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Chemical niche differentiation among sympatric species of orchid bees

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Abstract. Male Neotropical orchid bees (Euglossini) collect volatile substances (fragrances) from flowers and other sources (e.g., decaying wood) and store them in specialized hind tibial pockets. The accumulated chemicals are later emitted during courtship display, presumably to lure conspecific females for mating. We analyzed tibial fragrances of males of 15 sympatric Panamanian species in the genus *Euglossa* to test whether communities of euglossine bees are chemically structured, and to elucidate whether male fragrance signals evolve to convey premating isolation. Our analysis revealed substantial chemical disparity among all lineages. Disparity was mediated by compounds that were exclusive to certain species but also by differences in relative quantity of shared compounds. We mapped tibial fragrance compounds present in each species on a DNA-based phylogeny (reconstructed using partial sequences of *COI*, *EF1-α*, *ArgK*, and *Pol-II*) and found that most dominant compounds were highly homoplasious. In an analysis of chemical differentiation in relation to phylogenetic divergence through time, disparity was greater than expected from a null model at any point during evolutionary history, suggesting that diversifying selection has shaped fragrance phenotypes. Notably, chemical disparity was greater within recently diverged lineages than among them, suggesting that chemical preferences in orchid bees evolved rapidly in the early stages of species divergence. We postulate communication interference as the possible mechanism behind the observed fragrance differentiation, which may be the product of reproductive character (fragrance) displacement. Our findings are consistent with the hypothesis that male fragrance signals evolve to convey premating isolation.

Key words: Barro Colorado Island, Panama; chemical communication; diversifying selection; Euglossine bees; Euglossini; fragrance; mate recognition; pheromone; phylogeny; reproductive character displacement.

Introduction

Insects frequently use chemical signals to attract conspecifics for sex or resource exploitation. The chemical nature of the molecules emitted by insects is diverse, but the signals must be distinguishable from those used simultaneously by other species and from background noise (Wyatt 2003). In diverse communities, chemical signals may be subject to diversifying selection due to communication interference with sympatric species that use similar chemical signals (Groot et al. 2006). Such interference has been proposed for sex attractants, where the correct chemical signal is wholly or partly responsible for maintaining reproductive isolation (McElfresh and Millar 1999, Higgie et al. 2000, Gries et al. 2001, McElfresh and Millar 2001). The breakdown of chemically mediated mate recognition systems could impose high fitness costs due to either the waste of gametes or the production of inferior hybrids. Such fitness costs are thought to drive signal divergence in areas of sympatry (i.e., reproductive character displacement) (Butlin 1987, Coyne and Orr 2004).

Chemical distinctness may be achieved in two major ways, which frequently interact and can be considered as two extremes of a continuum. At the one end, a single type of molecule is used to transfer information, but because of its complex structure, it is unlikely to be duplicated by other species. At the other end, simple molecules occur in blends of distinctive quantitative proportions (Bjostad et al. 1987). Among the best-studied examples of the latter are the female sex pheromones of certain moths, which consist of variable blends of common fatty-acid derivatives (Bjostad et al. 1987, Morse and Meighen 1987, Roelofs and Rooney 2003).

Neotropical orchid bees (Apidae, Euglossini; ~250 species) are a group of conspicuous, long-tongued bees that share a unique system of chemical communication that is most likely used in the context of mate recognition (Roubik and Hanson 2004, Zimmermann

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et al. 2006). Males of all species of orchid bees possess voluminous pockets in their hind legs, which they use for the collection and accumulation of exogenous volatiles, mostly derived from flowers, fragrant plant exudates, decaying vegetative matter, or feces (Vogel 1966, Whitten et al. 1993, Eltz et al. 2007). This unique behavior has led to the evolution of one of the most intriguing pollination syndromes. Flowers of >700 species of Orchidaceae, among others, each produce scents that specifically attract males of one or a few orchid bee species. As males collect fragrances from these flowers, they act as their exclusive pollinators (Dressler 1982, Ramírez et al. 2002). Male orchid bees accumulate volatiles over much of their lives and thus acquire complex blends consisting of diverse terpenoids and aromatics (Eltz et al. 1999). These blends are later exposed by males during courtship display, which usually takes place in the forest understory where males defend nonresource display territories (Bembé 2004, Eltz et al. 2005*b*). Females have been observed to approach displaying males, in some cases from downwind, and copulate with males on the perch tree (Kimsey 1980, Stern 1991, Zimmermann et al. 2006). Although the direct proof of female attraction to male fragrances remains elusive, it is likely that fragrance signaling is involved in mate recognition and choice.

In samples of males of the same species, fragrances may vary substantially in both quantity and complexity. However, when we compared three species of Central American *Euglossa*, we found that the composition of individual blends was significantly more similar within species than between species, and that the distinction persisted even when samples from different localities and habitats were included in the analysis (Eltz et al. 2005*a*). Bioassays with hind-leg extracts confirmed that the information contained in individual chemical blends is sufficient to mediate species-specific attraction (Zimmermann et al. 2006).

Attaining chemical distinctness in male orchid bees seems a nontrivial task for three reasons. First, specific blends are accumulated from sources that vary in availability both spatially and temporally in the bees' habitat. This has been addressed in a previous study (Eltz et al. 2005*a*), which demonstrated that bees exert experience-dependent choices (negative feedback) to compensate for fluctuating availability. Second, specific blends must accumulate from sources that normally (but not always) produce blends of components themselves (e.g., Gerlach and Schill 1991, Whitten et al. 1993), thus limiting the degrees of freedom that are available for accumulating bee-specific blends. Third, chemical distinctness in orchid bees is made difficult by the elevated species diversity characteristic of Euglossini; a given community may contain up to 50 species, with a substantial fraction of them being active at any one time (Roubik and Hanson 2004).

In the present paper we analyzed male hind-leg fragrances of 15 sympatric species of Panamanian *Euglossa* to determine whether bees within a diverse community can acquire distinct chemical blends, and to elucidate the potential role of exogenous fragrances as premating isolation mechanisms. Specifically we asked (1) whether chemical distinctness between species collapses as more species are included in the analysis, (2) how overall chemical distinctness relates to phylogeny, and (3) to what extent major fragrance components are shared by closely related species. We found that chemical distinctness is substantial between all sympatric species, but most notably so between closely related taxa. Our findings support the hypothesis that male fragrances function as recognition cues and suggest that fragrance phenotypes evolve rapidly in response to chemical interference from congeners.

Materials and Methods

Sampling

From 6 February to 13 March 2006, 176 males of 15 species of *Euglossa* were sampled from an old-growth forest on the island of Barro Colorado (BCI) in the Panama Canal. Bees were netted during standardized baiting assays at the radio tower clearing in the center of the island using the following bait chemicals: 1,8-cineole, methyl salicylate, *p*-dimethoxybenzene, methyl cinnamate, skatole, vanillin, eugenol, benzyl acetate, 2-phenylethyl alcohol, and terpinene-4-ol. On a total of 24 mornings (8:30 to 12:30 hours), these substances were exposed on strips of filter paper housed in plastic "Wiffle" balls. The Wiffle balls were covered by nylon mesh to keep bees from gaining direct access to the chemicals and suspended by rope from branches around the clearing. Arriving bees were captured, cooled on ice in Eppendorf caps (Carl Roth, Karlsruhe, Germany), and later frozen in a laboratory freezer. Fragrance loads were sampled on the same day by extracting individual pairs of hind legs in 0.5 mL of hexane containing 1 mg/mL 2-undecanone as an internal standard. For reference, we also extracted individual heads of all species to gain information on compounds produced by male cephalic labial glands. Labial gland lipids are spat on fragrant surfaces by the males and serve as a carrier during the process of fragrance collection (Eltz et al. 2007). As both lipids and exogenous volatiles are present in hind legs, we used information on head extracts to identify exogenous compounds.

Chemical analysis

Gas chromatography/mass spectrometry (GC/MS) was done at the Department of Neurobiology, Düsseldorf, Germany, using a HP 5890 II GC fitted with a 30-m nonpolar DB-5 column and a HP 5972 mass selective detector (Hewlett Packard, Wilmington, Delaware, USA). Injection was splitless, the oven programmed from 60° to 300°C at 3°C/min with automatic pressure programming.

Mass spectra and associated retention indices of integrated peaks were compared and cross-referenced

TABLE 1. Collection data, taxonomic information, and National Center for Biotechnology Information (NCBI) GenBank accession numbers of samples of *Euglossa* bees used for phylogenetic analyses in the present study.

Taxon	DNA extraction ID	Country	Locality	Province	Collection date
E. allosticta	EU34	Colombia	La Virginia	Risaralda	4 Jan 2003
E. azureoviridis	EU52	Costa Rica	La Selva	Heredia	5 Apr 2003
E. bursigera	EU89	Colombia	Lloro	Chocó	25 Apr 2003
E. cognata	EU27a	Perú	Lagunas	Loreto	5 Mar 2005
E. cognata	EU27	Colombia	Mocoa	Putumayo	10 Jan 2003
E. crassipunctata	EU144b	Colombia	La Virginia	Risaralda	4 Jan 2003
E. deceptrix	EU58	Panamá	Cerro Azul	Panamá	May 2003
E. despecta	EU84	Colombia	Bahia Solano	Chocó	14 Apr 2003
E. dissimula	EU44	Colombia	Mocoa	Putumayo	10 Jan 2003
E. dodsoni	EU13	Costa Rica	La Selva	Heredia	9 Aug 2005
E. hemichlora	EU77	Colombia	Bahia Solano	Chocó	14 Apr 2003
E. heterosticta	EU19	Costa Rica	La Selva	Heredia	11 Aug 2002
E. igniventris	141d	Panamá	BCI	Panamá	Feb/Mar 2006
E. imperialis	EU5	Costa Rica	La Selva	Heredia	10 Aug 2002
E. mixta	EU8	Costa Rica	La Selva	Heredia	16 Aug 2002
E. tridentata	EU9	Costa Rica	La Selva	Heredia	13 Aug 2002
E. villosa	EU70	Guatemala	Chiquimula	Esquipulas	10 Sep 2003

Note: Abbreviations are as follows: BCI, Barro Colorado Island, Panama; *CO1*, cytochrome oxidase; *ArgK*, arginine kinase; *Pol-II*, RNA polymerase II; *EF1*-α, elongation factor 1-alpha.

with entries in the local user library (T. Eltz, *unpublished data*). New components were added over the course of the study. Chemical characterization of components was done by comparison with authentic standards or by matching spectra and retention times with those in the literature (Adams 2001). Mass spectral characteristics alone were not considered sufficient for compound identifications, but were used for an assignment to broader substance classes. Additional identifications were made by R. Kaiser (*personal communication*), who analyzed representative samples of some species. We excluded straight-chain lipids (alkanes, alkenes, alcohols, acetates, diacetates, and wax esters) from the analysis of fragrance composition, because such compounds are typically produced by the bees' labial glands and were prominent in the head extracts of the study species.

Fragrance similarity

Fragrance similarity was analyzed with nonmetric multidimensional scaling (MDS) and associated techniques (Legendre and Legendre 1998, Clarke and Warwick 2001). MDS is flexible concerning the similarity measure employed, which allowed us to use the Bray-Curtis index as a measure of chemical similarity/dissimilarity between samples (individuals). The value that this index takes between any two individuals is affected only by chemical compounds jointly present in the two individuals, but not by those jointly absent. This is desirable because similarities are fixed between pairs of individuals irrespective of the chemical phenotypes of other individuals in the data matrix (Clarke and Warwick 2001). Prior to calculating the index, absolute peak areas (integrated MS ion currents) were standardized to represent relative peak contributions to individual fragrance composition (in percent), and these were then square-root transformed. From these data we derived a triangular similarity matrix based on the mentioned Bray-Curtis index. Similarities (in percent) were ordinated in two or three dimensions using the nonmetric MDS algorithm in PRIMER v6 (Clarke and Gorley 2001). Ideally, MDS plots have interpoint distances that exactly match the rank order of dissimilarities between samples in the underlying similarity matrix. Deviations from this match are expressed in terms of "stress," with stress values <0.15 indicating a good fit concerning the overall structure of the plot. We tested the null hypothesis that the factor "species" had no effect on the rank order of between-individual similarities using ANOSIM one-way permutation tests (Clarke and Green 1988).

To determine whether chemical distinctness between species was affected by increasing the numbers of congeners present in fragrance space, we randomly assembled bee communities that differed in total species number. Average Bray-Curtis dissimilarity (1 − Bray-Curtis similarity) was calculated between the focal species and each of the other members of the communities, with a total of 15 focal species, 14 different communities per focal species (with one to 14 congeners), and 25 permutations of the order in which species were assigned to these communities. We plotted minimum community-wide dissimilarity to the focal species, averaged across communities, with the same number of coexisting species (Fig. 3).

Search for independent "building blocks" (motifs) in blends

The complexity of individual fragrance loads represents a major obstacle for discerning the more simple "building blocks" that compose male fragrance blends. We based our search on the assumption that compounds

TABLE 1. Extended.

Voucher no.	Collector	*CO1*	*ArgK*	*Pol-II*	*EF1-α*
SR52	S. Ramírez	EU421517	EU421648	EU421266	EU421389
DM1	D. McKenna	EU421541	EU421672	EU421288	EU421412
JCN.124	J. Neita	EU421573	EU421701	EU421319	EU421446
SR1189	S. Ramírez		EU421640	EU421261	
SR395	S. Ramírez	EU421510			EU421381
SR74	S. Ramírez	EU421494	EU421626	EU421246	EU421365
R1	D. Roubik	EU421546	EU421675	EU421293	EU421418
TA235	T. Arias	EU421568	EU421696	EU421314	EU421441
SR394	S. Ramírez	EU421530	EU421661	EU421277	EU421401
CS11	C. Skov	EU421484	EU421613	EU421234	EU421353
TA258	T. Arias	EU421561	EU421689	EU421306	EU421433
CS80	C. Skov	EU421500	EU421632	EU421252	EU421371
EU141d	T. Eltz	EU421492	EU421623	EU421243	EU421362
CS26	C. Skov	EU421537	EU421668	EU421284	EU421408
CS155	C. Skov			EU421309	EU421436
CS122	C. Skov	EU421574	EU421702	EU421320	EU421447
Yurr912	C. Yurrita	EU421556		EU421301	EU421428

derived from the same source would exhibit a tight positive correlation in quantity across individual males, with the proportion corresponding to that in the source (e.g., a certain species of orchid). However, a tight correlation would only be expected if the compounds in question are not also collected from alternative sources where they occur in different proportions. In the latter case, the composition of the original source is likely obscured. We first analyzed each species separately and selected components that were present in the majority (>60%) of individuals. Pearson R was calculated between square-root transformed peak areas of all pairs of these components across individuals. Only the top 5% of positive correlations per species were further analyzed and subjected to visual inspection by scatterplot. Finally, we analyzed whether convincing correlations would hold in cross-species analyses, suggesting shared fragrance sources. To do so we calculated R and visually inspected scatterplots across the individuals of two or more species.

Phylogenetic inference

The genus *Euglossa* comprises a relatively well-defined group of >100 species, of which 44 have been recorded from Barro Colorado Island, Panama (BCI) (Ackerman 1989). The 15 ingroup taxa here included represent a random (unbiased) sample of those taxa (all species with three or more individuals captured were included). To reconstruct the phylogenetic relationships of those taxa, we sequenced a total of ~4.0 kb from four different loci, including the mitochondrial protein-coding gene cytochrome oxidase (*CO1*, 1.2 kb) and the nuclear protein-coding genes elongation factor 1-alpha (*EF1-α*, ~1.2kb), arginine kinase (*ArgK*, ~0.7kb), and RNA polymerase II (*Pol-II*, 0.8kb). We used the species *Euglossa villosa* as our outgroup based on previous results (Ramírez 2008). We followed standard protocols of DNA extraction, amplification, and sequencing as indicated in Ramírez (2008). Voucher specimens for all sampled species were deposited in the Entomological Collection of the Museum of Comparative Zoology (Harvard University, Cambridge, Massachusetts, USA); GenBank (National Center for Biotechnology Information [NCBI]) accession numbers are indicated in Table 1.

We used the software ModelTest, Version 3.7 (Posada and Crandall 1998), to determine the best-fit model of sequence evolution for each locus separately. We implemented Bayesian phylogenetic analyses in the software package MrBayes, Version 3.1.1 (Ronquist and Huelsenbeck 2003). Tree searches were performed assuming a single model of sequence evolution for all loci, and two independent Markov chain Monte Carlo (MCMC) searches were made for 10 million generations, sampling every 1000 generations, for a total of 10 000 trees. Four different chains per search replicate were used. We estimated model parameters during runs and estimated Bayesian posterior probabilities as the proportion of trees sampled; the trees obtained in the first one million generations were discarded as "burnin," which we determined by plotting the log likelihood values against the number of generations. Additionally, we estimated the phylogenetic relationships among bee lineages with maximum likelihood (ML), as implemented in the software package Garli, Version 0.96 (Zwickl 2006). We determined node support using ML by running 100 nonparametric bootstrap replicates. Equivalent models of sequence evolution and estimated parameters were applied to both Bayesian and ML analyses. We calculated between-species genetic distances (nucleotide substitutions per site) via likelihood, optimized with the GTR + I + Γ (best fit) model of sequence evolution in the software package Paup* version 4.0b (Swofford 2003). In addition,

chemical compounds were coded into a data matrix (presence/absence) to determine fragrance motifs and to calculate character statistics. These analyses were performed with the software package Paup* version 4.0b (Swofford 2003).

To evaluate the evolutionary history of chemical variation among lineages in the genus *Euglossa* and determine how chemical disparity is partitioned within and among bee lineages, we calculated disparity-through-time (DTT) plots based on the method developed by Harmon et al. (2003, 2008). DTT plots allow one to calculate the diversification of multiple traits simultaneously by estimating the dispersion of points in multivariate space across time intervals in a phylogeny. This methodology first estimates trait disparity across the whole phylogenetic tree, and then, for each subclade in the phylogeny, disparities are calculated and standardized relative to the disparity observed in the whole tree. It should be noted that this method avoids the difficulties of inferring ancestral character states on the phylogeny. Instead, it relies on estimating the average relative subclade disparity for each point in time whose lineages were present at that time (Harmon et al. 2003). We used DTT plots to examine the time course of the observed phenotypic (chemical) variation in relation to the chemical disparity expected under a null model. Subclade disparities were calculated by moving up the phylogeny from the root node to the tips. We used pairwise Manhattan distance as a measure of chemical disparity, which we estimated as the average variance at each node.

Results

Fragrance distinctness

Individual fragrances were highly variable in complexity, ranging from five to 75 different components; we found a total of 514 different components in all our samples. There were significant effects of species affiliation on the total amount of fragrances (sums of integrated ion currents; Kruskal-Wallis $H = 91.09$, $P < 0.0001$, $df = 14$, $N = 176$) and the number of components ($H = 71.88$, $P < 0.001$, $df = 14$, $N = 176$; see Fig. 1). There was a significant positive correlation between the total amount and the number of compounds across all individuals (Spearman $R = 0.619$, $P < 0.0001$, $N = 176$), but the slope of that relationship was not the same in all species. E.g., males of *Euglossa allosticta* had large amounts of fragrance with low complexity, whereas males of *E. imperialis* were on the opposite end of the spectrum, having small amounts of comparatively diverse blends (Fig. 1). The chemical components were distributed nonrandomly among individuals, both qualitatively and quantitatively. Across all individuals and species, ANOSIM permutation tests showed highly significant effects of species affiliation on fragrance similarity (Global $R = 0.945$, $P < 0.001$). Fig. 2a shows a two-dimensional multidimensional scaling (MDS) plot in which interpoint distances reflect interindividual chemical dissimilarity. It should be emphasized that the two-dimensional view is only an imperfect (highly constrained; stress = 0.22) representation of the true between-species distinctness in the underlying multidimensional similarity matrix. Between-species overlap is reduced to almost zero by including a third MDS dimension, which can be confirmed by viewing the rotating three-dimensional representation (less constrained; stress = 0.15) provided as Appendix A: Fig. A1. Pairwise tests of species distinctness using ANOSIM were all significant at the 95% level (all R values >0.56) except the test between *E. mixta* and *E. dodsoni*, the latter of which had very low sample size ($N = 3$; $R = 0.25$, not significant). Average Bray-Curtis dissimilarity was high between all pairs of species (88.3% ± 7.86%; range 63.30% to 98.65%). Accordingly, minimum dissimilarity of sympatric species decreased only slightly with species richness in randomly assembled communities, asymptotically approaching ~75% in more speciose communities (Fig. 3).

Fig. 4 shows the quantitative representation of the 80 most abundant components in all the 15 *Euglossa* species (see Appendix A: Fig. A2 for an undivided version of Fig. 4). Restricting the similarity analysis to these 80 compounds provides a similar picture to that shown in Fig. 2 (data not shown). The percentage data are given in Appendix B.

It is evident that many of the abundant fragrance compounds were shared by two or more species, although species tended to differ in relative amount. An outstanding example is the sesquiterpenoid hexahydrofarnesyl acetone (hha), which occurred (at least occasionally) in each of the 15 species and was a major component of eight (see Fig. 2b). In contrast, exclusive major components (e.g., compounds that were abundant in one species but absent from all others) were relatively rare. For many of these, we were unable to make structural assignments. Major compounds that were abundant in a small set of species include Germacrene D-4ol (in *E. bursigera*, *E. crassipunctata*, and *E. igniventris*; see Fig. 2c) and an unknown sesquiterpene ketone, molecular weight 218 (in *E. cognata* and *E. mixta*; see Fig. 2d). Thus it is clear that chemical differentiation was mediated both by possession of rare (exclusive) compounds as well as by possession of specific proportions of common ones.

Fragrance motifs ("building blocks")

Individual amounts (peak areas) of components showed variable degrees of intercorrelation among individuals within the different species, but the average correlation coefficient was positive within each species (Appendix C). The lowest overall association between compounds was found in *E. deceptrix* ($R = 0.09$), where most components seemed to vary almost independently of each other, the highest in *E. dissimula* ($R = 0.71$), where individual profiles were most coherent. Due to these large differences in background intercorrelation,

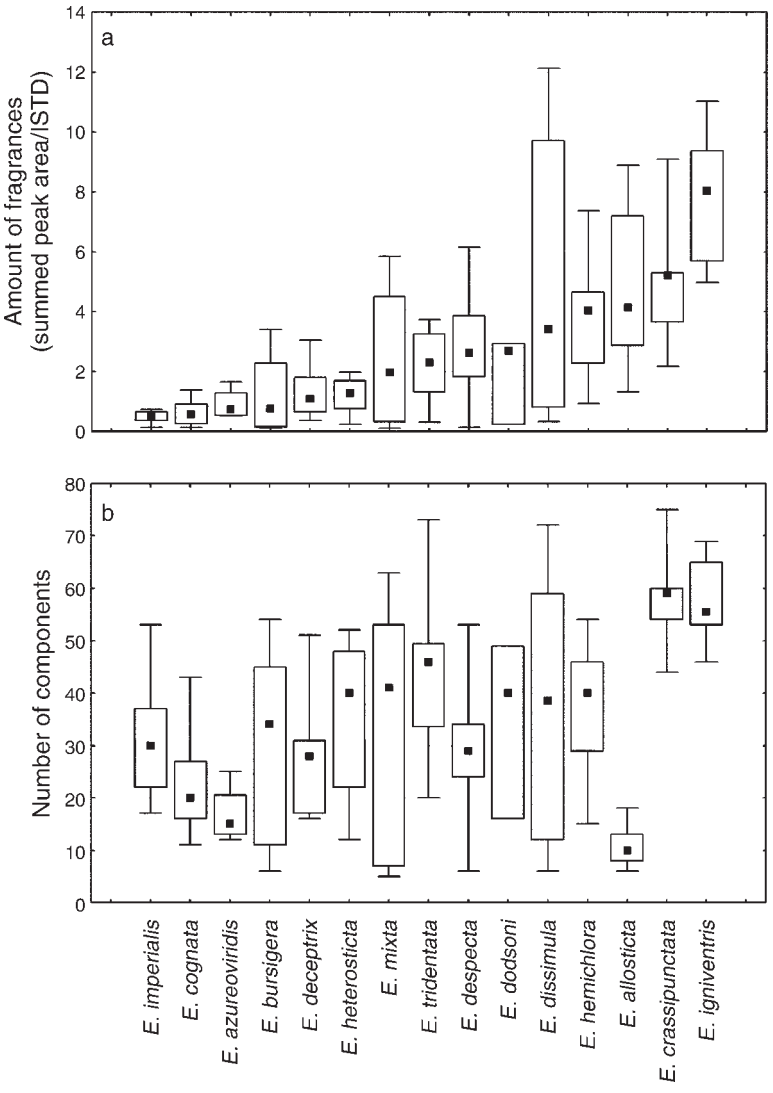

FIG. 1. (a) Median quantity of fragrances and (b) median number of different fragrance compounds detected in individual hind-leg extracts of males of 15 species of *Euglossa* from Barro Colorado Island (BCI), central Panama. Fragrance quantity is plotted as the ratio of the summed peak areas (integrated ion currents) of all fragrance compounds to the peak area of the internal standard (ISTD). Small black squares represent the median, the boxes extend to the 25% and 75% quartiles, and the whiskers show the minimum and maximum values.

we further analyzed only correlations that were outstanding for the respective species (>95th percentile of the distribution of correlation coefficients). In this way we inferred 22 fragrance motifs (i.e., associated groups of compounds that are likely derived together from specific sources). A list of these motifs and their distribution across species is given in Appendix C. Most motifs consisted of only a few (two to four) compounds, with one of them being quantitatively dominant, and frequently of structurally related compounds (e.g., a relatively widespread association between the (*E*) and (*Z*) isomers of methyl-4-methoxy cinnamate; motif 1). Four correlated stereoisomers of 2-hydroxy-6-nonadienyl benzaldehyde (motif 13) occurred in *E. mixta*. This motif was also found in males of *E. viridissima* in southern Mexico (not part of the present study) where it attracts *E. viridissima* males in bioassays (Eltz et al. 2008). Most widespread among species was motif 2, consisting of 97% hexahydrofarnesyl acetone (hha) and minor amounts of two structurally related compounds (the respective alcohol and, presumably, an unsaturated derivative of hexahydrofarnesyl acetone). This simple motif seemed to have been collected as such (i.e., without any other associated compounds) by at least eight species. This is confirmed by an individual of *E. despecta* that contained only motif 2 but no other fragrance compound. Most other motifs were not traceable between species.

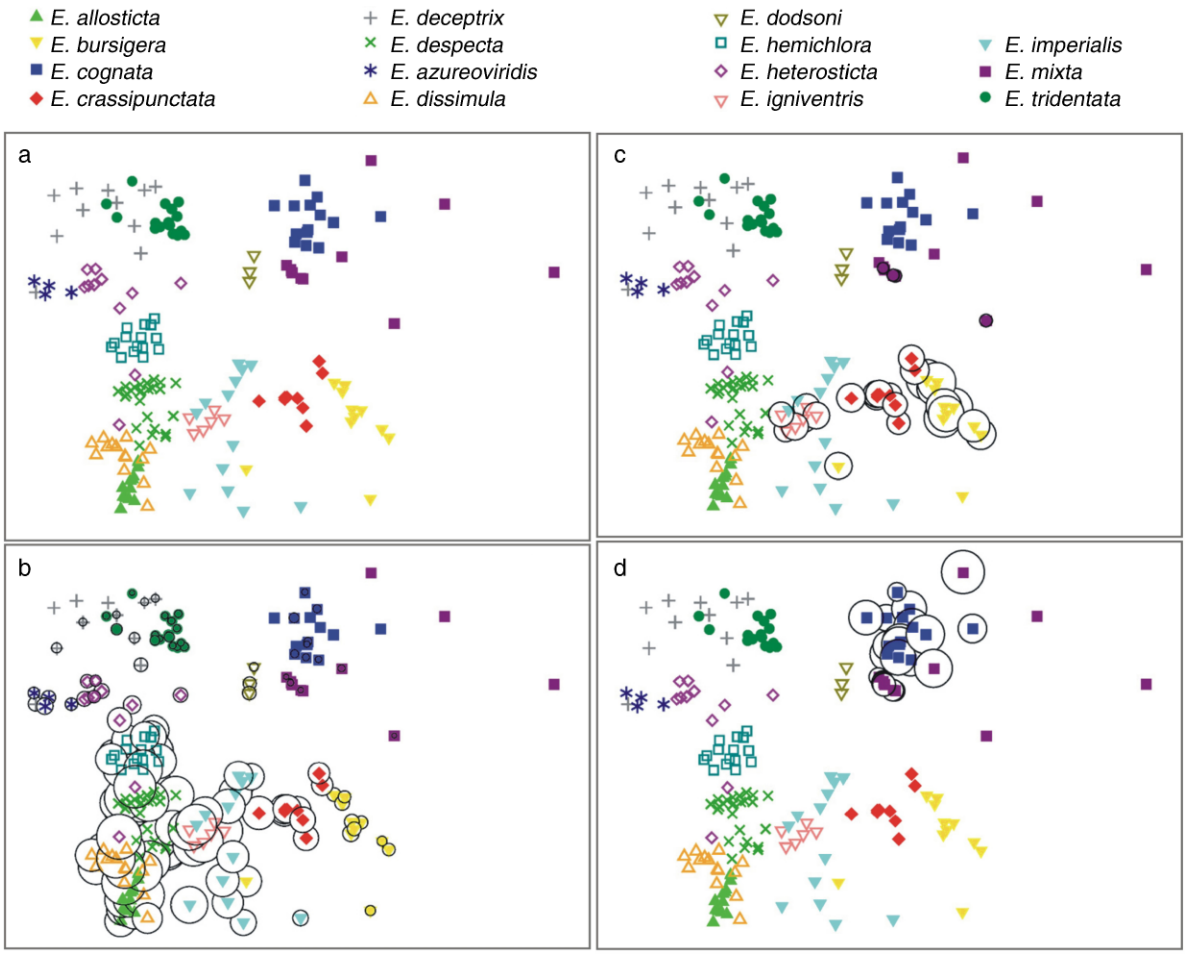

FIG. 2. Two-dimensional representation of the similarity/dissimilarity of fragrance mixtures of 176 individual male *Euglossa* belonging to 15 sympatric species from Barro Colorado Island (BCI), central Panama, and examples of how major compounds contributed to the observed pattern. (a) Multidimensional scaling (MDS) plot based on the Bray-Curtis index (stress = 0.22). Axes are simple distance dimensions without units. The proximity/distance of points represents the chemical similarity/dissimilarity of individual fragrances. Bubble overlays in panels (b)–(d) represent the relative contributions of (b) hexahydrofarnesyl acetone, (c) germacrene D-4-ol, and (d) an unknown sesquiterpene ketone, molecular weight 218, to individual blends. Bubble size reflects relative amounts between 0.1% and 99% (square-root scale).

Fragrance composition and phylogeny

Using the Bayesian DNA-based phylogeny, we estimated character statistics on the chemical traits alone. Of the total 514 chemical characters (fragrance compounds), 219 were variable but parsimony-uninformative, 293 were parsimony-informative, and only two were constant. Based on the tree topology obtained via Bayesian analysis with DNA data, the chemical data had a consistency index (CI) of 0.475, a retention index (RI) of 0.172, and a homoplasy index (HI) of 0.525. Most major fragrance components were present in two or more species, but the co-occurrences were often not congruent with phylogeny (Fig. 4, see also Appendix A: Fig. A2). The only convincing case of a synapomorphy is the unknown sesquiterpene ketone, molecular weight 218, which was present (and a major compound) only in the closely related *E. cognata* and *E. mixta*. In contrast, many other major compounds were clearly homoplasious, including gemacrene-D-4-ol, which is characteristic of the distantly related *E. bursigera*, *E. crassipunctata*, and *E. igniventris*. Also, the few species that did not possess hexahydrofarnesyl acetone as a major compound were not all close relatives.

Disparity-through-time plots show that chemical niche use in the genus *Euglossa* exhibits high values of average subclade disparity. Overall, the observed chemical disparity was greater than expected under a null model (Brownian motion) throughout the phylogenetic span of the taxa included (Fig. 5). We also observed that the average subclade chemical disparity peaks near the recent (Fig. 5), suggesting that much of the chemical disparity is concentrated among closely related taxa.

Discussion

Chemical niche differentiation

The present analysis corroborates the notion of nonrandom, species-specific accumulation of volatiles in male orchid bees. It confirms previous analyses that had been restricted to a few selected species (Eltz et al. 2005*a*, Zimmermann et al. 2006) and allows us to extrapolate how orchid bee communities partition their chemical environment. Of 15 species of sympatric *Euglossa* on Barro Colorado Island, all were sufficiently different in volatile composition to allow individuals to be assigned to their own species. The opportunities for between-species differentiation in "fragrance space" seemed unlimited; e.g., successive inclusion of species into "assemblages" only marginally increased the average chemical overlap between taxa. Instead, additional fragrance compounds were introduced along with additional species, and created additional niche space.

Between-species chemical differentiation is consistent with the hypothesis that fragrances are used for mate recognition. Previous studies have shown that male orchid bees expose and ventilate tibial fragrances during lengthy series of courtship display (Bembé 2004, Eltz et al. 2005*b*). The exposure of fragrances involves a range of cuticular structures shared by all male Euglossini, suggesting that active fragrance signaling is a basal trait of orchid bees (Eltz et al. 2005*b*). Although the attraction of conspecific females to male fragrances has yet to be demonstrated in bioassays, the high specificity of chemical signals is consistent with a role in mate recognition and premating isolation. Further support for this view comes from the pronounced chemical disparity among the most closely related species, evidenced by the disparity-through-time analysis. This finding suggests that male fragrances diverge rapidly between sibling species, possibly in response to potentially costly hybrid matings. Males of different euglossine species have been observed to display syntopically in the forest understory. For instance, males of the closely related *Eulaema meriana* and *E. bombiformis* display at the same time on the same forested hilltops in Panama (Stern 1991, Zimmermann et al. 2006; see also Kimsey 1980). The males of the two species are almost indistinguishable with respect to size, general morphology, and coloration. However, they differ in the circumference of their perch trees as well as in the composition of tibial fragrances (Stern 1991, Zimmermann et al. 2006). *Euglossa flammea* and *E. imperialis*, both in the Glossura species group (Cameron 2004), have also been observed to display in close proximity in the forest understory of the Azuero peninsula in Panama (Roubik and Hanson 2004:114). However, for most of the species of *Euglossa* analyzed in the present study, male display has never been observed.

Visual cues may assist in mate recognition. Most orchid bees, especially members of *Euglossa*, sport conspicuous structural colors ranging from green over hues of red to deep blue. However, body coloration often fails to distinguish closely related species, which may be difficult to separate for bee taxonomists (see Fig. 6).

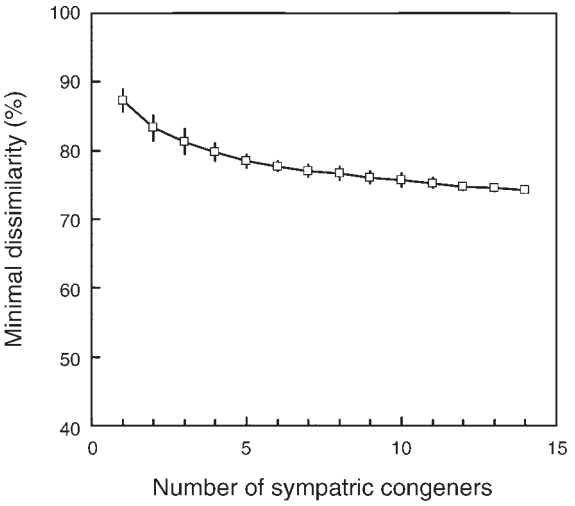

Fig. 3. Average observed minimum dissimilarity (measured as 1 − Bray-Curtis index, ±SD) between the fragrance blend of a focal species and that of the most similar species in randomly assembled communities of Panamanian *Euglossa* (with increasing species richness). Note that additional congeners would not further reduce the average dissimilarity in the community.

The costs of heterospecific matings in sympatry may range from waste of gametes in species that are already isolated by postmating barriers to the production of infertile (or less fit) hybrid offspring in species that are not. Such costs would result in natural selection to favor males with more distinct blends of chemicals. If such differentiation follows initial differentiation in allopatry, it represents "reproductive character displacement" (between already isolated species) or "reinforcement" (with some gene flow) (Coyne and Orr 2004).

As an alternative to chemical interference, fragrance differentiation could result from interspecific competition for limited chemical resources. In this case, natural selection would favor chemical niche differentiation between species because it reduces competition for chemicals among males ("ecological character displacement"), not because it reduces the risk of hybrid matings (Coyne and Orr 2004). Fragrances are likely scarce in the natural habitat, and male bees must allocate considerable time and energy to the acquisition of large quantities (Eltz et al. 1999). In agreement with competition for fragrances, males of some species have been observed to aggressively defend fragrance sources (e.g., flowers; Janzen 1981, Gracie 1993). However, it seems unlikely that interspecific competition alone is sufficient to create the extent of chemical differentiation and specialization found by the present study.

Fragrance sources and foraging

Many of the fragrance compounds found in male extracts are known from published euglossine sources.

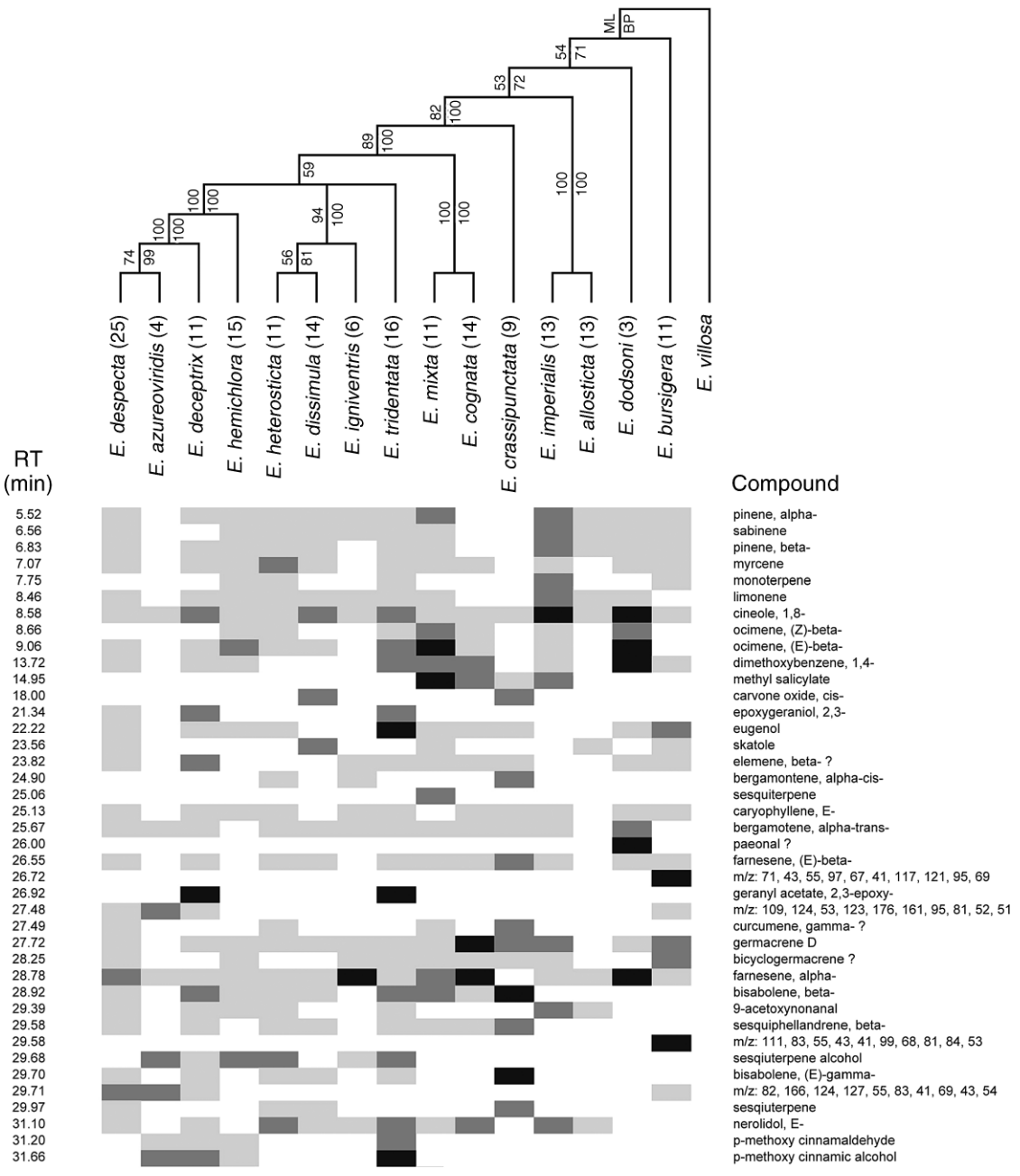

FIG. 4. Presence of fragrance compounds in hind-leg extracts of 15 sympatric species of *Euglossa* grouped by their phylogenetic relationships. The numbers of individual males analyzed per species are given in parentheses, and shades of gray indicate how much a compound contributed on average to the total peak area in a given species. Compounds are categorized as major (black, >5% of total peak area), minor (dark gray, 1–5%), and trace (light gray, <1%) components. The numerical data are given in Appendix B. Only the 80 most abundant compounds (across species) are shown, ranked from top to bottom by their retention time (RT) on a DB-5 nonpolar capillary column (30 m; 60°–300°C at 3°C/min). The 10 most abundant mass fragments (indicated by m/z) are given for unknown compounds. The cladogram corresponds to a 50% majority-rule consensus obtained from a Bayesian tree search; posterior probabilities are shown on the right side of the branches (along with bootstrap values of a maximum-likelihood analysis on the left side). See Appendix A: Fig. A2 for an undivided version of this figure.

The floral scents of euglossophilous flowers are normally chemical mixtures, although frequently of low complexity and characterized by pronounced dominance of one or two components (Williams and Whitten 1983, Gerlach and Schill 1991, Whitten and Williams 1992, Kaiser 1993). Single-component scents occur in decaying wood visited by male euglossines (Whitten et al. 1993, Eltz et al. 1999). The fragrance blends found in male

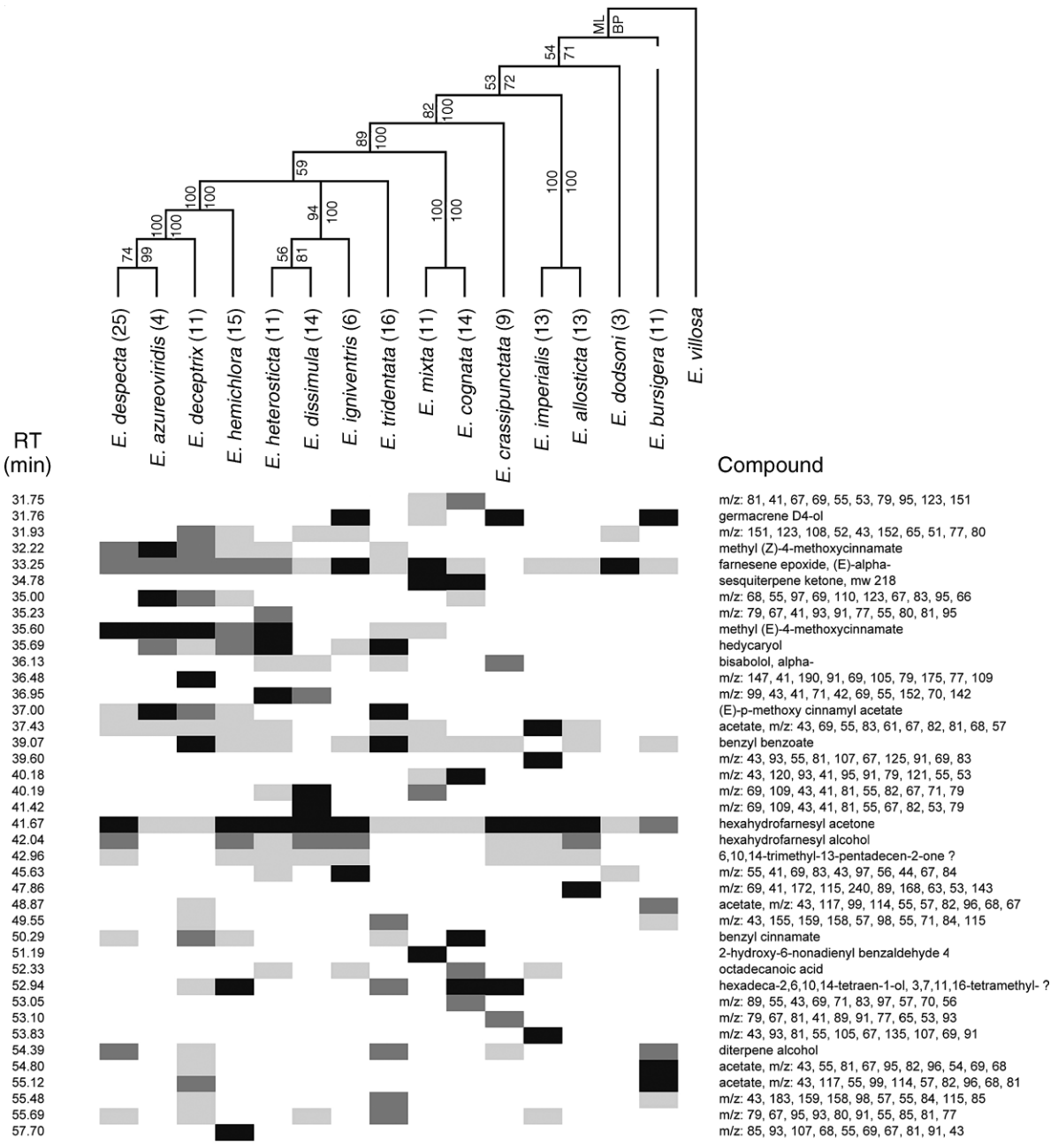

FIG. 4. Continued.

hind legs cannot be attributed to any known single source but are clearly assembled from subsets of compounds derived from different sources. However, the attempt to identify such subsets (i.e., motifs or building blocks) by an analysis of compound intercorrelation has had limited success. Nonetheless, a few simple motifs have been inferred. For instance, hexahydrofarnesyl acetone (hha) was found to be a major compound of eight species, representing 17% to 70% of total fragrances in their respective blends. Together with two structurally related trace compounds, hha represented a well-defined motif that appeared to be derived from the same source by all species. In recent bioassays, synthetic hha proved attractive to males of *E. imperialis* (T. Eltz, J. Andersson, J. Bång, and E. Hedenström, *unpublished data*). Males landed on and performed collecting behavior on the filter papers to which hha had been applied. Thus hha is a behaviorally active component in tibial fragrances of male *Euglossa*. Unfortunately, the source of hha remains elusive. Among the many floral scents and essential oils analyzed by R. Kaiser (*personal communication*), hha is relatively

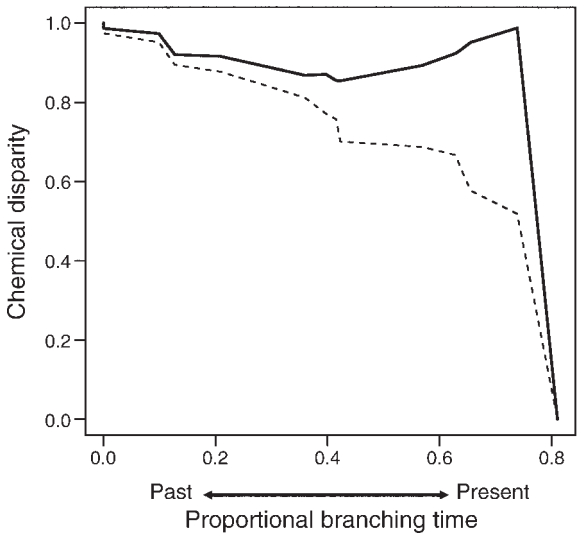

FIG. 5. Disparity-through-time (DTT) plot of chemical characters showing subclade dissimilarity in relation to relative branching times. The solid line corresponds to the observed empirical disparity, and the dashed line to the simulated null (1000 iterations; Brownian motion). Disparity was calculated via average Manhattan distance and is a unitless index.

widespread, but only as a minor or trace component. Notably, it occurs as a major compound in some euglossophilous orchids and an aroid (R. Kaiser and M. Whitten, *personal communication*). However, of these only one orchid species, *Kegeliella kupperi*, occurs in lowland central Panama, but due to its low population density is unlikely to be the source. Rather, the outstanding abundance of hha in male fragrances suggests that the hha source is highly available (i.e., not an orchid).

Other inferred fragrance motifs were also simple; i.e., they consisted of one highly dominant compound that appeared to be collected along with a few minor or trace compounds. While the predominance of simple motifs in our analysis may partly be an artifact (more complex motifs are increasingly likely to be obscured in complex blends), it is in general agreement with the prevalence of simple blends produced by most natural fragrance sources of orchid bees.

To accumulate complex fragrance blends, individual male bees must visit a range of different source types, presumably covering extensive areas of forest during their search. To attain specific blends, male bees appear to use innate fragrance preferences in combination with odor learning and experience-dependent choices (Ackerman 1989, Eltz et al. 2005*a*). Differences in innate preferences between species are evident from long-term baiting studies that used a range of synthetic chemicals to lure males. For instance, Ackerman (1989) used 16 different chemical attractants (single compounds) during a yearlong baiting program in Panama. Although there was considerable overlap in compound choices between species, each was attracted to a unique set of chemicals. The same author also found variation in choices of most species between geographic areas and in different seasons. In light of evidence from cage experiments (Eltz et al. 2005*a*), such differences were most likely due to differential availability of natural fragrances at the different localities and times. When caged males of *Euglossa imperialis* had repeated access to a fragrance compound (e.g., 1,8-cineole) in one experimental treatment, they satiated within a few days and finally stopped collecting this compound altogether. When a different compound (e.g., methyl salicylate) was given later, the males resumed their collecting activity. Thus it appears that male orchid bees learn the odor of visited sources and use a negative feedback mechanism to avoid overcollecting from abundant sources. Such modification of innate chemical preferences by experience would increase the species specificity of accumulated blends by compensating for fluctuating availability of fragrance source types (Eltz et al. 2005*a*).

There is controversy about the size of the foraging areas covered by male euglossines when searching for chemicals. Artificial chemical sources (baits) have been used to lure males over distances of one to several kilometers, even across open water or stretches of nonforest habitat (Ackerman 1981, Raw 1989, Tonhasca et al. 2003). While artificial baits emit volatiles in unnaturally high concentrations, observations at natural sources (orchids) support the notion of long-distance attraction (Janzen 1981). It has been discussed whether orchid bees are in fact vagabonds leading a nomadic life driven by their need for chemicals (Dodson et al. 1969). However, this view has been challenged by the observation that marked individuals revisit patches of nectar-offering food plants on consecutive days (Ackerman et al. 1982), and by perch site fidelity of males during their courtship behavior (Stern 1991). Furthermore, males appear to use spatial memory (not the scent plume) to revisit fragrance sources from which they have already collected on previous occasions. This is suggested by the observation that male bees continued to approach plants of the scent-offering *Dalechampia spathulata*, although these had already dropped their fragrant flowers (Armbruster and Webster 1979). Returning to the same source site by memory would be

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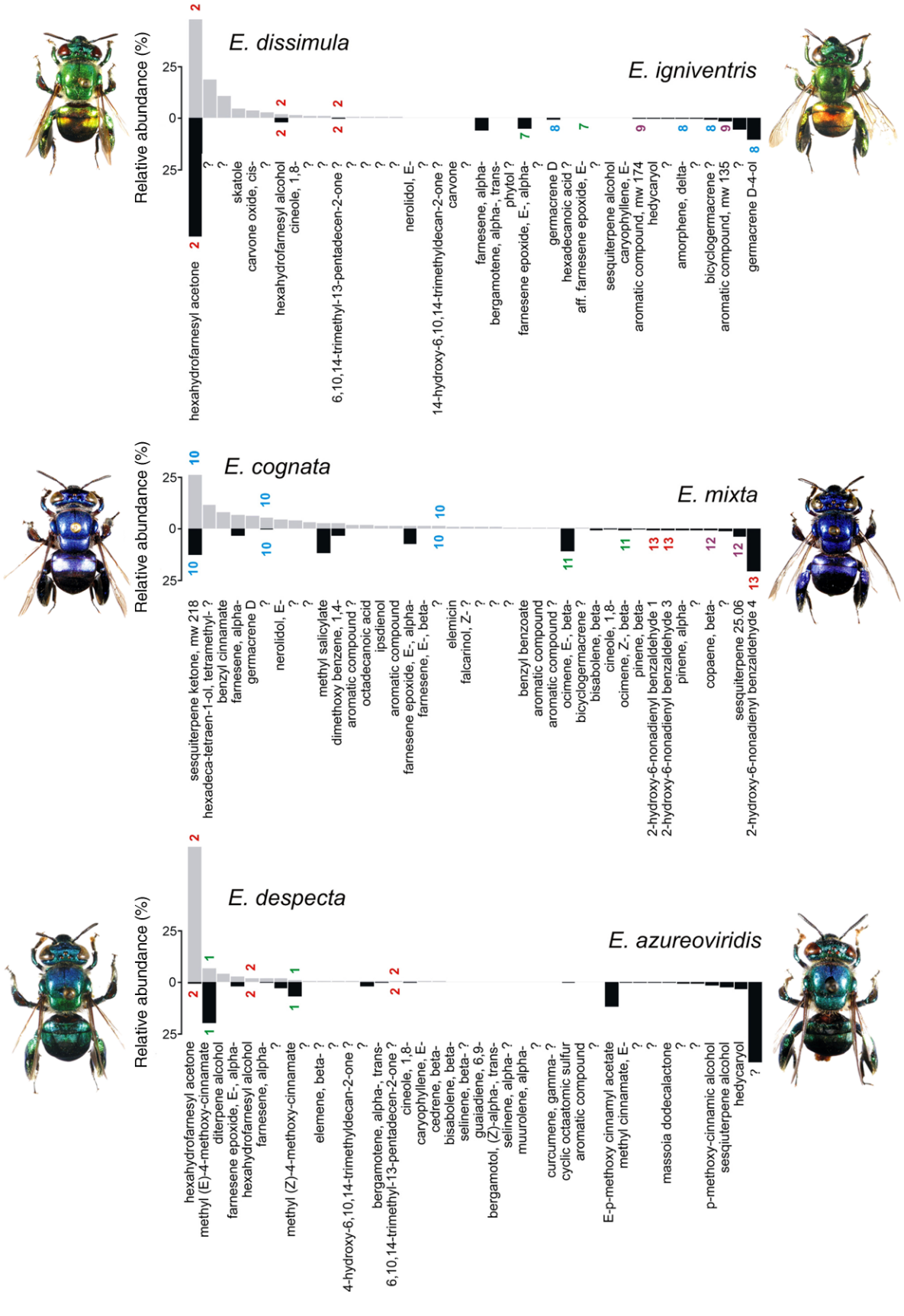
FIG. 6. Pairwise comparison of fragrance composition of closely related species of *Euglossa*. The 40 most abundant compounds of each pair of species are shown, and bars represent untransformed relative abundances (average percentage contribution to total peak area). Gray bars above the axis represent the bee species on the left side; black bars below the axis represent the species on the right side. Colored numerals refer to putative fragrance motifs (see Appendix C); i.e., the tagged compounds are likely co-derived from the same source, which are partly shared between species.


E. dissimula
E. igniventris
E. cognata
E. mixta
E. despecta
E. azureoviridis
Relative abundance (%)
25
0
25
hexahydrofarnesyl acetone
?
?
skatole
carvone oxide, cis-
?
hexahydrofarnesyl alcohol
cineole, 1,8-
?
?
6,10,14-trimethyl-13-pentadecen-2-one ?
?
?
?
nerolidol, E-
?
14-hydroxy-6,10,14-trimethyldecan-2-one ?
carvone
?
farnesene, alpha-
bergamotene, alpha-, trans-
phytol ?
farnesene epoxide, E-, alpha-
?
germacrene D
hexadecanoic acid ?
aff. farnesene epoxide, E-
?
sesquiterpene alcohol
caryophyllene, E-
aromatic compound, mw 174
hedycaryol
?
amorphene, delta-
?
bicyclogermacrene ?
aromatic compound, mw 135
?
germacrene D-4-ol
sesquiterpene ketone, mw 218
hexadeca-tetraen-1-ol, tetramethyl- ?
benzyl cinnamate
farnesene, alpha-
germacrene D
?
nerolidol, E-
?
?
methyl salicylate
dimethoxy benzene, 1,4-
aromatic compound ?
octadecanoic acid
ipsdienol
aromatic compound
farnesene epoxide, E-, alpha-
farnesene, E-, beta-
?
elemicin
falcarinol, Z- ?
?
?
?
benzyl benzoate
aromatic compound
aromatic compound ?
ocimene, E-, beta-
bicyclogermacrene ?
bisabolene, beta-
cineole, 1,8-
ocimene, Z-, beta-
pinene, beta-
2-hydroxy-6-nonadienyl benzaldehyde 1
2-hydroxy-6-nonadienyl benzaldehyde 3
pinene, alpha-
?
copaene, beta-
?
sesquiterpene 25.06
2-hydroxy-6-nonadienyl benzaldehyde 4
hexahydrofarnesyl acetone
methyl (E)-4-methoxy-cinnamate
diterpene alcohol
farnesene epoxide, E-, alpha-
hexahydrofarnesyl alcohol
farnesene, alpha-
?
methyl (Z)-4-methoxy-cinnamate
?
elemene, beta-
?
4-hydroxy-6,10,14-trimethyldecan-2-one ?
?
bergamotene, alpha-, trans-
6,10,14-trimethyl-13-pentadecen-2-one ?
cineole, 1,8-
caryophyllene, E-
cedrene, beta-
bisabolene, beta-
selinene, beta- ?
guaiadiene, 6,9-
bergamotol, (Z)-alpha-, trans-
selinene, alpha- ?
muurolene, alpha-
?
curcumene, gamma- ?
cyclic octaatomic sulfur
aromatic compound
?
E-p-methoxy cinnamyl acetate
methyl cinnamate, E-
?
?
massoia dodecalactone
?
?
p-methoxy-cinnamic alcohol
sesquiterpene alcohol
hedycaryol
?

expected if only small amounts of chemicals can be collected at any one time, or if intermittent collection from other sources renews interest in previously visited ones (see Eltz et al. 2005*a*). The use of spatial learning and memory during foraging opposes the idea that males are transient vagabonds. Instead, it suggests that males have foraging ranges, albeit large ones, and that communities of orchid bees are spatially structured. Such structuring is also suggested by detectable differences in the species composition of chemical bait samples taken at different sites within a given habitat (Armbruster 1993; but see Tonhasca et al. 2002).

Saltational mode of fragrance evolution?

There are currently two different views concerning the dynamics of chemical evolution in chemical mate recognition systems (Symonds and Elgar 2008). The traditional view emphasizes slow and gradual changes in sex pheromone blends due to the need for optimal recognition. Recently, however, it has been shown in moths that major saltational changes in signal chemistry can occur due to alterations in pheromone biosynthetic pathways, and that such shifts may initiate species divergence (Baker 2002, Roelofs et al. 2002). Studies in moths and bark beetles have revealed substantial differences in pheromones between closely related taxa, which appear to support a saltational mode of signal evolution (Löfstedt et al. 1991, Symonds and Elgar 2004). In orchid bees, we have also found substantial differentiation among closely related taxa. Furthermore, we have found that many major compounds or motifs co-occur in several, often unrelated species. The pattern of compound distribution shown in Fig. 4 (see also Appendix A: Fig. A2) indicates recurrent loss and gain of components in blends over time, consistent with saltational shifts in fragrance collection. Such shifts may be based on changes in behavioral preferences or on alterations in peripheral olfaction. Genes encoding odorant receptors (ORs) or odorant binding proteins (OBPs) are members of large multigene families. Genomic studies in *Drosophila* and *Apis* suggest they evolve in a birth-and-death mode, undergoing frequent changes in copy number due to duplication and pseudogenization (Robertson and Wanner 2006, Vieira et al. 2007). Loss and reactivation of odorant receptors may have induced shifts in fragrance collection in male orchid bees and caused the recurrent (homoplasious) pattern of signal evolution. A recent study on Mexican *Euglossa* has underlined the importance of peripheral olfaction for fragrance choice in males. In a pair of closely related sibling species, the male perfumes differ only in a set of four structurally very similar compounds, which are collected in large quantities by the males of only one lineage. The presence of these compounds in males of that lineage is associated with outstanding sensitivity of male antennae to that compound (Eltz et al. 2008). Future studies have to show whether there is a general congruence between male fragrances and antennal response profiles across the euglossine phylogenetic tree.

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APPENDIX A

Perfume differentiation in orchid bees. Fig. A1 shows a rotating, three-dimensional fragrance space of *Euglossa* spp. on Barro Colorado Island, Panama, in which the multidimensional scaling plot is based on the same data and analysis as the two-dimensional representation in Fig. 2a, but allows one additional dimension and thus accommodates more of the variation in the underlying similarity matrix (stress = 0.15); Fig. A2 is a full version of Fig. 4 (*Ecological Archives* E090-215-A1).

APPENDIX B

Average relative abundance of fragrance compounds in hind-leg extracts of 15 sympatric species of *Euglossa* from Barro Colorado Island, Panama (*Ecological Archives* E090-215-A2).

APPENDIX C

A list of 22 putative fragrance motifs inferred from intercorrelation analysis of male fragrance compounds and their occurrence in different species of *Euglossa* (*Ecological Archives* E090-215-A3).