An embellishment that became a mutualism: Inquiries on male bee tibial bouquets and fragrance-producing orchids in Panama and oceanic islands (Apidae: Apinae, Euglossini; Orchidaceae: Epidendroideae)

David W. Roubik [a],[*],[1], Jette T. Knudsen [b],[2]

[a] Smithsonian Tropical Research Institute, Balboa, Ancon, Panama
[b] Department of Biology, Lund University, SE-223 62, Lund, Sweden

ABSTRACT

We used comparative studies to investigate how and why floral and bee fragrances evolve, including courtship odors collected by male *Euglossa mixta* to form their "tibial bouquet", on Coiba Island and other Panama forests. Fragrances of four orchid genera, two used extensively by *E. mixta* – *Coryanthes* and *Mormodes* – and two never used, *Clowesia* and *Catasetum* – were also analyzed. From among 636 chemicals in 93 male tibiae, 66 were also found in 30 floral head-space samples of orchids, in which 315 total volatile compounds were detected. Geographic variation was noteworthy in *E. mixta*, but no significant difference was found between mainland and island populations. The aromatic benzenoids methyl salicylate, 2-hydroxy-6-nona-1,3-dienylbenzaldehyde (HNDB), and the monoterpene 1,8 cineole, nearly always occurred. *Coryanthes* or other orchids produce two of the chemicals, but no source of HNDB is known. No statistical evidence was found of bee preference for orchids with bouquets like those formed in bee hindlegs, yet *Coryanthes* and *Mormodes* produced the most monoterpenes and more resembled the bees, when compared to *Catasetum* and *Clowesia*. Coiba bee tibial bouquets averaged 56% as diverse as on mainland and Coiba has <50% the euglossine species of nearby mainland, but lacks those most similar to *E. mixta*, both in phylogeny and tibial bouquet. Coiba's diverse rain forest should contain many volatiles the bees seek. Because odor collection and production are costly, our findings strengthen hypotheses that odors are used to avoid interspecific reproductive interference. Despite finding large differences in the same orchid species, we do not know whether isolation of between 10^7 and 10^4 years produced differentiation. Fragrances seem analogous among orchids and bees, thus may lessen interspecific interference or competition, and promote outcrossing or favor embellishments, via female choice. Such adaptive reasons for fragrance variation within bee or orchid populations remain largely untested.

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1. Introduction

When attempting to understand function, structure and evolution of bees and flowers, we are indeed mindful of contributions by Dr. Stefan Vogel (1925–2015), who first understood the bee/floral fragrance interaction (Dressler, 1982) by witnessing male bees collect fragrances, both from orchids and non-orchids (Vogel, 1966). Here we examine consequences and correlates of the "tibial bouquet" formation, which that behavior represents. Coiba island,

$500 \, km^2$ of forested hills lying $\geq 25 \, km$ from the Pacific coast (Castroviejo, 1997; Ibañez, 2011; Roubik and Camargo, 2012), along with Panama's large, intact forest areas, offer natural settings to study ecological interactions and evolution, at different spatial and temporal scales. Although the 'orchid bees' are readily collected with chemical attractants (Dressler, 1982; Kaiser, 1993), orchids and their flowers in the forest are relatively rare, and their fragrances are not well studied (Ramírez et al., 2011).

Most "androeuglossophilous" orchids produce species-specific scents with a few dozen chemical components, of which one or two dominate (Williams and Whitten, 1983; Gerlach and Schill, 1991; Kaiser, 1993). Research on bees that pollinate such flowers, their tibial bouquets (Eltz et al., 2008; Ramírez et al., 2011), and how their behavior is organized (Mitko et al., 2016) continues apace. For orchids, fragrant compounds can include attractants,

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* Corresponding author.
  E-mail address: roubikd@si.edu (D.W. Roubik).
[1] Additional postal address at: MRC 705, PO Box 37012, Washington DC 20013-7012, United States.
[2] Present address: Nattaro Labs, Medicon Village, SE-223 81, Lund.

repellents, and a variety of compounds, allied with prevention of inbreeding and encouragement of "faithful" pollinators (Ackerman and Roubik, 2012; Milet-Pinheiro et al., 2015). In contrast, for the bees, the tibial bouquets have not been considered in outbreeding studies, but they possibly reflect "rare male advantage" or frequency-dependent selection via female choice, while also avoiding wasting time and investment in interspecies reproductive interference (see Discussion). The morphology of the flowers, in hermaphroditic species, as well as other morphological elements that prevent self-pollination, such as dimorphy in dioecious genera (Roubik, 2014; Milet-Pinheiro et al., 2015) encourage the scent collecting male bees to perform outcrossing. They first pass the stigma, after which a pollinarium may be glued on the bee, and then one or more pollinia may be removed by stigmata, and new pollinaria attached during subsequent floral visits (Dressler, 1993; Roubik, 2014). Both the orchids and bees commonly use two to several counterpart species, and some orchid genera or species, which have been studied sufficiently in normal field conditions (e.g. *Vanilla*, *Stanhopea*, *Mormodes*, *Gongora*, *Coryanthes*) are visited by more than one euglossine genus (Roubik and Hanson, 2004; Ackerman and Roubik, 2012).

Janzen (1981) reports high abundance, low richness and intense odor seeking behavior of *Euglossa* on a small, Costa Rican offshore island in the same biofaunistic region we consider. That observation implies island bee populations may have fewer fragrance resources available, and are perhaps less constrained by food competition, nesting sites, or natural enemies. Here we consider such general themes, on an island >100 times larger, and more generally isolated from mainland. Our fieldwork frames a theory of island biogeography and pollination ecology, intertwined with pollinator behavior. For Coiba Island, Republic of Panama, we had one principal subject – the use of fragrances in mating biology – and two principal taxa, *Euglossa mixta* Friese (Apidae: Euglossini), with its frequently visited "bucket orchid" fragrance host *Coryanthes* (Orchidaceae: Epidendroideae) to evaluate.

Our previous observations indicated that *Coryanthes* pollinaria were carried mainly by *E. mixta* on Coiba Island (Fig. 1). No *Coryanthes* and indeed, few orchids had then been documented on Coiba. Moreover, on the mainland other euglossine bee species dominate interaction with *Coryanthes* (see Ackerman and Roubik, 2012). We investigated the possibility that divergence in either the orchid or the bee, since the times of Coiba's periodic isolation from mainland populations, and its much smaller neighbor, Ranchería Island, occurred. Over 10,000 years have elapsed since the last mainland/island connection during a glacial maximum, and dozens of glaciations have caused similar events during the past three million years (Roubik and Camargo, 2012; O'Dea et al., 2016). Biological surveys on Coiba (Castroviejo, 1997; Ibañez, 2011; Roubik and Camargo, 2012; DWR unpublished) have uncovered an orchid bee and a stingless honey bee, *Melipona,* endemic to the island with several other organisms which now are endemic, relictual, or show disjunct distributions with mainland populations, and a strong affinity with the South American, Chocó biodiversity region. An ancient connection of Panama to South America, in Miocene times (10 mya), has been deduced (Montes et al., 2015) and would include Coiba. Disjunct portions of the Pacific mainland and islands harbor both large trees (*Peltogyne*, Fabaceae, and bees, *Ptilotrigona*, Apidae, see Roubik and Camargo, 2012) that are absent in almost all of Panama, but occur on Coiba, Pacific Costa Rica, and northwestern South America. This also may indicate organisms arrived in Panama's Pacific area from South America before the current isthmus was formed. Have chemical enticements provided by certain flowers to pollinators, or the chemicals used by bees in their mating behavior, changed with this complex pattern of colonization and isolation? Although unable to test more than a few hypotheses, we attempt to provide insight.

For comparative purposes, we also considered the orchid genera *Clowesia, Catasetum,* and *Mormodes* (Epidendroideae, Cymbidieae), each frequently associated by pollinaria with *Eg. mixta* or other euglossines. Our data confirm previous results (Zimmermann et al., 2009; Ramírez et al., 2011; Pokorny et al., 2013; Milet-Pinheiro et al., 2015; Mitko et al., 2016) that (1) volatile blends produced by orchid flower species or genera may be predictors of pollinator preference and bee tibial bouquets, (2) that a good portion of the bee bouquets are not derived from orchids that we or others have analyzed chemically, and that (3) one force driving the regulation of tibial bouquets is probably the reproductive costs that cause "odor partitioning" among sympatric species. We suggest bees collect a smaller variety of aromatic chemicals in less complex bee communities, because there is less potential interspecific mating interference.

2. Methods and materials

2.1. Rationale and sampling methods

We initially expected that orchids and bees on islands, compared to mainland populations, have different odor composition (Fig. 2). Most euglossine-pollinated orchids are epiphytic and often rare (Dressler, 1993; Ackerman and Roubik, 2012). However, the orchid relationship with particular bees is readily detected on bees collecting fragrances – revealed by pollinia they carry (Fig. 1a,b, and Roubik, 2014). Specimens of *Euglossa mixta* were collected and identified in the field by DWR, as were orchid pollinaria, using the field methods called "chemical baiting" (Roubik and Ackerman, 1987; Ackerman and Roubik, 2012). Importantly, the male bees were not allowed to collect chemical fragrances at the chemical baits. Live orchids were field-collected on Coiba Island and elsewhere, and identified by Mr. Bonarge Rodríguez (see Ibañez, 2011). Plants were maintained in orchid gardens, in Central Panama lowlands and the Caribbean foothills, until flowering occurred (see Methods, below). The most abundant orchid pollinaria and orchid bees carrying them, revealed by chemical baiting (Fig. 1), are *Coryanthes* and *Euglossa mixta*, respectively, throughout the year. This observation entailed 60 field days, with 11 trips to Coiba and Ranchería Islands, since 1979, up until February, 2015. Several *Euglossa* species besides *E. mixta* visit flowers of *Coryanthes* on the islands, and only one *Coryanthes* species is known there, *C. hunteriana* Schltr., also found on the mainland (Ibañez, 2011; DWR, unpublished).

Coiba is the largest island off tropical Pacific America, 500 km^2, and has not undergone extensive deforestation in historic times; its mean annual rainfall is 3400 mm, but reaches 6000 mm in the higher part of the island (Ibañez, 2011). The island, studied at several field sites for this work, in both lowland and the highest elevation areas ca. 420 masl, is separated from the closest point on the mainland by roughly 25 km. There are two small islands, Ranchería and the Channel Islands (Isla Canal de Afuera) (<230 ha area) 23 and 12 km from the mainland, respectively, and separating Coiba from the nearest mainland forest remnant at Punta Gorda (ca. 2500 ha) and the fishing village of Pixvae, all of which we sampled (Fig. 2).

Field studies through both wet and dry seasons (1979–2015) revealed consistently high numbers of *Eg. mixta* (Fig. 1), followed by *Eg. imperialis* Cockerell, *Eg. crassipunctata* Moure, *Eulaema marcii* Nemésio and *El. meriana* (Olivier) on Ranchería and Coiba islands; *Eg. mixta* and *Eg. imperialis* are abundant euglossines in the mainland at Punta Gorda (DWR, unpublished). The documented orchid flora in Coiba and Ranchería Island (B. Rodriguez and DWR, personal observations, see Ibañez, 2011) includes >70 species, with the 'androeuglossophilous' orchids *Coryanthes*, *Mormodes, Cycnoches,*
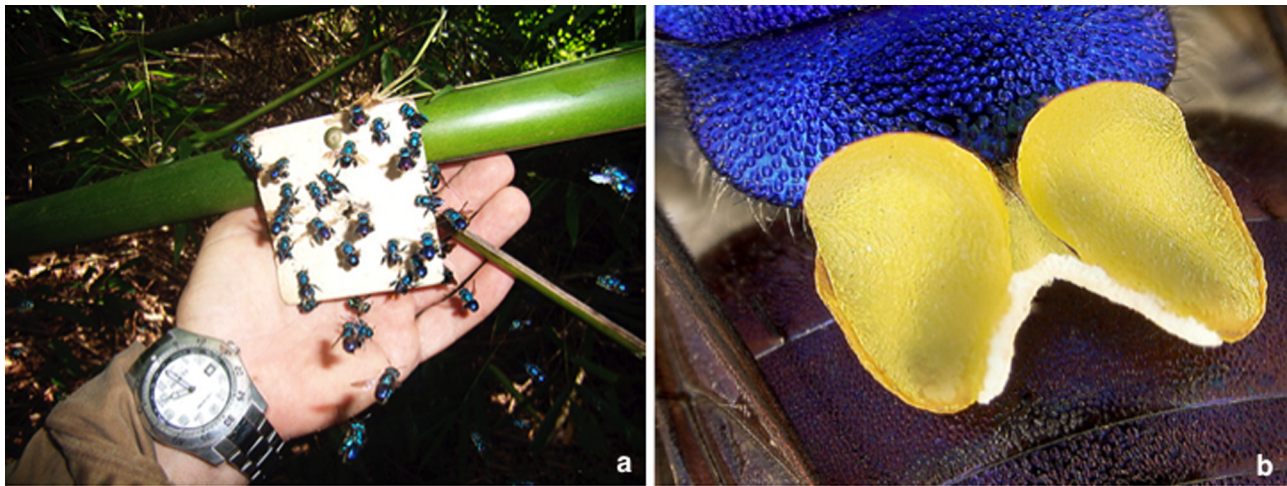
Fig. 1. a. Male *Euglossa mixta* visiting chemical bait (one of three presented simultaneously—all with similar bee activity), on Coiba Island. b. Pollinarium with two pollinia of *Coryanthes*, attached to *Euglossa mixta*.
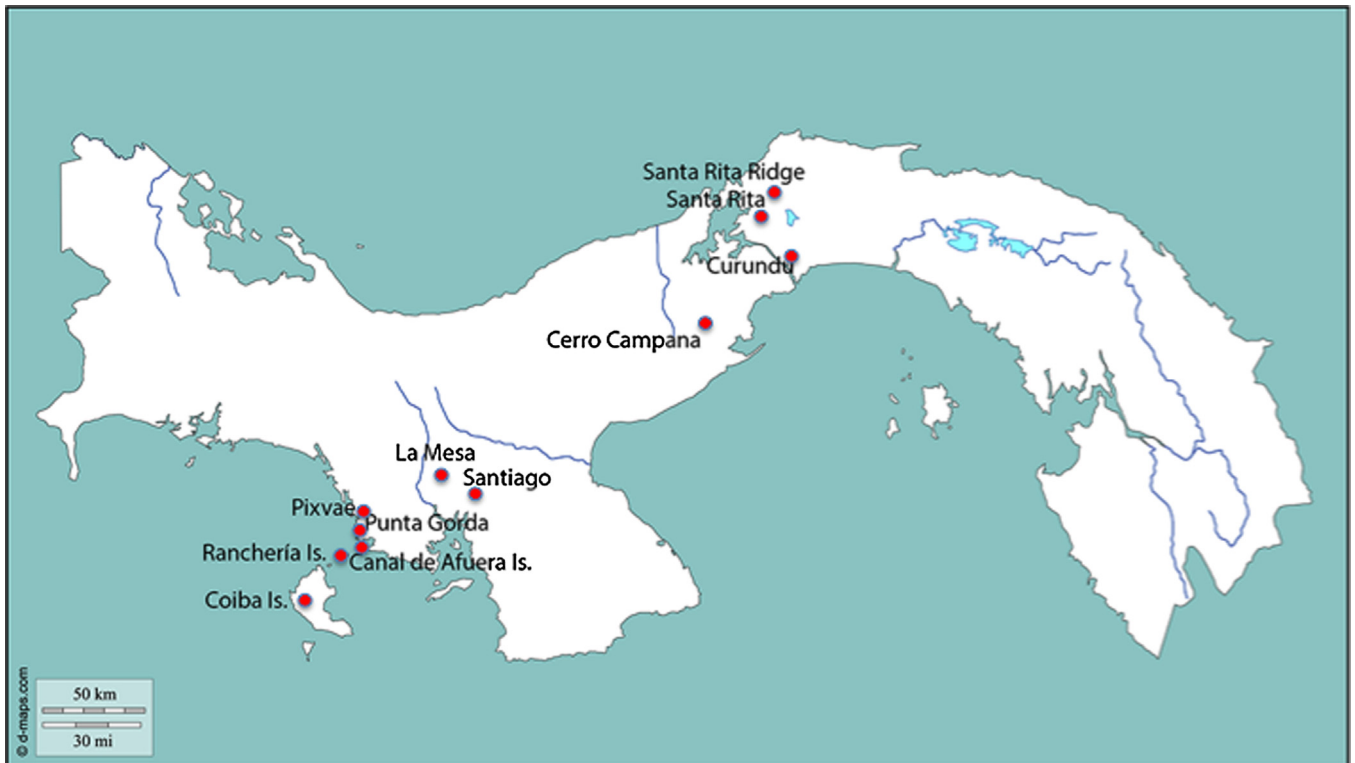

Fig. 2. Map of the Republic of Panama showing the principal specimen localities and rearing sites for orchids and bees.

Vanilla, Notylia, Aspasia and *Catasetum* among bees carrying pollinaria, with one species noted for each—and with no orchid plant yet collected for two genera.

Euglossa mixta principal collection localities (see also Table S1) were islands—Veraguas Province, Coiba Island (0–400 masl, 7°28′N, 81°45′W), Rancheria Island (0–120 masl, 3 km NW of Coiba); Channel Island, 15 km W of Coiba, and mainland Panama—Veraguas Province, Pixvae area, Punta Gorda (120–240 masl, 7°46′N, 81°35′W), Panamá Province, Cerro Campana (8°42′N, 79°54′W), 800 masl, and Colón Province, Santa Rita Ridge and Santa Rita home (300–450 masl, 9°22′N, 79°40′W). Santa Rita Ridge and Cerro Campana have been systematically censused for euglossines and orchid pollinaria they carry since 1980 and 1978, respectively (Roubik and

Ackerman, 1987; Roubik and Hanson, 2004; Ackerman and Roubik, 2012).

For floral and bee tibial fragrance analyses, island specimens were compared to samples from National Parks and old forest areas across the isthmus of Panama, in lowland and cloud forest (to 800 masl), of differing annual rainfall (ca. 1500 mm–4000 mm), near both Atlantic and Pacific coasts (Fig. 2). We made all collections for chemical analysis during 2007–2010.

2.2. Male tibial extracts

Males of *Euglossa mixta* were attracted using scent baits impregnated with three compounds they visit throughout the year, methyl salicylate, 1,8-cineole and skatole (Roubik and Ackerman, 1987;

Table 1

Collection locality data on orchid flowers used for head-space analyses. See Table S3.

CORYANTHES
Coryanthes hunteriana: Coiba, La Salina area, Panama—Veraguas, Santiago
Coryanthes cf. *mastersiana*: Panama—Veraguas, Santa Fe area; Arraiján, Rojas Garden
Co. panamensis: Panama—Nuevo Emperador
Co. panamensis: Panama—Curundu
MORMODES
Mormodes hookeri: Coiba
Mormodes sp. red flowers.
Mormodes cf. *flavida*: Panama—Veraguas, Pixvae
Mormodes flavida x hookeri: Panama—Veraguas, Pixvae
Mormodes hookeri: Panama—Veraguas, Pixvae
Mormodes powellii: Panama—Colón, Santa Rita Ridge
Mormodes sp.
CLOWESIA
Clowesia warsczewicii: Coiba, Panama—Veraguas, Santiago
CATASETUM
Catasetum maculatum: Coiba, Panama—Veraguas, lowland, Los Santos: La Mesa 1000m, El Oro 200 m

Roubik, 2001). The total hind tibial content from individual bees was extracted in 0.5 ml redistilled hexane, or in Licrosolv hexane (Merck 98% purity) to which 5 μg of furfuryl octanoate and/or methyl stearate was added as an internal standard. Bee samples were not concentrated before analysis, and some samples had to be diluted and re-analyzed to obtain acceptable separation.

2.3. Floral scent sampling

Specimens of the orchid genera and species are listed with locality and other data in Tables 1 and S2. After collection on the islands and mainland Panama, they were grown outdoors at the orchid collection of Bonarge Rodríguez, Panama Province, Arraiján, and at the nature preserve of D. W. Roubik, Colón Province, Santa Rita Arriba (here referred to as Santa Rita home) until flowers developed. Flower scents from two individuals of *Coryanthes* cf. *mastersiana* were taken from the collection of F. Rojas, Arraiján. At flowering an inflorescence was bagged in a polyacetate roasting bag (Toppits, Melitta Scandinavia AB, Box 504, SE-264 23 Klippan, Sweden). Depending on inflorescence size three different bag sizes (25 cm × 40 cm, 35 cm × 43 cm, 60 cm × 55 cm) were used. Teflon tubes (5 cm long, 3 mm inner diam. and 4 or 5 mm outer diam.) filled with 40 mg of Tenax Gr (Alltech, 2701 Carolean Ind. Drive State College PA 16801), mesh size 60-80, were inserted into the bag with flowers and via a silicon tube connected to a battery driven membrane pump. The airflow through the adsorbent plugs was between 130 and 150 ml per minute. Blank samples of either environmental air or plant parts (leaves and stems) were collected in parallel with all samples. Prior to their use, adsorbent plugs were cleaned with 1 ml of each of methanol, acetone and hexane, and dried with pressurized carbon filtered air. Floral scent was collected for approximately 2 to 6 h between 9:00 and 15:00 h. Individual samples were labeled with pencil on paper and wrapped in aluminum foil, then placed in a freezer in double roasting bags. Samples were brought to Sweden and extracted with 400 ml of either redistilled hexane or Licrosolve hexane (Merck, 98% purity). After extraction, 500 ng of methyl stearate and furfuryl octanoate, respectively, were added as internal standards to all samples. Prior to analyses, most samples were concentrated about 10 times at room temperature.

2.4. Chemical analysis

All samples were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) on a HP 6890 GC connected to a HP

5973 mass selective detector (MS) with Helium as carrier gas. Two 30 m long fused silica columns each with an inner diameter of 0.25 mm: a non-polar coated with HP-5MS and a polar coated with HP-Innowax, each at a film density of 0.25 mm, were used. The GC conditions for bee extracts were: injector temperature 225 °C and GC programmed for 3 min at 50 °C, increased by 8 °C/min to 230 °C, where it was held steady for 15 min and then increased by 5 °C/min to 280 °C. The scan parameters were 29 to 400 amu during the first 29 min then the upper mass was increased to 550 amu. GC-conditions for orchid samples were: injector temperature 225 °C and GC programmed for 3 min at 40 °C, increased by 5 °C/min to 225 °C, and then held steady for 5 min. The scan parameters were 29 to 400 amu. One microliter of each bee extract and 2 microliters of each scent sample were injected splitless into the GC by an automated injector (HP 7683 auto-sampler).

Extracts of the hind tibial pouches contains heavy, non-volatile compounds which only elute from a GC-column at high temperatures (Whitten et al., 1989; Mitko et al., 2016), and in order to prevent interference with future injections they were run on a non-polar column that allowed sufficiently high temperatures for a prolonged time. On the other hand, head- space samples of flowers only contain volatile compounds and can be analyzed on both polar and non-polar columns. Most floral scent samples are best analyzed on polar columns because the majority of the compounds are polar, thus are better chromatographed and separated on a polar column. Ideally, compound identifications are confirmed on two GC-columns differing in polarity, thus we ran all orchid samples on the polar column and most samples also on the non-polar column. Orchid samples were quantified from analysis made on the polar column and compared to analyses of bee extract quantified on the non-polar column. Because we obtained similar results comparing presence/absence with relative amounts we are confident that the results produced from the two different columns did not bias our results.

Several of the bee extracts were very strong and had to be diluted 10 times to obtain acceptable gas chromatographic separation of compounds. Identifications were made by comparing obtained mass spectra with those of authentic reference samples and tentative identifications by comparison with mass spectral libraries in Adams (2007), NIST and Wiley. Bee and orchid samples were quantified using peak areas and in orchids the total peak area of the compounds was compared to the internal standards, yielding an approximation of the total amount of volatiles produced. In bees this was not possible because of enormous range in concentrations and also one isomer of HNDB totally overlaid (and thereby obscured) the internal standard peak of methyl stearate used.

2.5. Data analysis

Differences in the number of compounds detected in tibial bouquets of bees from the different populations were tested using a 95% confidence interval. For bee data we used nonmetric multidimensional scaling (NMDS, Primer v6, Clarke and Gorley, 2006) to visualize differences between individuals in chemical content of the tibial pouch. Bray-Curtis distances were quantified on the basis of percentage chemical composition of individual bees. Data were 4th root transformed before analysis. In addition, the presence or absence of compounds was considered in analyses. Analysis of similarity ANOSIM has been applied to euglossine bee populations from different sites (Pokorny et al., 2013) and we employed the same method. To compare orchid bees and orchid genera we applied a One-Way Similarity percentage (SIMPER) analysis using Bray Curtis similarity of all compounds found in both orchids and bees. SIMPER analysis of the contribution of individual compounds to the similarity within each group and the dissimilarity between two groups, i.e., in our study the dissimilarity between any pair

of orchid genera and any pair of orchid genera and *E. mixta*, was thereby derived.

We performed ANOSIM tests using a non-parametric permutation procedure of rank similarities in the resemblance matrix produced by our classification of samples. A-priori we grouped bees as belonging to mainland Panama or islands, in different localities (Ranchería Island, Coiba Island, Cerro Campana, Santa Rita home, and Santa Rita Ridge). A two-way nested factorial design, with location and island as factors, was applied using ANOSIM, to test for differences grouped by island or mainland, and within the two groups. The 'tibial bouquet H_0' was that no differences occurred between bees of different localities. Because of meager sampling – only three individuals – bees from Pixvae and Channel Island were excluded from that particular analysis, but not from the general visualizations. ANOSIM produces a global R where an R-value close to zero indicates that the similarity within and between the groups, on average, is the same, and H_0 is accepted. Conversely, R = 1 indicates that all replicates within a locality are more similar to each other than to any replicates from different localities. Depending on the variability within and among the groups being compared, relatively low R-values may be significant for pairwise comparisons. Differentiation among populations as to which common chemicals bees collect was analyzed with principal component analysis (PCA), using, Simca-P 8.0 (Umetrics AB, Box 7960, Umeå, Sweden). The analysis was performed on the 36 chemicals that occurred in more than half of the 93 sampled bees. Using the most commonly occurring compounds presents the least variable picture and potentially underscores differentiation among populations. Again, PCA is a tool for organizing quantitative population data visually, accomplished by transforming the variables along orthogonal axes, then plotting them as scores, in multiple rotated axes. Plots in three dimensions can portray maximum variation in a fair proportion of the total variation. We do not offer interpretation of the new axes, but use the method to display information.

Only compounds with retention times below 31 min (about 300 amu or less in molecular weight) with our GC-programming were included, which corresponds to the limit where compounds are sufficiently volatile at environmental temperatures to attract bees, male or female, from a distance. Whitten et al. (1989) and Eltz et al. (2006) demonstrate that a range of long-chain saturated as well as unsaturated hydrocarbons, alcohols, acids, acetates and diacetates and ester lipids, although found in the hind tibia, in fact originate from the cephalic labial glands and are added during the process of fragrance collection. We found similar compounds in the hind tibiae and exclude them from our analysis (see also Ramírez et al., 2010a; Mitko et al., 2016).

To test for differences in chemical composition among the orchid genera, an ANOSIM analysis was applied to a resemblance matrix based on percentage (semi-quantitative) composition of all compounds of individual scent samples of all orchid samples, the latter grouped into their respective genera. Data were 4th root transformed before analysis.

To ascertain the degree of similarity between the orchids, and also to evaluate whether bees prefer orchids that resemble their tibial bouquet we made a combined analysis of bee and orchid samples. If bees prefer orchids that resemble their "full" tibial bouquet we reasoned they might prefer orchids that held more compounds in common. Total volatile amounts are only available for orchids, thus analysis of compounds in orchids and bees combined were by relative amounts, considered as percentages. We then used NMDS to visualize the relationship between compounds produced by orchids and those accumulated by bees. We used Bray-Curtis distances calculated on basis of 4th root transformed percentage composition of individual samples or we used presence/absence of the compounds. The combined dataset included 93 bee and 30 orchid samples and the 66 compounds that were shared between
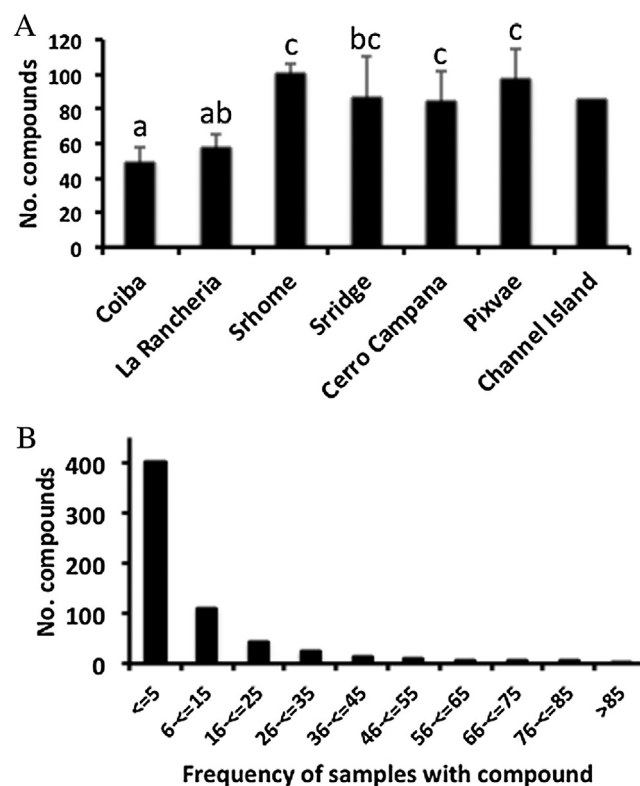

Fig. 3. a. Number of compounds available in the tibial bouquets of a bee (95% confidence interval indicated, with different letters indicating significant differences), at study sites (islands: Channel, Ranchería, Coiba; mainland: Pixvae, Cerro Campana and Santa Rita (home and ridge). b. Histograms of individual frequency of the 636 chemical components collected from hind tibial bouquets of 93 individual *Euglossa mixta* sampled at all localities.

orchids and bees. Applying this procedure may exaggerate similarity between bee perfumes and scents of the orchid genera, but differences are also more easily detected, by eliminating some 'noise'.

3. Results

3.1. Bee phenotypic variation

In total, 636 chemicals were detected in hind tibia samples of the 93 *Euglossa mixta* (Table S1). Most chemicals were found in ≤5 samples (Fig. 3b) and composition of the tibial bouquets varied both quantitatively and qualitatively, among bees within and between populations (Figs. 4 , S1–S3). The number of volatile compounds found in each bee varied between 11 and 132 (mean = 70 ± 29 SD). We analyzed all the compounds detected, and found bees on Coiba and Ranchería islands had 52 ± 22 (N = 55), significantly fewer (t-test, P < 0.001) than the 92 ± 24 (N = 38) for mainland Panama (95% CI, see Fig. 3a). Interestingly, bee samples from Ranchería Island had almost as many unique compounds as anywhere on the mainland (Table S1). The number of chemicals accumulated by bees on Coiba and Ranchería did not differ from each other, nor did those among mainland populations (Fig. 3a). Analysis of the relative amount of compounds provided similar locality effects. Four NMDS ordinations (see Figs. 4; S1–S3) included: a) 4th root transformed percentages of compounds (>5 samples) (Fig. S1 which on average included 96.9% of the tibial content (in 9 samples <90% total content), b) a similar ordination on 4th root transformed data which included all compounds (Fig. S2) and (c) an ordination based on presence/absence of all compounds (Fig. S3).
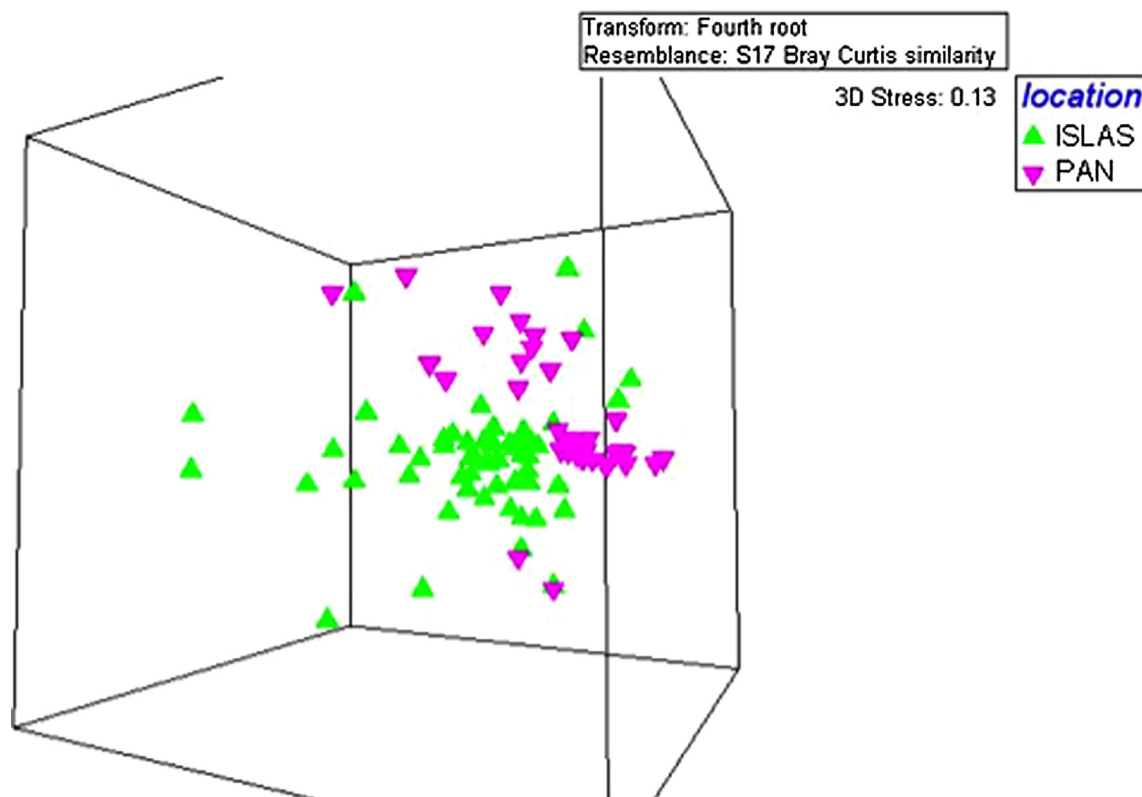
Fig. 4. MDS analysis based on all chemical components of the male *Euglossa mixta* hind tibia samples (93 bees, 636 compounds) 4th root transformed percentages, found on islands versus mainland sites.

Table 2

Pairwise R-values and associated significance of ANOSIM analysis of tibial volatile composition including 90 *Euglossa mixta* grouped by locality. Global R = 0.269. ***significantly different at 0.1% and * at 5% level. Values for compounds found in >5 samples are included. Percentage values were 4th root transformed before analysis. Channel Island and Pixvae not included because of few samples, one and two, respectively.

	Coiba Island	Ranchería Island	Santa Rita Ridge	Santa Rita home
Ranchería Island	−0.006 ns			
Santa Rita Ridge	0.29*	0.29*		
Santa Rita home	0.37***	0.29***	0.76***	
Cerro Campana	0.43***	0.36***	0.55***	0.74***

ANOSIM tested differences in tibial volatile composition of bees by locality. Irrespective of whether we examined compounds found only in >5 samples or included all collected compounds, all localities except Coiba and Ranchería differed significantly from each other (Table 2). Benzenoids and sesquiterpenes were most abundant and constituted between 26% and 47% and 33% to 37%, respectively, of the volatiles collected by the bees at five of the study localities (Fig. 6 and Table S1). However, at Santa Rita home and Santa Rita Ridge, monoterpenes constituted 27% and 29%, respectively, while they constituted ≤5% in the other populations. Chemicals constituting 4 to 17% of the volatiles could not be identified and classified according to biosynthetic pathways. From ANOSIM, with factors of location and islands, results indicated substantial differences between individual populations (P < 0.001, Global R = 0.18) but no significant difference between islands versus the mainland (P = 0.1, Global R = 0.5).

The principal components analysis of the 36 hind tibial compounds that occurred in >50% bee samples revealed substantial variation among bees from Ranchería and Coiba Islands, less among samples from Cerro Campana and little among bees from the Santa Rita area (Table 2, Fig. 5). The first two principal components accounted for 36.5% the total variation. The PCA loading plot (Fig. 5b) confirmed the influence of chemical classes on the different populations. The Santa Rita home and Santa Rita Ridge populations formed a group mostly defined by the monoterpenes cis- and trans-β-ocimene, myrcene and α-pinene, the Cerro Campana individuals displayed a high content of methyl salicylate (a benzenoid) and nerolidol (a sesquiterpene) and a low content of 1,4-dimethoxybenzene (a benzenoid), while the island populations contained higher amounts of the four isomers of 2-hydroxy-6-nona-1,3-dienylbenzaldehyde (HNDB, a benzenoid, see Eltz et al., 2008; Pokorny et al., 2013) than other populations. Finally, 1,8-cineole, and linalool occurred in substantial amount in bees and *Coryanthes* fragrances, independent of locality (see below).

3.2. Orchid scents

Scent samples included *Coryanthes* (9 samples; 3 species), *Mormodes* (10; 3), *Clowesia* (2; 1) and *Catasetum* (9; 1). Chemical composition of the samples was diverse; 315 compounds were detected, of which 47 were found in ≥5 samples (Table S2). The identity of about one-third of the chemicals (107) was verified against authentic standards, but two-thirds were classified to compound group on the basis of their mass spectrum (Table S2). Most (52) of the 99 unknown chemicals were detected in single samples, around 25% (24) in two samples, three in 15 samples, four in six samples, and seven in two samples.

The four genera differed from each other, irrespective of using quantitative (percentage, relative amount, Table 4) or qualitative (presence/absence, not shown) data of volatiles. Monoterpenes were the prominent chemical class, 42 to 94%, found in far larger amounts in *Clowesia* (92–94%) and *Catasetum* (0; 2 individuals) to between 92 and 98%. Benzenoid aromatics were also prominent
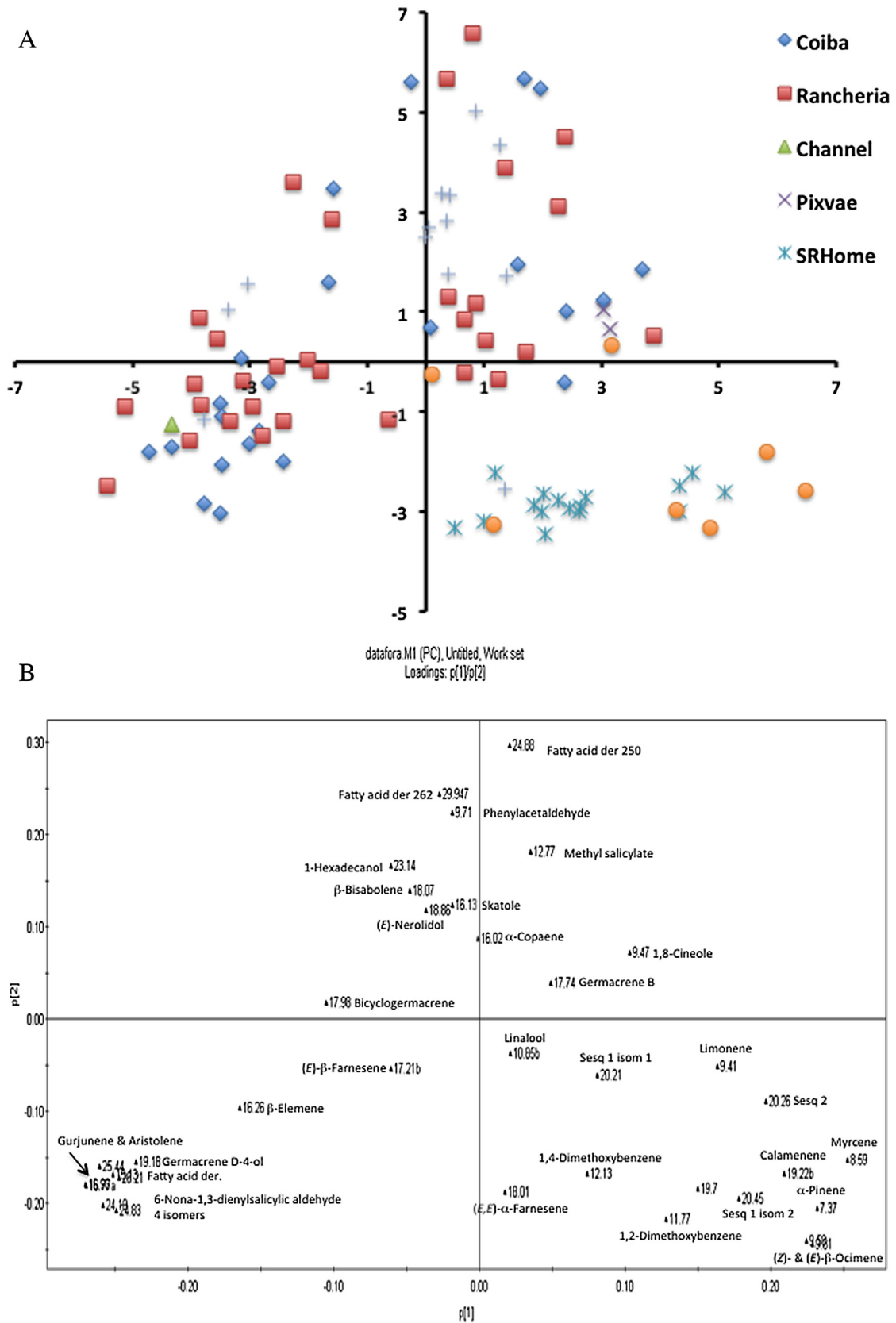
Fig. 5. Principal components (PCA) analysis based on the 36 compounds found in >1/2 of the samples of hind tibial bouquets of 93 bees from three islands and four mainland localities. PC1 and PC2 accounted for 20.1 and 16.4% of variation, respectively. 5a. Score plot showing the first two transformed axes ordinating individual male *Euglossa mixta* hind tibial bouquets and 5b. loading plot showing the influence of the 36 chemicals used to ordinate the samples.

Table 3
Pairwise tests of similarity of 66 shared compounds of *E. mixta* and orchids of four genera. Global R = 0.819. ***significantly different at the 0.1%, ** at the 1.0% and * at the 5% level. Percentage values were 4th root transformed before analysis.

	Euglossa	*Coryanthes*	*Mormodes*	*Clowesia*
Coryanthes	0.731***			
Mormodes	0.762***	0.463***		
Clowesia	0.928**	0.177 ns	0.635*	
Catasetum	0.961***	0.407**	0.707***	0.44*

Table 4
Pairwise tests of similarity in floral scent composition in the four orchid genera analyzed. Global R = 0.638. ***significantly different at the 0.1% and * at the 5.0% level. Percentage values were 4th root transformed before analysis.

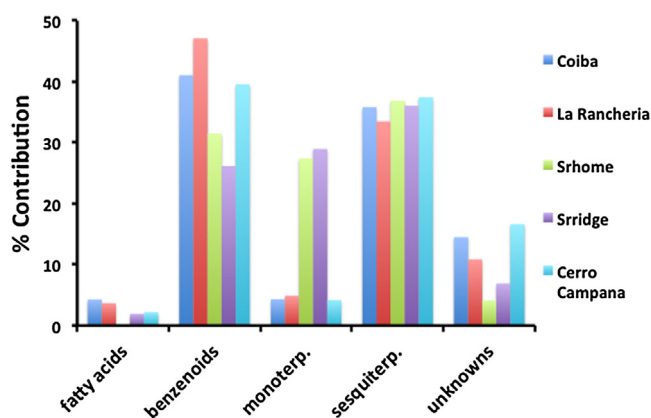|	*Coryanthes*	*Mormodes*	*Clowesia*
Mormodes	0.62***		
Clowesia	0.45*	0.81*	
Catasetum	0.57***	0.75***	0.54*

Fig. 6. Distribution of chemical compound classes—monoterpenes, sesquiterpenes, fatty acid derivatives, C5-branched compounds, benzenoids and unknowns in hind tibial extracts of *Euglossa mixta* in Panama, by locality, for two islands and three mainland localities (see Tables S1, 2).

in *Coryanthes* and *Mormodes*, the genera used by *Euglossa mixta*, but almost absent in the other two genera. Irregular terpenes were prominent in *Catasetum*. Fatty-acid derived compounds constituted a small fraction of the volatiles across the orchid genera.

The total absolute amount of scent produced varied from 10 ng to 62 μg per flower/hour within and among species and genera (Table S2). *Catasetum maculatum* produced the lowest (10 ng–12 μg/h/fl) and *Clowesia warsczewicii* the highest amounts (4.7–71 μg/h/fl), but the relative amounts varied among individuals of a species.

3.2.1. Coryanthes

Three stereoisomers of undecatriene were detected only in *Co. panamensis* and some nitrogen compounds only in *Co.* cf. *mastersiana* and *Coryanthes* sp. Methoxylated cinnamic compounds were found in both *Co. panamensis* and *Co. hunteriana*. *Coryanthes* 09.152 neither contained aromatic nor nitrogen containing compounds. The monoterpenes α-pinene, sabinene, myrcene, (*E*)-β-ocimene, limonene, 1,8-cineole and 2,3-epoxygeranyl acetate were detected in some of the *Coryanthes* samples, in high relative amounts (>10%), and in four samples (*Co. panamensis*, *Co. hunteriana* (10.22), *Co.* sp. 09.152 and 09.153) those compounds constituted between 45 and 88% of the scent. *Coryanthes panamensis* and *Co. hunteriana* shared methoxylated cinnamic compounds with *Mormodes flavida* and with its hybrid *M. hookeri* x *flavida*.

3.2.2. Mormodes

Monoterpenes accounted for more than 50% of the scent in five of the ten samples collected and in those ocimenes, geranyl acetate and epoxylated geranyl acetate accounted for the largest part. Four samples contained more than 50% of the aromatic 1,4-dimethoxybenzene, which in some samples was associated with 1,2-dimethoxybenzene. The last sample was characterized by the sesquiterpenes γ-muurolene and caryophyllene, and 31% of the scent in one sample was (*E*,*E*)-α-farnesene. Five samples also contained some amount of methyl 4-methoxy-(*Z*)- and (*E*)-cinnamate.

3.2.3. Clowesia warsczewicii

The two samples were characterized by monoterpenes in which myrcene and ipsdienol were found in the largest amounts, followed by geraniol and some sabinene

3.2.4. Catasetum maculatum

one sample contained exclusively (*E*)-4,8-dimethyl-1,3,7-nonatriene and another orchid sample contained a large proportion of this chemical, together with the sesquiterpene α-copaene. Both flowers produced low amounts of scent—10 and 33 ng per flower per hour. The remaining samples contained almost exclusively monoterpenes. Carvone oxides and related compounds constituted a large part of the scent in some individuals. The α-pinene, α- and β-phellandrene, and in one sample, also limonene, were produced in high relative amounts as was the sesquiterpene α-copaene which constituted 29% of the scent in one individual.

3.3. Bees and orchids combined

Most compounds shared by orchids and bees are listed in Table S3 and the NMDS of shared compounds, followed by an ANOSIM analysis, demonstrated that the bees and the orchid genera, except *Clowesia* and *Coryanthes*, divided into distinct groups (Table 3, P < 0.05, Global R = 0.819), irrespective of whether qualitative or quantitative data were applied (Fig. 7). However, most of the *Catasetum* samples, and those of the fewer samples from *Clowesia*, were distinctive from the 'bouquets' of *Eg. mixta*, *Coryanthes* spp. and *Mormodes* spp. In Supplemental Table 4 the compound similarities, and differences or similarities within and between *Euglossa mixta* and the four orchid genera are given, according to SIMPER analysis, using Bray-Curtis similarity. *Clowesia* and *Coryanthes* did not differ from each other. The results show primarily a high (0.91) similarity between *Clowesia* and *Catasetum*, a moderate similarity between *E. mixta* and *Mormodes* or *Coryanthes* (0.21–0.22), and relatively low similarities (0.07–0.11) among all other combinations.

4. Discussion

We sampled along a transect of intact forest habitats, national parks and protected areas, which included islands—of known history of separation and union to mainland habitats during glaciations, and ancient continental connections—to investigate variation in the chemicals of orchid fragrance hosts, and in the fragrances stored by bees that collect such plant products. We also applied the information gathered from >30 years of recording orchid associations with euglossines based on pollinaria, noted among >200,000 bees. The male bees are readily attracted to volatile chemicals in monthly field census studies in Panama (Roubik and Ackerman, 1987; Roubik, 2001; Ackerman and Roubik, 2012; Roubik, 2014). Taken together with fragrance sampling and analysis, we may explore bee chemical collection behavior via chemical and pollen proxy information, which draw from established field insights. Many odors common to the orchids and the bees are among the most abundant in floral scent samples, such as green leaf volatiles, ocimenes, pinenes, limonene, terpinenes, linalool, caryophyllene,
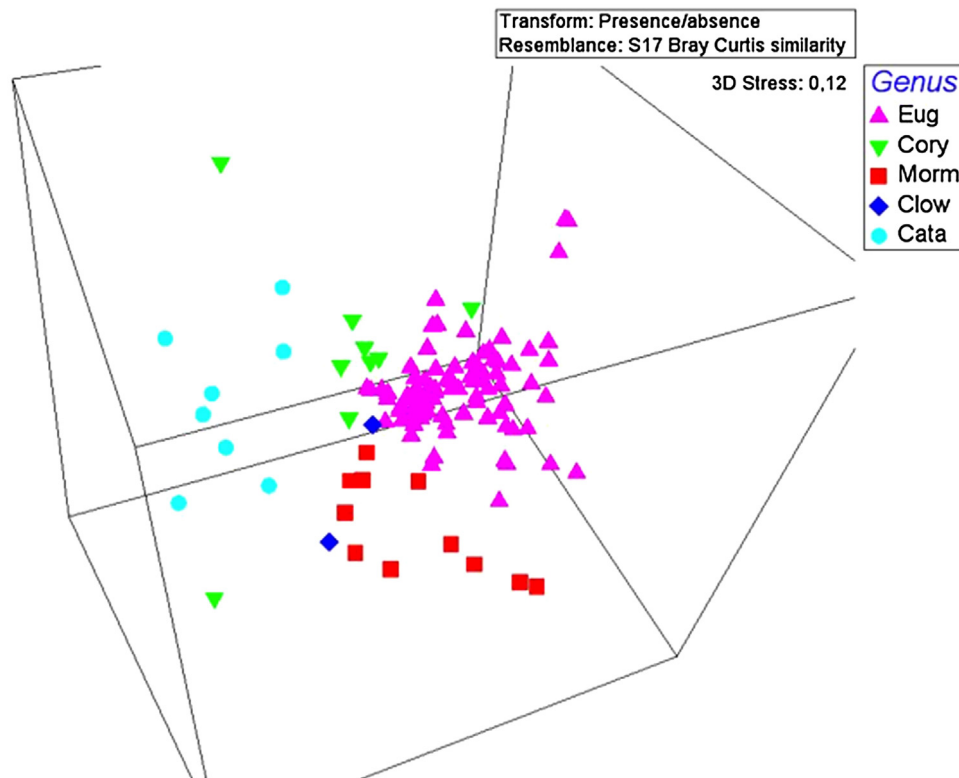
Fig. 7. Nonmetric multidimensional scaling (NMDS) similarity of bee and orchid fragrances. Of 66 compounds common to *Euglossa mixta* male tibial fragrances and flowers of four orchid genera, presence or absence data included (see text). Eug = *Euglossa*, Cory = *Coryanthes*, Morm = *Mormodes*, Clow = *Clowesia*, Cata = *Catasetum*.

and trans-α-bergamotene. However, a number of methoxylated and epoxylated chemicals are rare in floral scents of other plant species (Knudsen et al., 2006) but were found, in both the orchids and the bees, e.g., methyl methoxy-(Z)- and (E)-cinnamate. Furthermore, orchid bee species, most of which are active all year, collect different volatiles at different times and places (Ackerman, 1989).

To interpret our results, community survey data are useful. A maximum of euglossine species resident in Coiba, or that of nearby Ranchería island, of 220 ha, to which some bees the size of *Euglossa* fly from Coiba (Roubik and Camargo, 2012), is 21 (see below), much less (<50%) than mainland forest of similar elevation and rainfall (Roubik and Ackerman, 1987; Roubik, 2001, 2004). Lost from Coiba but on the mainland (Punta Gorda is the reference, 25 km from Coiba) are *Eg. cybelia, cyanura, flammea, deceptrix, cognata, bursigera, igniventris* and *milenae* (taxonomic authorities in Roubik and Hanson, 2004; Bembé, 2007). Forests in lowland Panama contain additional species not in the Pacific areas we sampled: *Eg. dissimula, sapphirina, ignita, hansoni, villosiventris, cordata, dressleri, gorgonensis* and *turbinifex* (see Roubik and Hanson, 2004, for taxonomic authorities) along with several species of *Eufriesea* and *Eulaema* (DWR, unpublished). The environment has presumably altered the evolutionary ecology of *Eg. mixta* on Coiba and associated islands. For example, *Eg. cognata*, a bee slightly larger than *Eg. mixta*, is evidently the most similar, local, tibial bouquet 'relative' to *Eg. mixta* (Zimmermann et al., 2009), while *Eg. cognata* and *Eg. villosiventris* are the closest phylogenetic relatives to *E. mixta* (Dick et al., 2004; Ramírez et al., 2010b). They are absent on Coiba. Nuclear genes reveal a younger age for *Eg. cognata*, roughly half that of *Eg. mixta* (Ramírez et al., 2010b). It is tempting to speculate that an early connection in the Miocene (Montes et al., 2015; and C. Jaramillo, pers. comm.) allowed *E. mixta* to reach Coiba then, before a sister species from mainland South America evolved, but other interpretations are available (O'Dea et al., 2016).

The investment in finding and sequestering fragrances (for bees), as well as producing them (for flowers) should be tuned to their necessity, and respond to natural selection. Male euglossine bees collect floral and additional fragrances apparently to demonstrate their longevity, skill and species (Eltz et al., 1999; Roubik and Hanson, 2004; Ramírez et al., 2011). We have not tested whether such substances are correlated with genotype, but they are certainly phenotypically informative (see Mitko et al., 2016). Although tibial fragrance breadth of an orchid bee species might tend to increase with food niche breadth or population size, or pollination performance (Ackerman and Roubik, 2012), "odor resource partitioning" (Zimmermann et al., 2009; Eltz et al., 2011) is proposed as essentially restrictive. There are limits in similarity between coexisting species, which collecting certain odors may violate. Novelty in orchid floral volatiles may, nonetheless, produce opportunity for coevolution with a principal pollinator (Gregg, 1983), yet the relative value of a particular compound within a fragrance is difficult to assess (Mitko et al., 2016). Judging from bees arriving at chemical baits with orchid pollinaria on their bodies, and field observations at orchid flowers, *Mormodes* and *Coryanthes* are often visited by *Euglossa mixta*, both on the mainland and Coiba and Rancheria Islands, and share more in common with the bee bouquets than *Catasetum* and *Clowesia* (Fig. 7, Table 3), never used by the bee (Roubik and Hanson 2004; Ackerman and Roubik, 2012). The total volatiles common to both the bee and *Coryanthes, Mormodes, Clowesia* or *Catasetum* were 15, 17, 4 and 16, respectively, and those found in ≥2% of both orchid and bee were 1,3, 0 and 0, of 27 compounds listed in Table S3. Moreover, as primary attractants frequently found in *Coryanthes* or other orchids (Kaiser, 1993) and also in the tibial bouquet of *Eg. mixta*, 1,8-cineole, methyl salicylate and linalool stand out. It is no coincidence that the most effective chemical attractants for male *Eg. mixta*, in baiting studies, are 1,8-cineole and methyl salicylate (Roubik and Ackerman, 1987). As our

limited generic orchid assays attest, however, from multivariate analysis (Table 3) *Euglossa mixta* does not seek an odor most like its own full tibial bouquet, at least when the fragrances emanate from flowers (see, in contrast, Roubik, 1999 – bees that strongly seek collected odors of their species). That implies a subset of fragrance compounds directs the behavioral response of the pollinator, and it likely changes over time (Eltz et al., 2005), yet one would predict that electroantennographic responses of the bee would be greater than the average response to the particular compound it prefers (Pokorny et al., 2013; Mitko et al., 2016). A complicating factor is the learned avoidance of chemicals already preferred and collected, despite heightened sensitivity to those volatiles (Eltz et al., 2005; Eltz and Lunau, 2005). This does not preclude "rare male advantage" – the basis for frequency-dependent selection and selective avoidance/attraction, based on experience and learning. The antennal sensitivity to fragrance compounds is likely to be modulated by the nervous system, before a behavioral response occurs (Schiestl and Roubik, 2002). We have still to devise a means of assaying repellents, attractants and visual cues for bees and orchids, using both field and laboratory systems.

Recently it was shown that the fragrance composition in the hind tibial bouquet of male euglossines is, at least functionally, species-specific among males from distant localities (Eltz et al., 2005; Zimmermann et al., 2006; Ramírez et al., 2010a). More importantly, a given, local assemblage contains *Euglossa* species that are completely distinguishable based on tibial bouquet. That may enforce premating isolation (Zimmermann et al., 2009; Pokorny et al., 2013), as a result of interspecific interference avoidance. By avoiding significant chemical interference, when displaying and dispersing chemicals at their mating perch sites, male bees of different species are better able to coexist. The bees are normally active all year, and form a large portion of the local bee community (Roubik, 1989). Males show species-specific EAG responses to total tibial extracts (Eltz et al., 2006; Mitko et al., 2016). Field tests, including reciprocal tests in different regions, indicate that the hexane extract of a tibial bouquet attracts conspecific males, in studies of the giant orchid bee, *Eulaema bombiformis* (Zimmermann et al., 2006; and DWR unpublished). In addition, two matings were witnessed by the first author at natural perch sites, in 38 h observation, and each mating sequence lasted ca. 8 s. These were in different areas and years but in each, many males sequentially approached the perching male, as did the single mating female, from downwind (DWR, unpublished data). There are very likely to be chemical profiles of each species, and tibial bouquets attract the rare female and rival males at the same time (Roubik and Hanson, 2004; Zimmermann et al., 2006; Ramírez et al., 2011, 2015). Additional variables such as ambient light and visual cues among bees will need greater understanding to simulate normal field conditions in controlled tests of euglossine mating.

We suggest that male tibial bouquets contain so many more chemical compounds than orchid flowers of a particular species because there is continuous selection to avoid interference between bee species, while there is much less likely to be a fitness penalty for resemblances between different orchid species. Indeed, mimicry and rewardless orchid flowers are common (summary in e.g., Roubik, 2014). As potential analogs, for orchids, we can hypothesize the value of particular chemical blends may be found both in preventing interspecific crosses and also in promoting outbreeding within a population. For bees, tibial bouquets conceivably permit genotypic recognition and thus serve genetic outbreeding purposes, while also preventing interspecific mating interference. All such propositions, unproven in the field, depend on bee sensitivity and response to chemical blends that are essentially similar but differ in minor components among individuals or populations. However, Coiba *Coryanthes* had far more chemicals in the bouquet than did a specimen from the nearby mainland, while the opposite

was true for one *Catasetum*. Those species in fact represent both unisexual and hermaphroditic flowers, and the unisexual flowers evoke contrasting bee responses, which may influence outbreeding (Milet-Pinheiro et al., 2015). More samples are required to appreciate such orchid scent variation (Table S2). Furthermore, fragrances are not the only floral attractants. The attractiveness of flowers includes form and appearance, phenology, rate of production of the volatile, or other functional variables yet to be elucidated (Willmer, 2011), while floral structure and anther cap further reduce inbreeding (Roubik, 2014). We have already alluded to the amounts of scent produced per unit time, which would be difficult to trace in any presence/absence comparison, or in the total amount or percentage, in complex compounds.

The comparison of bee similarity to the four orchid genera provided a novel analysis. The conclusions are only tentative, however, due to very limited taxon sampling within genera. For the local fauna and flora under consideration, the results are of interest. Two genera, *Catasetum* and *Clowesia*, have never been found associated with *Euglossa mixta* in Panama (Roubik and Ackerman, 1987; Ackerman and Roubik, 2012). *Clowesia*, however, was not statistically different (at a 5% level) from *Coryanthes* in similarity with scents collected by *E. mixta* (Table 3). Such a result likely reflects small samples and species number. Principal volatiles of the two orchid genera and bee tibial bouquets are similar with regard to terpenes, myrcene, and 6-methyl-5-hepten-2-one. The total fragrance blends among the orchid genera and *Eg. mixta* do, however, display similarity between the bees and the two genera whose flowers they regularly visit (Fig. 7, and SIMPER analysis, Table S4). The flowers of *Clowesia* do not have a complicated morphology, unlike that of the bucket orchids, *Coryanthes*, which would restrict visitation by bees. Therefore, it is plausible that chemical attraction is not so much the whole odor, but some of its parts. Any input of chemicals that repel visitors (see Milet-Pinheiro et al., 2015) remains to be analyzed.

We still have much to learn about variation in fragrance production within orchid and bee populations (see Knudsen, 2002; Eltz et al., 2008). Our conclusion is that a few compounds, such a benzenoids and monoterpenes among *Euglossa mixta, Coryanthes* and *Mormodes*, are more prominent in reduced island bee assemblages and point to avoidance of fragrance interference between congeneric bees. Further, they may constitute original components of the orchid/bee interaction, before "embellishments" were added, or be primordial embellishments themselves.

There is significant reduction of euglossine species on Coiba, but no likely reduction in potential tibial bouquet components, most of which probably originate at non-floral sources (see Courtois et al., 2009; Ramírez et al., 2011). Coiba has over 1000 angiosperm species, mean annual rainfall that varies from 3.4 m to 6.0 m in different habitats (Ibañez, 2011), and is within the realm of the most diverse plant fauna (Allen and Allen, 1977) in the region. We have no doubt that bees can encounter many more volatiles than those we detected in their hind tibiae. Furthermore, a major tibial compound, HNDB, did not appear in our orchid samples, yet is the major compound of tibiae in a *Euglossa* now living outside its historic range, in Florida, and also in the tibiae of *Euglossa mixta* (Eltz et al., 2008). Those authors suggest a fungal origin, which would certainly be favored by the seasonal yet humid and predominantly wet Coiba environment.

If our findings have generality, it is that in communities containing fewer euglossine species, the fragrance components of male bee tibial bouquets will be reduced. The bees are perhaps more compelled, by sexual selection driven by female acceptance of the male odor (Roubik and Hanson, 2004) to embellish their bouquets than are the orchids, but our orchid data were meager, at best. Coiba Island was potentially linked the mainland Darién and Chocó biota of South America in the Miocene, and in the Pliocene experienced repeated sea level shifts which united and separated island and

mainland habitats (Montes et al., 2015; O'Dea et al., 2016). We believe this coincided with the expansion of orchid bees from South to North America (Ramírez et al., 2010b). Nonetheless, the bees incorporated orchids in their chemical repertoires many millions of years after they existed, and likely collected other compounds to secure females (Eltz et al., 2007; Ramírez et al., 2011). Thus orchid floral fragrances acquired evolutionary significance as a novelty or embellishment useful in mating, and now maintain >650 mutualisms among large-flowered orchids and bees (Roubik, 2014).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.flora.2016.11.012.

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