RESEARCH ARTICLE

Olfactory specialization for perfume collection in male orchid bees

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ABSTRACT

Insects rely on the olfactory system to detect a vast diversity of airborne molecules in their environment. Highly sensitive olfactory tuning is expected to evolve when detection of a particular chemical with great precision is required in the context of foraging and/or finding mates. Male neotropical orchid bees (Euglossini) collect odoriferous substances from multiple sources, store them in specialized tibial pouches and later expose them at display sites, presumably as mating signals to females. Previous analysis of tibial compounds among sympatric species revealed substantial chemical disparity in chemical composition among lineages with outstanding divergence between closely related species. Here, we tested whether specific perfume phenotypes coevolve with matching olfactory adaptations in male orchid bees to facilitate the location and harvest of species-specific perfume compounds. We conducted electroantennographic (EAG) measurements on males of 15 sympatric species in the genus *Euglossa* that were stimulated with 18 compounds present in variable proportions in male hind tibiae. Antennal response profiles were species-specific across all 15 species, but there was no conspicuous differentiation between closely related species. Instead, we found that the observed variation in EAG activity follows a Brownian motion model of trait evolution, where the probability of differentiation increases proportionally with lineage divergence time. However, we identified strong antennal responses for some chemicals that are present as major compounds in the perfume of the same species, thus suggesting that sensory specialization has occurred within multiple lineages. This sensory specialization was particularly apparent for semi-volatile molecules ('base note' compounds), thus supporting the idea that such compounds play an important role in chemical signaling of euglossine bees. Overall, our study found no close correspondence between antennal responses and behavioral preferences/tibial contents, but confirms the utility of EAG profiling for discovering certain behaviorally active compounds.

KEY WORDS: Euglossini, EAG, Olfaction, Olfactory specialization, Fragrance, Pheromone

INTRODUCTION

Many insects use chemical signals to attract, locate and identify conspecific mates (Cardé and Baker, 1984; Roelofs, 1995; Wyatt, 2008). The chemical composition of mating pheromones ranges from single molecules to diverse, complex blends (Symonds and Elgar, 2008). Signal specificity may be achieved by the use of a single complex molecule that is rare in nature and therefore difficult to duplicate by other organisms (Chow and Wang, 1981). Alternatively, and more commonly, signal specificity can be attained through a blend of relatively simple and common components (Bjostad et al., 1987). Insect pheromones are usually synthesized *de novo* or modified from dietary precursors (Roelofs, 1995). In the case of orchid bees (Apidae, Euglossini; >200 species), however, blends of volatiles are harvested directly from the environment.

Male orchid bees collect and store volatile chemicals from flowers of orchids and other plants, as well as non-floral sources such as rotting wood, bark exudates, leaves and feces (Dodson et al., 1969; Vogel, 1966). This behavior of scent collection evolved approximately 38 million years ago (Engel, 1999; Ramírez et al., 2011) and a great number of neotropical plant species, many of them orchids, have adapted to male orchid bees as pollinators by producing floral scents (Dressler, 1982; Knudsen et al., 1999; Ramírez et al., 2002; Williams, 1982). These scents are highly attractive to euglossine males over long distances (Ackerman, 1983b; Janzen, 1971, 1981), thus ensuring efficient pollination of low-density plant taxa. The process of fragrance collection involves a range of morphological, biochemical and behavioral adaptations. Specialized hind-tibial pouches enable males to store volatile substances over long periods of time, ultimately accumulating complex blends of species-specific 'perfumes' (Eltz et al., 2005a; Zimmermann et al., 2009). Finally, the perfumes are actively exposed by males at the sites where mating takes place (Eltz et al., 2005b) and probably serve as pheromonal analogues (Zimmermann et al., 2006). At present, more than 40 synthetic chemical compounds have been reported to attract euglossine bee males and most of them are also known from natural sources (Ramírez et al., 2002; Roubik and Hanson, 2004; Williams and Whitten, 1983). Species-specific preferences for collecting certain compounds are evident (Ackerman, 1989), resulting in distinct tibial blends (Eltz et al., 2005a). There is, however, broad overlap in the range of chemicals collected by different species (Ackerman, 1983a; Janzen et al., 1982; Pearson and Dressler, 1985) as well as geographical and seasonal shifts of preferences (Ackerman, 1989; Pokorny et al., 2013).

The unique composition of mating pheromones can lead to prezygotic reproductive isolation among closely related lineages (Roelofs et al., 2002) and, because of communication interference among sympatric species with similar chemical signals, sexual chemical signals may be subject to diversifying selection (Coyne and Orr, 2004; Groot et al., 2006; Higgie et al., 2000). In fact, a pattern consistent with diversifying selection in sympatry was found for male orchid bee perfumes (Zimmermann et al., 2009). The chemical composition of blends collected by 15 sympatric species of *Euglossa* from central Panama were more different from each other than expected under a model of neutral phenotypic evolution, and perfume disparity between species was particularly pronounced

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between closely related lineages (Zimmermann et al., 2009). Based on these findings, we hypothesized that the outstanding divergence of fragrances observed among closely related species is mediated by an underlying species-specific sensory adaptation that evolved in response to reproductive character displacement at the sensory level. In order to reveal potential sensory adaptations, we used electroantennography (EAG) to screen overall antennal sensitivity for a large range of substances. This method is generally suitable to detect sensory specialization in the antennae (Roelofs, 1984) and has been used to investigate odor detection in diverse insects (Schiestl and Marion-Poll, 2002), including orchid bees (Eltz et al., 2006, 2008; Eltz and Lunau, 2005; Milet-Pinheiro et al., 2015; Schiestl and Roubik, 2003). We used two different approaches. In the first approach, we conducted a phylogenetically broad community-level comparison of 15 sympatric Panamanian species in the genus *Euglossa*, testing for species-specific sensory tuning for compounds that were present and abundant in a range of species, as well as compounds that were typical only for certain species. Next, we compared the antennal response of the sibling species *Euglossa purpurea* Friese 1899 and *Euglossa hansoni* Moure 1989 when presented with all the major compounds contained in the perfume mixture of *E. purpurea* in order to identify whether a sensory shift occurred between these two species. In addition to the EAG approach, a single-choice cage experiment was performed in order to determine the behavioral significance of individual compounds present in the perfume mixture of *E. purpurea*.

MATERIALS AND METHODS

Bees

We conducted a community-level comparison using 102 males of 15 sympatric species in the genus *Euglossa*: *Euglossa allosticta* (N=7), *Euglossa bursigera* (N=7), *Euglossa cognata* (N=7), *Euglossa crassipunctata* (N=7), *Euglossa despecta* (N=10), *Euglossa dissimula* (N=7), *Euglossa dodsoni* (N=7), *Euglossa hansoni* (N=7), *Euglossa hemichlora* (N=7), *Euglossa heterosticta* (N=4), *Euglossa igniventris* (N=4), *Euglossa imperialis* (N=7), *Euglossa mixta* (N=7), *Euglossa sapphirina* (N=7) and *Euglossa tridentata* (N=7). Males were collected from 30 March to 26 June 2010 on 10–15 km of 'Pipeline Road' near Gamboa, Panama, between 08:00 h and 12:00 h. Bees were lured with synthetic 1,8-cineole, p-dimethoxybenzene, benzyl benzoate, methyl salicylate, eugenol, vanillin, skatole and methyl cinnamate, and arriving bees were captured with hand nets and identified in the field. All individuals were introduced into a 50×50×60 cm mesh cage containing artificial feeders until they were subjected to EAG at the Smithsonian Tropical Research Institute (STRI) in Panama City.

In addition, we conducted a series of comparisons between males of the two sibling species *E. purpurea* and *E. hansoni* as well as the more distantly related *E. tridentata* (as an outgroup). Male bees for this study were baited in the forest near the botanical garden of the Tropenstation La Gamba, Costa Rica, in March 2012. Thirty individuals of each species were introduced into a 2×2×2 m flight cage containing sugar-water feeders and potted plants. After the bees were acclimated for a period of 1 week, behavioral bioassays were conducted with a set of test compounds (see below). Subsequently, 10 individuals of each species were used to conduct EAG recordings (see below).

Test substances

From a database of tibial contents that we previously assembled for 15 sympatric Panamanian species (Zimmermann et al., 2009), we selected a set of 18 single compounds for the community-level analysis of sensory specialization, which we either purchased from commercial suppliers or isolated from tibial bee extracts using preparative gas chromatography (see below and Table S1). These compounds included both common compounds that were present and abundant in a range of species as well as compounds that were present in only one or few species. To characterize the 18 compounds as 'minor' or 'major' (<5% or >5% of total fragrance peak area) components in a given species, we used quantitative information compiled in a yet unpublished database on tibial contents of males of 66 species of *Euglossa* (M.G.W., L.M., T.E. and S.R.R., unpublished results). In addition to these 18 compounds we also stimulated antennae with solvent (hexane) and unspecific odor (geraniol and 2-undecanone) controls. Both geraniol and 2-undecanone elicit significant EAG responses but are neither attractive to, nor known to occur in the tibial blends of males of the studied species.

For the sibling-species comparison, we used each of the eight major compounds present in the tibial perfume of *E. purpurea* (see below). We isolated these compounds using the preparative gas chromatography technique, which resulted in eight 2 ml test solutions with a concentration of 500 µg ml^{-1} dissolved in hexane. We isolated compounds from crude tibial hexane extracts following a preparative capillary gas chromatography protocol similar to that described by Nojima et al. (2008). Analytes were trapped with short pieces of megabore column (DB-1 in our case) connected to the end of the chromatographic column (DB-5, 30 m, 0.53 mm ID, housed in a HP 5890 II GC). This is a non-automated but relatively robust system that allowed us to isolate sufficient material to conduct EAG assays for the eight compounds. Trapping success, concentration and purity of isolated compounds was confirmed by gas chromatography/mass spectrometry (GC/MS) 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). Chemical identification of compounds was conducted by comparison of mass spectra and retention times with that of reference compounds or by cross referencing against a custom-built, local user library (T.E., unpublished data). As in the community-level comparison, we also stimulated antennae with solvent (hexane) and unspecific odor (geraniol and 2-undecanone) controls.

Electroantennography (EAG)

EAG studies were conducted at the Smithsonian Tropical Research Institute (STRI) in Panama City in 2010 (community-level comparison) and at the Tropenstation La Gamba, Costa Rica, in March 2012 (sibling species comparison). Male bee antennae were cut at the third segment and at the tip, and mounted between two glass pipettes filled with insect Ringer solution connected to silver electrodes. The test substances were applied in a set order with a solvent and unspecific odor control at the start and end of each test series. Every bee was tested once per compound. For every stimulus, 5 µl of the test sample was applied to a clean 2×10 mm strip of filter paper placed inside a clean pipette tip. Each stimulus pulse consisted of 200 µl of air puffed over the filter paper using an electronic pipette (Biohit eline, 50–1000 ml), injected into an air stream blowing over the antenna. The resulting EAG responses were amplified and recorded (in mV) using Syntech (Hilversum, The Netherlands) electrode holders, an IDAC acquisition controller and the EAG recording software (Syntech). The amplitude of the negative baseline deflection was used as a measure of response.

Bioassays

Attraction of male *E. purpurea*, *E. hansoni* and *E. tridentata* to chemical compounds was tested in a 2×2×2 m mesh cage at the Tropenstation La Gamba, Costa Rica, in March 2012. There were 30 male bees of each species present in the cage simultaneously. We used the same eight compounds/samples that were used in the EAG experiments. 50 µl aliquots of the samples were pipetted on clean filter paper squares and exposed consecutively for 15 min in the cage in a single-choice bioassay between 08:00 h and 12:00 h. Each compound was tested once per day for five consecutive days. Within this period individual bees collecting at the filter paper were counted. Volatile collection was defined as a male bee landing on the filter paper and performing stereotypical leg movements.

Data analysis

We first calculated paired *t*-tests for each species separately to determine whether a given compound elicited stronger responses relative to the mean response elicited by hexane (solvent) controls. Owing to variation in body size and quality of preparation, the EAG responses were then standardized across individuals and species, i.e. every response value in an individual male was divided by the average response of the same male to the two solvent controls and the two unspecific odor controls. This was done both for the community-wide comparison and for the sibling species comparison and all consecutive statistical analyses are based on the resulting standardized data sets. Single-factor ANOVA was calculated to test for the overall effect of the factor species on the EAG response to each test substance separately, and Scheffé *post hoc* tests were used to identify significant differences between pairs of species, as implemented in Statistica v.10 (StatSoft). For the community-wide analysis, Bray–Curtis similarities of response profiles were calculated between pairs of individuals based on similarity/dissimilarity of standardized EAG response profiles (including all 18 compounds) and the resulting similarity matrix was plotted in two dimensions using non-metric multidimensional scaling (nMDS) in the software Primer v.6 (Clarke, 1993; Clarke and Gorley, 2001). A one-way ANOSIM test was used to test the null hypothesis that the factor species has no influence on similarities between individuals, also with Primer v6.

To estimate the amount of phylogenetic signal as well as the degree and timing of divergence in EAG responses, we used a range of phylogenetic comparative methods and a trimmed euglossine phylogeny from Ramírez et al. (2011). First, we estimated the phylogenetic signal of the EAG response using K_{mult}, a generalized Blomberg's K statistic for multivariate data (Adams, 2014), and assessed the significance of the estimated K using 1000 permutations. We estimated K_{mult} based on the scores from a six-dimensional nMDS analysis, run on Bray–Curtis dissimilarities of standardized EAG response profiles, using the packages vegan v.2.3 (Community Ecology Package) and geomorph v.2.1.6 (Adams and Otárola-Castillo, 2013) in the R statistical framework (R Development Core Team). Second, we used Harmon et al.'s (2003) disparity through time (DTT) approach to test whether closely related lineages in *Euglossa* are more disparate in their EAG response than expected under a random-walk model of evolution. We calculated the DTT using Bray–Curtis dissimilarity, and compared the observed DTT with that of 1000 Brownian Motion simulations, using the package geiger v.2.0.3 (Harmon et al., 2008) with Bray–Curtis implementation code kindly provided by Luke Harmon.

RESULTS

Community-level comparison

All compounds tested, with the exception of β-pinene, elicited stronger-than-solvent responses in at least one species (paired *t*-tests; $P<0.05$), with some compounds eliciting strong responses in all species (Table 1). There was a significant effect of the factor species on the standardized EAG responses for all tested compounds (single-factor ANOVA; $P<0.05$) except for a terpenoid tentatively identified as 3,7,11,16-tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol (see Table S2). For three compounds, strong responses were clearly associated with species that had those compounds as a major component in their hind leg extracts (Fig. 1). (*E,E*)-α-farnesene epoxide is a major compound of *Euglossa dodsoni*, *E. igniventris* and *E. mixta*, and these three species exhibited the strongest responses to this compound (Fig. 1). *Euglossa dodsoni* exhibited significantly stronger EAG response to (*E,E*)-α-farnesene epoxide compared with all other species (ANOVA, Scheffé test; $P<0.05$), except *E. mixta*, *E. igniventris* and *E. imperialis*, which also showed above average antennal responses to that compound (those of *E. igniventris* being not significantly different from solvent control because of the low sample size, $N=4$). Second, 6-(4-methylpent-3-enyl)-naphtalene-1,4-dione is a major compound of *E. allosticta*, which induced significantly stronger EAG responses in relation to all other species, except *E. heterosticta* (ANOVA, Scheffé test; $P<0.01$). And third, 2-hydroxy-6-nonadienylbenzaldehyde (HNDB) is a major compound of *E. mixta* and induced significantly stronger EAG responses in that species compared with all other species (ANOVA, Scheffé test; $P<0.05$) except *E. dodsoni* from which the difference was only marginally significant ($P<0.07$). Response patterns of other compounds showed no remarkable associations with presence as major compounds in the hind tibiae of the different species. *P*-values of all Scheffé test pairwise comparisons between species are given in Table S3.

Although the overall response profiles broadly overlapped among most species, a 2-dimensional MDS plot revealed some species-specific clustering (Fig. 2) and the associated one-way test of similarity showed a significant effect of the factor species (ANOSIM test: Global $R=0.4$, $P<0.01$). Closely related species exhibited very similar EAG responses (see also Fig. 1). For instance, the closely related species *E. sapphirina* and *E. crassipunctata* revealed no significant differences in their relative responses to the 18 compounds when tested pairwise (ANOSIM: $R=0.069$, $P=0.186$). By contrast, we observed stronger differentiation among more distantly related species. Consistent with the above results, we found that EAG profiles displayed a strong, highly significant phylogenetic signal ($K_{mult}=0.9$, $P=0.009$), reflecting an underlying pattern where closely related species tended to have more (not less) similar EAG responses in the observed data (Fig. 3A). The DTT analysis confirmed this result, and showed that there was no overall distinction between a Brownian motion model of character evolution and the observed EAG responses across lineages of *Euglossa* (MDI=0.18, $P=0.87$, Fig. 3B).

Sibling species comparison

The EAG experiment conducted with eight major compounds characteristic for *E. purpurea* revealed significant differences in responses between the three species examined (*E. purpurea*, *E. hansoni* and *E. tridentata*) for three of the compounds (see Fig. 4): *p*-anisyl acetate (single-factor ANOVA; $F_{2,27}=10.17$, $P<0.001$), *p*-anisyl alcohol ($F_{2,27}=7.02$, $P<0.01$) and (*E*)-β-

Table 1. Paired *t*-test *P*-values between EAG responses to a given compound and the respective hexane solvent control in individuals of the different species of *Euglossa* orchid bees

	E. bursigera N=7	*E. allosticta* N=7	*E. imperialis* N=7	*E. dodsoni* N=7	*E. sapphirina* N=7	*E. crassipunctata* N=7	*E. cognata* N=7	*E. mixta* N=7	*E. igniventris* N=4	*E. heterosticta* N=4	*E. dissimula* N=7	*E. hansoni* N=7	*E. tridentata* N=7	*E. hemichlora* N=7	*E. despecta* N=10
α-Pinene	0.2356	**0.0014**	**0.0025**	0.0767	0.0618	0.8814	**0.0361**	0.1769	0.8467	0.3747	0.8777	0.4831	0.2953	0.9812	0.1193
β-Pinene	0.6570	0.1644	0.3730	0.6722	0.3348	0.5208	0.0660	0.1687	0.4437	0.9445	0.9162	0.6914	0.8307	0.9444	0.3185
Limonene	0.6373	**0.0006**	0.8016	0.1386	0.3386	0.5538	0.3087	0.3367	0.5927	0.8982	0.2517	0.2478	0.9629	0.2681	0.5688
1,8-Cineole	**0.0397**	**0.0004**	**0.0037**	0.0640	0.2202	0.0786	0.1315	0.8433	0.9999	0.4117	0.1397	0.2871	0.0721	0.7321	**0.0074**
Benzyl acetate	**0.0071**	**0.0003**	**0.0001**	**0.0004**	**0.0044**	**0.0009**	**0.0000**	**0.0029**	**0.0313**	0.0770	**0.0002**	**0.0001**	**0.0001**	**0.0018**	**0.0000**
Methyl salicylate	**0.0043**	**0.0000**	**0.0002**	**0.0020**	**0.0042**	**0.0053**	**0.0003**	**0.0100**	0.1378	0.0975	**0.0001**	**0.0001**	**0.0001**	**0.0426**	**0.0001**
Eugenol	**0.0079**	**0.0000**	**0.0000**	**0.0006**	**0.0004**	**0.0036**	**0.0010**	**0.0004**	**0.0282**	0.0781	**0.0049**	**0.0001**	**0.0000**	**0.0053**	**0.0000**
2,3-Epoxygeranyl acetate	**0.0077**	**0.0000**	**0.0002**	**0.0010**	**0.0029**	**0.0014**	**0.0001**	**0.0027**	0.3666	0.0973	**0.0110**	**0.0139**	**0.0006**	**0.0323**	**0.0000**
Nerolidol	**0.0425**	**0.0011**	**0.0029**	0.2330	0.0618	**0.0155**	**0.0001**	**0.0152**	0.7535	0.2289	**0.0028**	**0.0208**	0.0814	0.1180	**0.0039**
Germacren-D-4-ol	**0.0033**	**0.0000**	**0.0000**	**0.0023**	**0.0148**	**0.0010**	**0.0011**	**0.0011**	0.1192	0.1420	**0.0149**	**0.0199**	**0.0007**	0.1335	**0.0001**
(E,E)-α-Farnesene epoxide	**0.0317**	**0.0012**	**0.0001**	**0.0005**	0.1610	**0.0058**	**0.0005**	**0.0015**	0.1245	0.1131	**0.0029**	0.0952	**0.0004**	0.1903	**0.0000**
m/z:147, 190, 91, 105, 79, 69, 175, 77, 119, 148	**0.0048**	**0.0001**	**0.0001**	**0.0102**	**0.0029**	**0.0032**	**0.0004**	**0.0006**	0.1825	**0.0224**	**0.0013**	**0.0027**	**0.0014**	**0.0126**	**0.0000**
Benzyl benzoate	0.0704	**0.0260**	0.2026	**0.0049**	0.1903	0.0818	**0.0022**	**0.0177**	0.2329	0.5739	0.1822	**0.0224**	**0.0008**	**0.0286**	0.1668
Hexahydrofarnesyl acetone	**0.0216**	**0.0022**	**0.0135**	**0.0046**	0.2831	**0.0337**	**0.0002**	**0.0019**	0.7954	0.1421	**0.0111**	0.2588	**0.0034**	0.1893	**0.0004**
6-(4-Methylpent-3-enyl)-naphtalene-1,4-dione	0.4777	**0.0000**	**0.0211**	**0.0224**	0.0709	**0.0037**	**0.0133**	**0.0149**	0.1796	0.0689	0.7149	**0.0192**	0.0867	0.5268	**0.0127**
2-Hydroxy-6-nonadienyl benzaldehyde (HNDB)	0.5415	0.1406	0.2330	0.9332	0.4306	0.5068	0.4152	**0.0025**	0.6760	0.9972	0.5479	0.8339	0.7846	0.3428	0.1608
Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl- ?	0.9631	**0.0002**	0.1248	0.9756	0.8933	0.1025	**0.0320**	**0.0255**	0.2626	0.1776	0.2406	0.6716	**0.0332**	0.3032	**0.0056**

Significant values are shown in bold. Note low sample sizes (*N*=4) and resulting high frequency of non-significant results for *E. igniventris* and *E. heterosticta*. ? indicates predicted compound based on a spectral search in the NIST mass spectral library.
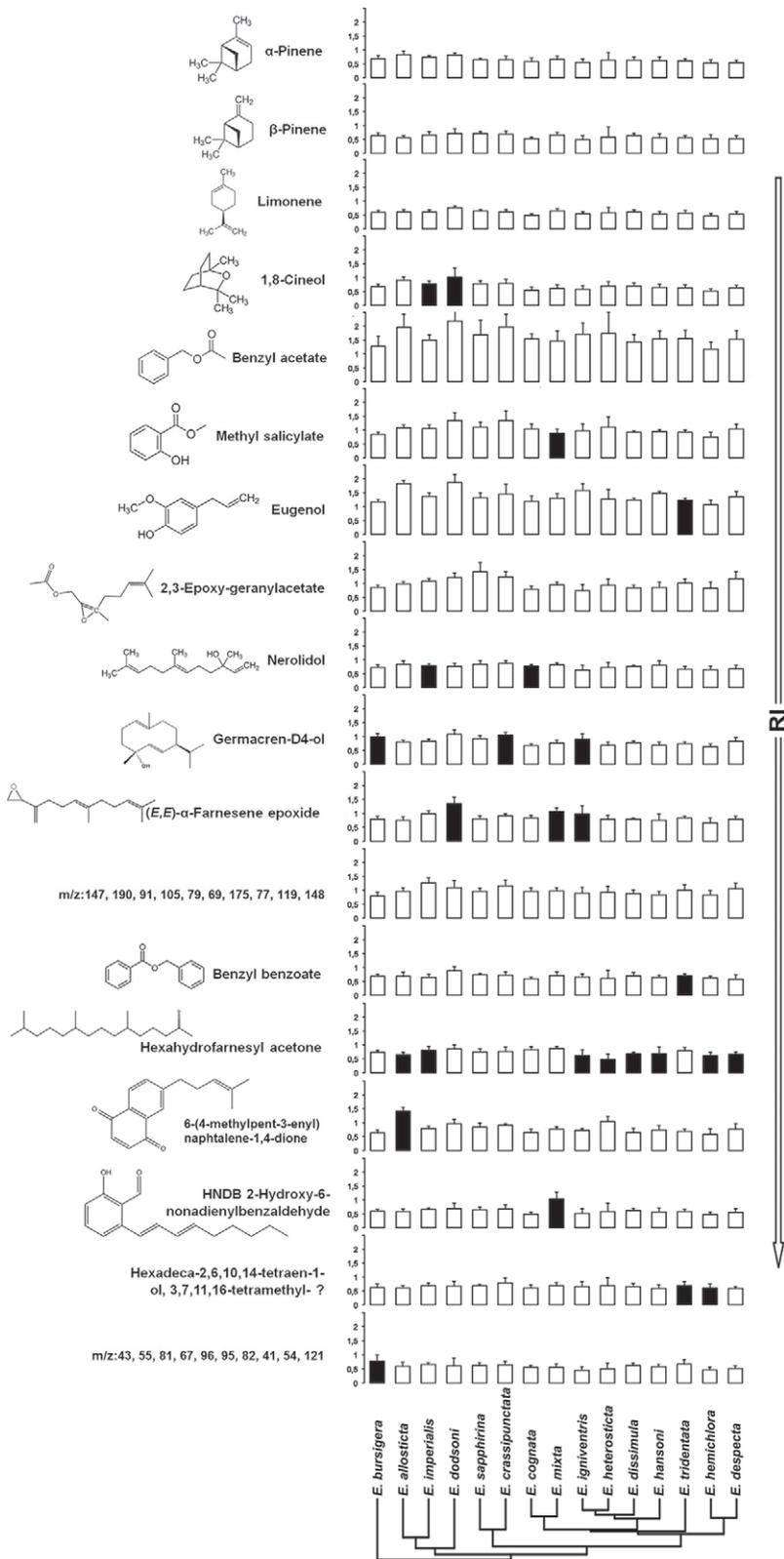
Fig. 1. Mean standardized EAG responses (+s.d.) of males of 15 species of *Euglossa* to 18 selected perfume compounds. Major compounds in tibial extracts of a tested species are indicated in black. Compounds are sorted from top to bottom by increasing retention index (RI). Phylogenetic relationships among tested species (trimmed from Ramírez et al., 2011) is displayed at the bottom.

ocimene ($F_{2,27}$=5.89, $P<0.01$). Of these, *E. purpurea* showed stronger responses than the other two species to *p*-anisyl acetate (pair-wise Scheffe tests: both $P<0.01$) and *p*-anisyl alcohol ($P<0.05$ to *E. hansoni*, $P<0.01$ to *E. tridentata*) while exhibiting comparatively weaker responses than *E. hansoni* to (*E*)-β-ocimene ($P<0.01$). The unknown compound 6 (m/z: 93, 79, 41, 121, 107, 91, 55, 43, 77, 53), which is the only chemical tested that is jointly present in the perfumes of both *E. purpurea* and *E. hansoni*, elicited EAG responses that were not significantly different between the species.
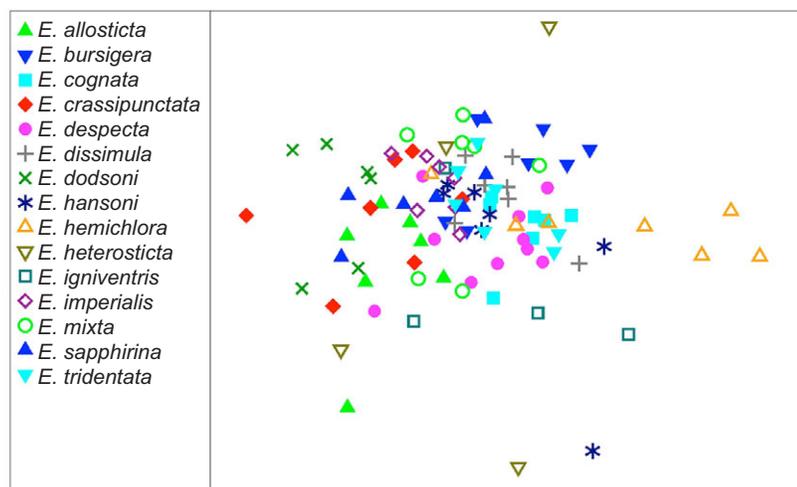
Fig. 2. Two-dimensional non-metric multidimensional scaling representation of Bray–Curtis similarities between EAG response profiles of 102 individual male _Euglossa_ belonging to 15 different species. The distance between points (individuals) represents their dissimilarity in EAG response profiles (stress=0.14).

Behavioral assays with the same compounds and the same three species were conducted for five consecutive days under similar weather conditions during the morning, and all bees remained active and exhibited volatile collection behavior during this time. Male _E. purpurea_ were attracted to five of the compounds, with 1,4-dimethoxy benzene, _p_-anisyl acetate and the unknown compound 6 (m/z: 93, 79, 41, 121, 107, 91, 55, 43, 77, 53) receiving most visits (Fig. 4). The compounds _p_-anisyl alcohol and (_E_)-nerolidol were also attractive, but generally received fewer visits from _E. purpurea_. Its sister species, _E. hansoni_, was not attracted to any of these compounds. _Euglossa tridentata_ was attracted to 1,4-dimethoxybenzene and (_E_)-nerolidol, both of which are present in the perfumes of _E. tridentata_.

DISCUSSION

Sensory specialization may evolve in response to selection for locating and identifying food and sexual partners, and avoiding predators (Dekker et al., 2006; Domingue et al., 2007; Goldman-Huertas et al., 2015; Schäffler et al., 2015). In the case of male orchid bees, olfactory specialization is expected because detecting and finding scattered perfume sources may entail traveling long distances (Pokorny et al., 2015). Species-specific perfume preferences were previously shown to have led to species- and compound-specific antennal specialization (Eltz et al., 2006, 2008; Schiestl and Roubik, 2003). The present study is the first, however, to compare antennal response profiles across a larger number of species in a phylogenetic framework. We found additional evidence for olfactory specialization to key compounds in certain species, but no general patterns of elevated divergent evolution of perfume compound perception among closely related species across the phylogenetic tree.

Although stimuli were presented at relatively low concentrations, most of the compounds tested elicited EAG responses in at least some species. Some frequently used bait compounds, especially benzyl acetate, methyl salicylate and eugenol, elicited strong responses across all species, but the amplitude of the response did not reflect whether the species is actually attracted to the respective compound (see Ackerman, 1989 for bait attractiveness). Other compounds, like α- and β-pinene, the putative 3,7,11,16-tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol or hexahydrofarnesyl acetone elicited no or very small responses across species, even in those that contained the compound in its perfume. A third set of compounds, however,

showed some interesting pronounced differences in responses between species. Perhaps the clearest evidence for compound-specific antennal tuning was found for 2-hydroxy-6-nonadienyl benzaldehyde (HNDB). This compound elicited a strong response exclusively in _Euglossa mixta_, which is the only species investigated here that contains HNDB in its hind legs. Antennal responses to HNDB were previously found to be high in Mexican _Euglossa dilemma_, which also collects and contains HNDB, but not in its sympatric sibling species, _Euglossa viridissima_, which does not (Eltz et al., 2008). Thus, _E. dilemma_, which is distributed from central Mexico to western Costa Rica, and _E. mixta_, distributed from southern Mexico to Brazil, uniquely share both behavioral preferences and high antennal sensitivity for HNDB. Interestingly, the two species are only very distantly related (Ramírez et al., 2010), suggesting that interactions with HNDB evolved independently in the two lineages. Next to HNDB, several other compounds elicited particularly strong responses in species that are known to collect them. 6-(4-Methylpent-3-enyl)-naphtalene-1,4-dione is an exclusive and highly dominant compound in tibial blends of Panamanian _Euglossa allosticta_, where it represented 54% of the perfume on average [m/z: 69, 41, 172, 115, 240, 89, 168, 63, 53, 143 (Zimmermann et al., 2009)]. The strong responses of _E. allosticta_ support the hypothesis that species-specific selection shaped antennal detection of that compound. Additional matching between EAG response and the presence of a particular compound in tibial extracts were found (albeit less clear-cut) for (_E,E_)-α-farnesene epoxide, which is most strongly perceived by the three species (_E. dodsoni_, _E. mixta_ and _E. igniventris_) that have it as major perfume component.

Finally, the sibling species comparison revealed that _Euglossa purpurea_ responded more strongly to _p_-anisyl acetate and _p_-anisyl alcohol than both its sibling species, _E. hansoni_ and the more distantly related _E. tridentata_. The two compounds are the most abundant components of hind tibial perfumes of _E. purpurea_, corresponding to 33% (alcohol) and 24% (acetate) of the blend, and are not present at all in either of the other two species, thus also supporting the hypothesis of antennal specialization. Generally, it should be noted that tibial perfumes are very different in composition between all three tested species, with only one of the test compounds shared between _E. purpurea_ and _E. hansoni_ (compound 6 in Fig. 4) and three between _E. purpurea_ and _E. tridentata_ [compound 1, (_E_)-β-
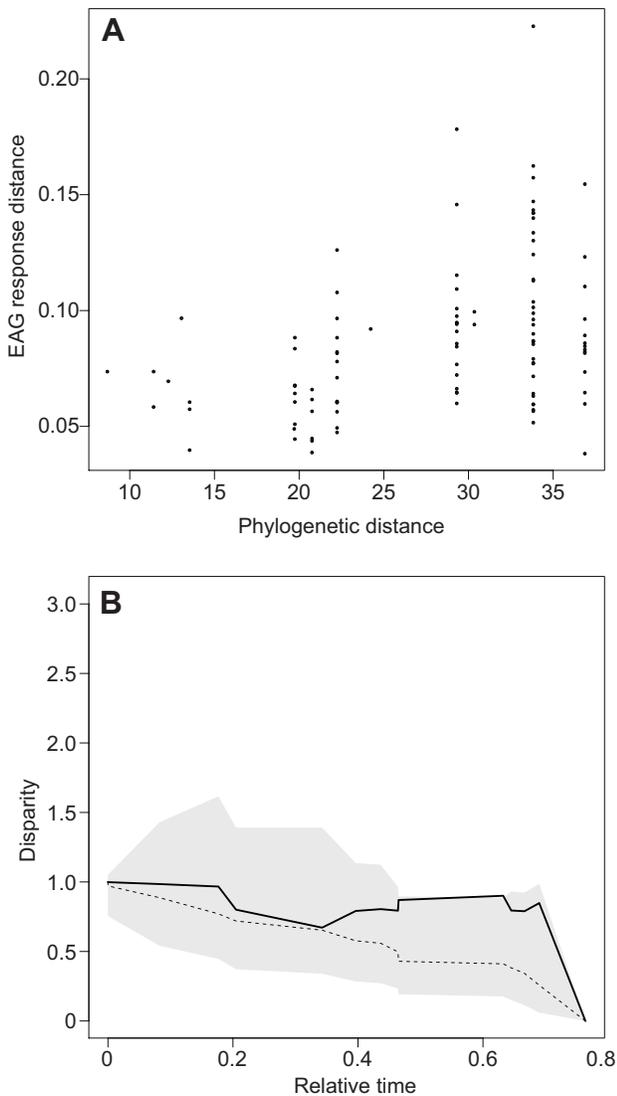
Fig. 3. Evolutionary patterns in EAG response across *Euglossa* based on the phylogeny from Ramírez et al. (2011). (A) The relationship between phylogenetic distance and EAG response Bray–Curtis dissimilarity, with each dot representing a pairwise comparison. (B) Disparity through time (DTT) plot showing the observed disparity among subclades through time for EAG response across *Euglossa* (black solid line) compared with the mean (dotted line) and 95% confidence intervals (grey box) of random walk, Brownian motion simulations.

ocimene; compound 2, 1,4-dimethoxybenzene; and compound 5, nerolidol; our unpublished results]. Male *E. purpurea* were strongly attracted to *p*-anisyl acetate in behavioral assays. Interestingly, *p*-anisyl alcohol, which probably originates from the same source as the acetate, was much less attractive in behavioral assays and also elicited somewhat smaller (but significant) antennal responses. This suggests that in nature, it is primarily the acetate that is targeted by male *E. purpurea*, whereas the alcohol probably excites some of the same receptor neurons because of structural similarities.

Generally, the response patterns observed with *p*-anisyl acetate confirm that EAG screening can be used to identify behaviorally relevant compounds among the great diversity of chemicals that are present in euglossine bee perfumes. The relationship between EAG response and behavioral attraction, however, is not a simple or linear one, neither is the relationship

between EAG response and tibial perfume composition. First, many compounds that are not present in the tibial extracts of a given species also elicited strong EAG responses. Second, certain compounds that are clearly highly attractive at the behavioral level for some species (like the very good baiting compound 1,8-cineole) do not show strong EAG responses (see Fig. 1) and may also not represent major compounds in tibial extracts even of the attracted species (see also Eltz et al., 1999). Thus, it is not generally possible to predict the attractiveness or presence or absence of a compound in tibial loads from EAG responses alone.

The amplitude of an EAG is believed to represent the number of receptor potentials that are elicited along the antenna upon stimulation, and thus the number of olfactory receptor neurons (ORNs) responding (Roelofs, 1984; Schiestl and Marion-Poll, 2002), i.e. overall antennal sensitivity of the antenna to a given substance. Classic examples of outstanding olfactory responses are those of male nocturnal Lepidoptera to female sex pheromone compounds (Kaissling and Priesner, 1970), with early comparative studies showing striking cases of species- and compound-specific antennal tuning using EAG (Priesner, 1968, 1975). Clearly, long-range signaling in combination with extremely low concentrations of female pheromones forced males to evolve substantial populations of olfactory neurons responding to the specific substance. Interestingly, most compounds that elicited distinct EAG responses in male orchid bees (this study) are semivolatile chemicals (Kovats index >1615). This may be explained by the particular challenge to localize sources of chemicals with high molecular weight and low vapor pressure, which necessitates substantial adaptations in antennal configuration. Once the respective behavioral preferences for a certain semivolatile compound have evolved in a species, the possession of large quantities of such chemicals could be an honest signal to choosy females due to the difficulty of acquiring them. In this case, strong selective pressures are expected to act on the male olfactory system to minimize the detection threshold.

Although the overall species-specific response profiles were rather similar among species, an MDS analysis revealed that each species did respond in a species-specific fashion. However, the segregation of species by EAG responses was much less pronounced than that based on perfume chemical composition found by Zimmermann et al. (2009). In particular, there was no pronounced divergence of EAG responses among recently diverged species. This is in contrast with the striking differences in perfume composition among those same species. This suggests that chemical divergence is often not based on changes in the sensory periphery, i.e. the antenna, but rather on changes in the central nervous system that modify odorant processing and/or behavioral preferences. Alternatively, changes in the olfactory periphery could be important for chemical divergence, as is clearly the case in some euglossine taxa (Brand et al., 2015; Eltz et al., 2008), but the EAG approach is too crude to reveal subtle olfactory tuning adaptations. As stated above, the amplitude of an EAG is believed to represent the number of receptor potentials that are elicited along the antenna upon stimulation, and thus the number of ORNs that respond to a given substance. The existence of few highly specialized ORNs that respond strongly to minute traces of relevant odors would not result in conspicuous EAGs, and may have gone unnoticed in the present study. Single-cell or single-sensillum recordings, possibly in combination with heterologous expression of euglossine odorant receptors, should be used to further elucidate the role of olfaction in shaping the evolution and diversification of perfume phenotypes in orchid bees.
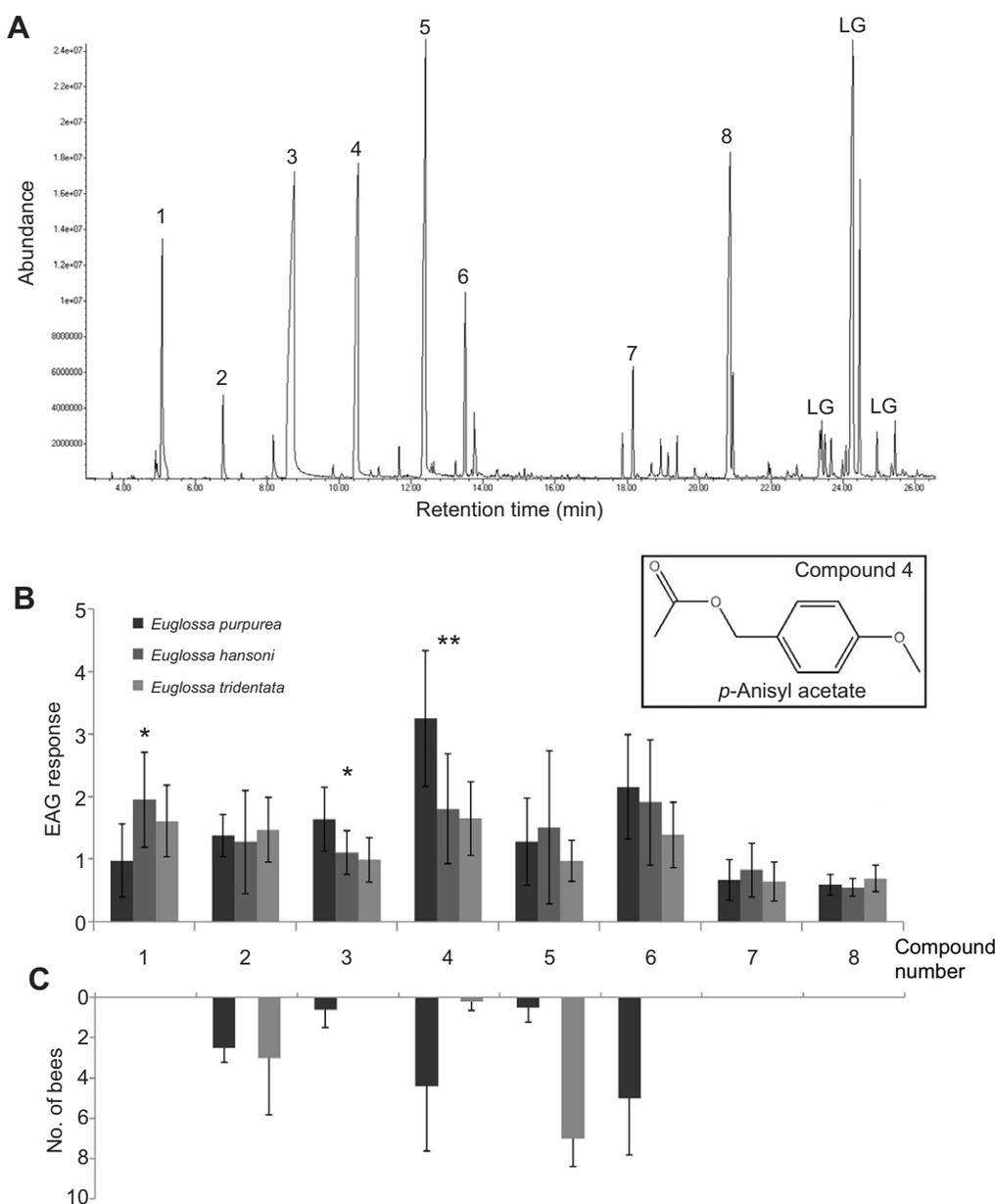
Fig. 4. Background and results of the sibling species comparison. (A) Total ion chromatogram of a typical hind leg extract of *Euglossa purpurea* highlighting the eight major perfume compounds which were used for EAG and behavioral assays [compound 1, (*E*)-β-ocimene; compound 2, 1,4-dimethoxybenzene; compound 3, *p*-anisyl alcohol, compound 4, *p*-anisyl acetate; compound 5, (*E*)-nerolidol; compound 6, unknown, m/z: 93, 79, 41, 121, 107, 91, 55, 43, 77, 53; compound 7, unknown, m/z: 135, 91, 77, 242, 92, 136, 65, 64, 63, 107; compound 8, unknown, m/z: 121, 135, 272, 77, 122, 92, 78, 91, 64, 63]. High molecular weight compounds originating from the male labial glands (LG) were excluded from the study. (B) Mean standardized EAG response (±s.d.) of male *E. purpurea* (*N*=10), *E. hansoni* (*N*=10) and *E. tridentata* (*N*=10) to the same compounds (1–8). Asterisks indicate significant differences between species (**P*<0.05; ***P*<0.01). (C) Mean number of bees (+s.d.) collecting a compound during single-choice behavioral assays.

Supplementary information

Supplementary information available online at
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Table S1. Compounds used for EAG in the community-level comparison. The table contains 18 test compounds plus the two control compounds, 2-undecanone and geraniol.

Compound	Concentration [µg/ml]	Origin	Extracted species
α-Pinene	511	Roth	-
β-Pinene	505	Aldrich	-
Limonene	581	Aldrich	-
1,8-Cineole	260	Roth	-
Benzyl acetate	734	Merck	-
Methyl salicylate	527	Sigma-Aldrich	-
Eugenol	530	Roth	-
2,3-Epoxygeranyl acetate	199	Preparative GC	*E. deceptrix*
Nerolidol	820	Aldrich	-
Germacren-D-4-ol	632	Preparative GC	*E. bursigera*
(*E,E*)-α-Farnesene epoxide	380	Preparative GC	*E. mixta*
m/z:147, 190, 91, 105, 79, 69, 175, 77, 119, 148	270	Preparative GC	*E. deceptrix*
Benzyl benzoate	453	Sigma-Aldrich	-
Hexahydrofarnesyl acetone	617	Preparative GC	*E. hemichlora*
6-(4-Methylpent-3-enyl)-naphtalene-1,4-dione	500	Synthesis (E. Hedenström, Sundsvall)	-
2-Hydroxy-6-nonadienyl benzaldehyde	514	Preparative GC	*E. mixta*
Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl- ?	508	Preparative GC	*E. hemichlora*
m/z:43, 55, 81, 67, 96, 95, 82, 41, 54, 121	518	Preparative GC	*E. bursigera*
2-Undecanone	635	Roth	-
Geraniol	464	Roth	-

Table S2. Single-factor ANOVA results for all test compounds in the community-level analysis (factor: species).

Compound	SQ	DF	MQ	F	p<
α-Pinene	0,79	14	0,06	4,31	0,001
β-Pinene	0,50	14	0,04	2,12	0,018
Limonene	0,51	14	0,04	4,27	0,001
1,8-Cineole	1,78	14	0,13	6,48	0,001
Benzyl acetate	6,92	14	0,50	3,31	0,001
Methyl salicylate	2,61	14	0,19	5,24	0,001
Eugenol	5,03	14	0,36	4,89	0,001
2,3-Epoxygeranyl acetate	3,51	14	0,25	7,61	0,001
Nerolidol	0,51	14	0,04	2,83	0,002
Germacren-D-4-ol	1,84	14	0,13	11,39	0,001
(*E,E*)-α-Farnesene epoxide	2,79	14	0,20	9,98	0,001
m/z:147, 190, 91, 105, 79, 69, 175, 77, 119, 148	1,61	14	0,12	3,99	0,001
Benzyl benzoate	0,54	14	0,04	2,75	0,002
Hexahydrofarnesyl acetone	0,91	14	0,07	3,80	0,001
6-(4-Methylpent-3-enyl)-naphtalene-1,4-dione	4,13	14	0,30	15,87	0,001
2-Hydroxy-6-nonadienyl benzaldehyde	1,74	14	0,12	6,66	0,001
Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl- ?	0,26	14	0,02	1,04	0,423
m/z:43, 55, 81, 67, 96, 95, 82, 41, 54, 121	0,66	14	0,05	2,43	0,006

Table S3. Error probabilities (P) of post-hoc Scheffe-test of pairwise species comparisons for each of the 18 test compounds.

Click here to Download Table S3