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LETTER

Macroevolution of perfume signalling in orchid bees

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Abstract

Theory predicts that both stabilising selection and diversifying selection jointly contribute to the evolution of sexual signalling traits by (1) maintaining the integrity of communication signals within species and (2) promoting the diversification of traits among lineages. However, for many important signalling traits, little is known about whether these dynamics translate into predictable macroevolutionary signatures. Here, we test for macroevolutionary patterns consistent with sexual signalling theory in the perfume signals of neotropical orchid bees, a group well studied for their chemical sexual communication. Our results revealed both high species-specificity and elevated rates of evolution in perfume signals compared to nonsignalling traits. Perfume complexity was correlated with the number of congeners in a species' range, suggesting that perfume evolution may be tied to the remarkably high number of orchid bee species coexisting together in some neotropical communities. Finally, sister-pair comparisons were consistent with both rapid divergence at speciation and character displacement upon secondary contact. Together, our results provide new insight into the macroevolution of sexual signalling in insects.

Keywords

Chemical communication, coexistence, *Euglossa*, macroevolution, orchid bees, sexual selection, sexual signalling, species recognition.

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INTRODUCTION

Sexual signalling traits display remarkable diversity across the tree of life (Darwin 1872; West-Eberhard 1983). Theory has long suggested that this striking variation is due in part to their role in mediating population divergence, speciation and pre-zygotic reproductive isolation (Lande 1981; Panhuis *et al.* 2001; Coyne & Orr 2004). Therefore, sexual signalling traits are expected to exhibit distinct patterns of diversity across species, including high species-specificity, accelerated rates of evolution across lineages and enhanced divergence among closely related, sympatric taxa. However, despite their known importance in mediating ecologically and evolutionarily significant processes, we know remarkably little about the broad-scale patterns of macroevolution of most sexual signalling traits (Sampson 1999), and studies that have been conducted have produced mixed results (e.g. Gleason & Ritchie 1998; Badyaev & Hill 2003; Symonds & Elgar 2004; Ord & Martins 2006; Lewis & Cratsley 2008; Arnegard *et al.* 2010). In particular, information is lacking on chemical signalling traits, which are widespread and crucial in mediating sexual interactions across taxa, but have received considerably less attention in comparative studies (Symonds & Elgar 2008).

Multiple hypotheses have been put forward to explain how chemical sexual signalling phenotypes evolve on macroevolutionary scales. A simple model suggests that female preferences for a signal (the receiver component) impose strong stabilising selection on male signal traits (the sender components), and thereby favour the stability and integrity of the signal among interbreeding populations (Cardé & Baker 1984). This should result in high species-specificity of chemical traits even across large geographic distances (Ord & Stamps 2009). These dynamics may or may not translate into a macroevolutionary signal of conservatism or stasis, whereby the rate of evolutionary change is relatively slow compared to null expectations (Sampson 1999). A second model proposes that disruptive or directional selection in the form of natural selection (e.g. reinforcement or reproductive character displacement) and/or sexual selection drives signal differentiation between closely related lineages (West-Eberhard 1983), leading to a macroevolutionary signature of high rates of signal evolution associated with either speciation events or related to species occurring in sympatry (e.g. Arnegard *et al.* 2010). A similar third model suggests that sexual signalling traits are under selection for increasing redundancy values as more closely related lineages fill a community (Rand & Williams 1970). This would result in a pattern where, as the number of closely related lineages increases in the community, signalling traits of new species must diverge into more distant unoccupied trait spaces to be distinct. Together these models may explain patterns of trait stasis, rapid divergence and community-related diversification, but are not mutually exclusive and may operate simultaneously to shape signal evolution at different temporal scales across the phylogeny.

In this study, we examine the macroevolutionary patterns of chemical signals in the neotropical orchid bee genus *Euglossa*, a

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Data accessibility statement: *Data is available on Dryad and upon request from sanram@ucdavis.edu.*

group of charismatic insects that have captivated the minds of biologists for over 150 years (Darwin 1886; Vogel 1966; Dodson *et al*. 1969; Dressler 1982; Williams & Whitten 1983). Male orchid bees collect odoriferous volatile substances from orchids and other non-floral sources (including decaying wood, resin, etc.). Once collected, these compounds are stored in specialised pouches located in the enlarged hind tibia of males (Fig. 1 inset) (Vogel 1966; Eltz *et al*. 1999). The chemical mixtures (hereafter referred to as perfumes) are subsequently emitted during courtship displays at perches in the forest understory (Eltz *et al*. 2005b), where males engage in ritualised flight contests (Kimsey 1980). Females approach display sites from downwind, at which point mating can occur (Dodson *et al*. 1969; Kimsey 1980; Zimmermann *et al*. 2006). Although the full functionality of male perfumes has not been demonstrated, behavioural data strongly suggest perfume compounds play a central role in sexual signalling, presumably by enabling species-specific recognition and/or acting as a signal of male fitness. Male perfumes thus represent an excellent behavioural and chemosensory phenotype with which to study the evolution of sexual signalling across a clade of closely related species.

Previous research suggests that the evolution of male perfumes in *Euglossa* was likely shaped by a combination of factors, including stabilising selection, rapid divergence and community-related diversification. For instance, a study by Zimmermann *et al*. (2009) showed that the perfume signals of a community of 15 sympatric species of *Euglossa* from central Panama display remarkably high levels of divergence, where chemical signals appear to evolve quickly in response to strong diversifying selection. On the other hand, stabilising selection may act to maintain perfume phenotypic integrity within species. For instance, studies by Zimmermann *et al*. (2006) and Ramírez *et al*. (2010) revealed remarkable intraspecific qualitative consistency in perfume composition even among populations inhabiting distant geographic regions and thus experiencing dissimilar habitats with high host-plant turnover. In fact, perfume specificity has even been used to identify cryptic species of orchid bees (Eltz *et al*. 2011).

While these patterns are suggestive, studies of perfume-trait variation in orchid bees have been restricted to few individual species pairs (e.g. Eltz *et al*. 2005a; Ramírez *et al*. 2010) or to a limited number of species in a particular sampling location (e.g. Zimmermann *et al*. 2009). To fill this gap, we conducted a geographically and taxonomically large-scale investigation of perfume signal trait variation across the orchid bee genus *Euglossa*. We assembled a dataset of > 880 individual orchid bees representing 65 species collected across the neotropics. We asked the following questions: (1) Does perfume phenotypic variation corresponds to species identity across large geographic distances, reflecting groups of reproductively isolated lineages? (2) Do macroevolutionary patterns support elevated rates of perfume evolution compared to non-signalling traits? (3) Is coexistence of close relatives within communities associated with the evolution of more complex or novel perfumes? And, (4) are patterns of perfume divergence among closest relatives consistent with signatures of rapid trait-divergence at speciation, evolutionary stasis and/or character displacement dynamics?

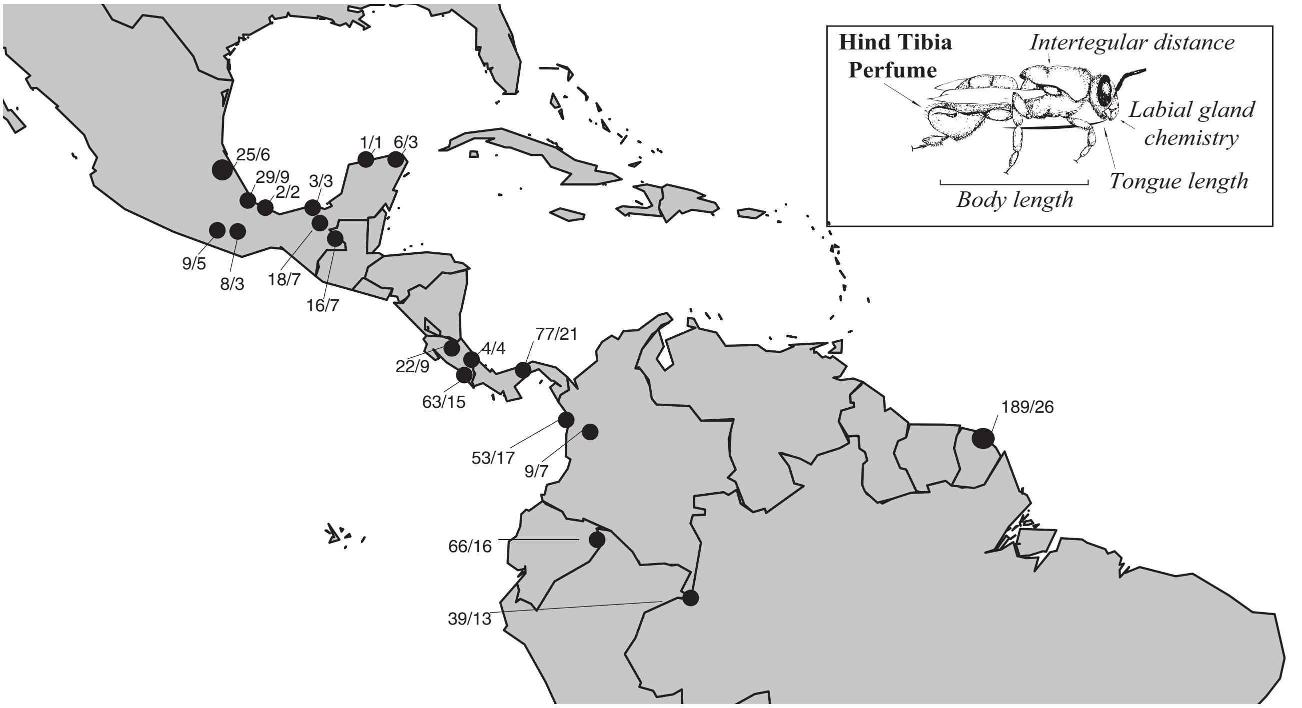

Figure 1 Sampling locations of bees used in this study, with the number of individuals/number of species reported for each site**.** Full specimen information, including the exact latitude and longitude of the collections, can be found in Appendix S1. Inset: Schematic of orchid bee illustrating the traits used in this study. A pouch in the enlarged hind tibia stores perfume blends (indicated in bold). Note the slit through which males deposit chemical fragrances. Other traits investigated in the study (indicated in italics) include the intertegular distance, the chemical compounds found in labial gland secretions, the length of the tongue and the length of the bee body.

MATERIAL AND METHODS

Chemical sampling

At study sites across the neotropics (Fig. 1), male *Euglossa* bees were lured with synthetic chemical baits and transported to the laboratory for chemical analysis. Perfume compounds were analysed using GC-MS from individual male bees following established protocols (Eltz *et al.* 1999; Ramírez *et al.* 2010), which we describe in the supplementary methods.

Male bees use cephalic labial gland secretions to facilitate exogenous volatile uptake and transfer to hind tibial pouches, and therefore hind leg extract contain both exogenous volatiles and endogenous secretions (i.e. lipid compounds). Thus, we analysed hind tibiae and labial gland extracts separately. Compounds present in both leg extracts and labial glands were not considered part of the perfume phenotype. This resulted in the creation of two datasets: (1) perfume signal compounds, which included compounds found in only the hind leg extracts but not in the labial gland extracts, and (2) the labial gland dataset, which included just those compounds found in the labial gland extracts.

Structure of variation: species-specificity of perfumes across geography

To assess whether orchid bee perfumes exhibit greater dissimilarity between species than within species, we analysed the perfume signals of species that were represented by over 10 replicate individuals in our dataset (34 species total, Appendix S1). Here, and in all downstream analyses, we quantified chemical distance using Bray–Curtis dissimilarity matrices based on the square root transformed standardised perfume signal profiles. We used the Bray–Curtis index due to its insensitivity to compounds that are jointly absent in sample pairs (i.e. pairwise dissimilarities are fixed). We tested for species-specificity by comparing the pairwise dissimilarities of conspecific and heterospecific individuals using a permutational multivariate analysis of variance analysis using distance matrices (ADONIS) and an analysis of similarity test (ANOSIM). For both the ADONIS and ANOSIM, we assessed significance via 1000 permutations using the R package 'vegan' (Oksanen *et al.* 2014). To test for a potential sampling effect, whereby variation was a function of the number of individuals sampled, we examined the relationship between intraspecific variation in perfume phenotype and the number of conspecific individuals sampled in our dataset using a linear regression implemented using the 'lm' function in the 'stats' package (Development Core Team 2015).

It is important to note that an overall pattern of perfume differentiation across species does not preclude the existence of substructure of perfume blends at lower levels (e.g. across populations of the same species). Thus, we investigated the relative contributions of (1) geographic distance and (2) phylogenetic distance between individual samples as predictors of perfume phenotype similarity using a multiple matrix regression (MMRR) (Wang 2013). We calculated the geographic distance between sampling locations of individual bees using the great circle distance (e.g. the distance as measured on the curved earth surface) between collection localities using the 'rdist.earth' function in the R package 'fields' (Nychka *et al.* 2014). For phylogenetic distance, and for all downstream phylogenetic analyses, we used a published, fossil time-calibrated distribution of molecular phylogenies of Euglossini estimated using BEAST (Ramírez, Eltz *et al.* 2011). We quantified the branch length between individual pairs using the 'distTips' function in the 'adephylo' package (Jombart *et al.* 2010) using 1000 time-calibrated trees randomly drawn from the post-burnin distribution. Using these 1000 trees, we calculated the mean pairwise phylogenetic distance of species, scoring individuals of the same species as having a phylogenetic distance of zero. Nine species were present in the dataset but were missing from the phylogeny, and were thus necessarily omitted from the MMRR analysis (and other downstream analyses necessitating a phylogenic hypothesis, Appendix S2). Chemical, geographic and phylogenetic distance matrixes were all mean centered and standardised prior to MMRR analysis. We assessed significance of our results via 1000 permutations of the regression using the MMRR function from Wang (2013).

Tempo and mode of perfume profile evolution

For phylogenetic comparative analyses, we created a species-level dataset by averaging relative peak areas for each compound across individuals of each species and then reducing the dimensionality using non-metric multidimensional scaling (nMDS) (see supplementary methods).

We visualised patterns of evolutionary divergence and phylogenetic structure using 1D phenograms, 2D phylomorphospace plots and 3D traitgrams (Revell 2012). For chemical traits, plots were made by projecting the trait values of extant species onto chemical trait space (represented by two-dimensional nMDS axes of variation with lines connecting species according to their phylogenetic relationships) using the phylomorphospace and 3D traitgram functions in the phytools package (Revell 2012). For morphological traits, plots were made by projecting the tips of the phylogeny onto single dimensional trait space using the traitgram function in phytools (Revell 2012).

We tested whether perfumes exhibited elevated rates of trait evolution compared to non-perfume traits using several comparative phylogenetic methods. First, we directly compared rates of evolution of chemical and morphological traits using a multidimensional approach to quantifying rates of evolution by Adams (2013). We utilised four-dimensional nMDS scores for chemical traits, and a three-dimensional scaled and centred matrix for morphological traits. Second, we investigated the divergence pattern of perfume phenotypes by calculating the morphological diversity index (MDI), which describes among-subclade vs. within-subclade trait variation (Harmon *et al.* 2003). A negative MDI statistic reflects a pattern where diversity is largely partitioned among subclades (closely related species have similar chemistry to one another). On the other hand, a positive MDI statistic reflects a pattern where chemical diversity is largely partitioned within subclades (closely related lineages differ considerably in their chemical phenotypes). We visualised these patterns using disparity through time (DTT) plots, which display the observed and simulated disparity as a function of evolutionary time. We calculated MDI and made DTT plots using Bray–Curtis dissimilarity of four-dimensional nMDS scores for chemical traits and Euclidean distance of morphological traits using 1000 replicate simulations per tree across each of the 1000 trees from the posterior distribution.

Third, we asked whether perfume signalling, labial gland chemistry, and morphological traits are consistent with either Brownian motion (BM), early burst (EB), or increasing rate (IR) models of multivariate trait evolution. Strong support for a BM model would accommodate a pattern where average trait disparity increases uniformly through time. Strong support for an EB model would accommodate higher rates of trait evolution occurring early after divergence among the major *Euglossa* lineages, consistent with a scenario where the rate of evolution decreases through time as niches become filled (frequently referred to as an 'adaptive radiation' scenario). And finally, strong support for an IR model would reflect a pattern where the rate of chemical evolution increases through time, consistent with a scenario where evolutionary rates accelerate with the number of *Euglossa* lineages filling niche space. All models of multivariate trait evolution were implemented in the 'MVmorph' package (Clavel *et al.* 2015), and compared using AICc scores.

Signatures of isolating mechanisms: chemical distance, phylogenetic distance and range overlap

To investigate community-wide patterns of perfume divergence, we used two approaches: (1) we tested whether, across the *Euglossa* phylogeny, perfume complexity and exclusiveness (defined below) are predicted by the number of sympatric congeners a species experiences in its range and (2) we compared perfume phenotypes among sister-species pairs to examine the relationship between time, chemical divergence and levels of sympatry following speciation.

Perfume complexity/exclusiveness and congeneric range overlap

We calculated geographic ranges for each species (see supplementary methods for details). Using these ranges, we estimated congener co-occurrence for each *Euglossa* species as the number of species in our dataset with which those species shares range overlap greater than zero. We quantified perfume complexity as the total number of compounds in a species' perfume, and perfume exclusiveness as the number of compounds in a species blend that are unshared with co-occurring *Euglossa* species. We tested for a relationship between the number of co-occurring *Euglossa* species and both perfume complexity and exclusiveness using phylogenetic generalised linear regressions implemented in the 'glm' function in the package 'caper' (Orme *et al.* 2013).

Sister-species correlations

We zoomed in on closely related sister-species pairs in our dataset to disentangle patterns of perfume evolution with respect to time since divergence and amount of range overlap experienced in recently diverged taxa. We selected 10 species pairs that were strongly supported as each other's closest relatives in the well-sampled phylogeny of Ramírez *et al.* (2011) (Appendix S2). We included only cases where closely related species pairs are unambiguously monophyletic in this well-sampled phylogenetic study, omitting any cases where taxonomically similar species remained unsampled, or where phylogenetic support for monophyly of the pair was not fully supported. However, it is important to note that one can never be completely certain that closely related species pairs are true sisters due to the possibility of intervening extinction (an assumption of all sister-pair studies). Using these pairs, we conducted three analyses: (1) To examine the geographic history of co-occurrence, we estimated the relationship between sister-pair age (node depth) and the amount of range overlap (percent of range overlapping) using linear regression (also known as age-range analysis). Range overlap was reciprocal log transformed to accommodate non-linearity in the relationship. (2) To investigate whether chemical divergence steadily accrues over time as species age, or whether species' chemistry diverges rapidly near the time of speciation events, we regressed species-pair age (node depth) against the evolutionary rate of chemical divergence (the net amount of chemical divergence experienced per unit time, calculated as pairwise chemical distance/mean node depth). Node depth was inversely transformed in this analysis to accommodate non-linearity of the relationship. (3) In order to test for patterns consistent with reinforcement/reproductive character displacement, we used *t*-tests to directly compare divergence in sympatric vs. allopatric sister species. To assess divergence, we used the residuals of this former analysis to ask whether, for their age, sympatric sister species had higher rates of profile divergence than allopatric sister species. To identify which aspects of perfume may be displaying the strongest displacement patterns, we followed this up by directly comparing the Bray–Curtis distances of sympatric and allopatric species pairs using whole perfume, minor perfume compounds only (compounds that represented < 10% of total blend), and major perfume compounds only (compounds that represented > 10% of total blend).

RESULTS

Compound characterisation

A total of 887 male orchid bees belonging to 65 species were included in this study (Appendix S1). The number of replicate individuals per species ranged from 1 to 47 bees/species (average: 13.44 ± 9.8). We found a total of 697 exogenous compounds in hind leg extracts (i.e. compounds extracted from the hind leg that are not found in the labial glands, Fig. S1) representing a large diversity of terpenoids and benzenoids including many oxygenated compounds such as alcohols, esters and epoxides, mostly with a molecular weight below 300 (Appendix S3). An additional 43 compounds were registered in labial gland extracts. These compounds were mostly long-chain unsaturated acetates, hydrocarbons, alcohols, acids and wax esters with molecular weights above 250.

Perfume complexity varied widely across the bees sampled. The per-bee number of exogenous compounds ranged from 1 to 34 compounds (average: 10.93 ± 5.49). The per-species diversity of exogenous compounds ranged from 3 in *Euglossa* *bidentata* to 102 in *E. intersecta* (average: 33.51 ± 20.81). Labial gland extracts displayed lower compound diversity: per-bee labial gland chemical diversity ranged from 1 to 13 compounds, but the mean was low (1.78 ± 1.07), and per-species labial gland compound diversity ranged from 1 to 14 compounds (average: 3.57 ± 2.48). *E. hansoni* had the highest number of labial gland extract compounds, whereas we detected only singular compounds in the labial gland extracts of nine species.

Structure of variation: species-specificity of perfumes across geography

Perfume phenotypes exhibited high levels of species-specificity. Bray–Curtis dissimilarity values were lower between conspecific individuals (mean = 0.52, SD = 0.27) than between individuals of different species (mean = 0.94, SD = 0.09). This difference was significant in both ADONIS ($R^2 = 0.62$, $P = 0.001$) and ANOSIM ($R = 0.87$, $P = 0.001$) permutation tests, reflecting significant and robust effects of species affiliation on fragrance similarity. However, although perfume phenotypes were significantly species-specific, the amount of intraspecific variation in perfume signals varied considerably across species (Fig. S2a). The variation in chemical phenotypes within species was not predicted by the number of individuals sampled for that species (i.e. was not due to a sampling effect) (linear regression, $F_{1,32} = 1.26$, $R^2 = 0.04$, $P = 0.26$, Fig. S2b). Multiple matrix regression, however, revealed that Bray–Curtis distance in scent profiles between individual orchid bees was a function of both geographic ($\beta_{geo} = 0.1$, $T_{geo} = 43.68$, $p_{geo} < 0.001$) and phylogenetic distance ($\beta_{phylo} = 0.33$, $T_{phylo} = 148.7$, $p_{phylo} < 0.001$) between individuals (overall model: $R^2 = 0.14$, $F = 12968.12$, $P < 0.001$) (Fig. S3). The relative contribution of phylogenetic distance to chemical differentiation was larger than that of geographic distance, suggesting that while both geography and species barriers contribute to perfume phenotypic differentiation, species identity and evolutionary history may play more prominent roles.

Tempo and mode of perfume profile evolution

Perfume traits exhibit an over twofold higher rate of evolution relative to both labial gland chemistry and morphological traits. Across the 1000 trees, perfume signalling had a mean rate of $\sigma^2 = 0.016 \pm 0.002$, whereas labial gland chemistry and morphology displayed much lower rates ($\sigma^2 = 0.003 \pm 0.0005$ and $\sigma^2 = 0.007 \pm 0.002$ respectively). The difference in the rate of evolution was illustrated when the traits were visualised using 'phylomorphospace' (Fig. S4). In labial gland chemistry and morphological traits, closely related species tended to cluster together in similar regions of multivariate space, with only infrequent occurrences of recently diverged lineages differing significantly. Conversely, in perfume signal profiles, expansion into distant regions of chemical phenotypic space frequently occurred between close relatives, resulting in a pattern of abrupt widening at the later end of the time axis. This pattern suggests that, in *Euglossa,* rapid chemical diversification may be common among close relatives for perfume phenotypes, but not in the labial gland chemistry or morphological phenotypes.

A disparity through time analysis revealed an exceptionally high level of disparity in perfume phenotypes through time relative to the other traits (Fig. 2, Table 1). Perfume disparity remained significantly higher than expected under simulated BM evolution throughout the evolutionary history of *Euglossa,* with a peak of high disparity occurring among the most recently diverged lineages. In contrast, labial gland chemical disparity was slightly higher than expected through time, but converged towards the Brownian expectation near the tips of the phylogeny. Morphological traits did not differ from the Brownian expectation. These results were congruent with the obtained MDI values, in which perfume MDI was 1.5 higher than that obtained for labial gland chemistry, and morphology traits displayed a non-significant MDI value (Table 1).

Multivariate model fitting analyses strongly favoured a model where rates of perfume evolution increased throughout the evolutionary history of *Euglossa* (Table 2). However, this pattern was not observed in labial gland chemistry or morphology, which both displayed a better fit to a single-rate BM models of evolution, whereby the rate of chemical evolution was constant over time (Table 2).

Signatures of isolating mechanisms: chemical distance, phylogenetic distance and range overlap

Range overlap ranged from 0 (no overlap) to 0.96 (almost complete overlap) across pairs of *Euglossa* species, with a mean overlap of 0.36. Across species, both the complexity (Fig. 3a) and the exclusiveness of a species perfume (Fig. 3b) were positively correlated with the number of congeners a species overlapped with in its range. This was the case in phylogenetic generalised regressions using the Maximum Clade Credibility (MCC) tree (complexity: $R^2 = 0.19$, $P < 0.001$, exclusiveness: $R^2 = 0.16$, $P = 0.002$), and across the distribution of trees (complexity: $R^2 = 0.19 \pm 0.03$, $P = 0.002 \pm 0.006$, exclusiveness: $R^2 = 0.16 \pm 0.03$, $P = 0.004 \pm 0.007$). Because the number of overlapping congeners was positively correlated with a species' range size ($R^2 = 0.44$, $P < 0.001$), we conducted a follow-up analysis using stepwise AICc model comparison to ask whether range size *per se* was a better predictor of perfume traits relative to models that include congener overlap. We implemented model comparison using the 'dredge' function in the package 'MuMIn' (Bartoń 2015) to compare models with range overlap, range size and interaction term predictor variables. In all cases, range size on its own was a poorer predictor of perfume complexity and exclusiveness compared to models that incorporated congener overlap, suggesting that these results were not an artefact of range size *per se*.

Age-range correlations using closely related species pairs revealed a significant positive relationship between the age of species pairs (node depth) and their percent range overlap ($R^2 = 0.26$, $P = 0.05$, Fig. 4a), where the probability of range overlap increased non-linearly with time. Chemical distance between sister-species pairs, however, was neither predicted by the age of the sister species (regression of chemical divergence and node depth: $R^2 = 0.05$, $P = 0.56$) nor by the amount of contemporaneous range overlap (regression of chemical divergence and percent range overlap $R^2 < 0.001$, $P = 0.99$),

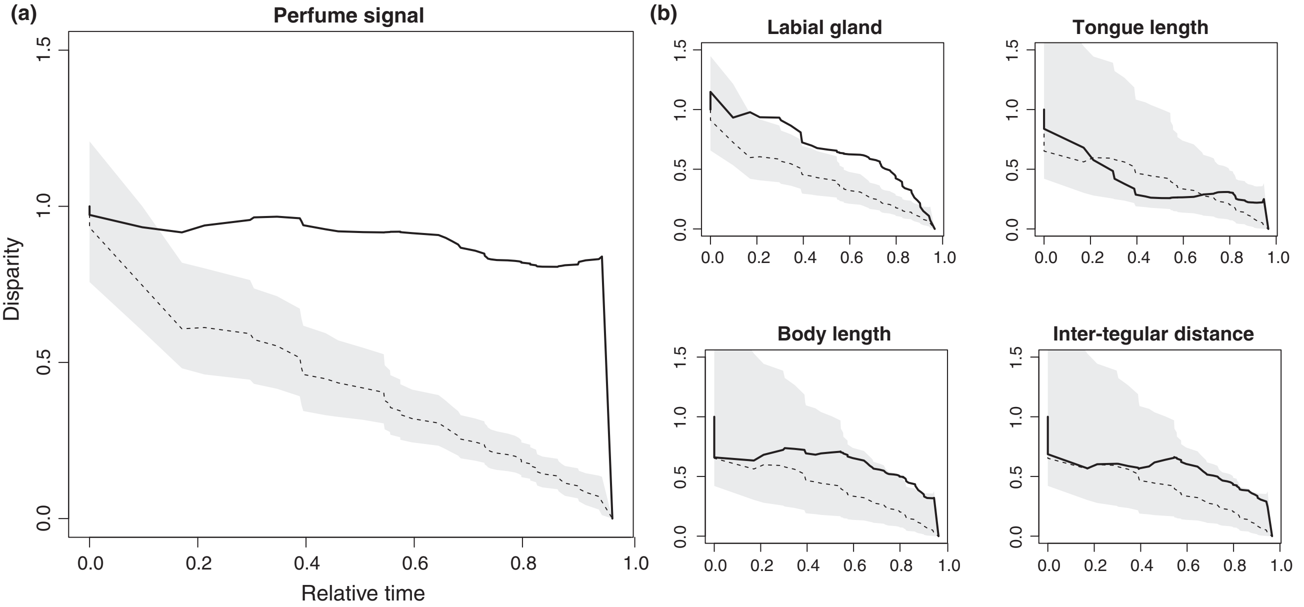

Figure 2 Disparity through time plots for (a) perfume and (b) labial gland and morphological traits. Observed disparity is indicated by a solid black line, and with the mean (dashed black line) and 95% confidence intervals (grey shading) from 1000 simulations per tree over the distribution of 1000 trees.

Table 1 Morphological disparity index (MDI) statistics for perfume and non-perfume traits in *Euglossa*

	MDI	P_{sim}	Interpretation
Perfume signal	**0.445 ± 0.026**	***P* = 0.034 ± 0.006**	**Disparity significantly higher than BM**
Labial gland chemistry	*0.255 ± 0.029*	*P = 0.058 ± 0.019*	*Disparity marginally higher than BM*
Tongue length	−0.002 ± 0.026	*P* = 0.292 ± 0.067	Not distinct from BM expectation
IT distance	0.13 ± 0.028	*P* = 0.146 ± 0.051	Not distinct from BM expectation
Body length	0.193 ± 0.029	*P* = 0.102 ± 0.045	Not distinct from BM expectation

Mean ± standard deviation of the MDI and *P*-values derived from 1000 simulations per tree over 1000 trees. Consistently significant MDIs below the 0.05 level are in bold, and marginally significant MDIs are italicised.

Table 2 Comparisons of multivariate models of evolution for perfume chemistry and non-perfume traits in *Euglossa*

	Perfume signal	Labial gland chemistry	Morphology
Brownian motion			
AICc	−79.865 ± 11.169	**−412.928 ± 13.556**	**−234.992 ± 9.618**
Delta	12.882 ± 9.679	**0.067 ± 0.558**	**0.185 ± 1.201**
AICc Weight	0.155 ± 0.262	**0.732 ± 0.107**	**0.644 ± 0.114**
Early burst			
AICc	−75.978 ± 11.155	−409.807 ± 14.424	−232.102 ± 9.682
Delta	16.77 ± 9.679	3.188 ± 1.27	3.075 ± 1.228
AICc Weight	0.022 ± 0.038	0.175 ± 0.117	0.153 ± 0.033
Increasing rates			
AICc	**−92.345 ± 13.045**	−408.59 ± 13.245	−232.503 ± 8.386
Delta	**0.403 ± 0.975**	4.406 ± 1.384	2.673 ± 1.132
AICc Weight	**0.823 ± 0.299**	0.093 ± 0.045	0.203 ± 0.142

Mean ± standard deviation of AICc scores (and weights) across 1000 phylogenetic trees. Three models were compared: a Brownian motion model with a single rate of chemical evolution through time; an early burst model with a rate of chemical evolution decreasing through time; and an increasing rates model in which the rate of chemical evolution increases through time. The best model for each set of traits is in bold.

suggesting that species pairs do not steadily accrue perfume divergence at a constant rate after speciation nor as a function of the amount of overlap they experience. This resulted in a pattern where the rate of chemical evolution (chemical divergence/node depth) was inversely proportional to the age of the species pair ($R^2 = 0.88$, $P = 0.0002$, Fig. 4b), whereby most of the divergence accrued early on in the history of a species pair with little additional divergence with added time.

We used the residuals of this linearised relationship to investigate whether, as predicted from theory on character displacement, sympatric sisters displayed a higher rate of profile evolution considering their age since divergence relative to allopatric sisters. We found that perfume evolution tended to be higher for sympatric species pairs as compared with allopatric pairs (marginally significant, $t(5.9) = 1.69$, $P = 0.07$, Fig. 4b inset). Finally, directly comparing Bray–Curtis distances of sympatric and allopatric species pairs revealed that sympatric species were more distinct than allopatric species in their low-abundance perfume compounds (compounds that made up < 10% of total blend, $t(7.89) = 2.32$, $P = 0.02$, Fig. 4c), but not in their whole perfume ($t(7.94) = 0.71$, $P = 0.25$, Fig. 4c) nor in their high-abundance compounds (> 10% of total blend $t(2.41) = 0.40$, $P = 0.36$, Fig. 4c).

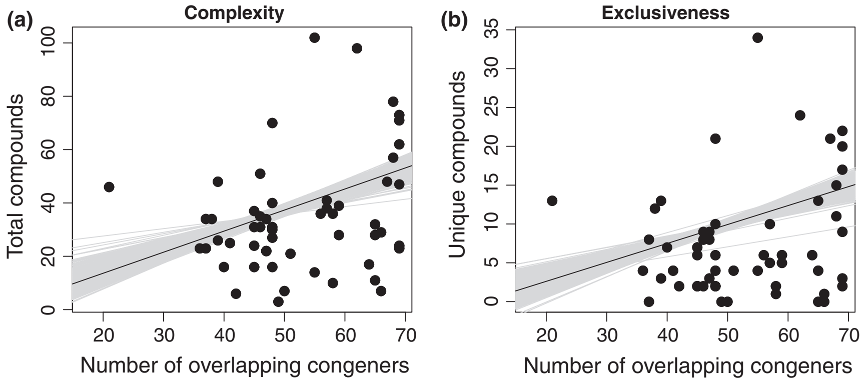

Figure 3 The relationship between range overlap with congeners and perfume complexity and exclusiveness. (a) Perfume complexity (the total number of compounds in a perfume) and (b) perfume exclusiveness (the number of compounds not shared with overlapping congeners) both increase with the number of congeners a species overlaps with in its. Each dot represents a species, solid black lines represent phylogenetic regression using the maximum clade credibility tree and grey lines represent the phylogenetic regressions using 1000 trees from posterior distribution.

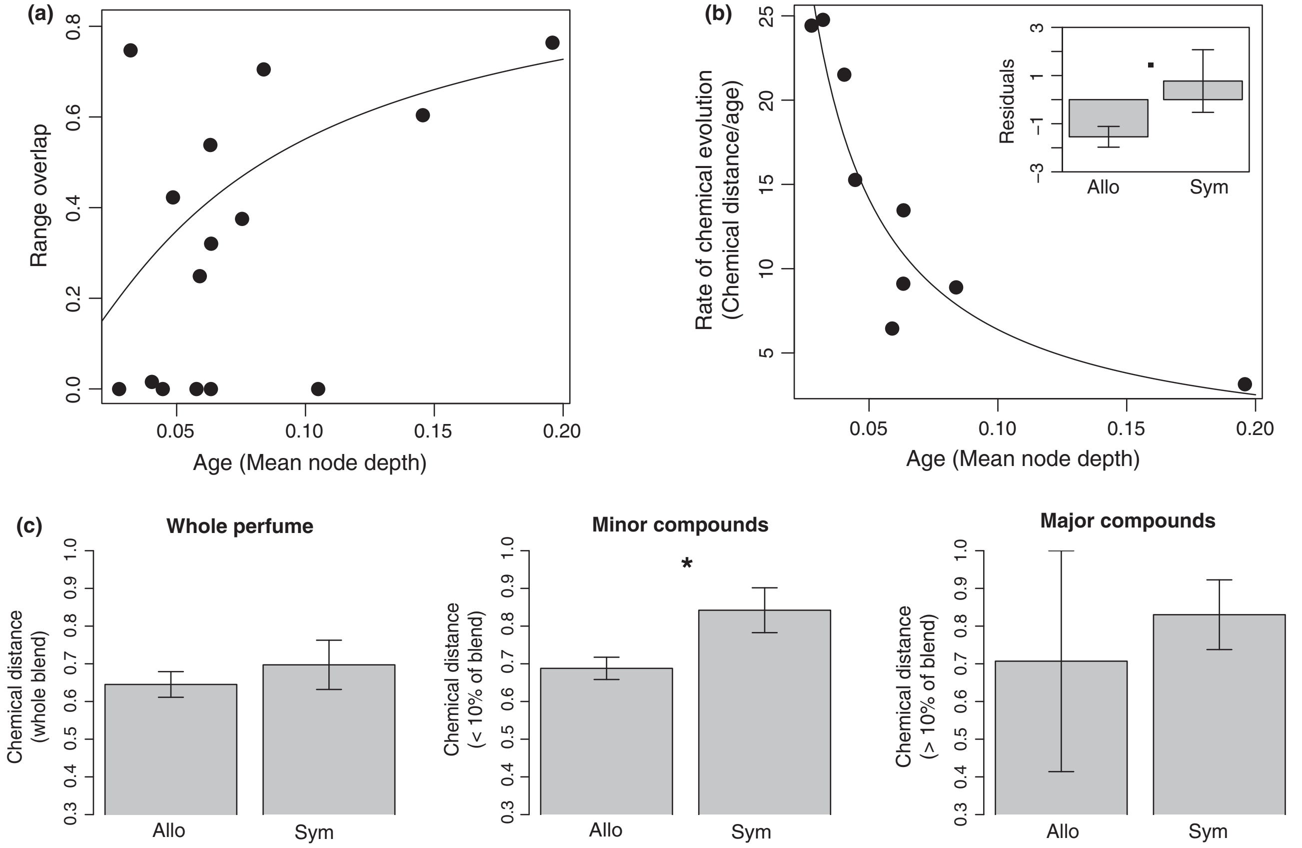

Figure 4 For closely related species pairs, the relationships between species age, chemical disparity and range overlap. (a) Age-range correlation (ARC) for *Euglossa* sister pairs, displayed as mean percent range overlap as a function of node depth (in percent of total tree length) across pairs of sister species. (b) The net rate of chemical evolution over time (note that mean node depth is both the predictor variable and the denominator of the response variable). Inset = Barplot of residuals from linearised regression for species pairs experiencing range overlap (Sym = sympatric) and those with no range overlap (Allo = allopatric). (c) Bray–Curtis distance of sympatric and allopatric species pairs for whole perfume blends, only low-abundance (minor) compounds and only high-abundance (major) compounds. All barplots represent means with standard errors. Dot reflects marginal significance $P < 0.1$, asterisk reflects $P < 0.05$.

DISCUSSION

Orchid bee perfumes are a fascinating example of chemical traits that mediate sexual communication among closely related species. Here, we examined phylogenetic patterns of perfume signal macroevolution across the genus *Euglossa*, testing whether perfume compounds evolve as predicted from theory on sexual signalling and communication. Our results revealed three strong evolutionary patterns. First, we found high species-specificity in perfume traits across *Euglossa*, despite large geographic distances between individuals sampled. Second, perfume signals showed elevated rates of evolution compared to other chemical and morphological traits across the *Euglossa* phylogeny, and phylogenetic models suggest that the rate of perfume evolution increased with orchid bee species diversity over time. And third, the relationship between species relatedness, range overlap and perfume divergence among closest relatives suggest that the majority of perfume evolution occurred early in the divergence of species pairs, with a slight increase in the rate of evolution upon secondary sympatry but otherwise small amounts of perfume divergence accumulating over time. Together, these results illustrate that the remarkable diversity of contemporary perfume signals in *Euglossa* has been shaped by a complex pattern of perfume macroevolution, with potential roles for rapid divergence at speciation, evolutionary stasis and reproductive character displacement among coexisting close relatives. Below, we elaborate on each of these findings in the light of major concepts in the fields of signal evolution and species coexistence.

Species-specificity of diverse chemical blends

Sexually reproducing organisms depend on their ability to reliably locate and recognise conspecific mates (Coyne & Orr 2004). In systems where closely related species interact, sexual signals are hypothesised to be under strong selection for species-specificity (Smadja & Butlin 2009). We sampled multiple, geographically distant populations of the same species to estimate the amount of intraspecific phenotypic variation present in perfume traits, testing whether species-specificity or geographic distance were predictive of perfume divergence. The resulting diverse dataset of over 700 compounds revealed high levels of species-specificity across taxa, with geography being an important factor in explaining some of the residual within-species variability. This is consistent with the hypothesis that species identity is a stronger driver of variation in perfume composition across individual orchid bees than local availability of perfume sources (e.g. fungi, orchids, decaying vegetation). It also strengthens previous studies suggesting that orchid bees employ experience-dependent choices to adjust their species-specific perfume blends (Eltz *et al.* 2005a,b; Pokorny *et al.* 2013).

While smaller than interspecific variation, the degree of intraspecific variability we observed was substantial and variable across species. These differences could be due to population differentiation due to limited gene flow, cryptic diversity across taxa, insipient speciation or variability in the heterogeneity of perfume sources across geography. Due to the cumulative nature of male perfumes, within-species variability could also depend on variability in the age structure of sampled populations, since perfumes become more complex as individuals age (Eltz *et al.* 2015) and similarity to the species centroid increases with the number of compounds included (Pokorny *et al.* 2013). Additionally, because within-species variability is required for sexual selection to act on male traits (e.g. through choosy females favouring mates with certain perfume traits such as complexity or presence of rare compounds), variation in the strength of sexual selection among populations could contribute to the range of intraspecific variation observed across species. Future work aimed at disentangling the drivers of within-species variation in perfume signalling will be crucial for understanding how macroevolutionary patterns are linked to microevolutionary mechanisms of divergence.

Tempo and mode of perfume profile evolution

Our analyses revealed that perfume signals exhibit elevated rates of evolution compared to traits not implicated in sexual communication, and that these elevated rates may increase through time. These phylogenetic results are consistent with a scenario of interspecific interactions shaping patterns of signalling divergence, for example, through interference of perfume signals among closely related species. This agrees with findings obtained for single locality communities of orchid bees (Zimmermann *et al.* 2009). Further, to the best of our knowledge, these results represent the first macroevolutionary evidence for increasing rates of evolution of chemical sexual signals through time (though other studies have found chemical signal divergence correlated with phylogenetic distance, e.g. Symonds & Elgar 2004; Symonds & Wertheim 2005). Rapid differentiation of sexual signalling traits among taxa could be due to sexual selection, as it is possible that perfume compounds function as indicators of male genetic quality. Alternatively, sexual signalling traits may diverge after allopatric populations reach secondary contact, and thus natural selection operating against hybrid mating could drive signal divergence. Distinguishing between these scenarios is difficult, but future work aimed at studying recently diverged specific species pairs in sympatry and allopatry may prove insightful.

Community interactions likely play a role in shaping sexual signal evolution, and conversely, evolutionary processes may shape patterns of coexistence of closely related species in communities. However, phylogenetic studies of signalling phenotypes rarely incorporate information on range overlap (but see Symonds & Elgar 2004; Tobias *et al.* 2014; Edwards *et al.* 2015). By analysing the range overlap among congeners, we found that both perfume complexity (the number of distinct compounds in a perfume) and exclusiveness (the number of compounds not shared with community members) of a species' perfume were positively evolutionarily correlated with the number of sympatric congeners a species experiences within its range. These results are consistent with research conducted in flower signalling, which support species diversity as a major driver of the evolution of floral signal specialisation (Armbruster & Muchhala 2009). Our approach cannot distinguish whether this pattern is due to a filtering process following divergence or *in situ* evolution of perfume phenotypes (e.g. character displacement), and future work focusing explicitly on community assembly and perfume divergence through time is needed. Regardless, this pattern suggests that, in *Euglossa,* chemical divergence may be linked to the degree of coexistence of congeners in the same community.

Signatures of isolating mechanisms: chemical distance, phylogenetic distance and range overlap

Zooming in on recently diverged species pairs, the relationship between range overlap, perfume divergence and time since divergence revealed intriguing patterns of evolution. The rate of perfume evolution was highest in young species pairs consistent with a scenario of rapid divergence near speciation with a dwindling rate of divergence through time thereafter. Additionally, there was a trend towards increased rates of perfume evolution in sympatric lineages, as predicted from character displacement or reinforcement scenarios (Brown & Wilson 1956). We also found a pattern of increased range overlap with increasing age of sister pairs, consistent with a model of primarily allopatric speciation followed by secondary sympatry. While this pattern is consistent with an allopatric speciation process, other mechanisms could also lead to this result, and work aimed at evaluating speciation in a microevolutionary framework is needed to confirm this interpretation (Warren *et al.* 2014). Finally, we found that sympatric species pairs of *Euglossa* displayed higher divergence in the minor compounds of their perfume compared to allopatric species pairs, suggesting that low-abundance compounds may play a disproportionately large role in driving speciation. While care should be taken in using pattern-based results as tests for specific processes, taken together, our sister species analyses strengthen the hypothesis that one function of male perfumes is recognition, and that other functions (e.g. perfumes conferring information on male attractiveness via sexual selection, Schemske & Lande 1984; Eltz *et al.* 1999), may be constrained by selection favouring species-specificity.

Overall, our results are consistent with multiple evolutionary forces – acting simultaneously and at different temporal scales – shaping the diversification of perfume signals across *Euglossa* orchid bees. Our data support the hypothesis that stabilising selection maintains signal integrity across populations within species, that perfume traits evolved rapidly at speciation, and that interactions with close relatives may contribute to diversification dynamics. Together, our study paints a picture of perfume phenotype playing a critical role in the early stages of speciation, and motivates future work evaluating the role of perfume in initiating, strengthening or maintaining reproductive isolation among populations.

CONCLUSIONS

Our study reveals phylogenetic patterns in orchid bee perfumes that are consistent with predictions from several long-standing theories on the evolution of signalling traits that mediate species recognition. Together, our results suggest that the evolution of species-specific perfume phenotypes in *Euglossa* is rapid, is impacted by co-occurring *Euglossa* species within communities and may be a function of both bursts of perfume divergence early in speciation and subsequent reproductive character displacement. Our study highlights the relative importance of pre-zygotic mechanisms in mediating reproductive isolation, and suggests that both stabilising selection and diversifying selection may promote signal integrity and rapid signal evolution, respectively, at different points in species divergence. It also reinforces the existing literature on the prominent role that sexual interactions can have in shaping the remarkable diversity of signalling traits across the animal phylogeny.

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AUTHORSHIP

SR and TE planned the research, LM, TE and SR collected samples and analysed output data. MGW designed and performed all statistical analyses and wrote the first draft of the paper, and SR and TE contributed to revisions.

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