An olfactory receptor gene underlies reproductive isolation in perfume-collecting orchid bees

P. Brand[1], I. A. Hinojosa-Díaz[2], R. Ayala[3], M. Daigle[1], C. L. Yurrita Obiols[4], T. Eltz[5], S. R. Ramírez[1]

[1]Department for Evolution and Ecology, Center for Population Biology, University of California, Davis, CA, USA.

[2]Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad de México, Mexico.

[3]Estación de Biología Chamela, Instituto de Biología, Universidad Nacional Autónoma de México, Jalisco, Mexico.

[4]Centro de Estudios Conservacionistas, Universidad de San Carlos de Guatemala

[5]Department of Animal Ecology, Evolution and Biodiversity, Ruhr University Bochum, Bochum, Germany.

Correspondence to: pbrand@ucdavis.edu and sanram@ucdavis.edu

Speciation is facilitated by the evolution of reproductive barriers that prevent or reduce hybridization among diverging lineages. However, the genetic mechanisms that control the evolution of reproductive barriers remain elusive, particularly in natural populations. We identify a gene associated with divergence in chemical courtship signaling in a pair of nascent orchid bee lineages. Male orchid bees collect perfume compounds from flowers and other sources to subsequently expose during courtship display, thereby conveying information on species identity. We show that these two lineages exhibit differentiated perfume blends and that this change is associated with the rapid evolution of a single odorant receptor gene. Our study suggests that reproductive isolation evolved through divergence of a major barrier gene involved in chemically mediated pre-mating isolation via genetic coupling.

Speciation, the formation of new species from a single ancestral species, is facilitated by the emergence of reproductive barriers between lineages and is considered the most fundamental process in the generation of biological diversity (*1*, *2*). While a growing number of studies have revealed that recently formed species often exhibit marked divergence across multiple genomic regions (*3*), the role of these genomic 'islands of divergence' in reproductive isolation remains controversial (*4*-*7*). Even when specific genomic regions can be associated with reproductive isolation, they usually encompass hundreds of genes of unknown function. As a result, few studies have successfully linked specific genetic loci to reproductive barrier traits or determined how they contribute to the speciation process (*7*-*9*). Here we combine a large-scale population level approach with high-resolution genome-wide diversification analyses to identify the genetic basis of a phenotypic trait that likely controls reproductive isolation in a pair of orchid bee species.

Male orchid bees actively collect volatile chemical substances from floral and non-floral sources to concoct highly species-specific perfume blends (*10*-*12*), which they subsequently expose during ritualized courtship displays (Fig. 1) (*13*, *14*). The exact type of information conveyed remains unknown, but perfumes are clearly involved in mating behavior and species recognition (*15*). Because orchid bees acquire volatile chemicals directly from the environment, their olfactory system is critical for both perfume concoction by males and perfume detection by females, which effectively creates a strong linkage between male trait and female preference. Thus, changes in genes underlying olfactory perception can simultaneously alter the male perfume signal and the female perfume preference through pleiotropic effects (*16*), a scenario that could lead to the rapid evolution of assortative mating (*17*, *18*). We hypothesize that the differentiation of chemosensory genes drives rapid shifts in chemical perfume composition during the speciation process, facilitated by genetic coupling of male trait and the associated female preference in orchid bees.

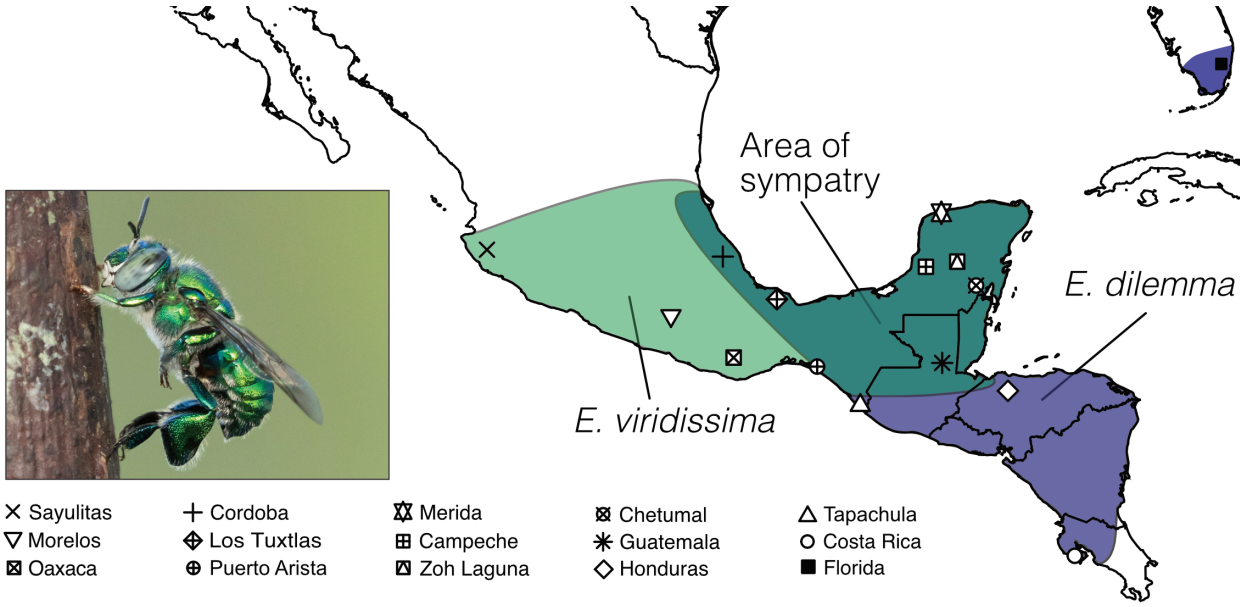


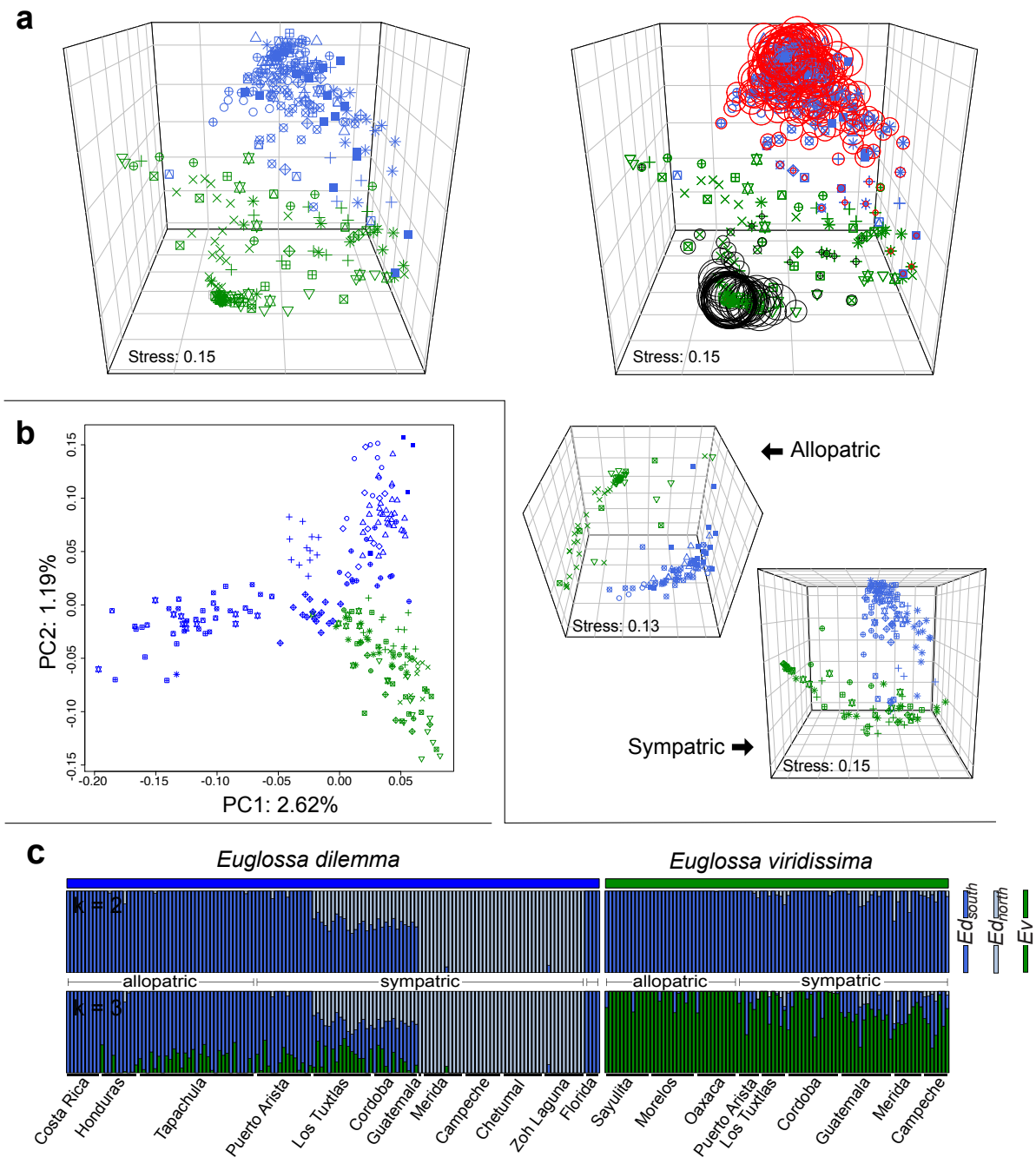
Fig. 1 Distribution range of *Euglossa dilemma* and *E. viridissima*. Bees were collected in 15 sampling sites (Table S1) throughout the distribution ranges of each species including both allopatric (blue: *E. dilemma*, light green: *E. viridissima*) and sympatric populations (dark green). Photograph shows *E. dilemma* male perching during perfume display.

To test whether perfume composition evolves rapidly during species formation, we conducted a population-level analysis of perfume chemistry in a pair of orchid bee lineages (*Euglossa dilemma* and *E. viridissima*) that diverged ~150,000 years ago (*19*). We collected male bees across the entire geographical range of each lineage throughout Central America (Fig. 1, Table S1-S2) and analyzed the perfume chemistry of 384 individuals via gas chromatography-mass spectrometry (Table S2). A non-metric multidimensional scaling analysis revealed strong differentiation of perfumes into two distinct lineage-specific chemical phenotypes independent of geography (Fig. 2a, ANOSIM R=0.8, p=0.001). This pattern was driven by both quantitative and qualitative differences of perfume chemistry and held true when using either the entire set of compounds or the 40 most prevalent compounds (Fig. S1, Supplementary Text). This observation supports the hypothesis that those compounds collected by a high number of individuals play a critical role in perfume specificity and private signaling in orchid bees.

We found that the most striking difference in perfume chemistry between *E. dilemma* and *E. viridissima* was the presence of two lineage-specific compounds that are highly prevalent and present in high relative abundance. The compound HNDB (2-hydroxy-6-nona-1,3-dienyl-benzaldehyde, (*16*)) was only present in perfume blends of *E. dilemma*, and the compound L97 (lactone-derivative of linoleic acid, (*20*)) was only present in perfume blends of *E. viridissima* (Fig. S2-S3, Table S3). These two molecules accounted for the highest average proportion of overall perfume content per species (relative abundance HNDB: 55%, L97: 37%, Table S4) and together contributed to 46.3% of the chemical differentiation between *E. dilemma* and *E. viridissima* (SIMPER analysis, Table S5).

Remarkably, perfume chemistry is the only trait that allows reliable identification and separation of these lineages. Close examination of morphological traits revealed that the number of teeth on the male mandible differs between the two lineages, with most but not all individuals segregating into two groups (*19*). Males of *E. dilemma* always have three mandibular teeth, whereas males of *E. viridissima* are polymorphic for the number of teeth with 89.4% of males exhibiting two teeth and the remaining fraction (10.6%) exhibiting three teeth that are hardly distinguishable from *E. dilemma* (Fig. S4, Supplementary Text). Together, these results demonstrate that species-specificity in perfume chemistry evolved rapidly through changes of few major compounds. These observations are consistent with the hypothesis that perfume chemistry is a mating recognition signal that functions as a pre-mating reproductive barrier among orchid bee lineages.

104



105

106 **Fig. 2 Phenotypic and genetic differentiation of *E. dilemma* and *E. viridissima*.** (**a**) Perfume phenotypes were species-specific
107 (upper left) independent of geography or co-occurrence (below) mainly due to the relative quantity of the major components
108 HNDB (red circles), and L97 (black circles) as indicated by circle size (upper right). (**b**) *E. dilemma* (blue) and *E. viridissima*
109 (green) are genetically differentiated over two PC axes explaining less than 4% of the genetic variation indicating that genetic
110 differentiation is low. (**c**) Populations within *E. dilemma* (k=2) were separated before species (k=3) in a genetic clustering
111 analysis, supporting population structure within *E. dilemma* (Fig. S6) and low interspecific genetic differentiation. Several
112 individuals drew ancestry from multiple genetic lineages suggesting admixture.

113

To contrast the divergence we observed in perfume chemistry with genetic differentiation between *E. dilemma* and *E. viridissima*, we genotyped 232 males sampled from across their geographic ranges (Fig. 1, Table S2). A principal components analysis of genetic variance (PCA) based on 16,369 single nucleotide polymorphisms (SNPs) revealed that these lineages are genetically distinct in both allopatric and sympatric populations (Fig. 2b, Fig. S5). *E. dilemma* and *E. viridissima* were not separated over a single PC axis (Fig. 2b, Fig. S6, Supplementary Text), which is consistent with a scenario of incomplete genome-wide separation between species. This observation was further supported by a genetic clustering analysis that first separated geographically distinct populations within *E. dilemma* before it separated species (ADMIXTURE, Fig. 2d, Fig. S7, Supplementary Text). In fact, this analysis revealed the existence of three main genetic lineages including 1) the entire *E. viridissima* population (*Ev*), 2) a southern *E. dilemma* population (Ed_{south}), and 3) a northern *E. dilemma* population (Ed_{north}). We also identified admixture between *E. dilemma* and *E. viridissima* (f_4-test: 0.001, z: 2.7, $p < 0.007$, Table S6, Supplemental Text), as suggested by individuals that draw ancestry from multiple genetic lineages in the clustering analysis (Fig. 2d). These results show that while perfume chemistry evolved into two discrete chemical phenotypes, genetic differentiation is incomplete and low between *E. dilemma* and *E. viridissima* (pairwise F_{ST}: 0.04 to 0.1, Table S7). These results support the hypothesis of ongoing gene flow during the early stages of speciation.

Next, to identify the genetic basis of perfume differentiation and reproductive isolation, we performed a genome-wide scan of divergence between *E. dilemma* and *E. viridissima* on the basis of 30 genomes from the three genetic lineages (N=10 for Ed_{north}, Ed_{south}, *Ev*, each, Fig. S8, Table S2, Supplemental Text). Genomic regions that contribute to a species-specific reproductive barrier trait should show high levels of differentiation due to diversifying selection between lineages but not within lineages. We capitalized on the fact that *E. dilemma* exhibits population structure to distinguish and identify regions of high differentiation between *E. dilemma* and *E. viridissima* but not within *E. dilemma*. Therefore, we estimated the net interspecific differentiation, which is calculated by subtracting the intraspecific F_{ST} from the interspecific F_{ST} (ΔF_{ST}), for non-overlapping 50 kilobase (kb) windows across the genome (*21*). The resulting windows of elevated ΔF_{ST} (>99th percentile) were clustered into eight distinct outlier peaks of varying size (0.05 to 1.7 Mb, Fig. 3b, Fig. S9, Table S8) that revealed elevated levels of genetic linkage compared to non-outlier windows (Mann-Whitney U Test, $p<0.0001$).

While these genomic regions are likely to have evolved under linked selection, we found that absolute sequence divergence (D_{xy}) was significantly reduced in ΔF_{ST} outlier windows (Mann-Whitney U Test, $p<0.0001$, Fig. 3c), suggesting that genetic differentiation in most outlier regions was not driven by diversifying selection and thus most likely unrelated to selection for species differences (*4*, *5*, *22*). Notwithstanding this general trend, we identified three outlier windows with elevated values of both ΔF_{ST} and D_{xy} (Fig. 3c), two of which exhibited a highly skewed differential in nucleotide diversity towards *E. dilemma* ($\Delta\pi$, Fig. 3c), suggesting strong unilateral positive selection in this lineage. We identified signatures of an *E. dilemma*-specific selective sweep in one of these windows based on allele frequency spectra and haplotypes of the three distinct genetic lineages (Fig. 4a). We did not identify any additional species-specific sweeps (Fig. S10), highlighting that this 50 kb window contains a unique, locally restricted signature of positive selection in *E. dilemma*. This observation is congruent with strong and

recent selective forces driving the divergence between *E. dilemma* and *E. viridissima* and suggests that the identified genomic region harbors loci mediating reproductive isolation between these nascent bee lineages.

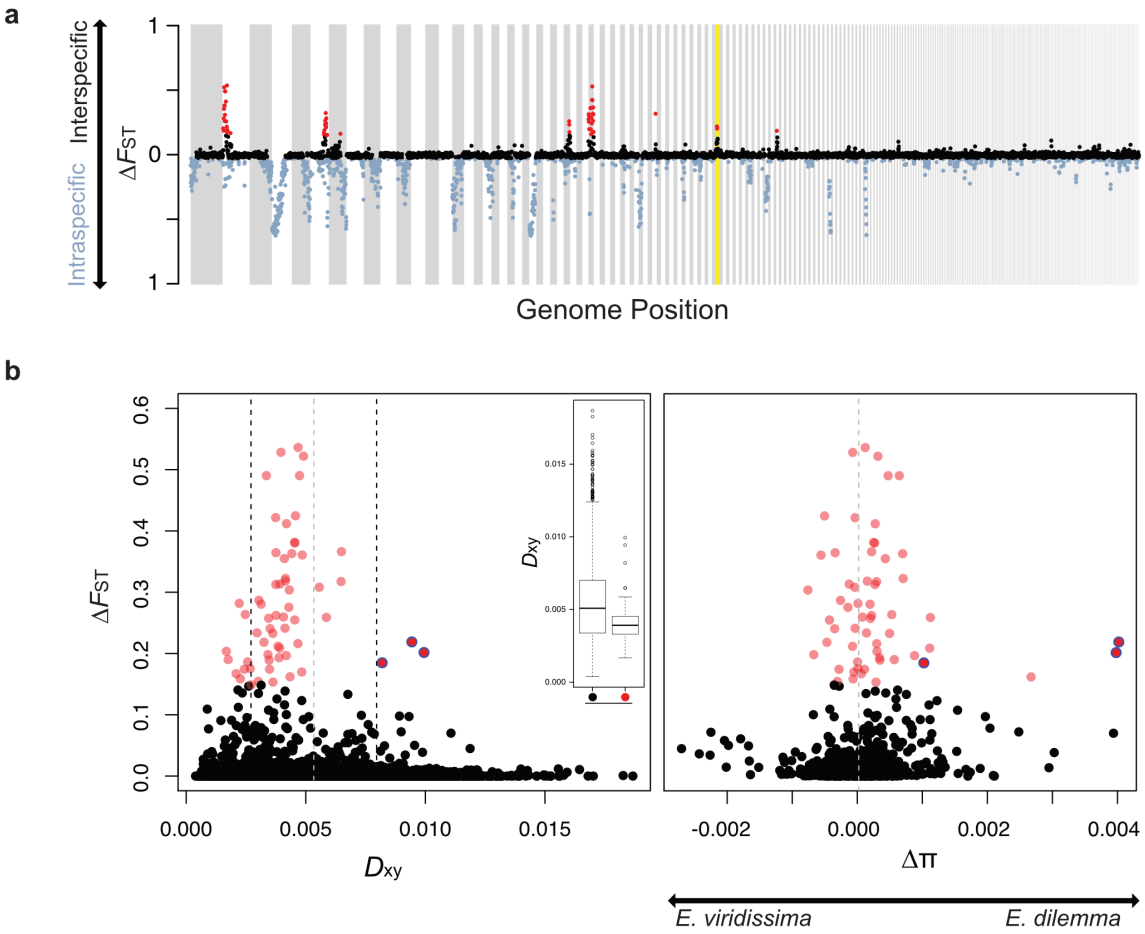


Fig. 3 Whole-genome differentiation. (**a**) Eight regions of the genome revealed higher interspecific (black) than intraspecific (blue) differentiation (ΔF_{ST} >99th percentile red). (**b**) Interspecific divergence (D_{xy}) was negatively correlated with ΔF_{ST} (left panel, r=-0.06, p=0) and significantly reduced in outlier windows (red) in comparison to non-outliers (black, inlet, Mann-Whitney U test, p<0.0001). Only two of three ΔF_{ST} outlier windows (circled blue) that revealed increased D_{xy} also had and a net differential of intraspecific nucleotide diversity ($\Delta\pi$) skewed towards *E. dilemma* (right panel), a pattern expected in genomic regions evolving under positive selection. Both correspond to the same outlier peak (yellow background in (**a**)). Grey dashed lines: mean D_{xy} and $\Delta\pi$. Black dashed lines: one standard deviation of mean D_{xy}.

Close inspection of the selective sweep region revealed the presence of 14 genes (Fig. 4a), all of which belong to the odorant receptor (OR) gene family and are located within a large ~170 kb tandem array of 39 ORs (*23*). The OR gene family is the largest chemosensory gene family in insects and is integral to the sensory detection of odorant compounds including pheromones (*24*, *25*). Olfactory tuning is controlled by the OR protein sequence and therefore amino acid substitutions can shift odorant binding properties and sensory perception (*26*, *27*). To identify the specific genetic targets of divergent selection, we mapped loci within the tandem array. We found that the region containing the selective sweep overlapped with both elevated interspecific

F_{ST} values and reduced nucleotide diversity (π) in *E. dilemma* centered around a single OR gene, *OR41*, that we previously identified as divergent between Ed_{north} and *Ev* (*28*) (Fig. 4a). This suggests that *OR41* evolved under strong positive selection in the common ancestor of *E. dilemma* after or during the split between *E. dilemma* and *E. viridissima*. Re-sequencing of *OR41* confirmed these results (N=47, Fig. 4b, Table S9-S10, Supplementary Text), and revealed that the protein coding sequences were fixed for 19 substitutions between species, 17 of which were non-synonymous leading to changes in the amino acid sequence of the resulting protein (Fig. 4c). A comparison with distantly related *Euglossa* species demonstrated that all fixed substitutions were derived (Fig. S11) and evolved under strong positive selection in *E. dilemma* ($d_N/d_S = 3.6$, $\chi^2 = 16.1$, $p < 0.0001$, Table S11, Supplemental Text) but not *E. viridissima* ($d_N/d_S = 0.3$), suggesting that strong selective forces fixed amino acid substitutions and possibly drove odorant perception changes in *E. dilemma*. Future studies should focus on elucidating the binding properties of this receptor and each allelic variant.

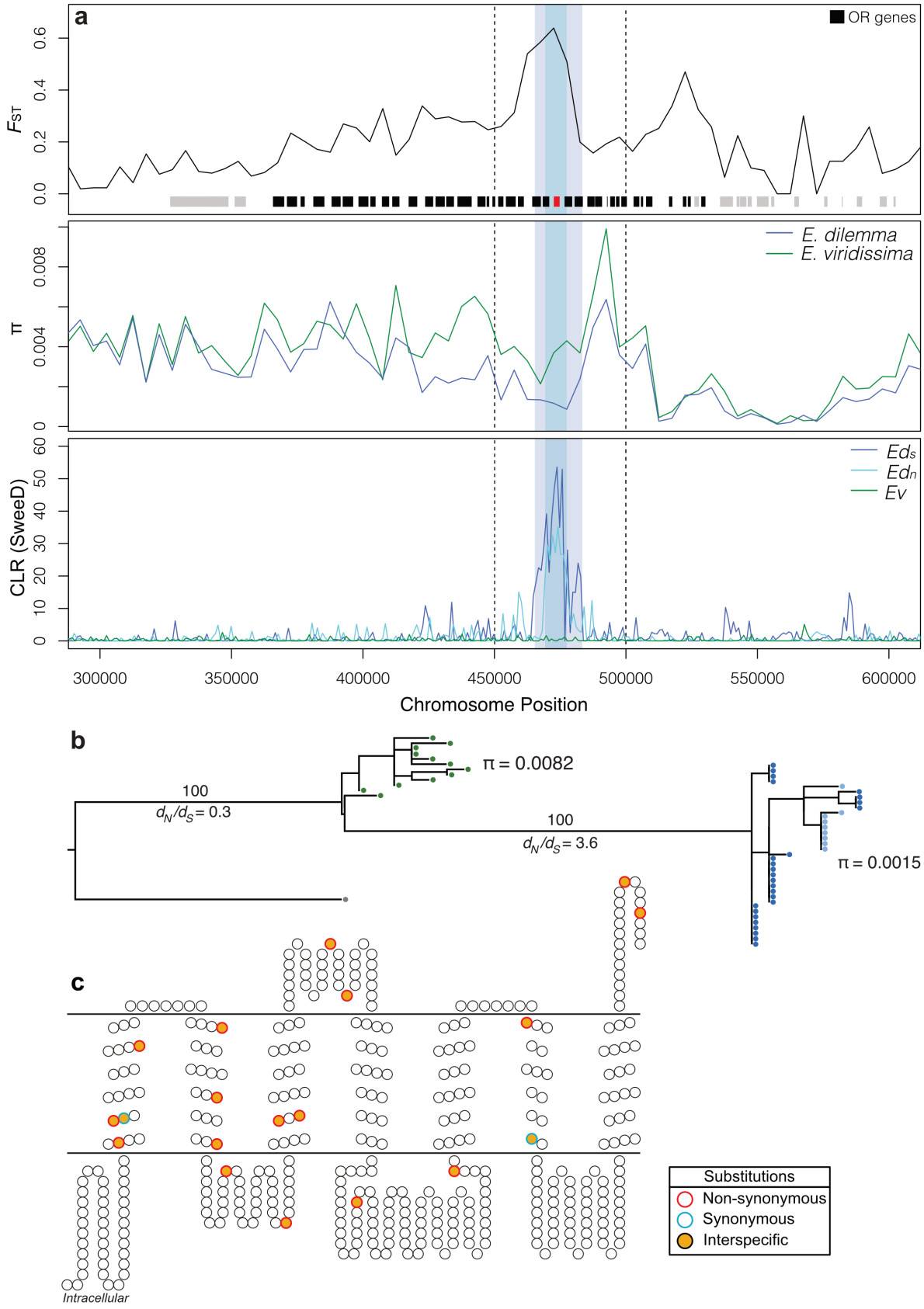


Fig. 4 Odorant receptor (OR) gene *OR41* evolved through a species-specific selective sweep. (**a**) The only species-specific selective sweep identified was located within an F_{ST} outlier window (dashed lines) overlapping with a high interspecific difference in π in the middle of a tandem array containing 37 OR genes. High composite likelihood ratios (CLR, bottom) within Ed_{north} (light blue) and Ed_{south} (dark blue) but not in *Ev* (green) indicate a selective sweep shared by both *E. dilemma* lineages that overlap with *OR41* in the center (shaded regions). (**b**) A Maximum Likelihood phylogeny of *OR41* (N=47 individuals) demonstrates that genotypes are species-specific. π was five times lower in *E. dilemma* in comparison to *E. viridissima*. A d_N/d_S analysis of species-specific genotypes with five outgroup species (grey dot) indicates positive selection on the *E. dilemma* branch (d_N/d_S=3.6), but purifying selection of the ancestral genotype in *E. viridissima* (d_N/d_S=0.3). Bootstrap support for tested branches is indicated. (**c**) 17 of 19 substitutions mapped on the predicted membrane topology of the OR41 protein were non-synonymous.

Our results show that a simple major phenotypic difference in a reproductive barrier trait between two lineages in the early stages of speciation is maintained despite low genetic differentiation and ongoing gene flow. Only strong selection can counteract such equalizing mechanisms, highlighting the adaptive value of the species-specific major perfume compounds in *E. dilemma* and *E. viridissima*. While genome-wide analyses often lack resolution to identify the genes that control barrier traits (*3*, *6-9*, *29*), we were able to identify a single genetic locus of adaptive interspecific divergence, leading to a unique opportunity to understand the genomic landscape of speciation on a fine genetic scale in a non-model system. Our findings provide a link between a discrete shift in perfume composition with a single olfactory receptor gene that evolved under strong positive selection, linking a chemosensory barrier trait with an olfactory gene. Perfume composition in orchid bees is intricately connected to the sense of smell (*16*, *30*, *31*). In fact, *E. dilemma* and *E. viridissima* are known to differ in the behavioral preference towards and the sensory detection of HNDB (*16*) the major perfume compound in *E. dilemma*. This observation lends support to the hypothesis that the 17 non-synonymous substitutions present in *OR41* in the *E. dilemma* lineage underlie functional changes in sensory perception between species. Accordingly, the data presented here are consistent with the genetic coupling of a reproductive trait and trait preference (*17*, *18*, *32*) that evolved through rapid divergent selection in a single gene leading to speciation.

References:

1. T. Dobzhansky, *Genetics and the Origin of Species* (Columbia University Press, 1937).

2. E. Mayr, *Systematics and the Origin of Species, from the Viewpoint of a Zoologist* (Harvard University Press, 1942).

3. O. Seehausen *et al.*, Genomics and the origin of species. *Nat. Rev. Genet.* **15**, 176–192 (2014).

4. T. E. Cruickshank, M. W. Hahn, Reanalysis suggests that genomic islands of speciation are due to reduced diversity, not reduced gene flow. *Molecular Ecology*. **23**, 3133–3157 (2014).

5. S. Stankowski, M. A. Chase, A. M. Fuiten, P. L. Ralph, M. A. Streisfeld, The tempo of linked selection: emergence of a heterogeneous genomic landscape during a recent radiation of monkeyflowers. *bioRxiv*, 342352 (2018).

6. J. B. W. Wolf, H. Ellegren, Making sense of genomic islands of differentiation in light of speciation. *Nat. Rev. Genet.* **18**, 87–100 (2017).

7. M. Ravinet *et al.*, Interpreting the genomic landscape of speciation: a road map for finding barriers to gene flow. *Journal of Evolutionary Biology*. **30**, 1450–1477 (2017).

8. C. A. Buerkle, Inconvenient truths in population and speciation genetics point towards a future beyond allele frequencies. *Journal of Evolutionary Biology*. **30**, 1498–1500 (2017).

9. D. Lindtke, S. Yeaman, Identifying the loci of speciation: the challenge beyond genome scans. *Journal of Evolutionary Biology*. **30**, 1478–1481 (2017).

10. T. Eltz, W. M. Whitten, D. W. Roubik, K. E. Linsenmair, Fragrance Collection, Storage, and Accumulation by Individual Male Orchid Bees. *J. Chem. Ecol.* **25**, 157–176 (1999).

11. Y. Zimmermann, S. R. Ramírez, T. Eltz, Chemical niche differentiation among sympatric species of orchid bees. *Ecology*. **90**, 2994–3008 (2009).

12. M. G. Weber, L. Mitko, T. Eltz, S. R. Ramírez, Macroevolution of perfume signalling in orchid bees. *Ecology Letters*. **19**, 1314–1323 (2016).

13. T. Eltz, A. Sager, K. Lunau, Juggling with volatiles: exposure of perfumes by displaying male orchid bees. *J. Comp. Physiol. A*. **191**, 575–581 (2005).

14. T. Pokorny *et al.*, Blown by the wind: The ecology of male courtship display behavior in orchid bees. *Ecology* (2017), doi:10.1002/ecy.1755.

15. Y. Zimmermann, D. W. Roubik, T. Eltz, Species-specific attraction to pheromonal analogues in orchid bees. *Behav Ecol Sociobiol*. **60**, 833–843 (2006).

16. T. Eltz *et al.*, An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees. *Curr. Biol.* **18**, 1844–1848 (2008).

17. R. K. Butlin, M. G. Ritchie, Genetic coupling in mate recognition systems: what is the evidence? *Biol J Linn Soc*. **37**, 237–246 (1989).

18. M. Kopp *et al.*, Mechanisms of Assortative Mating in Speciation with Gene Flow: Connecting Theory and Empirical Research. *Am. Nat.* **191**, 1–20 (2018).

19. T. Eltz *et al.*, Characterization of the orchid bee Euglossa viridissima (Apidae: Euglossini) and a novel cryptic sibling species, by morphological, chemical, and genetic characters. *Zoological Journal of* .... **163**, 1064–1076 (2011).

20. T. Pokorny *et al.*, Acquisition of species-specific perfume blends: influence of habitat-dependent compound availability on odour choices of male orchid bees (Euglossa spp.). *Oecologia*. **172**, 417–425 (2013).

21. N. Vijay *et al.*, Evolution of heterogeneous genome differentiation across multiple contact

zones in a crow species complex. *Nature Communications*. **7**, 13195 (2016).

22. R. Burri, Dissecting differentiation landscapes: a linked selection's perspective. *Journal of Evolutionary Biology*. **30**, 1501–1505 (2017).

23. P. Brand, S. R. Ramírez, The Evolutionary Dynamics of the Odorant Receptor Gene Family in Corbiculate Bees. *Genome Biol Evol*. **9**, 2023–2036 (2017).

24. L. B. Vosshall, H. Amrein, P. S. Morozov, A. Rzhetsky, R. Axel, A spatial map of olfactory receptor expression in the Drosophila antenna. *Cell*. **96**, 725–736 (1999).

25. B. S. Hansson, M. C. Stensmyr, Evolution of insect olfaction. *Neuron*. **72**, 698–711 (2011).

26. G. P. Leary *et al.*, Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 14081–14086 (2012).

27. C. S. McBride *et al.*, Evolution of mosquito preference for humans linked to an odorant receptor. *Nature*. **515**, 222–227 (2014).

28. P. Brand *et al.*, Rapid evolution of chemosensory receptor genes in a pair of sibling species of orchid bees (Apidae: Euglossini). *BMC Evol. Biol.* **15**, 176 (2015).

29. C. D. Jiggins, S. H. Martin, Glittering gold and the quest for Isla de Muerta. *Journal of Evolutionary Biology*. **30**, 1509–1511 (2017).

30. D. L. P. Schorkopf, L. Mitko, T. Eltz, Enantioselective Preference and High Antennal Sensitivity for (−)-Ipsdienol in Scent-Collecting Male Orchid Bees, Euglossa cyanura. *J. Chem. Ecol.* **37**, 953–960 (2011).

31. L. Mitko *et al.*, Olfactory specialization for perfume collection in male orchid bees. *Journal of Experimental Biology*. **219**, 1467–1475 (2016).

32. C. R. B. Boake, Coevolution of senders and receivers of sexual signals: Genetic coupling and genetic correlations. *Trends in Ecology & Evolution*. **6**, 225–227 (1991).

Acknowledgements:

We thank K.W. McCravy for sharing the Honduras bee samples; T. Pokorny, J.J.G. Quezada-Euan, R. Medina, M.A. Arteaga, and C. Pozo for help with field work; J. Fong for help with morphometry; the Ward lab for support with imaging; and M. Servedio and the Ramirez lab for helpful discussion. **Funding:** The project was supported by a UC Mexus Dissertation Research Grant (P.B.), the David and Lucile Packard Foundation (S.R.R.), the NSF (S.R.R., DEB-1457753), and the DFG (T.E., EL 249/11). **Author contributions:** Conceptualization: P.B., T.E., S.R.R.; Fieldwork: P.B., S.R.R., I.H.-D., C.L.Y.O., R.A.; Data generation: P.B., M.D.; Project administration, Data curation, Formal analysis, Investigation, Visualization: P.B.;

Funding acquisition: P.B., T.E., S.R.R.; Writing – original draft: P.B.; Writing – review & editing: P.B., T.E., S.R.R. with input from all authors; Supervision: S.R.R. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** Raw sequence data are available through NCBI (BioProject XXX), GCMS data are available through Dryad (xxx).