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REPRODUCTIVE CHARACTER DISPLACEMENT OF EPICUTICULAR COMPOUNDS AND THEIR CONTRIBUTION TO MATE CHOICE IN *DROSOPHILA SUBQUINARIA* AND *DROSOPHILA RECENS*

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Interactions between species can alter selection on sexual displays used in mate choice within species. Here we study the epicuticular pheromones of two *Drosophila* species that overlap partially in geographic range and are incompletely reproductively isolated. *Drosophila subquinaria* shows a pattern of reproductive character displacement against *Drosophila recens*, and partial behavioral isolation between conspecific sympatric versus allopatric populations, whereas *D. recens* shows no such variation in mate choice. First, using manipulative perfuming experiments, we show that females use pheromones as signals for mate discrimination both between species and among populations of *D. subquinaria*. Second, we show that patterns of variation in epicuticular compounds, both across populations and between species, are consistent with those previously shown for mating probabilities: pheromone compositions differ between populations of *D. subquinaria* that are allopatric versus sympatric with *D. recens*, but are similar across populations of *D. recens* regardless of overlap with *D. subquinaria*. We also identify differences in pheromone composition among allopatric regions of *D. subquinaria*. In sum, our results suggest that epicuticular compounds are key signals used by females during mate recognition, and that these traits have diverged among *D. subquinaria* populations in response to reinforcing selection generated by the presence of *D. recens*.

KEY WORDS: **Mate choice, pheromones, reinforcement, sexual selection, speciation.**

In natural populations, it is common for individuals of one sex to prefer certain trait values over others when choosing mates. Although an important source of sexual selection within populations, these mate preferences, and the sexual signals or displays they target, are also thought to play a central role in the origin of new species. In particular, the divergence among populations of signal traits and preferences may be an important cause of behavioral isolation during speciation (Coyne and Orr 2004). For example, when two populations that have been diverging in allopatry come into secondary contact, reinforcing selection can favor a strengthening of premating isolation in areas of sympatry in response to reduced hybrid fitness (Dobzhansky 1951; Howard 1993; Servedio and Noor 2003; Coyne and Orr 2004). The expected outcome is a pattern of reproductive character displacement (RCD) in which there is greater divergence of traits involved in behavioral isolation in areas of sympatry than in areas of allopatry (Howard 1993), although subsequent gene flow out of sympatry may erase this pattern.

Most demonstrations of RCD are based on mating probabilities, where behavioral isolation is shown to be stronger between

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sympatric than between allopatric populations of two species (Coyne and Orr 2004). However, a comprehensive understanding of how divergent natural and/or sexual selection generate behavioral isolation in nature requires knowledge of the male signal traits and female preferences that underlie mate choice and that diverge during reinforcement (Mendelson and Shaw 2012). In some cases, the male signal trait of interest is obvious, for example in the *Ficedula* flycatchers where male plumage differs dramatically between sympatric and allopatric populations (reviewed in Saetre and Saether 2010). However, in many cases it can be challenging to identify the traits that underlie behavioral isolation in nature, in part because females often assess multiple traits when choosing mates (Candolin 2003; Chenoweth and Blows 2006).

One indication that a male signal trait contributes to behavioral isolation, and thus may be a target of reinforcing selection, is if it varies in a pattern concordant with that of both female mate preferences and with the observed behavioral isolation. In other words, one might expect to see displacement not only in the pattern of female discrimination, but also in the male characters and female preferences that target them. Furthermore, this pattern may suggest that females from sympatric populations use population-specific rather than species-specific cues for mate discrimination. This could also lead to an additional layer of reproductive isolation between conspecific allopatric and sympatric populations, where conspecific allopatric males are no longer considered to be suitable mates (reviewed in Ortiz-Barrientos et al. 2009; Hosken and Higgie 2010).

Here we focus on the sexual signals involved in RCD between the fly *Drosophila subquinaria* and its sister species, *Drosophila recens*. *Drosophila subquinaria* and *D. recens* occur in western and eastern North America, respectively. Their geographic ranges overlap for about 1200 km in central Canada, and it is thought that this zone of sympatry reflects a secondary contact event that has occurred within the last 20,000 years after the retreat of the last glacial maximum (Jaenike et al. 2006). The two species are morphologically identical except for the male genitalia (Wheeler 1960). Where these species overlap, there are no known ecological differences between them: both can be collected at the same time of year and on the same mushrooms, which they use as a food source and mating substrate. Like other mushroom-feeding *Drosophila*, both species are thought to be generalists on fleshy basidiomycetes.

Drosophila subquinaria females show a pattern of RCD against mating with *D. recens*: in the geographic region where the species overlap, *D. subquinaria* females discriminate strongly against *D. recens* males, whereas *D. subquinaria* females from populations outside the zone of sympatry (i.e., allopatric) will mate with *D. recens* males at a moderate rate (Jaenike et al. 2006). In contrast to *D. subquinaria*, populations of *D. recens* that are sympatric and allopatric with *D. subquinaria* do not show a pattern of RCD (Jaenike et al. 2006). This asymmetric pattern in RCD is similar to many other species of *Drosophila* (Yukilevich 2012). A potential selective origin for this asymmetric pattern in this system is a *Wolbachia* infection present only in *D. recens*. The infection causes offspring survival from crosses between *D. subquinaria* females from any population and *D. recens* males to be low due to interspecific cytoplasmic incompatibility, whereas the offspring from the reciprocal cross survive and F1 females are fertile, although hybrid males are always sterile (Shoemaker et al. 1999).

In addition to displaying a pattern of RCD against *D. recens*, sympatric *D. subquinaria* females also discriminate against males of their own species from allopatric populations, although they mate freely with the conspecific males with which they co-occur (Jaenike et al. 2006). Although the performance of hybrids in nature is not known, there are no known postzygotic effects of these intra-specific crosses in the lab based on the fertility of F1 and F2 individuals (Jaenike et al. 2006; K. A. Dyer, unpubl. data). This suggests that this isolation is primarily, if not entirely, behavioral. The presence of gene flow among sympatric and nearby allopatric (i.e., "inland" allopatric, see Materials and Methods) *D. subquinaria* populations suggests that the pattern of between-species mate discrimination is the result of a history of reinforcing selection on *D. subquinaria* females to avoid mating with *D. recens* males (Jaenike et al. 2006, E. R. Bewick and K. A. Dyer, unpubl. data). Furthermore, this change in female mate choice may have also caused the behavioral isolation between sympatric *D. subquinaria* females and allopatric *D. subquinaria* males as a by-product, consistent with a pattern of "cascade reinforcement" (Hosken and Higgie 2010).

In many species of *Drosophila*, as well as in many other insects, epicuticular compounds are known to be important signals during courtship and mate discrimination (reviewed in Ferveur 2005). Several lines of evidence point to the importance of epicuticular compounds as male signals for female mate choice within these species. First, after their antennae are ablated, the third segment of which is necessary for the perception of male pheromones in *Drosophila* (Grillet et al. 2006), female *D. subquinaria* almost never mate with conspecific males from their own population (Giglio and Dyer 2013). This effect is also seen in *D. recens* females, although it is not as strong. In contrast, the removal of the female's arista, which is attached to the antennae and is necessary for hearing, or painting over the female's eyes, have no effect on mating frequency in either species. Second, epicuticular compounds are shared between the species, several are sexually dimorphic, and a few are found only in males (Curtis et al. 2013). Furthermore, the relative amounts of many epicuticular compounds differ between *D. recens* and *D. subquinaria* males, but not between females, suggesting that these traits are targets of sexual selection. Finally, in both species, female binomial

Table 1. Summary of populations used in this study. Sympatric (sym) populations contain both species, and allopatric (allo) populations are where one species only is present, with the allopatric region (coast or inland) indicated for *Drosophila subquinaria* populations. For each population, the number of isofemale lines used in this study, as well abundance of *D. subquinaria* relative to *Drosophila recens* at the time of collection, is included.

Population	Abbr.	Region	Year collected	No. of *D. subquinaria* lines	No. of *D. recens* lines	% D. *subquinaria* among wild flies
Portland, OR	POR	allo – coast	2010	24	0	100
Seattle, WA	SEA	allo – coast	2010	24	0	100
Missoula, MT	MIS	allo – inland	2010	5	0	100
Deary, ID	DEA	allo – inland	2009	7	0	100
Shuswap, BC	SHU	allo – inland	2010	3	0	100
Canmore, AB	CAN	sym	2010	3	4	47
Hinton, AB	HIN	sym	2010	12	7	27
Kawtikh, AB	KAW	sym	2010	6	22	8
Nordegg, AB	NOR	sym	2010	0	1	Unknown
Lake Nippising, ON	NIP	allo	2010	0	7	0
Peru, NY	PER	allo	2009	0	9	0
Smoky Mountains, TN	SMT	allo	2009	0	15	0

mate choice trials using males from the same species and population suggest there are differences in female mate preferences for these traits. Specifically, within each species there was strong directional sexual selection on male epicuticular profiles, and between species the sexual selection vectors differed significantly in multivariate trait space (Curtis et al. 2013).

As chemosensory cues are essential for *D. subquinaria* females to mate within species, and are apparent targets of sexual selection in both species, they may also underlie variation in behavioral discrimination across populations and species. Here we test whether male epicuticular compounds act as pheromonal signals used by sympatric *D. subquinaria* females to discriminate against both *D. recens* and their own allopatric *D. subquinaria* males. First, we use perfuming experiments to assay the contribution of divergent epicuticular profiles to the existing behavioral isolation both between the two species and between sympatric and allopatric populations within *D. subquinaria*. If these compounds are a target of female choice that underlies species and/or population discrimination, we hypothesize that perfuming normally unattractive *D. recens* males with *D. subquinaria* male pheromones will increase the receptivity of *D. subquinaria* females to these males, thereby increasing hybridization rates. In addition, perfuming normally unattractive allopatric *D. subquinaria* males with sympatric male pheromones will increase mating rates with sympatric *D. subquinaria* females. Second, we examine variation in male and female epicuticular compounds from populations throughout the ranges of *D. subquinaria* and *D. recens*. Using populations as replicates, we quantify variation in epicuticular compounds and test whether their composition follows the same geographic pattern as that of female mate discrimination. In particular, we hypothesize that if these traits act as sexual pheromones that are the subject of reinforcing selection, a pattern of RCD will be present in *D. subquinaria* but not in *D. recens*. Finally, given known genetic structuring among allopatric *D. subquinaria* populations on either side of the Coast Mountains (termed coastal vs. inland populations; Jaenike et al. 2006), we also test for differences between these regions.

Materials and Methods

DROSOPHILA STRAINS AND REARING

We used isofemale lines of *D. subquinaria* and *D. recens* collected from sympatric and allopatric populations during the summers of 2009 to 2010 (Table 1 and Fig. 1). In *D. subquinaria*, within the large allopatric region there is evidence for genetic differentiation among populations to the west (i.e., coastal) and east (i.e., inland) of the Coast Mountains (Jaenike et al. 2006). Thus, we sampled replicate sympatric, allopatric coastal, and allopatric inland populations of *D. subquinaria* (Table 1). Because *D. subquinaria* and *D. recens* are morphologically identical, flies were identified to species with multiple molecular markers, including *Wolbachia* infection, mtDNA *COI* haplotype, and Y-linked *kl-3* haplotype. The number of isofemale lines of each species used in this study, as well as the overall species composition of each population, is shown in Table 1.

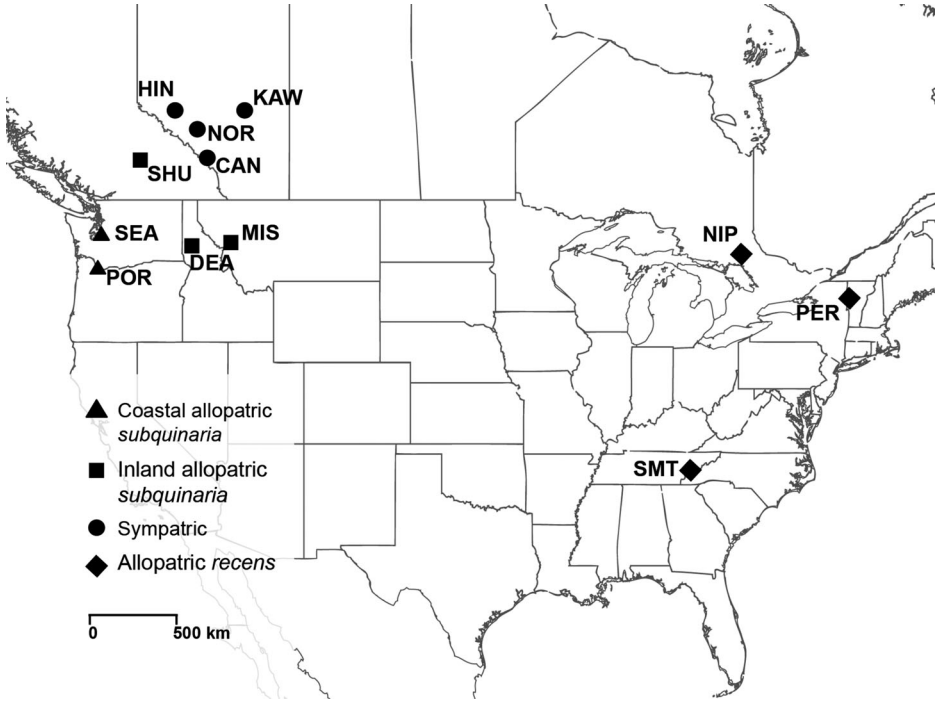

Figure 1. **Map of population locations used in this study. Locations are noted by their abbreviation in Table 1.**

We also created four "mixed" stocks by combining isofemale lines and allowing them to mass breed for at least four generations. These included two mixed *D. subquinaria* stocks: one made from four isofemale lines from three sympatric populations (Hinton, Canmore, Kawtikh), and the other made from six isofemale lines from four allopatric populations, which included flies from both coast (Portland, Seattle) and inland (Missoula, Shuswap) populations. The other two were mixed *D. rec*ens stocks. The first combined three isofemale lines from separate sympatric populations (Hinton, Canmore, Kawtikh) that were known to carry the *Wolbachia* infection, and the second was created from three isofemale lines all from the Kawtikh population, each of which was naturally uninfected with *Wolbachia*. (In *D. recens*, *Wolbachia* has a maternal transmission rate of ∼98% [Shoemaker et al. 1999], and thus these rare uninfected lines are assumed to be the result of imperfect maternal transmission.) Because no *Wolbachia* are present in this second *D. recens* stock, and there is thus no cytoplasmic incompatibility, the offspring of *D. subquinaria* females and *D. recens* are expected to survive. We verified this by pairing *D. subquinaria* allopatric females with these uninfected male *D. recens*; of 15 observed matings, 11 of the mated females produced viable offspring.

All fly cultures were maintained on Instant *Drosophila* food (Carolina Biological, Burlington, NC) supplemented with commercial mushroom (*Agaricus bisporus*) and reared at 20°C on a 14:10 light:dark cycle and 60% relative humidity. Flies for experiments were reared at a controlled density. Virgins were collected using light CO_2 anesthesia within 24 h of emergence and subsequently held separately by sex at 10–15 flies per vial.

EXTRACTION AND QUANTIFICATION OF EPICUTICULAR COMPOUNDS

Epicuticular compounds were extracted from single flies by washing individuals in 100 μL of hexane for approximately 3 min and then vortexing for 1 min, after which the fly was removed and discarded. All extractions were completed within 3 h of the lights turning on in the incubator, and were performed in a randomized block design to minimize effects from the time of day, day, and order of extraction. Extractions were stored at −20°C and were subsequently shipped from Athens, GA, to Ottawa, ON, for analysis.

Samples were analyzed on a dual-channel Agilent Technologies (Wilmington, DE) 6890 N fast gas chromatograph with flame ionization detector using the temperature program described in Curtis et al. (2013). Individual profiles were determined by integrating the area under 17 and 23 peaks in females and males, respectively, corresponding to those previously identified in Curtis et al. (2013) with the exception of hentria-*n*-*n*-contadiene ($C_{31:2}$), a very low concentration hydrocarbon that was undetectable in many individuals and which was therefore not included. The integrated peaks included 17 long-chain hydrocarbons composed only of odd carbon numbers (C_{29}, C_{31}, C_{33}, and C_{35}) and consisting of several methyl-branched alkanes, alkenes, and alkadienes, all of which were present in both sexes of both species

Table 2. Results of no-choice perfuming trials measuring the fraction of sympatric *Drosophila subquinaria* females that mated when confined with the target male for 3 h (with *D. subquinaria*) or 24 h (with *Drosophila recens*). Each target male had been perfumed with pheromones from either their own males or from sympatric *D. subquinaria* males.

Target male	Block	Perfumed with target males	Perfumed with sympatric *D. subquinaria* males
Allopatric *D. subquinaria*	1	7/23 (30%)	16/30 (53%)
	2	3/16 (19%)	6/12 (50%)
	3	3/15 (20%)	7/13 (54%)
	4	3/20 (15%)	4/13 (31%)
	5	6/41 (15%)	5/28 (18%)
	Total	22/118 (19%)	38/96 (40%)
Sympatric *D. recens*	1	0/18 (0%)	1/18 (6%)
	2	0/15 (0%)	0/24 (0%)
	3	0/12 (0%)	0/10 (0%)
	4	0/27 (0%)	2/21 (10%)
	5	0/51 (0%)	0/15 (0%)
	Total	0/123 (0%)	3/87 (3.4%)

(Curtis et al. 2013). Also present in males only of both species was 11-*cis*-Vaccenyl acetate (cVa), along with five fatty acids, provisionally identified as tri-acylglycerides (Curtis et al. 2013).

After integration, the relative abundance of each compound was calculated by dividing the area under each peak by the total area of all peaks for that individual. Working with relative abundances corrects for substantial technical error associated with quantifying absolute amounts via gas chromatography. To break the unit-sum constraint inherent in such compositional data and thus allowing multivariate analyses to be performed, proportions were transformed into log-contrasts (Aitchison 1986) using 2-methyl octacosane as the common divisor, as described in Curtis et al. (2013). The resulting 23 and 17 log-contrast traits for males and females, respectively, were used in all subsequent analyses.

PERFUMING ASSAYS

We conducted two types of perfuming experiments, both of which used the mixed *D. subquinaria* and mixed *D. recens* stocks described above and employed females that were always derived from the *D. subquinaria* sympatric mixed stock. In the first experiment, allopatric *D. subquinaria* mixed males were perfumed with 25 or 50 males from either their same allopatric *D. subquinaria* mixed stock or from the sympatric *D. subquinaria* mixed stock. In the second experiment, sympatric *D. recens* males that did not carry the *Wolbachia* infection were perfumed with 50 males from either the same *D. recens* mixed stock or from the sympatric *D. subquinaria* mixed stock. We used naturally uninfected flies to reduce any confounding effects of antibiotic treatment on the microbiome of the fly, which may affect pheromone composition (Sharon et al. 2010).

To perfume males, a single target male with unclipped wings was placed with the donor males (with clipped wings) in a standard food vial, and the cotton plug was pushed down to leave about 2 cm of space for the flies to move around. All male flies were 0–3 days old at the beginning of perfuming, and flies were perfumed for 9 days. Each target male was removed from the perfuming vial by aspiration and added to a vial that contained five virgin 7- to 10-day-old females from the *D. subquinaria* sympatric mixed stock. All males used in the mating trials had experienced the crowded perfuming environment, thus controlling for any potential effects on male activity. Mating trials used 1-dram vials that contained a blended mushroom-agar food, and were started within 1 h of the incubator lights on. Vials were observed for 3 h, and the time until copulation and copulation duration(s) were recorded. For trials with *D. recens* males, flies were left in the mating vial for an additional 21 h following the observation period, after which the male was discarded. These females were then placed together in a standard food vial and two weeks later scored for the presence of offspring. A total of five blocks of mating trials were completed for each perfuming experiment; within each block we included an average of 21 replicate test crosses (range 10–51) for each type of perfumed male (Table 2).

To test whether female mating preferences differed when males were perfumed with attractive versus unattractive males, we used a logistic regression with the male treatment type and block as effects in the model. In the within-species perfuming experiment, more than one copulation occurred in some vials during the observation period, and thus we also tested whether the total number of copulations per male differed using a Wilcoxon rank sum test. The time to first copulation from being placed in the vial and the duration of the first copulation were also compared between male types using a Wilcoxon rank sum test. We combined the results for 25 vs. 50 perfuming males in the within species treatment because there was no effect of the number of perfuming

males in a vial. Analyses were performed using JMP version 10 (SAS Institutes, Cary, NC).

We tested the extent to which perfuming altered the epicuticular compounds of the target males, specifically asking whether the cuticular hydrocarbon (CHC) profiles of these males changed to more closely resemble their respective donor males. This experiment was completed at a different time than the perfuming experiments, but used the same stocks. We completed the perfuming procedures exactly as for the mating trials, but instead of placing the perfumed male with females, we extracted and quantified each male's epicuticular compounds as described above. We did this for both the within and between species perfuming experiments, and we also simultaneously extracted the epicuticular compounds from virgin nonperfumed males for reference. An average of 21 males (range 11–34) of each type was used in this experiment. To provide a simple, visual interpretation of the effects of perfuming, a canonical discriminate analysis was conducted on all log-contrast epicuticular compounds including only males from the three nonperfumed (i.e., pure) types: sympatric *D. recens*, allopatric *D. subquinaria*, and sympatric *D. subquinaria*. Perfumed individuals were then scored for the first two canonical variates and all individuals (perfumed and nonperfumed) were plotted in the resulting trait space, allowing the effects of perfuming to be examined within the context of the major axes of variation that distinguish the sympatric and allopatric forms of the two species (note that sympatric and allopatric *D. recens* do not differ, see Results).

Our perfuming experiments used *D. recens* that were not infected with *Wolbachia*, whereas most flies in the wild harbor the infection. We therefore also tested whether the presence of *Wolbachia* alone affected *D. subquinaria* female mating preferences. The assay involved placing a single 7- to 10-day-old virgin female from the *D. subquinaria* sympatric mixed stock and either an infected or uninfected *D. recens* virgin male from their respective mixed stocks together in a 1-dram vial that contained a blended mushroom-agar food. Each pair was observed for 3 h to determine whether copulation occurred.

VARIATION IN EPICUTICULAR COMPOUNDS

Epicuticular compounds were sampled from an average of 25 (range 21–28) virgin females and 26 (range 19–31) virgin males from a variable number of isofemale lines from each species and population, as outlined in Table 1. Extraction and quantification were performed as described above. All flies were 7–9 days postemergence. Analyses were conducted separately by sex because the suite of epicuticular compounds varies qualitatively between males and females. To visualize RCD within the context of the total among population and between species variation in epicuticular compounds, we extracted individual scores from a canonical discriminant analysis that differentiated among all combinations of species and populations, separately for each sex. We then plotted the first two discriminate functions for both males and females. Working with the canonical variates had the advantage not only of reducing dimensionality, allowing subsequent tests for RCD to be performed on the majority of the among-individual variation in epicuticular compounds, but also avoided statistical issues arising from a moderate degree of multicolinearity among the log-contrast traits. We therefore tested the effects of sympatry/allopatry on the first five canonical variates via multivariate analysis of variance (MANOVA), separately by sex, using the following linear model:

$$\text{Canonical variates } 1-5 = sym + spp + sym \times spp + pop(sym \times spp), \quad (1)$$

where *sym* and *spp* are the fixed effect of sympatry/allopatry and species (*D. recens* and *D. subquinaria*) respectively, and *pop* is the random effect of population nested within the fixed effects interaction. The *sym*×*spp* interaction was highly significant in both sexes, indicating species-specific effects of the presence versus absence of the other species. We therefore repeated the discriminate analysis separately for each sex and species, extracting the individual-level canonical scores for the first four canonical variates in each case, accounting for 92% or more of the total variation. Differences in epicuticular compounds between sympatry/allopatry on the first four canonical variates of each sex and species were then tested via MANOVA, with sympatry (i.e., *sym*) as a fixed effect and population (i.e., *pop*) as a random effect nested within *sym*. Finally, given the known genetic structuring of allopatric *D. subquinaria* populations into coastal versus inland groups, this analysis was also repeated in *D. subquinaria* males and females after replacing the sympatry/allopatry effect with the three-level designation of sympatric/allopatric-inland/allopatric-coastal, with population again as a random effect nested within this. The above analyses treat populations as the unit of replication. In doing so, we seek to demonstrate that any pattern of RCD detected is not specific to a particular population, but rather is detected across multiple populations. Separate populations are clearly not phylogenetically independent, and the presence of RCD across multiple populations does not imply that it evolved independently in each (i.e., multiple origins).

Results

PERFUMING ASSAYS

Perfuming of allopatric *D. subquinaria* males successfully altered their epicuticular profiles as expected. In particular, when these allopatric males were perfumed with sympatric *D. subquinaria* males, their epicuticular profiles shifted to more closely resemble that of the sympatric males (Fig. 2). In contrast, perfuming

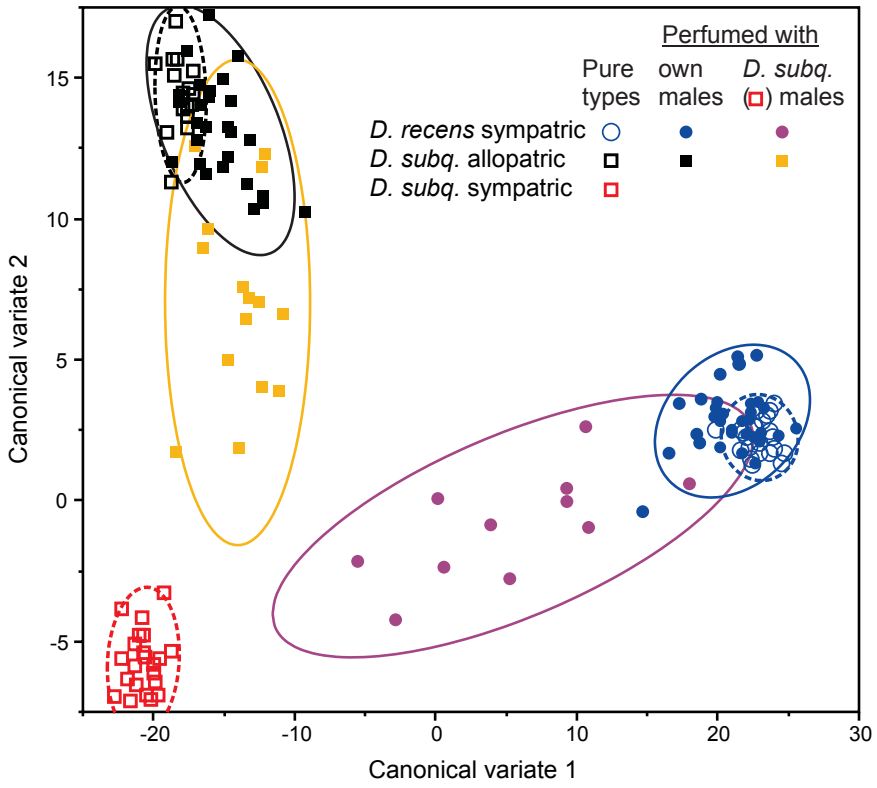

Figure 2. **Individual variation in epicuticular compounds among male *Drosophila recens* (circles) and *Drosophila subquinaria* (squares) in response to perfuming. Pure (i.e., nonperfumed) individuals (open symbols) are shown in relation to perfumed individuals (filled symbols), with the latter including those individuals perfumed with their own (conspecific) males and those perfumed with sympatric *D. subquinaria* males. Ninety percent bivariate normal density ellipses are also shown for the various combinations of perfumed (solid lines) and nonperfumed (broken lines) groups.**

of these males with other allopatric *D. subquinaria* males as a control produced little change in their epicuticular profiles (Fig. 2). In response to this perfuming, the acceptance by sympatric *D. subquinaria* females of the allopatric *D. subquinaria* males approximately doubled, from 19% (n = 118) to 40% (n = 96) (Table 2), when these males were perfumed with sympatric as opposed to control (i.e., allopatric) males. In a logistic regression, male perfume type was highly significant (Likelihood ratio test [LRT]: $\chi^2 = 9.5$; df = 1; $P = 0.002$). Similarly, considering the total number of copulations per male during the 3-h observation period, allopatric *D. subquinaria* males perfumed with sympatric *D. subquinaria* pheromones attained twice as many copulations as did these males when perfumed with *D. subquinaria* allopatric pheromones (mean ± SE per male: allopatric = 0.40 ± 0.09, sympatric = 0.80 ± 0.12), a difference that is significant overall (Wilcoxon rank sum $\chi^2 = 10.3$; df = 1; $P = 0.0013$). The time to the first copulation did not vary significantly depending on which type of male was used to perfume (allopatric males = 34.2 ± 7.9 min, sympatric males = 25.3 ± 4.2 min; Wilcoxon rank sum $\chi^2 = 0.46$; df = 1; $P = 0.5$), nor did the duration of the first copulation (allopatric males = 6.9 ± 0.65 min, sympatric males = 8.3 ± 0.56 min; Wilcoxon rank sum $\chi^2 = 2.3$; df = 1, $P = 0.12$).

The perfuming of *D. recens* males also altered their epicuticular profiles as expected. In particular, the *D. recens* males perfumed with sympatric *D. subquinaria* shifted to more closely resemble these latter males, whereas those in the control treatment that were perfumed with their own *D. recens* males showed little change (Fig. 2). The *D. recens* males that had been perfumed with sympatric *D. subquinaria* males were also found to be more attractive to sympatric *D. subquinaria* females than were the controls. In particular, none of the 123 *D. recens* males perfumed with their own *D. recens* male pheromones mated during the 3 h observation period, and none of these vials produced any offspring after an additional 21 h together. Of the 87 *D. recens* males perfumed with sympatric *D. subquinaria* pheromones, one mated during the 3-h observation period and an additional two vials produced offspring indicating that mating occurred during the following 21 h. This yielded a total of 3.4% mated across the 24-h mating period (Table 2). A logistic regression of the incidence of copulation indicates that the male perfume type was significant (LRT: $\chi^2 = 4.61$; df = 1; $P = 0.032$). Thus, there was a moderate, though significant, increase in female mate acceptance of heterospecific *D. recens* males when these males were perfumed with sympatric *D. subquinaria* male pheromones.

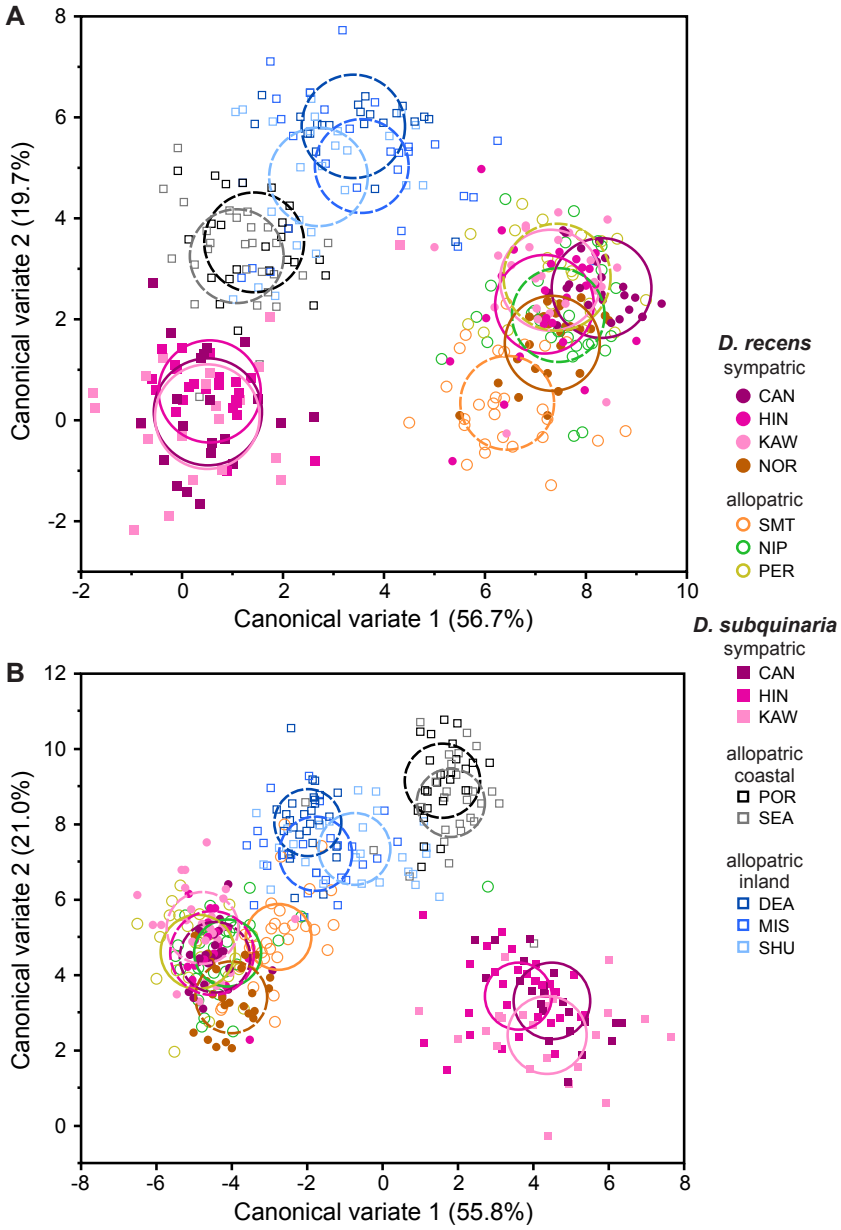

Figure 3. **Individual variation in epicuticular compounds of (A) female and (B) male *Drosophila recens* (circles) and *Drosophila subquinaria* (squares) collected from sympatric (filled symbols) and allopatric (open symbols) locations. Axes are the first and second canonical variates from a discriminate function analysis, conducted separately by sex, which discriminated among individuals according to species and population. Circles depict the 95% confidence limits for the means of the various sympatric (solid line) and allopatric (broken line) populations.**

Finally, we found that *Wolbachia* infection itself did not have a significant effect on the patterns of female mate discrimination of *D. subquinaria* against *D. recens*. Of 38 trials in which sympatric *D. subquinaria* females were confined with *Wolbachia*-infected *D. recens* males, and 40 trials in which they were confined with *Wolbachia*-uninfected *D. recens* males, no copulations occurred within the 3-h observation period in either case (Fisher's exact test, $P = 1.0$).

VARIATION IN EPICUTICULAR COMPOUNDS

In *D. recens*, there was little indication of any pattern of RCD of epicuticular compounds, with extensive phenotypic overlap among sympatric and allopatric populations for the first two canonical variates of the among-population variation in both males and females (Fig. 3B; see Table S1 for trait loadings). In *D. subquinaria*, however, a pattern of RCD was apparent in both sexes, with little to no overlap between sympatric and allopatric

Table 3. Results of multivariate analyses of variance testing for differences in epicuticular compounds separately for male and female *Drosophila recens* and *Drosophila subquinaria*. Analyses were conducted on the first four canonical variates from a discriminate function analysis among individuals based on their population of origin.

Species	Sex	Pillai's trace	*F*	df	*P*	% variance[1]
Sympatry vs. allopatry						
D. recens	Female	0.388	0.32	4,2	0.849	95.3
D. recens	Male	0.622	0.82	4,2	0.613	92.4
D. subquinaria	Female	0.992	98.17	4,3	0.002	95.9
D. subquinaria	Male	0.988	62.55	4,3	0.003	97.4
Sympatry vs. allopatric inland vs. allopatric *coastal*						
D. subquinaria	Female	1.965	41.96	8,6	<0.001	95.9
D. subquinaria	Male	1.976	62.44	8,6	<0.001	97.4

[1]Percent of the among-individual variance accounted for by the first four canonical variates.

populations of the first two canonical variates (Fig. 3A; see Table S1 for trait loadings). In a multivariate test of the first five canonical variates of the among-population variation, accounting for 95.5% and 94.5% of the total variation in epicuticular compounds among females and males respectively, this contrasting pattern generated a highly significant species × sympatry/allopatry interaction overall in females (MANOVA: Pillai's trace $= 0.957, F_{5,7} = 31.08, P < 0.0001$) and in males (MANOVA: Pillai's trace $= 0.937$, $F_{5,7} = 20.92$, $P = 0.0004$), indicating that the effect of sympatry varied between species. Given this interaction, we repeated the discriminate analyses separately by species (and sex), extracting the first four canonical variates in each case and then testing for RCD in them in a multivariate analysis. In *D. recens*, there was again little indication of any consistent difference in epicuticular compounds between sympatric and allopatric populations in either sex (Table 3), demonstrating the absence of RCD of epicuticular compounds in this species. In contrast, in *D. subquinaria*, highly significant differences in epicuticular compounds were detected between sympatric and allopatric populations in both sexes, with allopatric populations also differing in conjunction with known genetic structuring on either side of the Coast Mountains (i.e., allopatric-coastal vs. allopatric inland; Table 3; Fig. S1).

Discussion

The divergence of mate recognition systems can generate the behavioral isolation that has long been thought to be key in both initiating and completing (i.e., reinforcing) speciation in nature (Howard 1993; Coyne and Orr 2004). Mate recognition systems are the product of the underlying mate preferences and the signal traits they target, and these can evolve in response to sexual selection within populations, due to ecological differences among populations, and in response to species interactions (e.g., hybridization and/or reproductive interference). How these different processes interact to affect the evolution of mate recognition systems, and the consequences this has for diversification, are not well understood (Andersson 1994; Coyne and Orr 2004; Hoskin and Higgie 2010; Mendelson and Shaw 2012). Here we were interested in identifying and characterizing variation in sexual signals for two sister species of *Drosophila* in which one of the pair (*D. subquinaria*) shows a pattern of RCD consistent with reinforcing selection to avoid mating with the other (*D. recens*), as well as a pattern consistent with cascade reinforcement (Jaenike et al. 2006). Previous work suggested that epicuticular compounds were critical for *D. subquinaria* to mate, and were also potential targets of sexual selection arising from female mate choice within each species (Giglio and Dyer 2013; Curtis et al. 2013). We tested whether these signals are also involved in discrimination among populations and between species, and characterized their variation among populations of both species.

From direct manipulations using perfuming experiments, our results suggest epicuticular compounds serve as pheromonal signals that contribute to behavioral isolation both among *D. subquinaria* populations and between the two species. Although in neither case did perfuming recover the mating rates seen within populations (e.g., about 80% of sympatric *D. subquinaria* females mate with conspecific sympatric males within a 2-h period; Jaenike et al. 2006; E. R. Bewick and K. A. Dyer, unpubl. data), our manipulations did increase the among-population conspecific mating rate by two-fold to about 40%, and the between species mating rate from zero to approximately 3.4% of pairs. Given the exceptionally strong behavioral isolation between sympatric *D. subquinaria* females and *D. recens* males, it is notable that any heterospecific matings occurred after perfuming. In another study that completed more than 700 mating trials between *D. subquinaria* sympatric females and *D. recens* males, we did not observe a single successful copulation within 2 h, and even when

flies are crowded for an extended period of time it is difficult to get these flies to mate (E. R. Bewick and K. A. Dyer, pers. obs.). Perfuming studies are somewhat crude as pheromone profiles likely only transfer to a limited extent and will also mix with the existing profile of the recipient. In our case, perfuming did generate a substantial, but not complete, shift in multivariate epicuticular profile towards that of the donor males (Fig. 2), and therefore likely underestimate the contribution of these traits to mate choice. Our results are comparable to perfuming effects in other *Drosophila* in which epicuticular compounds have been shown to be important in mate choice. For example, in isolated populations of *Drosophila mojavensis*, perfuming males nearly doubled the rate of mating between populations (Etges and Ahrens 2001). Between species, Blows and Allan (1998) found that interspecific perfuming of *D. serrata* and *D. birchii* increased mating rates from 0% to 8% using 3-day-long mating trials. Furthermore, Coyne et al. (1994) found that perfuming *D. simulans* females with heterospecific *D. sechellia* females reduced the attractiveness of these females to *D. simulans* males, as the number of copulations fell from 10% to 3% during 30 min trials. In future studies of *D. subquinaria*, it will be interesting to perfume sympatric *D. subquinaria* males with *D. recens* males to ask if mating rates decrease, as was found by Coyne et al. (1994). As with other species, chemosensory signals are likely not the only sexual displays involved in mate choice, and between species there are likely additional layers of discrimination as even allopatric *D. subquinaria* females mate with *D. recens* only about 30% of the time (Jaenike et al. 2006; E. R. Bewick and K. A. Dyer, unpubl. data). Nevertheless, following past studies, our results demonstrate that epicuticular compounds contribute to this isolation.

We also used wild-derived isofemale lines to characterize the natural variation among populations of *D. subquinaria* and *D. recens* for both males and females from across the geographic range of each species. Before conducting our assays, all lines were raised in a common laboratory environment for several generations so that differences in trait means among populations could be attributed to genetic rather than to the environment differences. We found strong differences between the species, with almost no overlap in pheromonal profiles between them for either sex (Fig. 3). Within *D. recens*, epicuticular compounds were also very similar between populations that are allopatric versus sympatric with *D. subquinaria*, providing no evidence of any pattern of RCD (Fig. 3). In fact, there was little evidence of variation among populations from across the entire, geographically large, range of *D. recens* (Fig. 1), with the possible exception of a single population from the Smoky Mountains (Fig. 3). This allopatric population lies at the southern edge of the geographic distribution of *D. recens* and also exhibits moderate genetic differentiation from the rest of the range (Jaenike et al. 2006; Dyer et al. 2007). It is currently unknown whether there is any sexual isolation between *D. recens* from the Smokies and flies from the rest of the range, nor what has driven the differentiation of this population.

Finally, *D. recens* is infected with *Wolbachia* whereas *D. subquinaria* is not, and other studies have shown an effect of *Wolbachia* infection on mate preferences (Koukou et al. 2006; Miller et al. 2010). All of the isofemale lines of *D. recens* we assayed for CHCs were infected with *Wolbachia*, and we have not tested for a direct effect of *Wolbachia* on the CHC profile in this species. However, our experiment that compared mating rates of sympatric *D. subquinaria* females with infected versus uninfected *D. recens* males showed no increase in acceptance of uninfected males, indicating that if *Wolbachia* infection has an effect on CHC profile it is not a change that affects the patterns of behavioral discrimination by *D. subquinaria* females.

In contrast to *D. recens*, *D. subquinaria* showed substantial geographic variation in epicuticular profiles, with a striking and highly significant difference between populations that are sympatric versus allopatric with *D. recens*. Specifically, epicuticular composition was extremely similar among three replicate sympatric populations, and these differed consistently from the three replicate and nearby inland allopatric populations. This trait divergence was multivariate, involving the contribution of multiple epicuticular compounds (Table S1), and was substantial, even relative to the between-species differences for both sexes (Fig. 3). This pattern mirrors that previously shown for mate discrimination among sympatric vs. allopatric populations (Jaenike et al. 2006; E. R. Bewick and K. A. Dyer, unpubl. data) and is consistent with the presence of *D. recens* in sympatric populations generating reinforcing selection on these pheromones. Previous work on the genetic structure of these populations indicated the presence of gene flow between sympatric and these nearby inland allopatric populations (Jaenike et al. 2006; E. R. Bewick and K. A. Dyer, unpubl. data), suggesting the pattern observed in the CHCs is unlikely to be the product of genetic drift and must be maintained by some form of selection.

In addition to differences between sympatric and allopatric populations of *D. subquinaria*, we also found significant differences in epicuticular composition between allopatric populations that occur to the west (coastal) versus east (inland) of the Coast Mountains. There is no behavioral isolation between populations of *D. subquinaria* from these two regions (Jaenike et al. 2006; E. R. Bewick and K. A. Dyer, unpubl. data), at least that is detectable in no-choice mating trials, although as previously noted sympatric *D. subquinaria* females do differentiate between them. This inland-coastal divergence appears to have occurred in a multivariate combination of pheromones that is almost orthogonal to that separating the sympatric from the inland allopatric populations (Fig. S1), inconsistent with gene flow out of sympatry into nearby allopatric populations.

The origin of this allopatric inland-coastal divergence is currently unknown and there are several possible explanations. First, it may have arisen in allopatry as product of genetic drift, as this divergence is consistent with previous population genetic work that found a similar pattern of very strong genetic differentiation between these regions (Jaenike et al. 2006). Second, and potentially more likely given the magnitude of the differences, it may have arisen as a result of divergent selection caused from abiotic and/or biotic differences between the regions (e.g., Frentiu and Chenoweth 2010). Epicuticular hydrocarbons are well known to be important for desiccation resistance and temperature tolerance (reviewed in Howard and Blomquist 2005), and the coastal populations of *D. subquinaria* in Seattle, WA, and Portland, OR, experience much more rainfall than populations to the east of the Coast mountains, which are mesic in climate during the summer. However, *D. recens* occupies a broad range of climatic conditions yet shows no such variation among populations, suggesting that climatic conditions alone may be insufficient to explain these differences. In other species, desiccation selection also tends to favor increases in the relative concentration of the longest chain-length hydrocarbons (Kwan and Rundle 2010), but this is not the primary axis of trait differences between the two allopatric regions in *D. subquinaria* (Fig. S1). Alternatively, or in combination with other differences, this variation in epicuticular composition may be caused by the presence in only one of these regions of a third species, driving character displacement between the coastal and inland allopatric populations of *D. subquinaria*. Closely related species in this region include *Drosophila suboccidentalis*, *Drosophila occidentalis*, *Drosophila rellima*, and *Drosophila falleni*, although fine-scale distributions are not well characterized in this region and patterns of behavioral isolation with *D. subquinaria* have not been investigated. A separate RCD involving another species is therefore a distinct plausibility that will require further work to evaluate.

All else being equal, in *D. subquinaria* we expect that reinforcing selection should target male pheromones more strongly than female pheromones because the fitness consequences of hybridization are greater for females than for males. However, it is striking that both male and female epicuticular compounds vary in a fairly consistent pattern of RCD. This may simply represent a correlated response to selection, whereby reinforcing selection on males caused changes in pheromone profiles in females due to a shared genetic basis of the traits between the sexes. In *D. subquinaria*, all epicuticular compounds present in females are hydrocarbons and all are also found in males (Curtis et al. 2013). Although the intersexual correlations for these traits have not been measured in this species, they are sufficiently low in at least two other *Drosophila* to allow at least partially independent evolution of the sexes (e.g., Chenoweth et al. 2008; Bedhomme et al. 2011). An alternative explanation is that female epicuticular hydrocarbons may be under sexual selection arising from male mate choice. Male preferences for hydrocarbons in females have been shown in *D. serrata* and *D. simulans* (Coyne et al. 1994; Chenoweth and Blows 2006; Rundle and Chenoweth 2011), and the removal of the male antennae from sympatric *D. subquinaria* males, causing them to be unable to smell, was observed to decrease the mating rate (Giglio and Dyer 2013). It is not known whether sympatric males prefer their own sympatric females over allopatric conspecific females, although if this is the case it may contribute to maintenance of divergence in epicuticular composition between sympatric and allopatric populations of *D. subquinaria*. However, male mate choice likely does not contribute to the divergence in female epicuticular pheromones between inland and coastal allopatric populations of *D. subquinaria*, as there was no effect on mating rates after removing the antennae of allopatric *D. subquinaria* males (Giglio and Dyer 2013). Instead, as with the differences in males between these regions, genetic drift or divergent selection is more likely driving divergence in male epicuticular pheromones among these populations.

In summary, in combination with previous work in this system, our results suggest that females use epicuticular compounds as pheromonal signals during mate recognition and that this contributes to behavioral isolation between conspecific populations within *D. subquinaria* as well as between the species (this study; Giglio and Dyer 2013; Curtis et al. 2013). Epicuticular pheromones have also been shown to be critical to mate discrimination in other insect systems, often differing between closely related species (Jallon and David 1987; Howard et al. 1993; Coyne et al. 1994; Noor and Coyne 1996; Mullen et al. 2007; de Oliveira et al. 2011) and among recently diverged populations within a species (e.g., Etges and Ahrens 2001; Higgie and Blows 2007). Furthermore, in laboratory selection experiments these traits can evolve rapidly (e.g., Higgie et al. 2000; Rundle et al. 2005; Hunt et al. 2012), and in some cases are thought to be the target of reinforcing selection (Higgie et al. 2000; Ortiz-Barrientos et al. 2004). CHCs have also been shown to depend on diet (Etges et al. 2009; Delcourt and Rundle 2011; Gosden and Chenoweth 2011). Thus, although *D. recens* and *D. subquinaria* are thought to be generalists on mushrooms, a comprehensive understanding of the role of epicuticular compounds in mate choice and reproductive isolation in the wild will therefore also require knowledge of their diets and whether this varies among populations and between species. It will also be interesting to test whether epicuticular compounds vary depending on exposure to other species in the rearing substrate. Finally, characterizing among-population variation in mate preferences for these traits in *D. subquinaria* and *D. recens*, including testing for RCD of preferences, will be an additional important next step, with further studies aimed at providing a manipulative test of the origins of behavioral isolation, and the operation of reinforcement, in the wild.

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DATA ARCHIVED

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Loadings of the log-contrast transformed epicuticular compounds on the first two canonical variates (CV1, CV2), conducted separately by sex, discriminating among all combinations of populations and species.

Figure S1. Individual variation in epicuticular compounds of (A) female and (B) male *Drosophila subquinaria* collected from sympatric (filled symbols) and allopatric (open symbols) locations.

Table S1. Loadings of the log-contrast transformed epicuticular compounds on the first two canonical variates (CV1, CV2), conducted separately by sex, discriminating among all combinations of populations and species. Females lack any acetates and tri-acylglycerides.

			Females		Males	
Log-contrast trait[a]	Trait #[b]	ECL[b]	CV1	CV2	CV1	CV2
Acetates						
11-*cis*-Vaccenyl acetate (cVa)	1	21.90	NA	NA	0.580	2.760
Hydrocarbons						
(*Z*,*Z*)-5-9-nonacosadiene and 7-nonacosene	3	28.78	-2.285	1.083	-0.200	-0.864
5-nonacosene	4	28.85	-0.109	-2.423	1.730	-1.882
2-methyl triacontane	5	30.66	3.594	0.775	-1.394	-3.040
(*Z*,*Z*)-5-11-hentriacontadiene	6	30.74	-0.794	0.778	0.338	0.238
5-hentriacontene	7	30.81	-1.179	0.836	1.019	1.974
n-methyl dotriacontane	9	32.40	1.750	-0.364	-1.209	0.024
n-tritriacontene	10	32.47	-0.974	-0.497	0.574	-1.413
n-tritriacontene	11	32.56	1.507	-2.407	-0.682	0.806
(*Z*,*Z*)-5-13-tritriacontadiene	12	32.66	-3.567	6.335	3.379	4.713
(*Z*,*Z*)-5-11-tritriacontadiene	13	32.74	4.486	-2.648	0.639	1.656
(*Z*,*Z*)-*n*-*n*-tritriacontadiene	14	32.83	0.710	0.380	-2.819	-1.158
n-methyl tetatriacontane	17	34.40	-0.247	1.975	-1.050	0.937
n-pentatriacontene	18	34.47	-3.393	-1.542	0.457	-0.065
(*Z*,*Z*)-*n*-*n*-pentatriacontadiene	19	34.56	0.872	0.929	2.150	-0.100
(*Z*,*Z*)-5-13-pentatriacontadiene	20	34.66	1.786	1.974	-3.775	1.823
(*Z*,*Z*)-5-11-pentatriacontadiene	21	34.74	0.408	-1.349	1.336	-2.621
Tri-acylglycerides						
Tri-acylglyceride #1	15	33.46	NA	NA	0.638	-2.719
Tri-acylglyceride #2	16	33.58	NA	NA	-0.472	-0.765
Tri-acylglyceride #3	22	35.35	NA	NA	-0.918	-2.111
Tri-acylglyceride #4	23	35.44	NA	NA	0.065	-0.234
Tri-acylglyceride #5	24	35.56	NA	NA	0.190	3.248

[a]log-contrasts calculated using 2-methyl octacosane (trait #2) as the divisor
[b]Trait # and equivalent chain length (ECL) values for use in compound identification in Fig. S1 and with reference to Curtis et al. (2013)

Figure S1. Individual variation in epicuticular compounds of A) female and B) male *D. subquinaria* collected from sympatric (filled symbols) and allopatric (open symbols) locations. Axes are the first and second canonical variates from a discriminate function analysis, conducted separately by sex, which discriminated among individuals according to population. Circles depict the 95% confidence limits for the various population means. Vectors depict loadings of the log-contrast traits on each canonical variate, with labels as given in Table S1. For clarity, labels have been omitted for a few traits vectors with the lowest loadings.