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Patterns of reproductive isolation in the *Drosophila subquinaria* complex: can reinforced premating isolation cascade to other species?

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Abstract

The reinforcement of premating barriers due to reduced hybrid fitness in sympatry may cause secondary sexual isolation within a species as a by-product. Consistent with this, in the fly *Drosophila subquinaria*, females that are sympatric with *D. recens* mate at very low rates not only with *D. recens*, but also with conspecific *D. subquinaria* males from allopatry. Here, we ask if these effects of reinforcement cascade more broadly to affect sexual isolation with other closely related species. We assay reproductive isolation of these species with *D. transversa* and find that choosy *D. subquinaria* females from the region sympatric with *D. recens* discriminate strongly against male *D. transversa*, whereas *D. subquinaria* from the allopatric region do not. This increased sexual isolation cannot be explained by natural selection to avoid mating with this species, as they are allopatric in geographic range and we do not identify any intrinsic postzygotic isolation between *D. subquinaria* and *D. transversa*. Variation in epicuticular hydrocarbons, which are used as mating signals in *D. subquinaria*, follow patterns of premating isolation: *D. transversa* and allopatric *D. subquinaria* are most similar to each other and differ from sympatric *D. subquinaria*, and those of *D. recens* are distinct from the other two species. We suggest that the secondary effects of reinforcement may cascade to strengthen reproductive isolation with other species that were not a target of selection. These effects may enhance the divergence that occurs in allopatry to help explain why some species are already sexually isolated upon secondary contact.

Key words: epicuticular hydrocarbons, hybrid sterility, mate choice, reinforcement, speciation, *Wolbachia*.

Speciation is rarely an instantaneous event. When 2 populations have been diverging in allopatry, differences may accumulate so that when they come into secondary contact the hybrid offspring have reduced fitness (Dobzhansky 1937; Coyne and Orr 2004). Such postmating isolation can occur because of genetic incompatibilities that affect hybrid fertility or viability and/or because hybrid phenotypes are a poor fit to local ecological conditions or make them poor competitors for mates. As a consequence, individuals that preferentially mate with members of their own species will have increased fitness relative to those individuals that mate indiscriminately. In this way, reduced hybrid fitness can select for increased premating isolation in a process termed reinforcement (Butlin 1989; Howard 1993; Noor 1999; Servedio 2000). In addition to this classic or "narrow-sense" reinforcement, selection for increased sexual isolation may also arise from other consequences of heterospecific mating interactions such as efficient mate location or partitioning of mating signal space (Servedio and Noor 2003; Groning and Hochkirch 2008; Pfennig and Pfennig 2012). Outside of the context of mating, ecological interactions

between sympatric species (e.g., interspecific resource competition) may also drive ecological character displacement that strengthens sexual isolation as a by-product (Rundle and Nosil 2005; Stuart and Losos 2013). Based on empirical, comparative, and theoretical studies, it is now generally accepted that the reinforcement of species boundaries is not uncommon in nature and may be a key step in the latter stages of many speciation events (Coyne and Orr 2004).

Selection for increased premating isolation may not only help complete the speciation process for the now sympatric taxa, but it may also initiate secondary speciation events as a by-product in a process known as "cascade reinforcement" (Howard 1993; Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010). Cascade reinforcement may occur if sympatric females become more choosy or are better able to discriminate between conspecific and heterospecific males. This increased mate discrimination arises from changes in female sexual preferences and the male sexual displays or signals they target (Mendelson and Shaw 2012), for instance if sympatric females evolve to use population-specific rather than species-specific cues in mate discrimination (Hoskin and Higgie 2010). If these changes to mate discrimination cause sympatric females to discriminate against their own (i.e., conspecific) males from allopatry as a side effect, then sexual isolation can arise and potentially initiate a secondary speciation event in the absence of any postmating isolation between the sympatric versus allopatric populations (Howard 1993; Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010). Evidence for such cascading effects of reinforcement comes from several recent studies in insects, fish, and amphibians (e.g., Hoskin et al. 2005; Jaenike et al. 2006; Higgie and Blows 2007; Lemmon 2009; Porretta and Urbanelli 2012; Kozak et al. 2015; Pfennig and Rice 2015).

Although previously unconsidered, at a broader level the effects of reinforcement could also cascade further beyond the taxa involved. For example, if females evolve increased discrimination due to mate preferences that are more restrictive and/or target unique signals or displays that have evolved in males in sympatry, then this may also increase discrimination by these females against males from other closely related species. Reinforcement may thus have more widespread consequences via the strengthening of sexual isolation among multiple species, including those that were not the target of reinforcing selection and may not even currently be sympatric with the focal species. This may be particularly important in groups in which reproductive barriers between closely related taxa are not complete (e.g., *Drosophila*, Coyne and Orr 1989, 1997; Yukilevich 2012), including those that have not yet come into secondary contact or in rapidly diversifying lineages.

In this study, we focus on the evolution of reproductive isolation and the cascading consequences of reinforcement among three closely related members of the quinaria group of *Drosophila*: *D. subquinaria*, *D. recens*, and *D. transversa*. The quinaria group is a holarctic species complex found in temperate and boreal forests and all of their life stages revolve around mushrooms. *Drosophila subquinaria* and *D. recens* occur in western and eastern North America, respectively, and their ranges overlap for about 1,200 km eastward from the Canadian Rockies. This region of sympatry is likely a secondary contact event that occurred within the last 12,000 years since the end of the Wisconsin glaciation (Jaenike et al. 2006). Where these species co-occur, they can be found on the same mushrooms, and there are no known ecological differences between them. *Drosophila transversa* is allopatric to the other 2 species and has been found in northern and eastern Europe, Russia, Mongolia, and China (Patterson and Stone 1952; Wheeler 1960; Sidorenko 2009). All 3 species are morphologically identical except for the internal male genitalia (Wheeler 1960). A mtDNA phylogeny indicated that *D. subquinaria* and *D. recens* are most closely related and *D. transversa* is the outgroup (Perlman et al. 2003), while an analysis that used a locus on the Y chromosome suggested *D. recens* was the outgroup to the other 2 species (Dyer et al. 2011).

Patterns of reproductive isolation between *D. subquinaria* and *D. recens* are well characterized. When *D. recens* females mate with *D. subquinaria* males, the hybrids are viable and females are fertile (Shoemaker et al. 1999). However, in the reciprocal cross in which *D. subquinaria* females mate with *D. recens* males, nearly all the hybrids die as embryos due to a *Wolbachia* infection that occurs in nearly all wild *D. recens*. If the *Wolbachia* infection is cured with antibiotics, then the production of fertile daughters is restored (hybrid sons are always sterile, Shoemaker et al. 1999). The cost of mating with the wrong species is, therefore, much greater for *D. subquinaria* females than for *D. recens* females, and the patterns of premating isolation are consistent with this asymmetry in postmating isolation (Jaenike et al. 2006). *Drosophila recens* females from populations throughout the geographic range discriminate moderately against *D. subquinaria* males. In contrast, *D. subquinaria* females show a strong pattern of reproductive character displacement consistent with reinforcement: females from populations where both species occur discriminate strongly against mating with *D. recens* males, whereas females from outside this range will mate with *D. recens* at moderate rates (Jaenike et al. 2006; Bewick and Dyer 2014). Occasional hybridization occurs between species as about 2.5% of wild *D. subquinaria* harbor a *D. recens* mtDNA haplotype (implying the mating of *D. recens* females with *D. subquinaria* males, Jaenike et al. 2006; Bewick and Dyer 2014).

Selection for heightened discrimination in sympatry may have cascading effects that initiate sexual isolation within one (or both) of the species, and this is exactly what we observe in *D. subquinaria* (Jaenike et al. 2006; Bewick and Dyer 2014). The choosy sympatric *D. subquinaria* females not only discriminate against mating with heterospecific *D. recens* males, but they also mate at much reduced rates with conspecific males from allopatric populations. In contrast, allopatric *D. subquinaria* and both sympatric and allopatric populations of *D. recens* show no similar pattern. This sexual isolation within *D. subquinaria* occurs despite the absence of any detectable postzygotic barriers between conspecific crosses and the presence of some gene flow among populations that are sympatric and allopatric with *D. recens* (Jaenike et al. 2006; Bewick and Dyer 2014). Furthermore, there is indirect evidence that discrimination against *D. recens* and allopatric *D. subquinaria* males has at least some shared genetic basis (Bewick and Dyer 2014).

Based on the evidence currently available, *D. subquinaria* is an example of reinforcement in the classic sense: it appears that interactions with *D. recens* have changed the mate recognition system of sympatric *D. subquinaria*, and as a side product, these females now discriminate against their own allopatric males. We have shown that contact pheromones, which consist of epicuticular hydrocarbons and their derivatives, are important male signal traits for mate choice in *D. subquinaria* (Curtis et al. 2013; Giglio and Dyer 2013). These epicuticular hydrocarbons may be a target of reinforcing selection, as geographic variation in these compounds, as well as female preferences for them, show a pattern of reproductive character displacement that is largely consistent with the observed patterns of sexual isolation (Dyer et al. 2014; Rundle and Dyer 2015).

In this study, we investigate whether these changes in mate recognition in sympatric *D. subquinaria* cascade more broadly to alter sexual isolation with a closely related allopatric species.

Specifically, we characterize patterns of pre- and postmating isolation between *D. transversa* and both *D. subquinaria* (sympatric and allopatric) and *D. recens*. First, we ask whether allopatry of *D. transversa* from both of the other species corresponds with increased sexual isolation, or in contrast whether secondary contact and the subsequent reinforcement in sympatry have greater effects, as has been found in other *Drosophila* species (e.g., Coyne and Orr 1989, 1997; Yukilevich 2012). Second, we ask whether the reinforced mate discrimination of sympatric *D. subquinaria* females has cascaded to cause increased sexual isolation with *D. transversa* relative to that of allopatric *D. subquinaria* females. Third, we assay divergence in epicuticular compounds, which are known mating signals for *D. subquinaria* and *D. recens*, and ask whether their divergence matches the patterns of sexual isolation we observe. Finally, we assay the rate of synonymous substitutions (K_s) at 20 autosomal loci to ask if patterns of genetic divergence among these 3 species mirror patterns of pre- and postmating isolation.

Materials and Methods

Drosophila strains and rearing

We created mixed stocks to provide healthy outbred populations from which to infer general insights into mating behaviors in these species. We created a mixed stock for *D. transversa* using 6 lines from Uppsala, Sweden, for allopatric *D. subquinaria* using two 2 from Portland, Oregon, and 4 lines from Seattle, Washington, and for sympatric *D. subquinaria* using 4 lines from Hinton, Alberta, and 2 lines from Kawtikh, Alberta. We also constructed 2 mixed stocks for sympatric *D. recens*, including 1 stock with naturally *Wolbachia*-infected lines using 1 line each from Hinton, Kawtikh, and Canmore in Alberta, and 1 stock with 3 naturally *Wolbachia*-uninfected lines from Kawtikh, Alberta. Only populations that had naturally high levels of gene flow and similar cuticular hydrocarbons (CHC) composition were combined in a mixed stock (Bewick and Dyer 2014; Dyer et al. 2014). Each mixed stock was maintained for at least 3 generations before being used in experiments. *Drosophila subquinaria* and *D. transversa* are not known to be infected with *Wolbachia* in the wild, and we verified the *Wolbachia* infection status of each stock using *Wolbachia*-specific PCR.

Flies were reared at 20 °C on a 12-h light: 12-h dark schedule on Instant *Drosophila* food (Carolina Biological, Burlington, NC) supplemented with fresh commercial *Agaricus bisporus* mushroom. All flies used in experiments were reared at a controlled density. Virgins were collected using light CO_2 anesthesia within 24 h of emergence and stored 10–15 flies per vial on standard media.

Premating isolation

We used no-choice mating trials to quantify patterns of premating isolation. We paired females and males from each of 4 mixed stocks (*D. transversa*, allopatric *D. subquinaria*, sympatric *D. subquinaria*, *Wolbachia*-infected *D. recens*) for a total of 16 combinations, and used a randomized block design to obtain an average of 32 trials per combination (range 25–36). Mating trials took place in 4.5 cm long x 1 cm diameter vials that contained a blended mushroom–agar medium, and commenced within an hour of the incubator lights turning on. All flies were virgin and 7 days post adult emergence, and were transferred into the mating vials by aspiration without the use of CO_2. We observed each vial for 3 h, and if copulation occurred we noted the latency to copulation (the time from when the male was introduced to when copulation commenced) as well as the duration of the copulation.

For each species/type pair we used a logistic regression to assess the effect of female type, male type, female x male interaction, and block on mating rates. We calculated the Coyne and Orr sexual isolation index as 1 (frequency heterotypic matings/frequency homotypic matings)—for each species/type pair (Coyne and Orr 1989), where homotypic matings are within a type (i.e., within a mixed stock) and heterotypic matings are between types. We also used Fisher's exact tests (FET) to ask if females of each species/type mated with each opposite species/type less than with their own males. As a direct test of the broad-scale effects of reinforcement, we tested whether sympatric and allopatric *D. subquinaria* females mate at different rates with homotypic (i.e., from the same mixed stock) versus *D. transversa* males using a logistic regression, with female type (allopatric or sympatric), male type (homotypic or *D. transversa*), and male x female type interaction as fixed effects in the model. We excluded the interaction effect in the final analysis because it was not significant ($\chi^2_{1,1} = 0.07$, $P = 0.8$) in the initial analysis.

We tested whether copulation latency and duration differed between homotypic and heterotypic crosses using a Wilcoxon rank sum test, including only pairs that mated. We used nonparametric statistics here because the data violated the assumption of normality. Within each female type we tested whether the homotypic cross was different from each heterotypic cross using the Steel method for nonparametric comparisons. All statistical analyses were completed in JMP version 11 (SAS Institute, Cary NC).

Postmating isolation

We tested whether *D. transversa* produced viable hybrids when paired with the other species by pairing 7-day-old virgin females and males and counting the number of offspring that resulted. We placed 1 female with 2 males in a standard food vial and then transferred them to a fresh vial 5 days later. After an additional 5 days the adults were removed, and then all of the offspring from both vials were counted. In this way, we paired *D. transversa* females and males reciprocally with allopatric and sympatric *D. subquinaria* and reciprocally with *Wolbachia* infected and uninfected *D. recens*. We set up 15 replicate vials of each type of cross. We tested for reduced production of hybrid offspring of *D. transversa* with each of the 3 other species/stocks using an analysis of variance, with female type, male type, female x male interaction as fixed effects in the model. We also used unpaired *t*-tests to ask specifically if male *Wolbachia* infection in *D. recens* had an effect on offspring production. Because we did not observe the vials to ensure that matings took place, vials with no offspring could result from flies that did not mate, females that ejected sperm, or from the death of the hybrid offspring. These factors may affect whether hybrids result at all as well as how many survive to adulthood. Thus, we did these analyses both with and without the vials that produced no offspring.

We tested whether the hybrids were fertile for each of the eight heterospecific crosses. To do this, we first intercrossed the F1 male and female hybrids together (5 flies of each sex per vial, 3–11 vials/cross) and assayed for the presence of offspring. When no F2 offspring were produced we backcrossed the F1 hybrids of each sex to *D. transversa* and assayed for the presence of offspring, and we also tested whether the sperm were motile using the methods of Coyne (1984).

Epicuticular hydrocarbons

The epicuticular compounds (often called cuticular hydrocarbons or CHCs) of 7- to 9-day-old virgin flies were extracted from each of 4

isofemale lines of *D. transversa* (three from Uppsala, Sweden and one from Lahti, Finland; the latter was kindly provided by J. Jaenike), and from the mixed stocks of *D. transversa*, allopatric *D. subquinaria*, sympatric *D. subquinaria*, and *D. recens* that were used in the reproductive isolation trials. The CHCs from 8-32 flies/sex/stock were extracted by placing an individual fly in 100 uL of hexane for 3 min and then vortexing the sample for 1 min, after which the fly was removed and discarded (Dyer et al. 2014). CHCs were stored at −20 °C until they were shipped from Athens, GA, to Ottawa, ON, for analysis. Extractions were completed in a block design to minimize the effects of day, time of day, and order of extraction.

Samples were analyzed on an Agilent 6890N dual channel "fast" (220 V oven) gas chromatograph (Agilent Technologies, Wilmington, Delaware) that used H_2 as the carrier gas and employed flame ionization detection. Preliminary analyses of *D. transversa* samples using a wide temperature ramp (20–310 °C) revealed a chromatographic profile that qualitatively matched that previously observed in both *D. subquinaria* and *D. recens*, uncovering no peaks with retention times outside of those seen in these other species. Subsequent gas chromatography of all samples (i.e., *D. transversa*, *D. subquinaria*, and *D. recens*), therefore, used the method parameters previously optimized for *D. subquinaria*/*D. recens*, as described in Curtis et al. (2013).

In both sexes, the pattern of peaks in *D. transversa* precisely matched that seen in both *D. subquinaria* and *D. recens*. Individual profiles were, therefore, determined by integration of the area under 13 peaks corresponding to those hydrocarbons previously identified and quantified in male and female *D. subquinaria* and *D. recens* (Supplementary Figure S1). In *D. subquinaria* and *D. recens*, these consist of 13 long-chain hydrocarbons composed of odd carbon numbers (C_{29}, C_{31}, and C_{33}) that include several methyl-branched alkanes, alkenes, and alkadienes, all of which are shared between the sexes. The terminal group of five 35-carbon compounds (peaks 17–21 in Supplementary Figure S1) were not integrated because an unidentified problem with the gas chromatograph caused poor resolution of these peaks in the majority of samples. The male-specific 11-*cis*-Vaccenyl acetate (cVa) and five triacylglycerides were also integrated. However, our data analyses revealed very similar multivariate patterns in males whether or not these additional compounds were included (H.D. Rundle, unpublished results), so we present results for only the 13 CHCs that are shared between the sexes, thereby allowing species and sex-specific variation to be quantified in the same phenotypic space.

After integration, the relative concentration of each CHC was calculated by dividing the area under each peak by the total area of all peaks for a given individual. This corrects for technical error associated with quantifying absolute abundances via gas chromatography. Proportions such as these are a form of compositional data to which standard statistical methods should not be applied (Aitchison, 1986). Following past studies (Bonduriansky et al. 2015; Rundle and Dyer 2015), we calculated centered log-ratio (CLR) coefficients as:

$$CLR_n = ln\left(\frac{p_n}{\left(\prod_{n=1}^{k} p_n\right)^{1/k}}\right), \quad (1)$$

where p_n is the relative concentration (i.e., proportional area) of CHC_n and the divisor is the geometric mean of the proportions of all $k = 13$ CHCs within an individual (Aitchison 1986). A multivariate analysis of variance was then used to test for differences in CLR-transformed CHCs between the sexes, species/type (i.e., *D. recens*, allopatric *D. subquinaria*, sympatric *D. subquinaria*, and *D. transversa*), and the interaction of these effects. Multivariate CHC variation between the sexes and among species/types was then visualized using the canonical variates from a discriminant function analysis that discriminated CHCs by all 8 combinations of sex and species/type. The discriminant function analysis was performed using all individuals of both sexes from all 4 species/types, and then repeated using only *D. subquinaria* and *D. transversa* males and females to depict finer-scale variation among these groups.

Genetic divergence

To assess genetic divergence between strains/species, we sequenced 20 protein-coding autosomal loci from a single strain each of *D. transversa*, allopatric *D. subquinaria*, sympatric *D. subquinaria*, and allopatric *D. recens*. DNA sequencing used standard methods. These loci span all of the autosomal Muller Elements as characterized in *D. melanogaster* (www.flybase.org); there is a general conservation of locus to Muller Element across the *Drosophila* genus (Bhutkar et al. 2008). In total, we sequenced 2,607 synonymous sites from each strain, with an average of 130 synonymous sites/locus (Supplementary Table S2). For each locus we calculated synonymous divergence (K_s) between each pair of strains using a Jukes–Cantor correction, as implemented in MEGA (Tamura et al. 2013).

We first used an analysis of variance to ask whether *D. recens* had higher genetic divergence relative to the other species. We included whether or not a species pair included *D. recens* and the species pair combination nested with this as fixed effects in the model. We also used an analysis of variance to ask whether divergence was different between the 3 pairwise combinations of sympatric *D. subquinaria*, allopatric *D. subquinaria*, and *D. transversa*.

Results

Premating isolation

For five of six species/type combinations we identified a highly significant female × male interaction effect due to the reduced mating of heterotypic pairs (each LRT $\chi^2_1 > 10$ and each $P < 0.0003$; Supplementary Figure S2), indicating the presence of strong premating reproductive isolation. The exception was *D. transversa* and allopatric *D. subquinaria*, where interspecific pairs do not mate significantly less than conspecific pairs (female × male interaction LRT $\chi^2_1 = 2.4$, $P = 0.13$). The Coyne and Orr index of isolation was very low for this pair (0.12), and was highest for *D. recens* and sympatric *D. subquinaria* (1.0), for which we never observed any heterospecific matings. For the remaining pairs the sexual isolation index was between 0.48 and 0.60 (Supplementary Figure S2), with sympatric *D. subquinaria* approximately equally isolated from allopatric *D. subquinaria* (0.51) and *D. transversa* (0.48).

For each female type, we also compared the mating rate of each heterotypic cross to homotypic males (Figure 1). For pairings that included *D. transversa* females, the mating rate was reduced only with *D. recens* males (FET $P < 0.0001$); *D. transversa* females mated with allopatric and sympatric *D. subquinaria* about the same amount (FET $P = 0.3$). *Drosophila subquinaria* allopatric females also had a reduced mating rate only when paired with *D. recens* males ($P = 0.0014$; Figure 1). In contrast, matings of sympatric *D. subquinaria* and *D. recens* females were reduced against all other male types except their own (FET $P < 0.004$ for each; Figure 1). The frequency of matings between sympatric *D. subquinaria* females and

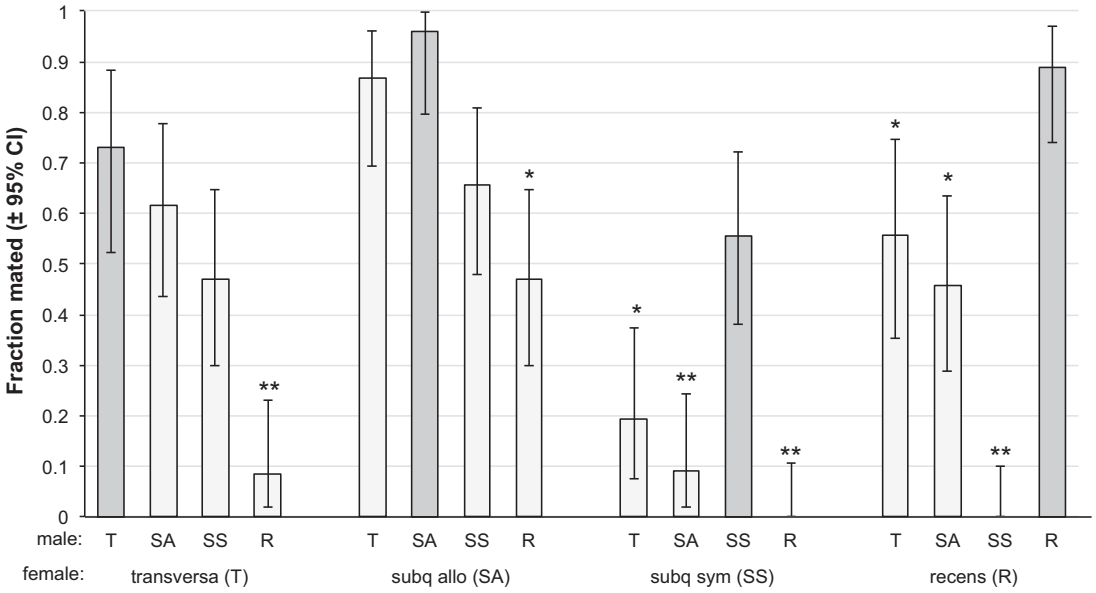

Figure 1. Premating isolation by female species/type. *D. subquinaria* allopatric and sympatric types are noted "subq allo" and "subq sym," respectively. Homotypic pairings (i.e., within a type/strain) are shaded in dark gray and heterotypic pairings (i.e., between types/strains) are in light gray. The sample size for each cross type is 25–36 pairs, and the error bars indicate the 95% confidence intervals, as calculated using a binomial distribution. Each heterotypic pair was compared to the homotypic pair that used the same female type with a Fisher's Exact Test; significance is indicated as * ($P < 0.004$) and ** ($P < 0.0001$), where $\alpha = 0.05/12 = 0.0041$ with a Bonferroni correction.

allopatric *D. subquinaria* versus *D. transversa* males was similar (FET $P = 0.3$; Figure 1), and it was also similar between *D. recens* females and *D. transversa* versus allopatric *D. subquinaria* males (FET $P = 0.6$). We note that while males courted females no matter the cross (D. Humphreys, pers. obs.), in a no-choice mating scenario the mating rate may reflect not only female but also male choice.

As a direct test for the broader cascading effects of reinforcement, we compared the mating rate of sympatric and allopatric *D. subquinaria* females with *D. transversa* versus homotypic (i.e., their own) males. We found a significant male effect (*D. transversa* versus the homotypic male; LRT $\chi^2_1 = 11$, $P = 0.0009$), which is due to a high rate of mating of allopatric but not sympatric *D. subquinaria* with *D. transversa* males. This pattern is expected if changes in female mate preferences of sympatric *D. subquinaria* extend to *D. transversa* males. The female effect was also significant (LRT $\chi^2_1 = 45$, $P < 0.0001$), which was due to the overall lower mating rates of sympatric *D. subquinaria*.

Across all species/type combinations, homotypic pairs that mated had a shorter latency to copulation than did the heterotypic pairs (homotypic median = 6 min, heterotypic median = 15 min; Wilcoxon rank sum test $\chi^2_1 = 23$, $P < 0.0001$; Supplementary Figure S3). The homotypic crosses also copulated for a longer duration than did heterotypic crosses (homotypic median = 11 min, heterotypic median = 10 min; Wilcoxon rank sum test $\chi^2_1 = 7.9$, $P = 0.005$; Supplementary Figure S4). With respect to cascade reinforcement of sympatric *D. subquinaria* with *D. transversa*, the copulation latency of sympatric and allopatric *D. subquinaria* females was not significantly different when paired with homotypic versus *D. transversa* males (Wilcoxon rank sum test, each $P > 0.1$). The copulation duration of allopatric *D. subquinaria* with *D. transversa* males was significantly longer than with homotypic males (Wilcoxon rank sum test $\chi^2_1 = 9.4$, $P = 0.002$), but there was no statistical difference in the copulation duration of sympatric *D. subquinaria* males with *D. transversa* versus homotypic males (Wilcoxon rank sum test $\chi^2_1 = 1.2$, $P = 0.28$). We note that the sample sizes of sympatric *D. subquinaria* that mated with heterotypic males are small, which limits the power of these analyses.

Postmating isolation

There was no evidence of post-mating isolation between *D. transversa* and *D. subquinaria*. When *D. transversa* is paired with allopatric *D. subquinaria*, hybrid crosses in both directions produced a similar number of offspring as the parental crosses (male × female interaction: $F_{1,55} = 0.18$, $P = 0.67$ using all replicate vials; $F_{1,48} = 0.05$, $P = 0.81$ including only vials with offspring; Figure 2). When *D. transversa* was paired with sympatric *D. subquinaria*, there was no evidence of postmating isolation (male × female interaction: $F_{1,56} = 1.6$, $P = 0.22$ using all replicate vials; $F_{1,37} = 0.03$, $P = 0.85$ including only vials with offspring), but sympatric *D. subquinaria* females produced fewer offspring overall (female type: $F_{1,56} = 13$, $P = 0.0005$ including vials without offspring; $F_{1,37} = 11$, $P = 0.0016$ including only vials with offspring). Furthermore, with both allopatric and sympatric types, and in both reciprocal directions, the male and female hybrid offspring were fertile, as every F1 intercross produced an abundance of offspring ($n = 6$–11 vials/cross).

Post-mating isolation between *D. transversa* and *D. recens* depends on the direction of the cross and the *Wolbachia* infection status of *D. recens*. For either *Wolbachia*-infected or uninfected *D. recens* crossed with *D. transversa*, the only significant effect is the male x female interaction when all vials are included (*Wolbachia* infected: $F_{1,56} = 28$, $P < 0.0001$; *Wolbachia* uninfected: $F_{1,56} = 5.4$, $P = 0.003$). The low mating rate between these species likely affected the results: when only vials that produced offspring are included, this effect (and the general model) is no longer significant (whole model $F_{3,26} = 1.8$, $P = 0.18$). However, for infected *D. recens* crossed with *D. transversa*, both the male and interaction terms are significant (male: $F_{1,39} = 6.9$, $P = 0.012$; interaction: $F_{1,39} = 29$,

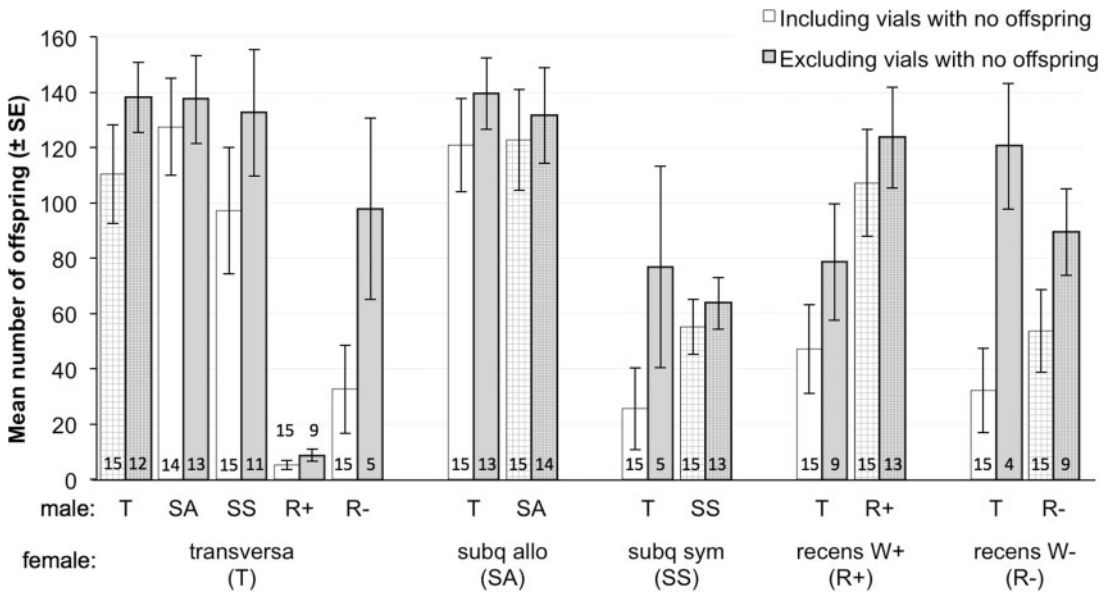

Figure 2. Mean offspring counts from crosses with *D. transversa,* separated by female type. *D. subquinaria* allopatric and sympatric types are noted "subq allo" and "subq sym," respectively. *Drosophila recens* stocks with and without *Wolbachia* infection are noted W+ and W-, respectively. Homotypic crosses (i.e., within a type) have grid shading, and heterotypic crosses (i.e., between types) are solid in color. Samples sizes are shown within each bar; white bars include the vials that did not produce any offspring, and grey bars exclude these vials.

$P < 0.0001$). This is due to lower hybrid production, which is especially pronounced when *D. transversa* females mate with *Wolbachia*-infected *D. recens* males (Figure 2). This is suggestive of cytoplasmic incompatibility due to *Wolbachia* infection. Additional observations support the presence of unidirectional cytoplasmic incompatibility due to *Wolbachia* infection in *D. recens*. First, we find that many fewer offspring were produced from *D. transversa* females × *Wolbachia*-infected *D. recens* males compared to *Wolbachia*-uninfected *D. recens* (t-test $t_{12} = 3.8$, $P = 0.0028$ excluding vials without offspring; $t_{28} = 1.7$, $P = 0.09$ including all replicate vials; Figure 2). Furthermore, this pattern is asymmetric, as the offspring produced by the reciprocal cross (*D. recens* female × *D. transversa* male) did not differ significantly depending on whether the female was infected with *Wolbachia* (*t*-test $t_{11} = 1.2$, $P = 0.3$ excluding vials without offspring; $t_{28} = 0.7$, $P = 0.5$ including all replicate vials).

Finally, when hybrids are produced between *D. transversa* and *D. recens*, the males are sterile. When we intercrossed the hybrid males and females no offspring were produced from any of the four cross types ($n = 3$–10 vials/cross). The hybrid females produced many offspring when backcrossed to pure *D. transversa* males and were thus fertile. The hybrid males produced no offspring, and dissection showed they lack any mature motile sperm (> 10 F1 males dissected/cross). This pattern of unidirectional cytoplasmic incompatibility and hybrid male sterility is the same that is seen between *D. subquinaria* and *D. recens* (Shoemaker et al. 1999).

Epicuticular hydrocarbons

The chromatographic profiles of male and female *D. transversa* corresponded qualitatively to that of both *D. subquinaria* and *D. recens* (Supplementary Figure S1), strongly suggesting that the same epicuticular compounds are all shared among these three species. Despite qualitative similarity of CHC identity, the relative concentrations varied significantly between the sexes and among the species (MANOVA, sex × type interaction: Wilks' lambda = 0.208, $F_{26,414} = 18.95$, $P < 0.0001$). The dominant axis of among sex and species/type variation distinguished *D. recens* individuals (males and females) from the other 2 species (Figure 3A; Supplementary Table S1). Within *D. subquinaria* and *D. transversa*, the first canonical variate largely reflected the effect of sex, with males of all three types having lower values than females, although it also separated *D. transversa* from allopatric *D. subquinaria* (Figure 3B; Supplementary Table S1). Sympatric *D. subquinaria* differed from allopatric *D. subquinaria* and *D. transversa* along the second canonical variate, with males being more divergent than females.

Genetic divergence

Average pairwise synonymous divergence (K_s) was highest between pairs involving *D. recens*, suggesting *D. recens* is the outgroup to *D. subquinaria* and *D. transversa* (mean $K_{s(R\text{-}T)} = 0.081$, $K_{s(R\text{-}SA)} = 0.079$, $K_{s(R\text{-}SS)} = 0.069$; Figure 4; Supplementary Table S2). This higher divergence of *D. recens* is statistically significant ($F_{1,114} = 8.0$, $P = 0.0055$), and variation among species pairs within this effect were not significantly different ($F_{4,114} = 0.4$; $P = 0.8$). We also found that the pairwise divergence among sympatric *D. subquinaria*, allopatric *D. subquinaria*, and *D. transversa* were not statistically different from each other (mean $K_{s(T\text{-}SA)} = 0.053$, $K_{s(T\text{-}SS)} = 0.060$, $K_{s(SA\text{-}SS)} = 0.050$; $F_{2,57} = 0.24$, $P = 0.48$; Figure 4).

Discussion

The reinforcement of premating barriers in sympatry may cause secondary premating isolation as a byproduct (Howard 1993; Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010). In *D. subquinaria*, females that are sympatric with *D. recens* mate at very low rates not only with *D. recens*, but also with conspecific *D. subquinaria* males from allopatry (Jaenike et al. 2006; Bewick and Dyer 2014). Here, we find evidence that the effects of reinforcement between *D. subquinaria* and *D. recens* cascade more broadly to affect sexual isolation with the closely related species *D. transversa*. Specifically,

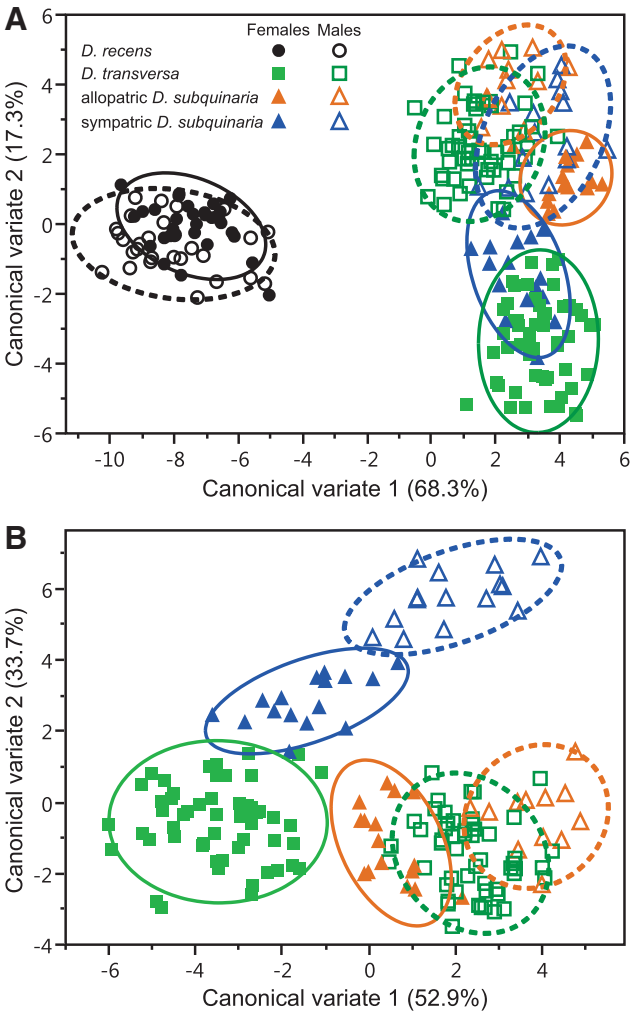

Figure 3. Among-individual variation in epicuticular compounds (CHCs). Analyses include females (filled symbols/solid lines) and males (open symbols/dashed lines) of **(A)** all four species/types and **(B)** only *D. subquinaria* (allopatric and sympatric) and *D. transversa*. Axes are the first and second canonical variates from separate discriminant function analyses that discriminated among individuals according to species/type and sex. Lines are 90% bivariate normal density ellipses.

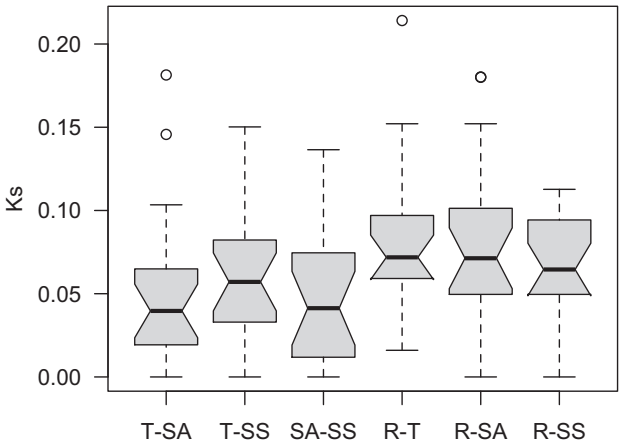

Figure 4. Pairwise synonymous divergence (K_s) across 20 autosomal loci. Notched Tukey boxplot where the bold horizontal line indicates the median, the box indicates the 25th and 75th percentiles, the whiskers indicate 1.5 * the interquartile range, and the open circles indicate outliers. Species are abbreviated *D. transversa* (T), allopatric *D. subquinaria* (SA), sympatric *D. subquinaria* (SS), and *D. recens* (R).

allopatric *D. subquinaria* females mate with *D. transversa* males as readily as with their own males, while sympatric *D. subquinaria* females discriminate as strongly against *D. transversa* males as they do against males from allopatric *D. subquinaria* and nearly as strong as against *D. recens*. Since *D. transversa* is completely allopatric to sympatric populations of *D. subquinaria*, this increased discrimination is not due to natural selection on these females to avoid mating with *D. transversa* males. In addition, the hybrids of crosses between *D. transversa* and *D. subquinaria* are abundant and both sexes are fertile, suggesting the absence of intrinsic postzygotic isolation required for reinforcement (although ecologically dependent forms have not been tested).

We also characterized the patterns of reproductive isolation between *D. transversa* with *D. recens*, which were not previously known. We find that *D. transversa* females mate at a low frequency with *D. recens* males, whereas they mate with allopatric and sympatric *D. subquinaria* at a rate only slightly reduced relative to conspecific males (Figure 1). In the reciprocal direction, *D. recens* females mate a moderate amount with *D. transversa* males, similar to their acceptance of allopatric *D. subquinaria* males. At the postmating level, *D. transversa* and *D. recens* show a pattern of hybrid male sterility and asymmetric hybrid death consistent with *Wolbachia*-induced cytoplasmic incompatibility (though egg hatching rates have not been measured). In contrast, there appears to be no intrinsic genetic postzygotic isolation between *D. transversa* and *D. subquinaria* (Figure 2).

Male epicuticular compounds (CHCs) are a mating signal used by female *D. subquinaria* (Curtis et al. 2013; Giglio and Dyer 2013; Dyer et al. 2014) and thus we hypothesized that variation in CHCs should largely reflect patterns of premating isolation. Two observations are consistent with this. First, the dominant axis of CHC variation among sexes and types distinguished between *D. recens* and *D. transversa*/*D. subquinaria* (Figure 3A). This is consistent with the reduced mating observed between *D. transversa* and *D. subquinaria* females and *D. recens* males (Figure 1). Second, males of *D. transversa* and allopatric *D. subquinaria* are more similar to each other than they are to sympatric *D. subquinaria* males, especially for the second canonical variate (Figure 3B). This is consistent with our finding that sympatric *D. subquinaria* females mate at reduced rates with both of these types of males, and would be expected if these choosy females used the same male signal trait to discriminate against each type of male. However, variation in CHCs does not explain all the patterns of premating isolation that we see. For instance, matings between *D. recens* females and allopatric *D. subquinaria* will occasionally occur in spite of the divergence in their CHCs. It is, therefore, likely that mating signals in these species involve traits beyond just CHCs (see Giglio and Dyer 2013; Rundle and Dyer 2015).

Reinforcement may not only select for changes in female preferences (e.g., Hoskin et al. 2005; Pfennig and Ryan 2006; Kozak et al. 2015), but may also result in changes in the male mating signals which females use to choose mates. Sexual isolation may thus also be strengthened if allopatric females reject the altered male signal traits of sympatric males (e.g., Higgie and Blows 2007; Higgie and Blows 2008). Our behavioral and CHC data support a scenario in which the mate preferences of sympatric *D. subquinaria* females

have become more specific or restrictive, but that the sympatric *D. subquinaria* males have not changed their mating signals in a way that renders them highly unattractive to allopatric *D. subquinaria* or *D. transversa* females. Thus, in this system, the effects of reinforcement have cascaded only in one direction: sympatric *D. subquinaria* females have narrowed their preferences substantially, and while sympatric *D. subquinaria* males have altered their sexual displays (Dyer et al. 2014), they are still within the range that is accepted by the other types of females in no-choice situations (this study; Bewick and Dyer 2014). The topic of male mate choice has not been investigated in this system, but it may also contribute to patterns of isolation. While *D. subquinaria* males will court and mate with females from any other population in no choice trials, it would be interesting to ask if males prefer to mate with females from their own population and also if males differ in the suite of female CHCs they prefer. No-choice trials as conducted here reflect both male and female mate preferences, and whether more sensitive multiple-choice mating trials may detect any additional isolation remains to be determined.

Our study used strains of *D. transversa* that are allopatric and very geographically distant from *D. subquinaria*, but the ranges of these 2 species are not fully described and the potential for sympatry exists. *D. subquinaria* is thought to extend northward into Alaska (Wheeler 1960), but to our knowledge there has been no recent sampling north of Vancouver, British Columbia. *D. transversa* has been described as being present in eastern Russia (Sidorenko 2009), although again there is a lack of recent sampling in this region. It will be important to survey these regions, as well as around the Bering Sea and the Aleutian Islands, to determine if there are areas of sympatry and if so, how much gene flow occurs and whether a pattern of reproductive character displacement is present. These were initially described as different species based on the morphology of the internal genitalia (specifically the shapes of the aedeagus and hypandrium shelf, Wheeler 1960). At this time, the possibility exists that allopatric *D. subquinaria* and *D. transversa* are a single biological species with a continuous range over which CHCs show some variation.

Patterns of genetic divergence have not been well characterized in these species, but may shed light on patterns of reproductive isolation and the species status of allopatric *D. subquinaria* and *D. transversa*. We sequenced 20 autosomal loci from each strain/species, and the results follow the patterns of postzygotic isolation and variation in CHCs discussed above. First, the data support a model where *D. recens* is the outgroup to *D. transversa* and *D. subquinaria*. This pattern is consistent with data from the Y chromosome (Dyer et al. 2011) but contrary to previous results from the mtDNA (Perlman et al. 2003). However, mtDNA has been shown to introgress repeatedly from *D. recens* into *D. subquinaria* and thus it may not reveal the true species history (Jaenike et al. 2006). Second, we find that the heightened mate discrimination of female sympatric *D. subquinaria* is not simply due to increased genetic divergence. Further work on the demographic history of these species is necessary, as patterns of divergence are likely complicated by ongoing and past hybridization as well as incomplete lineage sorting due to their generally large effective population sizes. An investigation that incorporates divergence between species and polymorphism within species across regions of the genome that vary in effective population size is therefore underway.

Based on results from comparative studies, premating isolation is expected to be strong when a species pair is sympatric or when genetic divergence is high (Coyne and Orr 1989, 1997; Yukilevich 2012). Here we show that mate discrimination can be high even when a species pair is allopatric and genetic divergence is low. Previous studies suggest that selection arising from the presence of *D. recens* has caused changes within *D. subquinaria* such that females sympatric with *D. recens* now differ in species discrimination, sexual displays, and mate preferences (Jaenike et al. 2006; Bewick and Dyer 2014; Dyer et al. 2014; Rundle and Dyer 2015). This selection has also likely caused these females to discriminate against their own allopatric males as a side effect. We show that these changes in the mate recognition system appear to cascade more broadly to affect sexual isolation with the closely related and allopatric species *D. transversa*. In sum, we suggest that the secondary effects of reinforcement could cascade more broadly than previously appreciated, especially in rapidly diversifying groups, to include not only diversification within the focal species but also strengthening of isolation with other species that were not a target of selection. This process may augment the basal divergence that occurs in allopatry and explain why some species are already reproductively isolated upon secondary contact.

Supplementary Material

Supplementary material can be found at http://www.cz.oxfordjournals.org/

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