excluded from the analysis were straight-chain lipids (alkanes, alkenes, alcohols, acetates, diacetates, and wax esters), contained in the bees' labial glands and prominent in head extracts of the studied species. Only individuals with at least six

perfume compounds above the detection threshold (on average 26) were further analysed ($N = 176$), thereby excluding 12 presumably young bees that had almost no perfume. Differences in perfume composition between individuals were calculated as Bray-Curtis distances based on relative compound contributions (percentage of total ion current) to individual blends. These distances were visualized in three dimensions by non-parametric Multidimensional Scaling (MDS) using the software Primer v6 (Clarke & Gorley, 2001).

### Microsatellite genotyping

Males subjected to perfume analysis were also genotyped at three polymorphic microsatellite loci, ann02, ann08, and Egc17 (Souza *et al*., 2007; Paxton *et al*., 2009). DNA was extracted from thoraces of ethanol-preserved specimens using the protocol given in Hunt & Page (1995). Multiplex-PCRs were conducted with fluorescent dye-labelled primers (VIC, 6-FAM, and NED; Applied Biosystems). Four microlitres of DNA template was used with 12.5 μL HotStar Taq™ Master Mix (Qiagen), and the reaction volume was filled up to 25 μL in total with RNase-free water (Qiagen). PCR reactions were performed in an Eppendorf Mastercycler with the following temperature program: 95 °C for 15 min, 22 cycles of 94 °C for 30 s, 52 °C for 90 s, and 67 °C for 90 s, followed by 67 °C for 10 min. Fragment analysis of PCR products was carried out with an ABI Prism 310™ Sequencer (PE Applied Biosystems) at the BMFZ in Düsseldorf. For visualization and allele assignment we used the software GENEMARKER V1.71. We tested for differentiation using exact tests implemented in the web version of GENEPOP (Raymond & Rousset, 1995).

### Quantitative morphometrics

A total of 245 males from 12 localities (all on the Yucatán peninsula) were measured. Individual heads, thoraces, and hind tibiae were gently squeezed into plasticine to keep them fixed during measurements. We took 15 measures of each individual: forewing length, forewing width, hind wing length, hind wing width, hind tibia length, hind tibia width, thorax length, thorax width, eye width, eye length, clypeus length, clypeus width, head length, head width, and distance between mandibular bases. The measurements were based on methods used by Hartfelder & Engels (1992), Ken *et al*. (2003), and Quezada-Euan *et al*. (2007). The length and width of the fore and hind wings were measured with an inverted microscope (Indumex), a digital table (Summasketc Professional Plus), a cold light (Indumex) and the program Afusda 7 (Rubink, dates not published). Stereoscopic (Motic®) images were used, in combination with the software Motic Image (Advanced 1.3), to perform additional measurements. We tested for differences in sizes of the various structures between bidentate and tridentate males using ANOVA (Statgraphics Plus 5.1). Furthermore, to test for an overall morphometric separation of the two lineages we performed a principal components analysis (PCA), including those morphometric variables that showed significant univariate differences (Ken *et al*., 2003; Quezada-Euan *et al*., 2007). The individual scores of the first four components were tested for differences between the two kinds of males using ANOVA. The scores were also plotted in two-dimensional space, with the axis representing the two principal components that showed a statistically significant difference.

### Phylogenetic analysis

We explored the phylogenetic position of *Euglossa viridissima* and *Euglossa dilemma* sp. nov. based on an 889-bp fragment of the mitochondrial cytochrome oxidase 1 gene (CO1). A DNA matrix was assembled using the software package MacClade v4.06 (Maddison & Maddison, 2003), and a parsimony analysis was conducted using the software package PAUP* v4.10b (Swofford, 2003) by assuming unordered transitions and weighting all characters equally. Heuristic tree searches consisted of 100 random addition sequences, using the TBR swapping algorithm. In addition, we implemented Bayesian analyses using the software package MrBayes v3.1.1 (Ronquist & Huelsenbeck, 2003) with models of sequence evolution partitioned by codon positions. Parameters were estimated separately during runs for first, second and third codon positions. Markov Chain Monte Carlo searches were run for 10 million generations, sampling every 1000 generations.

We estimated the age of the most recent common ancestor (MRCA) of *E. viridissima* and *E. dilemma* using molecular clock methods as explained in Ramírez *et al*. (2010b). We used penalized likelihood as implemented in the software package r8s v1.71 (Sanderson, 2002). A molecular phylogenetic tree of the genus *Euglossa* from a previous study (Ramírez *et al*., 2010b) based on both nuclear and mitochondrial loci was used. Based on the fossil-calibrated molecular clock analysis of Ramírez *et al*. (2010b), we fixed the age of the MRCA of *Euglossa* at 15–20 Mya.

## RESULTS

### Genetic, chemical, and morphological characterization

Our MDS analysis of male tibial perfumes produced two distinct clusters of individuals that were characterized by either the presence or the absence of

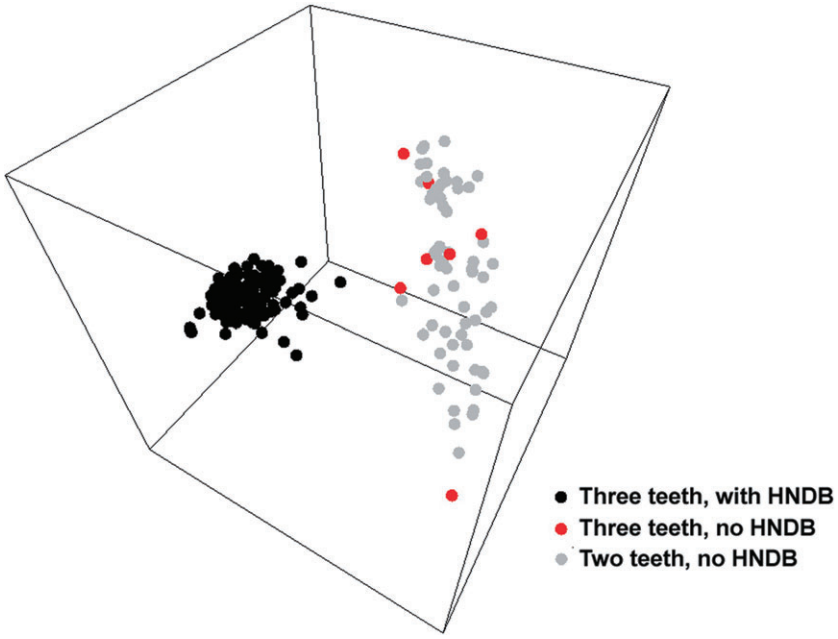


**Figure 1.** Differences in the chemical composition of tibial perfumes between tridentate (black circles) and bidentate (grey circles) *Euglossa viridissima*-like males as revealed by a multidimensional scaling (MDS) analysis. Only tridentate males contained HNDB. Tridentate males without HNDB are highlighted (red symbols).

HNDB (Fig. 1). In individuals with HNDB (black circles in Fig. 1), the HNDB isomers represented the dominant component of the individual perfume blend (mean ± SD, 77.8 ± 9.25% of the total peak area; minimum 44.5%), whereas all other individuals had no HNDB at all. Whereas individuals with HNDB were all strictly tridentate, the individuals lacking HNDB fell into two groups. Most were bidentate, but seven individuals had three mandibular teeth (red circles in Fig. 1). This confirms the findings by Eltz *et al*. (2008), and, given the extended sample size, we are now able to characterize these aberrant individuals further (tridentate males without HNDB).

Allele frequencies were significantly different between bidentate and tridentate males at each of the three screened loci (exact probability test, $N = 176$; $P < 0.001$ for ann02 and Egc17, $P < 0.05$ for ann08). However, only one of the markers, ann02, showed sufficiently little overlap in fragment sizes to allow genetic assignment of individuals to either lineage (Fig. 2). Unambiguously, these individuals all have small fragment sizes (179–183 bp) characteristic for the bidentate lineage. In contrast, tridentate males with HNDB, with a single exception, have fragment sizes above 199 bp. Thus, it is evident that males of the bidentate lineage occasionally express a third mandibular tooth. Based on this finding we carefully examined the shape of the male mandible, in particular the relative position of the middle tooth. The seven tridentate individuals lacking HNDB had interdental distance ratios consistently and significantly lower than tridentate males with HNDB (*t*-test, $t = 5.1$, $P < 0.01$, $N = 49$), i.e. their middle tooth was displaced towards the tip of the mandible (Fig. 3B). The seven aberrant individuals represented 10.3% of bait-captured males of the predominantly bidentate lineage on the Yucatán peninsula.

Of the 245 males examined for quantitative morphometrics, 152 had three mandibular teeth and 93 had two. However, among the 152 tridentate males examined, we found six that had their middle tooth displaced towards the tip of the mandible, and according to the findings reported above, these six males were grouped with the bidentate individuals. There were significant differences between the two groups of males in the size of six of the 15 measured characters (Table 1). All these characters were smaller in males of the tridentate lineage, but in each case there was considerable overlap between the two groups of males. The clearest size differences were found in the width and length of the hind tibia (ATP and LTP), reflecting a subtle but consistent difference in hind tibial shape (see Fig. 5 below). In the PCA, component 1, explaining 58% of the variability, and component 3, explaining 9% of the variability, were significantly different between lineages (ANOVA; $P < 0.01$), but again there was substantial overlap between them (Fig. 4).

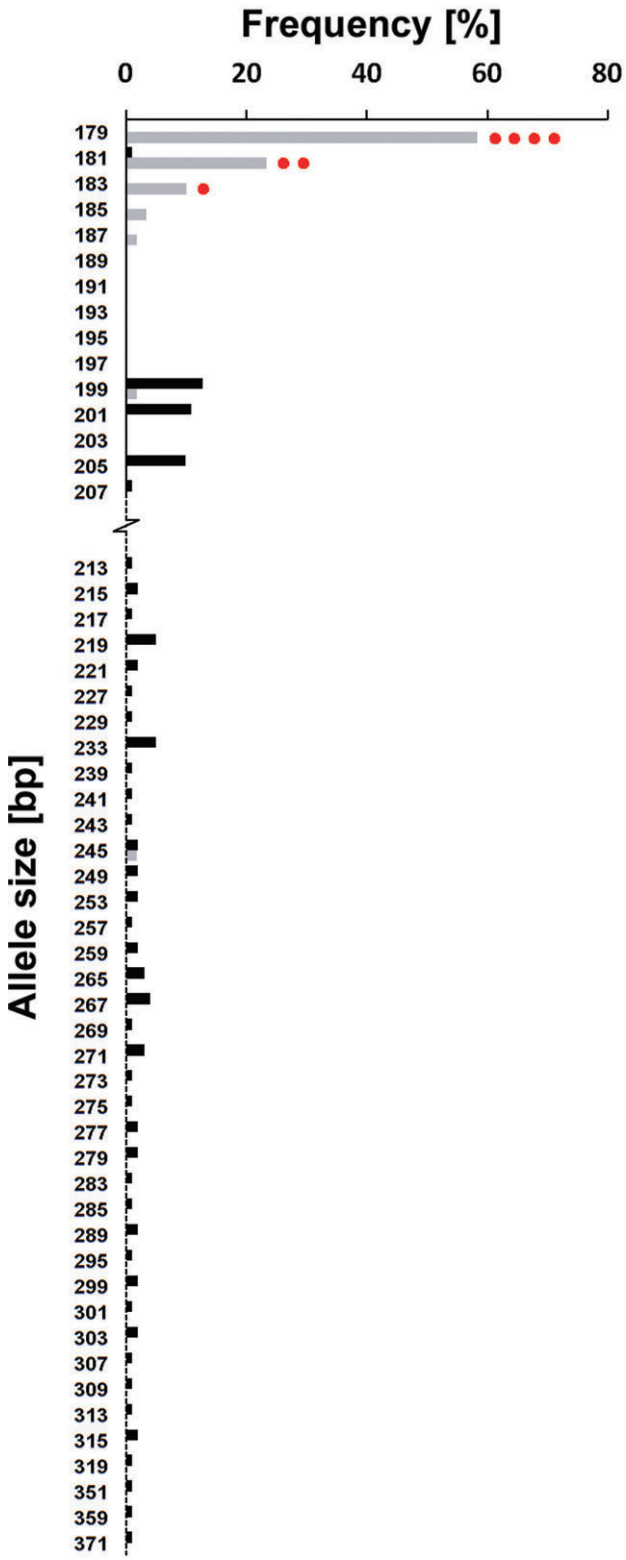


**Figure 2.** Allele size distribution of *Euglossa viridissima*-like males from the Yucatán peninsula, Mexico, at the microsatellite locus ann02. Overall, bidentate males (grey bars) had significantly smaller allele sizes than tridentate individuals (black bars), and there was little overlap in allele size. The seven individuals indicated as red circles were also tridentate, but had been clustered with bidentate males in the analysis of perfume similarity (see Fig. 1), lacking HNDB. These seven individuals had the third (central) mandibular tooth significantly displaced towards the tip of the mandible (nearer to the distal tooth, see Fig. 3B), unlike in other tridentate males. Their ann02 allele size suggests that they in fact belong to the bidentate lineage. See text for further explanation.

### *EUGLOSSA* (*EUGLOSSA*) *DILEMMA* BEMBÉ & ELTZ, SP. NOV.

The chemical, molecular, and morphological dichotomies described above require a re-evaluation of the taxonomic status of what was previously considered *E. viridissima* Friese. The male lectotype of that taxon at the Natural History Museum in Vienna has two mandibular teeth (see below) and, unambiguously, belongs to the less common, predominantly bidentate lineage. As a consequence this lineage has priority over the name *E. viridissima* Friese, and we describe the more common tridentate lineage as a novel species.

*Material and methods:* The studied specimens belong to the entomological collections of the Zoologische Staatssammlung München (ZSM), Germany, the Smithonian Institution (SI) in Washington, DC, USA, the Natural History Museum of Kansas (Snow Entomological Collections, SEC), the Collection of Thomas Eltz (CTE), Bochum, Germany, and to the Collections of G. Gerlach (CGG), Munich, Germany. The type series comprises 255 male specimens. *Euglossa dilemma* has been compared with the male lectotype of *E. viridissima* from the Naturhistorisches Museum Wien, Austria. The lectotype, which is also referred to by Moure (1970), is dirty, and missing the right antenna above the scape, left hindleg, and left forewing. Its right mandible, which bears two pointed teeth, is clearly visible. It has tree labels: 'Bilimek Mexico 1871 // Euglossa § viridissima det. Friese 1898 // LECTOTYPE viridissima Friese, J. S. Moure 1958'. In the following, terga and sterna are referred to as T1, T2, T3, etc., and S1, S2, S3, etc. Integument and setae coloration were described by eye using a Leica MZ 6 dissecting microscope.

*Diagnosis:* Males of *E. dilemma* can be distinguished from those of *E. viridissima* by two main morphological features: males of *E. dilemma* have three man-

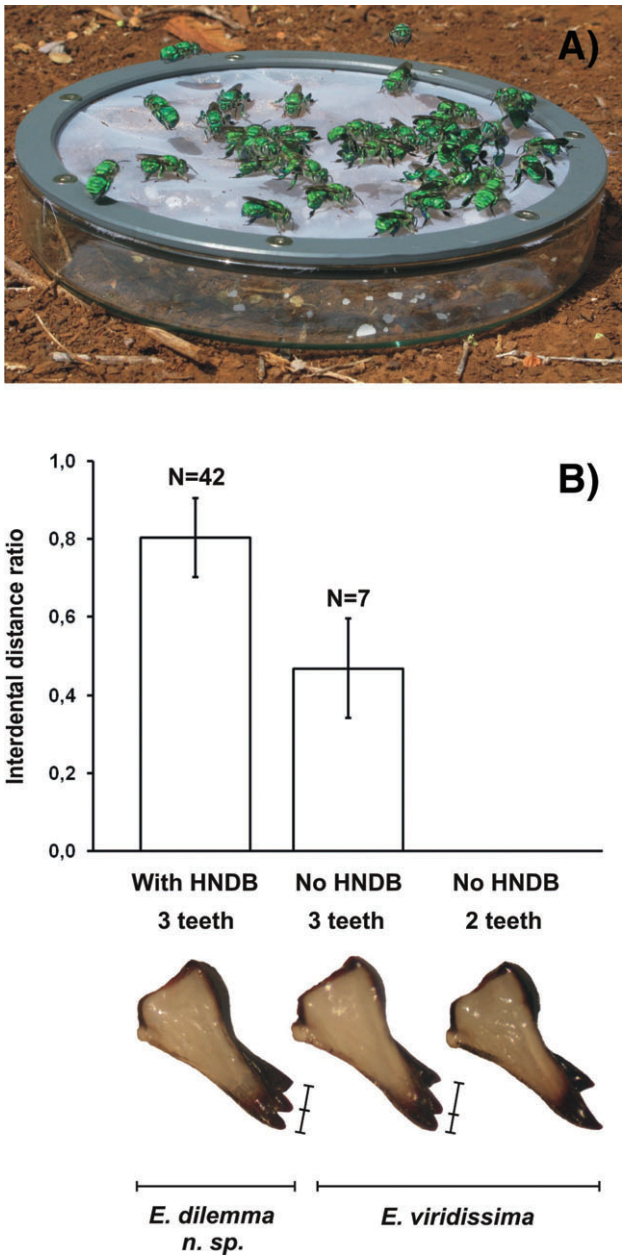


**Figure 3.** A, *Euglossa viridissima*-like males attracted to a bait dish at Xmatkuil, Yucatán, Mexico. B, mandibular morphology of males of tridentate ***Euglossa dilemma* sp. nov.**, and tridentate and bidentate males of *E. viridissima*. The position of the central mandibular tooth in tridentate individuals is expressed as the ratio of the distance between the distal and the central tooth to the distance between the central and the basal tooth. Means and standard deviations are given.

dibular teeth, with the intermediate tooth placed approximately at half distance between the other two teeth (Fig. 3B), and their hind tibia is less inflated, with its distal/posterior edge pointing in a more acute angle; in *E. viridissima* the hind tibia is slightly more obtuse and rounded (Fig. 5).

## Description

*Male:* Colour and vestiture. Whole bee metallic green, darkest green on front of clypeus (seldom with bluish hue) and hind legs, lighter green on face near antennal scape and behind the ocelli, on propodeum, pleurae, and on T7. Violet stripe in front of velvet area on mid tibia. Clypeus and front of head with white setae, on top of head mixed with black setae; scutum and scutellum dorsally covered with a mix of many white and some black setae, end of scutellum only white; terga with tiny white setae, on top of T2–T5 some black setae; sterna with white setae, centre of S2 with two large joining cushions of dense brownish hairs; wings transparent, covered with short black setae, wing venation dark brown. Jugal comb at base of hind wing with 16 blades.

Head. Width 4.6 mm, height from labrum to front 2.6 mm. Mandibles with three teeth, with the intermediate tooth placed approximately at half distance between the other teeth (Fig. 3B). Tongue in repose reaching S2. Front of clypeus densely punctuated, with a complete medial rigde; ivory paraocular marks well developed, reaching the malar area; forward side of antennal scape with white stripe occupying two-thirds of its length. Labrum white with a medial ridge and two oval transparent windows.

Thorax and metasoma. Total body length 10.5–11.5 mm; anterior wing 9.2 mm; scutum 2.9 mm wide (intertegular distance) and 2.7 mm long; scutellum 2.7 mm wide and 1.3 mm long. Punctuation on scutum dense and regular, all punctures have nearly the same size. Scutellum rounded on posterior margin. Punctuation on scutellum not as dense as on scutum. Anterior rim of scutellum with small punctures, at the centre and towards the posterior margin with larger punctures as scutum, nearly as many micropunctures as punctures. Median depression covering about two-thirds of scutellum, depression without punctures or with small punctures only. Punctuation on metasoma. Anterior half of T1 with sparse, large punctures; posterior half with dense, small punctures; T2–T4 with dense, smaller punctures as scutum; punctures in T5–T7 larger, and sparser.

Legs. Mid tibia, anterior tuft smaller than posterior tuft, triangular or comma-like. Posterior tuft oval or with drop shape, very close to anterior tuft. Generally, mid-tibial tufts indistinguishable in size and shape from those of *E. viridissima*. Velvet area dense on anterior end, sparse and incomplete on posterior end.

**Table 1.** Morphological measurements taken from males of the two lineages

| Measurement (mm) | *E. viridissima* (*N* = 99) | *E. dilemma* sp. nov. (*N* = 146) | *F* | *P* |
|---|---|---|---|---|
| Forewing length | 9.082 ± 0.043 a | 9.038 ± 0.035 a | 0.63 | 0.429 |
| Forewing width | 2.833 ± 0.014 a | 2.824 ± 0.011 a | 0.22 | 0.639 |
| Hind wing length | 4.414 ± 0.022 a | 4.415 ± 0.018 a | 0.00 | 0.973 |
| **Hind wing width** | **1.789 ± 0.009a** | **1.712 ± 0.008b** | 37.67 | 0.000* |
| **Hind tibia length** | **4.107 ± 0.025a** | **3.978 ± 0.020b** | 15.74 | 0.000* |
| **Hind tibia width** | **2.730 ± 0.016a** | **2.641 ± 0.013b** | 17.70 | 0.000* |
| Clypeus length | 1.102 ± 0.008 a | 1.122 ± 0.007 a | 3.47 | 0.063 |
| **Clypeus width** | **1.007 ± 0.006a** | **0.982 ± 0.005b** | 8.32 | 0.004* |
| Thorax width | 3.395 ± 0.013 a | 3.422 ± 0.011 a | 2.20 | 0.139 |
| Thorax length | 3.013 ± 0.015 a | 3.011 ± 0.012 a | 0.02 | 0.891 |
| Eye width | 1.451 ± 0.008 a | 1.452 ± 0.007 a | 0.02 | 0.876 |
| Eye length | 2.867 ± 0.012 a | 2.897 ± 0.010 a | 3.45 | 0.064 |
| **Head length** | **2.754 ± 0.016a** | **2.692 ± 0.013b** | 8.25 | 0.004* |
| Head width | 5.083 ± 0.020 a | 5.090 ± 0.016 a | 0.08 | 0.774 |
| **Distance mandibular base** | **2.242 ± 0.012a** | **2.185 ± 0.010b** | 12.09 | 0.000* |

Measurements are given as mean ± SD. Results of an ANOVA (d.f. = 1, 243) are also given and significant differences ($P < 0.01$) are highlighted in bold letters and numerals.

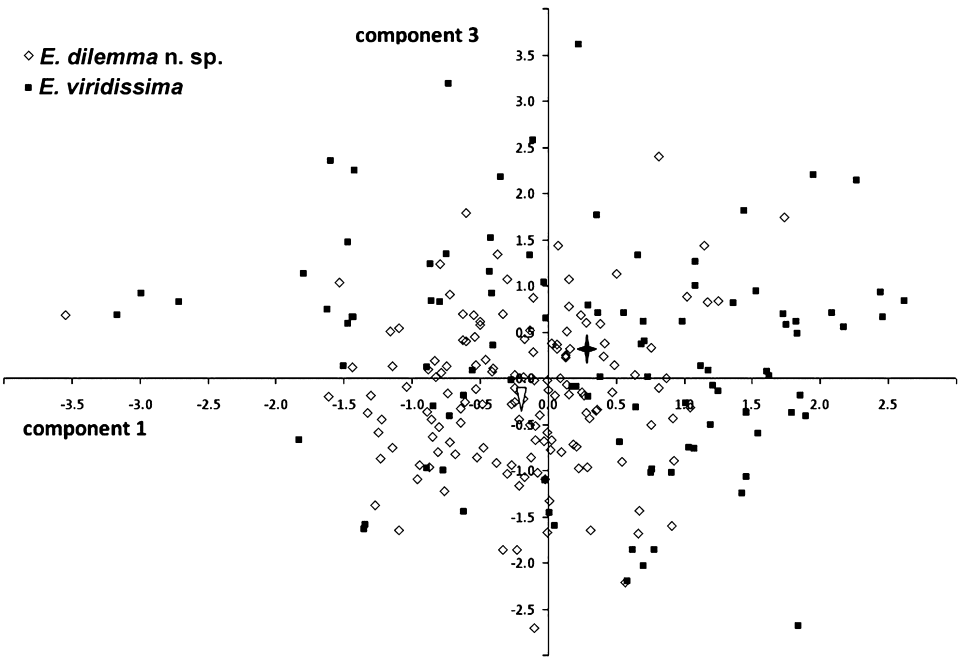


**Figure 4.** Results of a PCA of 15 morphological variables measured in male *Euglossa viridissima* and ***Euglossa dilemma* sp. nov**. Components 1 and 3, which showed significant differences between the species, are used for this two-dimensional representation. Note that *E. dilemma* shows slightly less variability and is essentially nested within *E. viridissima* morphospace. Centroids of distributions are shown.

Hind tibia triangular, 3.3 mm long and 2.6 mm wide. Basal third densely punctured. The distal two-thirds sparsely punctured; distance between punctures one or two diameters of punctures; punctures long, as many micropunctures as punctures. Hind tibia not as obtuse and distal/posterior edge less rounded as in *E. viridissima* (Fig. 5). Post-glandular area fringed with medium-sized hairs.

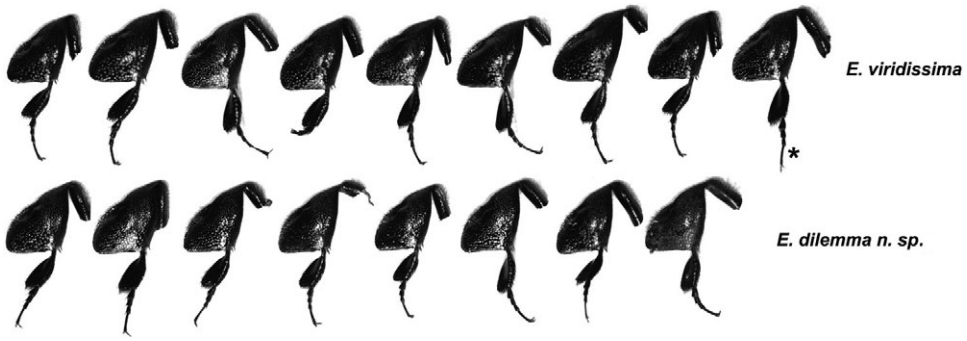


**Figure 5.** Series of right hind legs of males of the two species. Male *E. viridissima* (top line) have slightly larger, more obtuse, and distally less punctured hind tibiae than male ***Euglossa dilemma* sp. nov**. (bottom line). The asterisk indicates an exceptional tridentate individual of *E. viridissima* exhibiting typical *E. viridissima* hind tibial shape.

*Female:* Similar to male in size, coloration, punctuation, and vestiture. Paraocular marks, white stripe on antennal scape, or tufts on legs and S2 absent. Tongue in repose reaching S2. Mandible tridentate, distal and middle tooth ending in a point or rounded, basal tooth flat and broader than other two teeth. Scutellar tuft one-third to nearly half scutellar length. Hind tibia rounded with strong black or white setae, shiny but covered with micropunctures.

*Type locality:* Holotype collected at Xmatkuil near Mérida, Yucatán, Mexico (20°52′11.47″N, 89°37′ 10.03″W), April 2006, coll. Thomas Eltz.

*Distribution:* From Mexico to Costa Rica. Recently introduced into southern Florida, USA (see also Geographical distribution below).

*Etymology:* The species epithet refers to the dilemma of having to describe a taxon as a novel species although this taxon had been widely considered as an existing species (dilemma is a noun in apposition).

*Type material:* HOLOTYPE. Male, with the following label data: 'Mérida (Xmatkuil) Yucatán, Mexico April 2006, coll. Thomas Eltz // Holotypus, male, Euglossa dilemma, Bembé & Eltz, 2010' (ZSM). PARATYPES. 254 males, with the following labels data: 1 § 'MEXICO. Quintana Roo, 12 km NW. Reforma, 14 October 1986, C. D. Michener *et al*.' (SEC); 1 § 'MEXICO. S. Luis Potosí, Tlamaya Falls nr. Xilitla, 600 m, 1-IX-1991, D. Yanega' (SEC); 1 § 'MEXICO. Chiapas, 5 km S Cacahoatan, 340 m, 14°57′N, 92°09′W, 18-IV-1993, E. Tovar' (SEC); 2 §§ 'MEXICO. Veracruz, 5km S Boca del Rio . . . Jan. 2, 1982, B. H. Smith' (SEC); 2 §§ 'MEXICO. Veracruz, "Dunas Costadas" . . . Jan. 2, 1982, E. M. May' (SEC); 10 §§ 'GG-1739 – 1748, Mexico, 8/99, Quintana Roo, Las Panteras (bosque alto), coll. G. Gerlach, Köder: p-Dimethoxybenzen' (CGG); 1 § 'Mexico, Bacalar, 18°45′n. B., 88°40′w. L., 10 m ü NN, 10. 1. 1998, leg. B. Bembé' (ZSM); 1 § 'MEXICO, Veracruz' 'XI-1963, NLH Krauss' (SI); 23 §§ 'Merida (Xmatkuil) Yucatán, Mexico, 13.–27. 10. 2006, coll. Thomas Eltz' (6: CTE, 15: ZSM, 1: Collection Linz, Austria, 1: Universidade Federal de Minas Gerais, Belo Horizonte, Brazil); 1 § 'Mayapán, Yucatán, Mexico, 13.–27. 10. 2006, coll. Thomas Eltz' (CTE); 7 §§ 'Xmatkuil, Yucatán, Mexico, at chemical bait, September 2009, coll. Thomas Eltz' (1: CTE, 6: ZSM); 4 §§ 'Merida (Xmatkuil) Yucatán, Mexico, April 2006, coll. Thomas Eltz' (1: CTE, 3: ZSM); 10 §§ 'Chiquilá, Quintana Roo, Mexico, at chemical bait, September 2009, coll. Thomas Eltz' (4: CTE, 6: ZSM); 3 §§ 'Chetumal, Quintana Roo, Mexico, at chemical bait, September 2009, coll. Thomas Eltz' (CTE); 1 § 'Atasta, Campeche, Mexico, at chemical bait, September 2009, coll. Thomas Eltz' (CTE); 3 §§ 'Mexico, Veracruz, UNAM/Catemaco, VIII 92, coll. Thomas Eltz' (2: CTE, 1: ZSM); 1 § 'EL SALVADOR, San Salvador, 23. May 1958, OL Cartwright' (SI); 1 § '. . . ?' (SI); 1 § 'Qualan Quat, J E 4. 22. 1912' (SI); 2 §§ 'Belize, Sartaneja, Shipstern Reserve, 19°30′n. B., 88°20′w. L., 10 m ü NN, 12. 1. 1998, leg. B. Bembé' (ZSM); 2 §§ 'Guatemala, Santa Rosa, Monterrico, 25. VIII. 92, coll. Thomas Eltz' (1: CTE, 1: ZSM); 2 §§ 'GG-1459 – 1460, Guatemala, 4/98, San Lucas Toliman, Finca Faser, coll. P. Josefidou, Köder Eugenol' (CGG); 2 §§ 'Honduras, Utila, 16°15′n. B., 86°50′w. L., 20 m ü NN, 15. 2. 1998, leg. B. Bembé' (1: ZSM, 1: CTE); 1 § 'Honduras, Copan Ruinas, 14°35′n. B., 89°08′w. L., 3. 2. 1998, leg. B. Bembé' (ZSM); 1 § 'Honduras, la Ceiba, 4. 2003, leg. H. Heider' (ZSM); 1 § 'HONDURAS: Atlantida, Lancetilla Bot. Grd., Tela, 10 m, 22 VI 1994, 15°64′N, 87°27′W, Ashe, Brooks, ex: methyl salicylate' (SEC); 1 § 'HONDURAS: Atlantida, La Ceiba, 15 km W, 175 m, June/July 1996, R. Lehmann, ex: methyl salicylate' (SEC); 3 § 'GG-1125 –

1127, Nicaragua, 12/96, L. de Nicaragua, Insel Ometepe, coll. Anette Müller' (CGG); 5 §§ 'Nicaragua (-See), Insel Ometepe, Altagracia, 11°30′n. B., 85°42′w. L., 35 m ü NN, 22. 2. 1998, leg. B. Bembé' (ZSM); 64 §§ 'COSTA RICA: Guanacaste, Palo Verde Nat'l Park, 34 km SW Canas, 1 February 1984, at eugenol, S. A. Cameron' (SEC); 10 §§ 'COSTA RICA: Guanacaste, Palo Verde Nat'l Park, 34 km SW Canas, 1 February 1984, at methyl salicylate, S. A. Cameron' (SEC); 7 §§ 'COSTA RICA: Guanacaste, Palo Verde Nat'l Park, 34 km SW Canas, 1 February 1984, at cineole, S. A. Cameron' (SEC); 3 §§ 'COSTA RICA: San José, San Antonio de Escazu, 26 March 1984, at cineole, Sydney A. Cameron' (SEC); 3 §§ 'COSTA RICA: Heredia (La Selva Biol. Res.) nr. Puerto Viejo, 3–10 Mar 1984, at cineole, Sydney A. Cameron' (SEC); 2 §§ 'COSTA RICA. San José Pro., 4 km E. San Ignacio de Acosta, 8 July 1963, 4000ft. (C. D. Michener *et al*.)' (SEC); 1 § 'COSTA RICA. Guanacaste Prov., El Coco, 21 June 1963 (C. D. Michener)' (SEC); 1 § 'COSTA RICA. San José Pro., San José, 13 August (Michener & Kerfoot)' (SEC); 35 §§ 'COSTA RICA: San José Prov., San Antonio de Escazú, 13–17 Apr 1985, W. T. Wcislo' (SEC); 7 §§ 'COSTA RICA: San José Prov., San Antonio de Escazú, 22–24 Apr 1985, W. T. Wcislo' (SEC); 7 §§ 'COSTA RICA: Puntarenas Prov., Monteverde, 1500 m. January 1978, visiting Ipomoea, L. A. Real' (SEC); 3 §§ 'COSTA RICA: Heredia Santo Domingo, Hotel Bougainvillea, 9°58′30″N, 84°4′36″W, 14 May 1999, D. Brzosca, CR1B99 004 ex: on vervain' (SEC); 1 § 'COSTA RICA: Alajuela . . . 10°16′24″N, 84°31′24″W, 16 May 1999, D. Brzosca, CR1B99 006 attracted to methyl salicylate/eucalyptus oil' (SEC); 2 §§ 'San José, Costa Rica' (ZSM); 1 § 'Prov. San José, Costa Rica, SN-Jeronimo-Sabanilla, 21-V-1967, col. Wille' (ZSM); 4 §§ 'San José, Costa Rica' (SI); 1 § 'Finca Gibraltar, Spt. 1910, MEX.' (SI).

### Phylogenetic analysis

Both parsimony and Bayesian analyses supported the monophyly of *E. viridissima* + *E. dilemma* with high bootstrap and posterior Bayesian support (Fig. 6). However, neither lineage was recovered as monophyletic due to the lack of parsimony-informative characters within lineages. Only eight characters (from a total of 889) were variable between the two lineages, but all of these were parsimony-uninformative. The phylogenetic position of *E. viridissima* + *E. dilemma* within the genus *Euglossa* was congruent with the placement recently proposed based on several nuclear and mitochondrial genes (Ramírez *et al*., 2010b). We estimated the age of the MRCA of both *E. dilemma* and *E. viridissima* by implementing a molecular clock analysis. Our results suggest that these two lineages shared a MRCA between 110 000 and 150 000 years ago. Because our phylogenetic analysis based on CO1 did not support these two lineages as reciprocally monophyletic, the MRCA age estimates represent a maximum bracket for the actual divergence of these two species.

### Geographical distribution

As *E. viridissima* and *E. dilemma* have been lumped by previous authors and collectors, we reassessed distributional ranges by compiling information from recent baiting assays and from museum material that we have reanalysed. The two species occur sympatrically in most of central and southern Mexico. However, they appear to have different southern distributional limits. Whereas *E. dilemma* is commonly collected in western and central Costa Rica, the southernmost range limit of *E. viridissima* appears to be Honduras (Fig. 7).

## DISCUSSION

Dressler (1978: 189) stated the following regarding *Euglossa viridissima*: 'This species appears to be polymorphic in the number of teeth. It is possible, of course, that there are two sibling species, and the number of teeth is the only distinction we have found between them.' It turns out that both alternatives are actually true. *Euglossa viridissima* is indeed polymorphic in the number of male mandibular teeth (rare males expresses a third tooth), and it coexists with a widespread sibling species, *E. dilemma*, that has a fixed number of three mandibular teeth. Thus, one cryptic sibling species has been obscured by a morphological variation (polymorphism or plasticity) in the other. It is evident from our microsatellite, morphological and chemical data that the two lineages are reproductively isolated species, thus warranting the establishment of *E. dilemma* as a novel species. The first hint to this cryptic diversity was provided by chemical dichotomies in male perfumes, which probably play an important role in mate/species recognition in orchid bees (Zimmermann *et al*., 2009). Secondary sexual characters involved in mate recognition are expected to be most divergent in closely related species due to diversifying selection in sympatry (Andersson, 1994). Such diversifying selection can occur before postzygotic barriers are established between lineages due to lower fitness of hybrids, and is then equivalent to 'reinforcement' (e.g. Butlin, 1987). Alternatively, it can occur after the process of speciation is essentially complete. In this case, recognition errors could be selected against due to costs measured in loss of time, energy, and gametes, as well as the predation

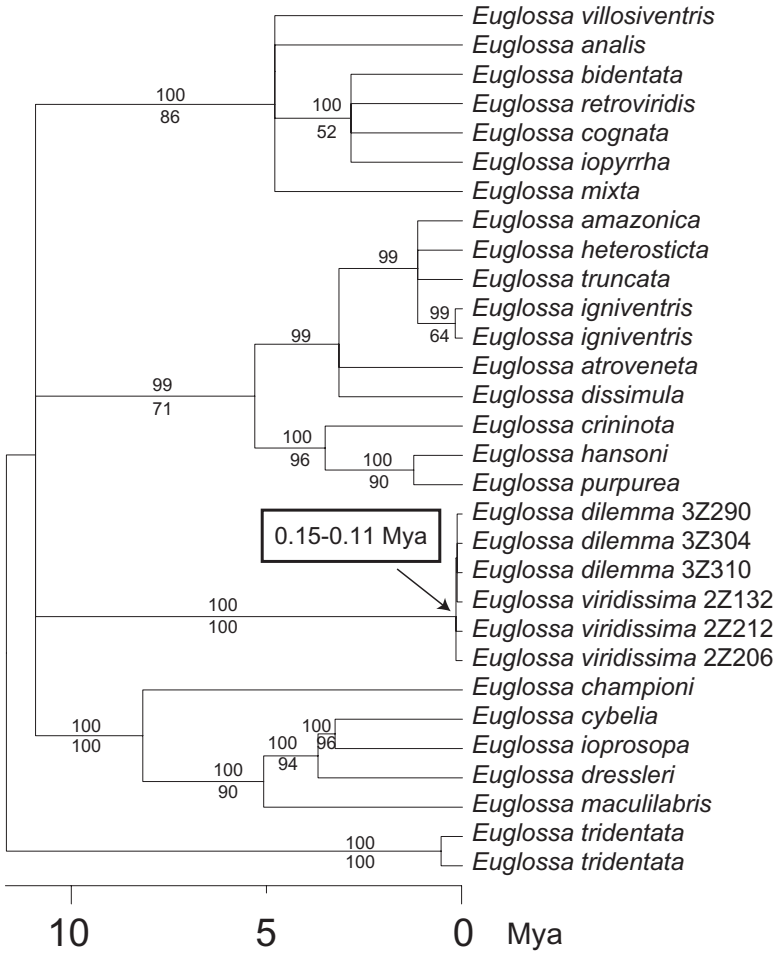


**Figure 6.** Chronogram showing divergence times and phylogenetic relationships of selected lineages in the genus *Euglossa* and the sibling species ***Euglossa dilemma*** **sp. nov.** and *E. viridissima*. The tree topology corresponds to that obtained via Bayesian methods. Bayesian posterior probabilities and parsimony bootstrap values are shown for the sister species only. Divergence times were obtained via penalized likelihood using the fossil-calibrated molecular clock procedures described in Ramírez *et al*. (2010b). The maximum and minimum age estimates for the MRCA of *E. dilemma* and *E. viridissima* correspond to the molecular clock analyses in which the MRCA of the genus *Euglossa* was assigned a fossil calibration of 20 and 15 Myr, respectively.

risk involved in spurious mate searching (Coyne & Orr, 2004). In both cases selection is expected to favour signals that allow unambiguous recognition of mates in sympatry. Although direct evidence that perfumes affect mate localization/choice in orchid bees is still lacking, there is evidence for diversifying selection on perfumes (Zimmermann *et al*., 2009). Among 15 sympatric species of the genus *Euglossa* from Barro Colorado Island in central Panama, the chemical composition of male perfumes was found to be non-random. All species had predictable blends, in terms of both diagnostic compounds and relative compound proportions. Furthermore, the difference in blends between species was larger than expected from a neutral null model of chemical evolution, and especially conspicuous amongst the most recently diverged lineages. This suggests that the perfumes have diverged quickly during or after the speciation process, as expected for recognition characters involved in mate finding (Zimmermann *et al*., 2009). The chemical dichotomy we found between *E. viridissima* and *E. dilemma* in southern Mexico may also have resulted from such diversifying selection. However, in this particular case, the chemical dichotomy is mainly determined by a single compound, HNDB, present only in perfumes of male *E. dilemma*, and is associated with a sensory adeptness to detect this compound in male *E. dilemma*. It remains unclear whether this sensory shift was a cause or a consequence of the speciation process.

*E. viridissima* and *E. dilemma* are broadly sympatric throughout most of their distributional range. In

**Figure 7.** Geographical distribution of tridentate ***Euglossa dilemma* sp. nov.** (black) and predominantly bidentate *Euglossa viridissima* (white) as inferred from recent baiting assays (circles) as well as museum material (diamonds). Note lack of *E. viridissima* in the south-eastern part (Costa Rica) of the range. Museum material included paratypes of *E. dilemma* and additional specimens of one or both species in the collections of D. W. Roubik, T. Eltz (CTE), G. Gerlach (CGG), the Zoologische Staatssammlung München (ZSM), the Smithsonian Institution (SI), and the Snow Entomological Collection (SEC). Only unambiguous and non-redundant localities were plotted. Localities of baiting assays are (from west to east): Chamela (Jalisco, Mexico), El Chote (Veracruz, Mexico), Ayozinthepec (Oaxaca, Mexico), Monte Pio and Poza Azul (both Veracruz, Mexico), Tuxtla Gutiérrez, Esquintla, Tapachula, Ocosingo and Palenque (all Chiapas, Mexico), Atasta (Campeche, Mexico), Retalhuleu (Guatemala), Lacanjá (Chiapas, Mexico), Escarcega (Campeche, Mexico), El Remate (Campeche, Mexico), Chablekal, Xmatkuil (Yucatán, Mexico), Tikal (Guatemala), San Crisanto (Yucatán, Mexico), Chetumal and Coba (both Quintana Roo, Mexico), Chinandega, Chacocente, Escameca Grande, Jinotega, Ometepe and Las Pampas (all Nicaragua), and Area de Conservación Guanacaste (Costa Rica).

the majority of Mexican localities where we have baited, individuals of both species were present at the same individual baits, although bait captures were often biased towards *E. dilemma* (T. Eltz, J. Ramírez Pech, S. Ramírez unpubl. data, Eltz *et al*., 2008). In fact, bait captures of *E. dilemma* were outstanding in some localities on the Yucatán peninsula, with up to 300 individual bees captured and marked on a single morning at a single baiting site (T. Pokorny, M. Hanibal & T. Eltz, unpubl. data). Both species appear to have similar ecological requirements, with a clear preference for hotter and drier habitats than is usual for euglossine bees. Both species thrive in heavily degraded dry forests in western Yucatán (Eltz *et al*., 2008), are surprisingly abundant in suburban parks and gardens (J. Ramírez Pech, unpubl. data), and were present in pastures devoid of forest remnants in Veracruz (Zimmermann *et al*., 2011). In summary, *E. viridissima* and *E. dilemma* appear less dependent on intact tropical forest than most other Central American species of euglossines.

Although the two species appear relatively similar in their ecological requirements, their distributional ranges do not overlap completely. Repeated and intensive baiting in north-western Costa Rica and Nicaragua failed to lure *E. viridissima*. Also, not a single *E. viridissima* was found among the museum specimens collected in Costa Rica. Thus, it seems likely that *E. viridissima* does not occur in Costa Rica, and that *E. dilemma* is therefore allopatric with respect to its sister species in its south-eastern distributional range. The ecological or historical reasons for this difference in geographical range remain unknown. Florida, where *E. dilemma* was recently introduced (Skov & Wiley, 2005; Pemberton & Wheeler, 2006), was also not reached by *E. viridissima* (Ramírez *et al*., 2010a). However, this may be explained by chance alone. Population genetic analyses have shown that Floridan populations of *E. dilemma* have reduced genetic diversity compared with populations from the Mexican mainland, e.g. appeared to have gone through a genetic bottleneck (Zimmermann *et al*., 2011). Such a bottleneck would be expected if the colonization was accomplished by only a few individuals that were accidentally relocated to Florida by a yet unknown mechanism, and from a yet unknown source population (Zimmermann *et al*., 2011).

Our phylogenetic analysis based on the mitochondrial CO1 confirmed that *E. dilemma* and *E. viridissima* are each other's closest relatives. Although CO1 alone failed to provide sufficient resolution to distinguish and resolve these lineages as reciprocally monophyletic, our analysis based on microsatellite, morphological, and chemical data strongly supports the hypothesis that they are distinct. The lack of species-level resolution in our CO1 dataset may be explained by the relatively low mutation rates known to occur in CO1 in orchid bees (Ramírez *et al*., 2010b), but also supports the hypothesis that the two species diverged in the recent past. CO1 has failed before to distinguish morphologically well-defined but closely related species in the genus *Euglossa*, possibly because those populations were very large and species coalescence was extensive (Dick *et al*., 2004). Because our phylogenetic analysis did not resolve *E. dilemma* and *E. viridissima* as separate, our molecular clock age estimates should be considered as an upper age estimate of the actual divergence time between *E. dilemma* and *E. viridissima*. Thus, it is possible that the two species diverged more recently than 150 000–110 000 years ago.

Finally, orchid bees constitute an important group of insect pollinators in the Neotropical Region. In fact, hundreds of plant species (mainly orchids) are known to depend exclusively on male orchid bees for pollination (Williams, 1982), and thousands of angiosperm species from diverse families use the pollination services of both male and female orchid bees (Ramírez, Dressler & Ospina, 2002). To date, about 200 species of orchid bees have been described, mainly based on external morphological characters. However, our results may indicate that a great deal of cryptic diversity in orchid bees, and possibly other insect groups, awaits discovery. Describing this cryptic diversity is crucial to better understand the evolutionary origins and ecology of species interactions.

## ACKNOWLEDGEMENTS

We thank David Roubik (STRI, Panama), Günter Gerlach (Botanical Garden, Munich), Dominique Zimmermann (Naturhistorisches Museum, Vienna), Brian Harris (Smithonian Institution, Washington), and Ismael Hinojosa-Diaz (Natural History Museum, Kansas) for providing specimens or allowing access to their collections. John Plant gratefully made a preliminary examination of the lectotype *E. viridissima* in Vienna. The study was supported by grants of the Deutsche Forschungsgemeinschaft (EL 249/3 and EL 249/6) to T.E.

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