



# Reinforcement shapes clines in female mate discrimination in *Drosophila subquinaria*

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Reinforcement of species boundaries may alter mate recognition in a way that also affects patterns of mate preference among conspecific populations. In the fly *Drosophila subquinaria*, females sympatric with the closely related species *D. recens* reject mating with heterospecific males as well as with conspecific males from allopatric populations. Here, we assess geographic variation in behavioral isolation within and among populations of *D. subquinaria* and use cline theory to understand patterns of selection on reinforced discrimination and its consequences for sexual isolation within species. We find that selection has fixed rejection of *D. recens* males in sympatry, while significant genetic variation in this behavior occurs within allopatric populations. In conspecific matings sexual isolation is also asymmetric and stronger in populations that are sympatric with *D. recens*. The clines in behavioral discrimination within and between species are similar in shape and are maintained by strong selection in the face of gene flow, and we show that some of their genetic basis may be either shared or linked. Thus, while reinforcement can drive extremely strong phenotypic divergence, the long-term consequences for incipient speciation depend on gene flow, genetic linkage of discrimination traits, and the cost of these behaviors in allopatry.

**KEY WORDS:** Hybridization, mate choice, selection, speciation.

When closely related species come into secondary contact following divergence, the opportunity for hybridization may arise. Natural selection can strengthen premating isolation in zones of sympatry in response to reduced hybrid fitness (Dobzhansky 1951), and this reinforcement of species boundaries may be a key step to completing the speciation process when postmating barriers are incomplete (Howard 1993; Noor 1999; Servedio and Noor 2003; Coyne and Orr 2004). There is now considerable theoretical and empirical evidence for reinforcement (reviewed in Coyne and Orr 2004), and recent work suggests it may play an important role in the diversification of certain taxa, for example, fruit flies (e.g., Yukilevich 2012).

Natural selection to avoid mating with the wrong species may alter mate recognition systems in a way that introduces effects on mate choice within species (Howard 1993; Coyne and Orr 2004; Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010; Mendelson and Shaw 2012). This has been observed in some frogs and *Drosophila* species, in which sympatric females reject heterospecific males, but also will not mate with conspecific allopatric

males (Hoskin et al. 2005; Jaenike et al. 2006). For instance, this pattern may result if females that co-occur with the other species evolve to use population-specific rather than species-specific cues in mate discrimination (Hoskin and Higgie 2010). Reinforcing selection may also alter sympatric male mating signals in a way that makes them less attractive to allopatric females, which has been found in *Drosophila serrata* (Higgie and Blows 2007). Thus, natural and sexual selection may interact during reinforcement, with a possible outcome of secondary isolation or “cascade reinforcement,” whereby the processes that complete speciation between two species also initiate isolation within a species (Howard 1993; Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010). We are in the early stages of understanding how widespread these effects are, but if they are common their consequences for diversity may be significant.

Thus far, the genetic basis of reinforced discrimination has been identified in only a few systems, and there is no case of cascade reinforcement in which the genes that underlie both reinforced and within-species discrimination are known. Even

without the genetic loci in hand, assaying the phenotypic distribution of reinforced discrimination through a cline from allopatry to sympatry with the other species may give valuable information about the nature and strength of selection on mate discrimination (Jiggins and Mallet 2000; Gay et al. 2008), inform what maintains differences in mate discrimination between sympatric and allopatric populations, and provide information about the trait's genetic basis (Barton and Hewitt 1985; Barton and Gale 1993). This approach has not been used to understand the process of reinforcement, but mirrors studies that have assayed variation in traits related to reproductive isolation across the hybrid zone of two diverging species (e.g., Orr 1996; Gay et al. 2008; Gompert et al. 2010; Bimova et al. 2011). The shape of the cline for a given trait is determined by the strength of selection against hybrids, the extent of gene flow across the cline, and the amount of time since contact (Endler 1977). A narrow cline compared to neutral expectations suggests that selection maintains the cline despite gene flow (Lewontin and Krakauer 1973). For example, in the case of a tension zone, selection against hybrids is balanced by dispersal of parental types into the zone (Barton and Hewitt 1989).

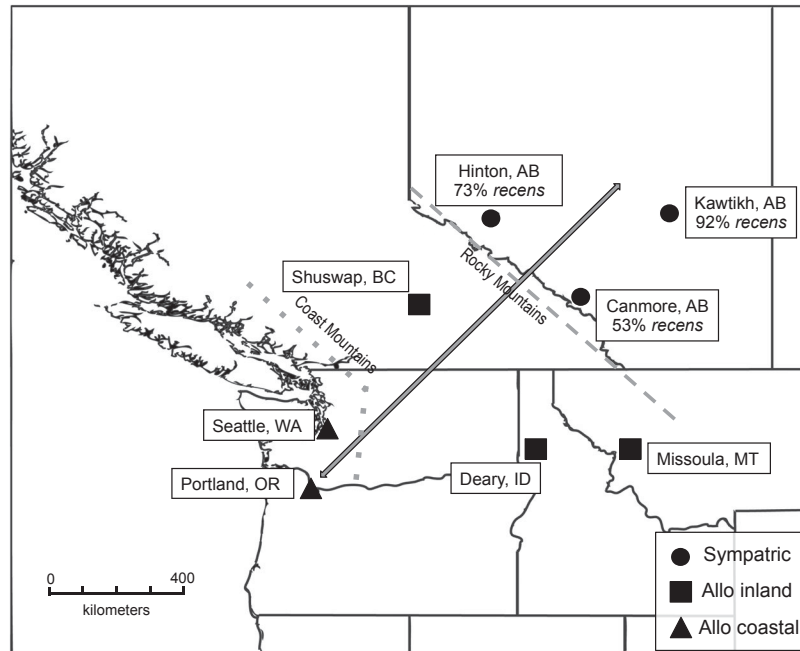
Comparing clines of different traits can also inform our understanding of how these traits are related to each other. If secondary isolation (i.e., mate discrimination among populations within a species) is a byproduct of reinforcement of species boundaries, we would expect that clines and patterns of variation in intra- and interspecific discrimination would mirror each other. Clines for different traits may become coupled if the same genes underlie the two traits or if different but linked genes are involved. Even without a genetic relationship between the traits, cline coupling can result from similar strength and type of selection on each cline. On the other hand, clines may also be coupled because different isolating barriers tend to attract each other. For instance, tension zones move toward environmental barriers, and different types of incompatibilities can be coupled together to strengthen overall isolation (Barton and Bengtsson 1986; Barton and Gale 1993; Barton and de Cara 2009; Bierne et al. 2011).

To date, there has been very little work to understand how much genetic variation segregates within sympatric and allopatric populations for reinforced discrimination against another species. Most studies focus on patterns of mate discrimination of sympatric versus allopatric populations, and often either combine multiple genetic lines to obtain a snapshot of a population or assay only one or a few lines from a population. To better understand the causes and consequences of selection for reinforced discrimination as well as its downstream effects on patterns of sexual isolation within species, in this study we use a cline theory approach to study the process of reinforcement in the fly *Drosophila subquinaria*. This species shows a pattern of reinforcement against its sister species, *D. recens*, as well

as behavioral isolation among conspecific populations (Jaenike et al. 2006). *Drosophila subquinaria* and *D. recens* occur in western and eastern North America, respectively, and are in secondary contact for about 1200 km east of the Rocky Mountain range in Canada. These species are morphologically nearly identical and have no known ecological differences; in sympatric populations they can be found on the same mushrooms that are used for all life stages. Where these species co-occur, *D. subquinaria* females discriminate strongly against *D. recens* males, whereas *D. subquinaria* females from allopatric populations will mate with *D. recens* males at a moderate frequency (Jaenike et al. 2006). Reinforcing selection on *D. subquinaria* may be due to a *Wolbachia* infection in *D. recens*, which causes offspring to die as a result of cytoplasmic incompatibility when a *D. subquinaria* female mates with a *D. recens* male (Werren and Jaenike 1995; Shoemaker et al. 1999). When *Wolbachia* is cured from *D. recens*, the resulting hybrid male offspring are sterile, indicating these are distinct species.

*Drosophila subquinaria* females from the region of sympatry with *D. recens* also strongly discriminate against conspecific males from allopatric populations (Jaenike et al. 2006). This isolation is primarily behavioral, as there are no known postzygotic effects based on laboratory assays of fertility of F1 and F2 individuals from intraspecific crosses (Jaenike et al. 2006; K. Dyer, unpubl. data). The postzygotic effects in between-species crosses but not within-species suggest that the behavioral isolation between conspecific populations may be a byproduct of reinforcement (i.e., cascade reinforcement), rather than the other way around. The basis for this female mate discrimination may be epicuticular compounds that have been shown as important signals in mate recognition both within- and between-species, and which show reproductive character displacement among populations that are sympatric versus allopatric with *D. recens* (Curtis et al. 2013; Giglio and Dyer 2013; Dyer et al. 2014).

In this study, we explore geographic patterns of variation of behavioral isolation in *D. subquinaria* to investigate the relationship between inter- and intraspecific reproductive isolation and patterns of selection on these traits. We measure female discrimination of *D. subquinaria* both within and among populations against *D. recens* males and *D. subquinaria* males, and ask: (1) How much variation is there in female mate discrimination, both within and among populations? (2) How do these behaviors change across the geographic range, from allopatry into sympatry with *D. recens*? (3) How steep are the geographic clines in discrimination behaviors, and what is the strength of selection on these traits? and (4) is there evidence of a shared genetic basis or genetic linkage between these behaviors? Throughout, we address these questions within the framework of cline theory and the genetic structure among populations of *D. subquinaria*.



**Figure 1.** Relative locations of populations used in this study. Sympatric populations are represented by circles, with the percent of each collection that was *Drosophila recens* noted within the label. Inland allopatric and coastal allopatric sites are represented by squares and triangles, respectively. Gray dotted and dashed lines indicate significant population genetic breaks, which correspond with the Coast and Rocky Mountains, respectively. The gray arrow indicates the 50° transect angle used to construct clines.

**Table 1.** Summary of the populations used in this study.

Site	Abbr.	Region	Longitude	Latitude	Year	Percentage of <i>Drosophila recens</i> among Wild Flies (Total <i>recens</i> and <i>subquinaria</i> )	<i>N</i> of mtDNA	<i>N</i> of Microsats	<i>N</i> of Mating Trial Lines
Portland, OR	POR	allo-coast	-122.68	45.52	2010	0 (70)	18	56	5
Seattle, WA	SEA	allo-coast	-122.33	47.61	2010	0 (40)	11	53	5
Deary, ID	DEA	allo-inland	-116.56	46.80	2009/2011	0 (7)	4	4	3
Missoula, MT	MIS	allo-inland	-113.99	46.87	2010/2011	0 (13)	12	56	13
Shuswap, BC	SHU	allo-inland	-119.71	50.79	2010	0 (4)	4	8	4
Canmore, AB	CAN	sympatric	-115.35	51.09	2010	53 (20)	6	19	2
Hinton, AB	HIN	sympatric	-117.57	53.41	2010	73 (118)	18	34	16
Kawtikh, AB	KAW	sympatric	-112.93	53.43	2010	92 (390)	13	32	7

For each population, the location, abbreviation (Abbr.), and region with respect to overlap with *D. recens* is indicated. For each allopatric (allo) population, we indicate whether it is to the west (coast) or east (inland) of the Coast Mountains. Also shown is the abundance of *D. recens* relative to *D. subquinaria* at the time of collection with the total number of flies collected of both species, the number of alleles sampled for mtDNA sequencing and microsatellite genotyping, and the number of isofemale lines assayed for behavior.

## Materials and Methods

### FLY COLLECTIONS AND SPECIES DIAGNOSIS

Flies were collected from natural populations during the summers of 2009–2011 by sweep netting over baits of commercial mushrooms (Fig. 1, Table 1). We established laboratory isofemale lines, and maintained cultures at 20°C on a 12-h light:12-h dark cycle

on Instant *Drosophila* Medium (Carolina Biological, Cary, NC) supplemented with commercial *Agaricus bisporus* mushrooms. To identify flies as *D. recens* or *D. subquinaria*, we used multiple molecular markers. Nearly all *D. recens* harbor *Wolbachia* (Shoemaker et al. 1999; Jaenike et al. 2006), which we screened for by polymerase chain reaction (PCR) of the *wsp* gene (Zhou

et al. 1998). Using population genetic data of individuals of known species composition (Jaenike et al. 2006; Dyer et al. 2011; K. Dyer, unpubl. data), we designed restriction fragment assays for the mtDNA gene *Cytochrome Oxidase I (COI)* and the Y-linked gene *kl-3* based on fixed differences between species.

### PATTERNS OF POPULATION GENETIC DIFFERENTIATION

We characterized patterns of genetic diversity and population differentiation of *D. subquinaria* at the mtDNA *COI* gene and at eight autosomal unmapped microsatellites. In most cases, we used wild-caught individuals; for a few populations, we increased the sample size by adding single flies from isofemale lines, and for these individuals, we randomly sampled one allele per microsatellite locus (Table 1). We sequenced 549 bp of *COI* as described previously (Jaenike et al. 2006), and we did not sequence any *D. subquinaria* that carried a *D. recens* mtDNA haplotype. The eight microsatellite markers are listed in Table S1 and were initially developed from *D. recens* (Ross et al. 2003). To genotype microsatellite loci, we used the three-primer fluorescent amplification method of Shuelke (2000) as implemented in Miles et al. (2008). Amplicons were run concurrent with a size standard, and fragments were scored with GeneMarker (SoftGenetics, State College, PA).

We calculated allelic richness, observed and expected heterozygosity, and tested for departures from Hardy–Weinberg Equilibrium (HWE) and for linkage disequilibrium (LD) using Arlequin version 3.5.1.2 (Excoffier and Lischer 2010), with significance determined by 10,000 permutations of the data. Based on these results, we used all eight microsatellite loci in our analyses because we found no consistent deviation from HWE or LD between pairs of loci across populations (Table S2). We estimated pairwise  $F_{ST}$  using Arlequin and pairwise  $S_{nn}$  using DnaSP version 5.10.01 (Librado and Rozas 2009). We resolved an mtDNA haplotype network using SplitsTree (Huson and Bryant 2006), implementing a neighbor-joining algorithm and a Kimura two-parameter model. We also investigated patterns of genetic differentiation among geographic regions using an analysis of molecular variance (AMOVA) and the associated  $\Phi$  statistics, as implemented in Arlequin. For the mtDNA, we also repeated the analyses including *COI* sequences from flies collected between 1997 and 2005 (Jaenike et al. 2006). Finally, to infer the number of genetic clusters ( $K$ ) in the microsatellite data, we used the program Structure version 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009). We used an admixture model with correlated allele frequencies, and ran the program five times at each value of  $K$  with a burn-in of 100,000 and run length of 250,000 steps. We identified the most likely  $K$  based on the mean  $\ln(\text{Pr}|K)$  across values of  $K$  and also using the  $\Delta K$  method of Evanno et al. (2005).

### MATING ASSAYS FOR FEMALE DISCRIMINATION

We used no choice mating assays to measure mate discrimination by each of 55 isofemale lines from the eight sampled populations of *D. subquinaria*, which included two to 16 isofemale lines/population (Table 1, Fig. 1). Lines were maintained in the laboratory for at least 10 generations before being used in mating assays. Females from each line were tested against each of four male types: coastal allopatric, inland allopatric, sympatric *D. subquinaria*, and sympatric *D. recens* (Fig. 1). The subdivision of allopatric populations into coastal and inland regions is based on previous work in this system (Jaenike et al. 2006; Dyer et al. 2014). All the males used in mate trials were taken from large mixed stocks; each was created by combining virgin females and males in equal numbers from 10 to 25 isofemale lines. The sympatric stock combined 25 lines from Kawtikh, Hinton, and Canmore, the inland allopatric stock combined 10 lines from Deary, Missoula, and Shuswap, and the coastal allopatric stock combined 10 lines from Portland and Seattle. A mixed stock of *D. recens* was created in the same way, using eight lines from the same three sympatric populations. Each mixed stock was maintained for at least four generations, mixing flies across vials each generation, before being used in a mate trial.

Virgins were collected under CO<sub>2</sub> anesthesia and stored 10–15 flies/vial, and all flies used in mating experiments were seven days old. Mating assays took place in 4 mL vials that contained a blended mushroom-agar food, and commenced within an hour of the incubator lights turning on. Flies were placed into vials by air aspiration, and we recorded whether each pair copulated within 2 h. Isofemale lines were randomized among blocks, and within a block each isofemale line was tested against every male type. Each isofemale line was tested in at least three blocks for a minimum of 18 mating assays per male type.

### VARIATION IN MATE DISCRIMINATION WITHIN AND AMONG POPULATIONS

Each isofemale line was tested in several blocks but not in every block; thus, we first tested for a block effect using a logistic regression for each individual isofemale line, considering block and male type as fixed effects. Because only one line had a significant block effect (Shuswap 3), we combined data across blocks, and the results did not differ if we excluded this line from the analyses. We tested for large-scale variation in mate discrimination using a logistic regression with male type, female region, and their interaction as fixed effects in the model. To investigate fine-scale geographic variation in mate discrimination, we used logistic regressions with female population and isofemale line nested within population as fixed effects in the model. We completed this analysis separately for each combination of male type and female region. This approach results in more statistical tests than a single model with all of the different factors; however, in

spite of extensive efforts we were unable to obtain consistent convergence when we used fewer but more complicated models. This is likely because large portions of the mating data show no variation. Statistical analyses used JMP version 10 (SAS Institutes, Cary, NC).

We also asked whether females from lines that were less likely to mate with *D. recens* were less likely to mate with coastal allopatric *D. subquinaria*. As we found no variation for female mating rate of sympatric *D. subquinaria* against *D. recens*, we used the 20 lines from the nearby inland allopatric *D. subquinaria* populations (Deary, Missoula, Shuswap). We used linear regression to test for a correlation of mating rate between male types, and used the propensity of these lines to mate with their own males as a control for general level of discrimination.

### CLINE AND SELECTION ANALYSES ON MATE DISCRIMINATION

We analyzed cline shapes using the simulated annealing algorithm implemented in Cfit7 (Gay et al. 2008). We fit sigmoidal curves to four phenotypic clines (discrimination against *D. subquinaria* coastal allopatric males, inland allopatric males, sympatric males, and *D. recens* sympatric males) and two genetic clines (multilocus microsatellite and mtDNA *COI*). For each behavioral trait, we treated each mean isofemale line mating rate as an individual trait value. For the microsatellites, we used the multilocus assignment values of each genotyped individual to one of the clusters under  $K = 2$ , and treated the data as a quantitative trait in the analyses. For the mtDNA, we scored whether each individual carried a coastal or inland haplotype based on the minimum spanning network (Fig. S1), and fit a simple sigmoid curve with right tail.

We used the phenotypic variation to test the fit of three cline models that correspond to different levels of introgression across the range, as implemented in Cfit7 (Jiggins and Mallet 2000; Gay et al. 2008). In the null unimodal model, there is high migration across the cline center with all individual phenotypic values drawn from a single distribution. In this model the distribution of phenotypes in the center resembles a hybrid swarm. In the bimodal model, phenotypic values come from two separate distributions that are on either side of the cline center, with very few intermediates. The trimodal model allows a third distribution for individuals in the center of the cline, thus these individuals have mixed phenotypes from the two opposite distributions. We used the Akaike information criterion (AIC) to determine the best-fitting model for each cline and checked for convergence via multiple runs with different random seeds. In a few cases, our runs did not converge despite increasing iterations, thus we also report the SD across at least five runs. This lack of convergence was likely due to high variation and low sample sizes within some populations.

We compared the clines in female discrimination against coastal allopatric males and *D. recens* males by asking whether

they share a cline center (i.e., are coincident) and width (i.e., are concordant). We used Cfit7 to constrain the slopes, centers, or both slope and center of these clines. We assumed the bimodal distribution for each cline because it is preferred for both (see Results), and used the AIC to determine the best-fitting model.

We used the ecotone model to estimate the strength of selection acting on each cline. This model assumes a step-like change in the selection environment and that dispersal distance is similar to or larger than the cline width (Slatkin 1973; Endler 1977). Selection,  $s$ , is estimated following  $w = \sigma/\sqrt{s}$ , where  $w$  is the cline width (estimated as  $w = 4/\text{slope}$ , as in Gay et al. 2008) and  $\sigma$  is the SD of adult-offspring dispersal distance (Haldane 1948; Bazykin 1969; Slatkin 1973). We estimated the variance in dispersal to be about 1.5 km based on a mark-recapture study of *D. falleni*, a sympatric mushroom-feeding member of the quinaria group (Montague 1985). We calculated selection under a range of dispersal variances, including 1, 2, 5, and 10 km, as longer range wind dispersal is likely to occur occasionally.

## Results

### SPECIES COMPOSITION AND PATTERNS OF GENE FLOW

*Drosophila recens* and *D. subquinaria* were sympatric in the three sampled populations east of the Rocky Mountains, where the percentage of *D. recens* ranged from 53 to 92% of the total composition of the two species (Table 1). Of the total 208 sampled *D. subquinaria* individuals, three (1.4%) carried a *D. recens* mtDNA haplotype, indicative of hybridization and subsequent introgression of the mtDNA from *D. recens* into *D. subquinaria*. We also identified one individual of *D. subquinaria* from Missoula infected with *Wolbachia*, though sequencing revealed this strain is not closely related to the one found in *D. recens* (data not shown).

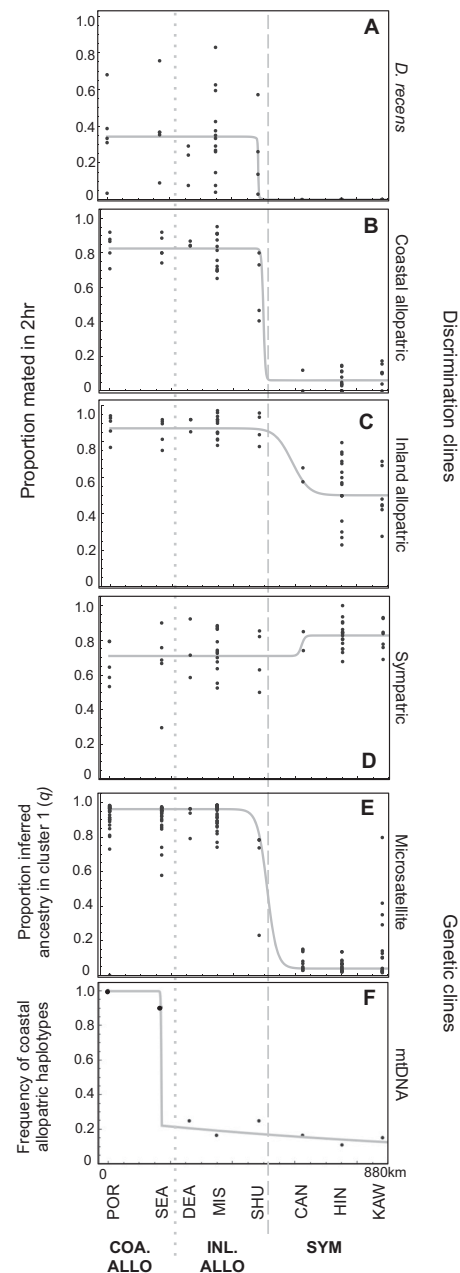
Using the mtDNA *COI* gene, the primary axis of genetic differentiation in *D. subquinaria* is to the east and west of the Coast Mountains, and not between sympatry and allopatry with *D. recens*. The largest percentage of variation among groups is explained when populations are separated into two groups, consisting of inland (allopatric inland and sympatric populations) and coastal populations ( $\Phi_{CT\text{-coast vs. inland}} = 0.38, P = 0.04$ ; Table S3). An AMOVA that excludes coastal populations indicates there is substantial gene flow between sympatric and nearby allopatric populations ( $\Phi_{CT\text{-sym vs. inland allo}} = 0.012, P = 0.30$ ; Table S3). Similar patterns were obtained using pairwise  $F_{ST}$  and  $S_{nn}$  (Table S4), and also visually from a haplotype network (Fig. S1). When we combine our data with those from an earlier study (Jaenike et al. 2006), we find consistent patterns of differentiation, indicating stable genetic structure across years (Table S3).

In contrast to the mtDNA, the strongest axis of population differentiation for the eight microsatellite loci is on either side of the Rocky Mountains, consistent with the division between allopatry and sympatry with *D. recens*. First, seven of nine pairwise  $F_{ST}$  comparisons between sympatric and inland allopatric populations were significant, whereas two of six comparisons between coastal and inland allopatric populations were, suggesting a stronger break between sympatry and allopatric regions (Table S5). Other measurements of genetic distance gave similar results (results not shown). Second, using the program Structure, the most probable number of genetic clusters was  $K = 2$ , using either the highest averaged likelihood (average LnL =  $-3502.52$ ) or  $\Delta K$  ( $\Delta K = 256.64$ ; Table S6). The two clusters primarily separate the sympatric and allopatric populations (Table S7; Fig. S2A). We also ran Structure excluding the two coastal allopatric populations, with consistent results (Tables S6 and S7; Fig. S2B). Finally, using an AMOVA with all eight populations, the largest percentage of variation among groups (13%) is explained when populations are separated into two groups based on sympatry with *D. recens* ( $\Phi_{CT-sym\ vs.\ allo} = 0.13$ ,  $P = 0.02$ ). However, excluding the coastal allopatric populations from the AMOVA, as these are the most distant from sympatry with *D. recens*, indicates the presence of gene flow between sympatric and nearby allopatric inland populations ( $\Phi_{CT-sym\ vs.\ inland\ allo} = 0.14$ ,  $P = 0.10$ ; Table S3).

#### VARIATION IN MATE DISCRIMINATION WITHIN AND AMONG POPULATIONS

There is tremendous genetic variation in mating rate that depends on the geographic origin of both the male and the female (Figs. 2A–D, S3). Using a model that included all of the data and tested for the effects of male type, female region, and their interaction, we found that all three effects were highly significant (male type:  $\chi^2 = 1440$ ,  $P < 0.0001$ ; female region  $\chi^2 = 897$ ,  $P < 0.0001$ ; interaction  $\chi^2 = 814$ ,  $P < 0.0001$ ). We subdivided the mating assay data by the three female regions and the four male types, and for each male–female combination, we tested for variation in mating rate among populations and among lines (nested within population; Table 2). We did not perform this analysis for the sympatric females with *D. recens* males, as none of the 689 pairs of this type copulated.

Within species, the mating rates for “home” combinations where the females and males are from the same geographic region are generally high and show no significant genetic variation (Table 2; Fig. S3), as expected if female preferences and male traits are at regional optima. In contrast, there is significant genetic variation in mating rates when conspecific females and males from different geographic regions are paired. Most of the genetic variation in mating rate appears to be among lines rather than among populations within a region, though



**Figure 2.** Clines in behavioral discrimination by male type (panels A–D) and in genetic distance (panels E and F). Source populations of isofemale lines used in mate trials are indicated along the x-axis, with the abbreviations from Table 1 used and their placement proportional to their distance along the cline from west to east. Black dots indicate the mean fraction mated of each isofemale line in the phenotypic clines (A–D), the individual assignment values to cluster 1 in the multilocus microsatellite cline (E), and the proportion of coastal allopatric haplotypes in each population for the mtDNA cline (F). Each cline is drawn from the best-fitting model of the phenotypic distribution (*Drosophila recens*: bimodal, allopatric coastal: mean of bimodal and unimodal estimates, allopatric inland: trimodal, sympatric: unimodal, microsatellite: trimodal, mtDNA: simple sigmoid). The dotted and dashed vertical lines indicate the relative locations of the Coast and Rocky Mountains, respectively.

**Table 2.** Geographic variation in female discrimination against each male type.

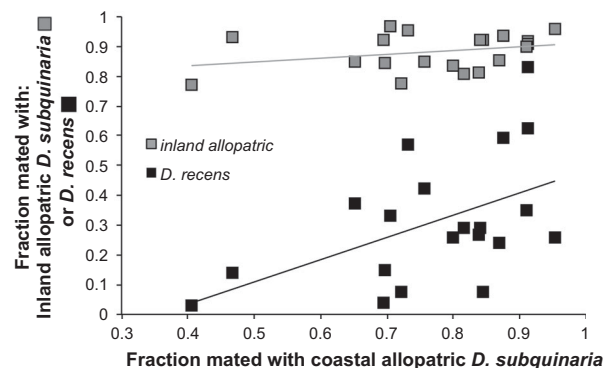
Female Region	Model Effect	Degrees of Freedom (df)	Male Type			
			Coastal Allopatric	Inland Allopatric	Sympatric	<i>Drosophila recens</i>
Allopatric coastal	Whole model	9	11.2 ( <i>P</i> = 0.26)	12.5 ( <i>P</i> = 0.19)	<b>36.7</b> ( <i>P</i> < 0.0001)	<b>63.9</b> ( <i>P</i> < 0.0001)
	Population	1	0.02 ( <i>P</i> = 0.88)	0.43 ( <i>P</i> = 0.51)	0.019 ( <i>P</i> = 0.89)	1.1 ( <i>P</i> = 0.29)
	Line (population)	8	11.2 ( <i>P</i> = 0.19)	12.0 ( <i>P</i> = 0.15)	<b>36.6</b> ( <i>P</i> < 0.0001)	<b>62.1</b> ( <i>P</i> < 0.0001)
Allopatric inland	Whole model	19	<b>61.2</b> ( <i>P</i> < 0.0001)	21.9 ( <i>P</i> = 0.29)	<b>53.5</b> ( <i>P</i> < 0.0001)	<b>139.5</b> ( <i>P</i> < 0.0001)
	Population	2	<b>22.2</b> ( <i>P</i> < 0.0001)	0.023 ( <i>P</i> = 0.99)	0.76 ( <i>P</i> = 0.68)	8.4 ( <i>P</i> = 0.015)
	Line (population)	17	<b>39.8</b> ( <i>P</i> = 0.0014)	21.5 ( <i>P</i> = 0.21)	<b>53.4</b> ( <i>P</i> < 0.0001)	<b>124.9</b> ( <i>P</i> < 0.0001)
Sympatric	Whole model	24	<b>52.3</b> ( <i>P</i> = 0.0007)	<b>76.1</b> ( <i>P</i> < 0.0001)	39.7 ( <i>P</i> = 0.0375)	na
	Population	2	0.000003 ( <i>P</i> = 1.0)	2.293 ( <i>P</i> = 0.23)	1.447 ( <i>P</i> = 0.49)	na
	Line (population)	22	<b>46.4</b> ( <i>P</i> = 0.0018)	<b>73.2</b> ( <i>P</i> < 0.0001)	37.2 ( <i>P</i> = 0.0227)	na

For each female geographic region and male type, shown are the likelihood ratio test statistic  $\chi^2$  for the whole model and for variation among populations and lines, and the associated *P* values. The Bonferroni corrected *P*-value is 0.05/11 = 0.0045; values in bold are significant at this level. Sympatric females with *D. recens* males were not analyzed because none of the pairs of this combination mated.

the number of populations surveyed in each region is small (Table 2).

Between species, while no sympatric *D. subquinaria* female mated with a *D. recens* male, *D. subquinaria* females from outside of this region were willing to mate with these heterospecific males. Across the 37 allopatric lines we surveyed, females mated with *D. recens* males on average 26% of the time (range 0.03–0.83 across lines). The genetic variation among lines in both the inland and coastal parts of allopatry is highly significant (coastal:  $\chi^2 = 64$ , *P* < 0.0001; inland:  $\chi^2 = 140$ , *P* < 0.0001; Table 2). The lack of any mating within sympatry coupled with the high level of variation in allopatry suggests that selection against mating with *D. recens* in sympatry may be very strong.

Comparing across lines, our data indicate there may be some genetic linkage or shared genetic basis of female discrimination against both *D. recens* males and *D. subquinaria* coastal males. Inland allopatric females that mate less often with *D. recens* males also mate less often with *D. subquinaria* coastal males ( $r^2 = 0.25$ ,  $F_{1,18} = 6.1$ , *P* = 0.024; Fig. 3). This is not because these females are generally more choosy, as there is no correlation between mating rate between coastal allopatric males and their own inland allopatric males ( $r^2 = 0.09$ ,  $F_{1,18} = 1.8$ , *P* = 0.20; Fig. 3).



**Figure 3.** Correlations of mate discrimination between coastal *Drosophila subquinaria* and *D. recens* males (black squares and black line) and between coastal *D. subquinaria* and inland allopatric *D. subquinaria* males (gray squares and gray line). Only isofemale lines from inland allopatric populations (Missoula, Deary, Shuswap) are included. The lines indicate the regressions (black:  $r^2 = 0.25$ ;  $F_{1,18} = 6.1$ , *P* = 0.024; gray:  $r^2 = 0.09$ ;  $F_{1,18} = 1.8$ , *P* = 0.20).

### CLINE AND SELECTION ANALYSIS OF MATE DISCRIMINATION

A single model did not fit for all clines (Tables 3 and S8), suggesting that the biological basis of the clines differs. For

**Table 3.** Summary of the characteristics of clines in female mate discrimination.

Cline	Model	N of Parameters	Llmax	Center (km)	Width (km)	$\Delta AICc$
Allopatric coastal	<b>Unimodal</b>	<b>7</b>	<b>64.35</b>	<b>501</b>	<b>159</b>	<b>1.19</b>
	<b>Bimodal</b>	<b>8</b>	<b>66.32</b>	<b>485</b>	<b>4</b>	<b>0</b>
	Trimodal	12	69.52	469	183	6.91
Allopatric inland	Unimodal	7	52.99	622	10	2.62
	Bimodal	8	52.76	623	5	5.84
	<b>Trimodal</b>	<b>12</b>	<b>62.19</b>	<b>589</b>	<b>110</b>	<b>0</b>
Sympatric	<b>Unimodal</b>	<b>7</b>	<b>44.29</b>	<b>618</b>	<b>22</b>	<b>0</b>
	Bimodal	8	44.25	486	10	2.82
	Trimodal	12	49.15	86	971	5.86
<i>Drosophila recens</i>	Unimodal	7	154.86	484	4	4.93
	<b>Bimodal</b>	<b>8</b>	<b>158.09</b>	<b>484</b>	<b>4</b>	<b>0</b>
	Trimodal	12	160.10	482	4	8.12
Microsatellites	Unimodal	7	128.21	484	4	84.66
	Bimodal	8	165.68	512	89	11.73
	<b>Trimodal</b>	<b>12</b>	<b>175.15</b>	<b>509</b>	<b>62</b>	<b>0</b>
mtDNA	Sigmoid	4	-28.05	171	4	Na

The cline centers and widths are with respect to the 50° transect angle in Figure 1, with the distance from the westernmost sampled point. The best-fitting models based on  $\Delta AICc$  are shown in bold. See the text for a description of the three different models, and see Gay et al. (2008) for a description of the parameters included in each model.

discrimination against *D. recens* males, the bimodal model was the best fit ( $\Delta AICc > 2.0$ ). The bimodal model was also the best fit for the cline in discrimination against coastal allopatric males, though it was not a statistically significant improvement over the unimodal model ( $\Delta AICc = 1.19$ ). Both the microsatellite cline and the cline in discrimination against inland allopatric males were best described by the trimodal model ( $\Delta AICc > 2.0$ ). Finally, the sympatric cline was best fit by the unimodal model ( $\Delta AICc > 2.0$ ).

Using the best-fitting models, all four of the behavior clines as well as the microsatellite cline have centers that fall in in the middle of the Rocky Mountains and also in the region where allopatry turns into sympatry with *D. recens* (Fig. 2A–E). The cline of discrimination against sympatric males is weaker than the others and in the opposite direction to the other behavior clines, consistent with slightly lower mating rates of allopatric females with sympatric males. The center of the mtDNA cline, in contrast, falls at the Coast Mountains (Fig. 2F). The clines vary in width, with the discrimination cline against *D. recens* males being the steepest at 4 km (Table 3). The other phenotypic cline widths are generally wider, for instance the cline in discrimination against inland allopatric males and the microsatellite cline range from 62 to 110 km wide (Table 3). The cline in discrimination against allopatric coastal males differs in width depending on the model, with 4 km under the bimodal model up to 159 km under the unimodal model (Table 3). For this cline, we use the average of the slopes of these two models (Table S9) to calculate the width in Figure 2B and in the selection analysis (Table 4). Finally,

**Table 4.** Summary of selection estimates.

Cline	Width (km)	Dispersal ( $\sigma^2$ )			
	( $=\sigma/\sqrt{s}$ )	1 km	2 km	5 km	10 km
Allopatric coastal	8.37	0.0143	0.0285	0.0714	0.1425
Allopatric inland	109.87	0.0001	0.0002	0.0004	0.0008
Sympatric	22.74	0.0019	0.0039	0.0043	0.0193
<i>Drosophila recens</i>	4.00	0.0625	0.1250	0.3125	0.6241
Microsatellites	62.48	0.0003	0.0005	0.0013	0.0026
mtDNA	4.06	0.0606	0.1212	0.3033	0.6056

The selection coefficient ( $s$ ) was inferred from the estimated cline width ( $w$ ) under four estimates of variance in dispersal ( $\sigma^2$ ), using the formula  $\sqrt{s} = \sigma/w$ . For the allopatric coastal cline, the unimodal and bimodal models were indistinguishable statistically, thus we used the mean of the slope parameter estimates of both models to calculate width.

we find that the clines in discrimination against *D. recens* males and coastal allopatric males are both concordant and coincident. Using a bimodal model, the best-fitting model for these clines constrains both width and center simultaneously ( $\Delta AICc > 3$ ; Table S9). This indicates that these clines in discrimination may be coupled.

Using a range of dispersal values (variance 1–10 km) and the width from each best-fitting cline model, the selection estimates vary from very weak to extremely strong across clines (Table 4). For the behavioral traits, discrimination against *D. recens* and coastal allopatric males is strong ( $s = 0.06$ – $0.62$  and  $0.014$ – $0.14$ , respectively). In contrast, selection against inland allopatric males



and sympatric males is weak, with the highest values of  $s$  at 0.008 and 0.02, respectively. At the molecular markers, the barrier to gene flow at the Coast Mountains for the mtDNA is strong, with  $s$  as high as 0.6. In contrast, selection on the microsatellite cline is weak ( $s$  up to 0.003), supporting some gene flow among populations of *D. subquinaria* across the Rocky Mountains and also across the boundary between sympatry and allopatry with *D. recens*.

## Discussion

Reinforcement involves the interaction of selection and gene flow to strengthen prezygotic isolation between hybridizing species (Servedio and Kirkpatrick 1997; Noor 1999; Servedio and Noor 2003; Coyne and Orr 2004). As a consequence of reinforcement, changes in mate recognition systems may create isolation among populations within a species (Howard 1993; Coyne and Orr 2004; Hoskin et al. 2005; Ortiz-Barrientos et al. 2009). How natural and sexual selection interact to alter mate recognition systems and subsequently drive reproductive isolation is not well understood (Andersson 1994; Coyne and Orr 2004; Hoskin and Higgie 2010; Mendelson and Shaw 2012). Here, we use a cline theory approach to provide a window into the processes that underlie behavioral isolation between *D. recens* and *D. subquinaria* and among populations of *D. subquinaria*. Studying the variation in mate discrimination both within and among populations may help illuminate both the causes and consequences of selection for reinforced species boundaries, including how selection drives the divergence of mate recognition systems and thus ultimately speciation. We place this variation in phenotypic traits within the context of background genetic variation to reveal where selection acts and how strong that selection is.

### GENETIC STRUCTURE OF THE SYMPATRIC–ALLOPATRIC BOUNDARY

For the nuclear genome, genetic differentiation among populations of *D. subquinaria* is moderate and primarily between populations on either side of the Rocky Mountains, coincident with the boundary for risk of hybridization with *D. recens* (Fig. 2E). This pattern of population differentiation is in contrast to what has been found using the mtDNA, which identifies a major phylogeographic break at the Coast Mountains (this study; Jaenike et al. 2006). Why the mtDNA and nuclear markers have such contrasting patterns of genetic differentiation is unknown. Ecological selection may promote divergence on either side of the Coast Mountains (Dyer et al. 2014; M. Stribos and K. Dyer, unpubl. data), but this is hard to interpret with respect to mating patterns because female preferences and male traits generally map to nuclear loci.

Tension zones can be pushed toward regions of low population density, and cause otherwise unlinked loci to accumulate genetic differences on either side of this barrier (reviewed in Barton and Hewitt 1985; Abbott et al. 2013). Coincident with this, divergent selection in mate discrimination may be contributing to differentiation between sympatric and allopatric populations of *D. subquinaria*. However, this differentiation appears to be in the early stages. Specifically, in the AMOVAs that consider only sympatric populations and the nearby inland allopatric populations, the  $\Phi_{CT}$  values are low and nonsignificant (Table S3). Furthermore, the microsatellite cline fits a trimodal distribution, indicating differentiation with the potential for introgression of alleles across the cline (Gay et al. 2008). Thus, we find that across the sympatry–allopatry boundary there is some gene flow, though not too much, as is necessary for reinforcing selection to operate (Kirkpatrick 2000; reviewed in Coyne and Orr 2004).

### REINFORCED DISCRIMINATION AGAINST *D. RECENS*

A striking result of our study is that we find no genetic variation in rejection of *D. recens* within and among sympatric populations of *D. subquinaria*. This is independent of the frequency of *D. recens*, which ranged from approximately 50–90% across populations at the time of sampling. Others have suggested that the frequency of the sister species may affect the strength of reinforcing selection (Yukilevich 2012; Nosil 2013), and thus in the future it would be useful to sample populations where *D. recens* is rare relative to *D. subquinaria*. Our estimates of selection to discriminate against *D. recens* males are very strong ( $s$  up to 0.62), which is consistent with the death of all hybrid offspring of *D. subquinaria* females. The upper values are among the highest selection coefficients estimated in natural populations (e.g., Hoekstra et al. 2001; Kingsolver et al. 2001). Importantly, the width of this cline is much narrower than for the microsatellites, even though the centers are near each other, suggesting that selection is operating in the face of gene flow to maintain it. Our cline analysis also indicates that a bimodal distribution is the best fit for the phenotypic distribution of this trait. In the tension and hybrid zone literature, bimodality is considered a strong indicator of nearly complete speciation and occurs when prezygotic barriers are present and/or postzygotic selection is strong (Jiggins and Mallet 2000). We note that in this case we are analyzing a cline within a single species and not across two species, which highlights the potential for reinforcement to generate strong phenotypic divergence within *D. subquinaria*.

In contrast to the area of sympatry, we find substantial genetic variation among allopatric *D. subquinaria* lines for rejection of *D. recens*, from complete rejection to nearly complete acceptance, with a range of values in between (Figs. 2A, S3). Without knowing the genetic basis of the reinforced discrimination, we cannot determine whether this variation is a result of segregating

ancestral variation or due to migration from sympatric populations. However, we suggest it is more likely the latter, given that our genetic markers indicate there is migration between regions that are sympatric and allopatric with *D. recens*.

The finding of segregating genetic variation among allopatric lines for reinforced discrimination suggests three things about reinforcement in this system. First, the broad distribution of discrimination found among lines in allopatry suggests that reinforced discrimination may not be a simple all-or-nothing trait. Instead, the genetic basis may be more complicated than a single locus of large effect and, as has been found in *Drosophila pseudoobscura*, there may be multiple genetic routes to reinforced discrimination in *D. subquinaria* (Ortiz-Barrientos et al. 2004). Second, alleles that confer reinforced discrimination against *D. recens* may not be very costly to harbor outside of sympatry. However, they do not seem to be spreading to fixation, which suggests that they are probably not beneficial and there are likely trade-offs between species recognition and mate quality recognition, as has been suggested in other systems (Pfennig 2000; Higgie and Blows 2007). Third, the presence of these alleles in allopatry suggests that these populations harbor the genetic material to respond to selection to increase discrimination and maintain species boundaries. Thus, if *D. recens* were to expand its range westward or if levels of gene flow were high, the combination of strong selection due to the death of hybrid offspring and the presence of genetic variation in discrimination against *D. recens* suggests these alleles would increase in frequency to prevent hybridization between species. These findings are only evident because we assayed within-population variation in discrimination; in contrast, most other studies of reinforcement combine across lines to estimate the population level of discrimination.

#### BEHAVIORAL ISOLATION WITHIN *D. SUBQUINARIA*

Within *D. subquinaria*, our analyses indicate that female preferences are locally adapted, differ across the geographic range of the species, and are maintained in the face of gene flow. Consistent with previous work (Jaenike et al. 2006), sympatric females show the highest preference for their own males and discriminate against males from other geographic regions. Sympatric females mate with coastal allopatric males less than 10% of the time, though there is variation across lines in the level of discrimination (Table 2). This creates a steep cline and strong selection ( $s$  up to 0.14). There is a slight preference for the bimodal model, as for the *D. recens* cline, which indicates strong phenotypic divergence in mate discrimination across the cline and reflects the presence of premating barriers. In contrast, these sympatric females do not discriminate nearly as strongly against inland allopatric males as against coastal males. This results in a cline against inland allopatric males that is much wider than for coastal males, and which supports a trimodal phenotypic distribution such that there

are abundant hybrid phenotypes in the contact zone (Barton and Hewitt 1985; Gay et al. 2008). This inland allopatric region may be a “melting pot” where alleles from the coastal and sympatric regions come together by migration.

Females from throughout the geographic range of *D. subquinaria* will mate with males from the region of sympatry with *D. recens*. Selection against these males is weak ( $s < 0.02$ ), and a unimodal phenotypic distribution is preferred, indicative of significant introgression across the cline. There is a slight decrease in mating rates of allopatric females with sympatric males relative to their own males (Fig. 2D), which could result from a change in the mating signals of these males due to reinforced selection by *D. recens*, similar to what is seen in *D. serrata* (Higgie and Blows 2007). Indeed, in *D. subquinaria* the epicuticular profiles of sympatric individuals differ from allopatric individuals, consistent with reinforcement driving differences in male mating signals across populations (Dyer et al. 2014). Even at a low cost, conditions of strong sexual selection may be sufficient to prevent the sympatric male signals from spreading to high frequency in allopatric populations.

#### CASCADE REINFORCEMENT AND THE POTENTIAL FOR INCipient SPECIATION

One way that cascade reinforcement can be facilitated is if the genes responsible for reinforced prezygotic isolation between species also cause sexual isolation within species. The genetic basis of reinforced discrimination is known in only a few systems (e.g., Ortiz-Barrientos et al. 2004; Saether et al. 2007; Hopkins and Rausher 2011), and to our knowledge the genetic basis of cascade reinforcement has not been identified in any system. Complicating this, there may be multiple genetic mechanisms of reinforced female mate discrimination within a species, as is the case in *D. pseudoobscura* (Ortiz-Barrientos et al. 2004), and the same may be true for within-species discrimination due to cascade reinforcement. This underscores the importance of accounting for variation not only among populations, but within them as well. If the genetic basis of reinforced and within species discrimination is the same or tightly linked, we predict that at the broad scale the between- and within-species discrimination clines should be tightly correlated, and that at the fine scale, we should see an association of between- and within-species discrimination among isofemale lines. We find support for both predictions. First, the bimodal model is the best-fitting phenotypic distribution for both the *D. recens* and coastal allopatric clines, whereas this model does not fit any other cline (Table 3). Furthermore, these clines share the same shape and are both centered at the same location (Table S9). Second, females from inland allopatric lines that are more likely to mate with *D. recens* males are also more likely to mate with allopatric coastal conspecific males (Fig. 3). Because the lines that are more choosy against both types of males do

not also mate less against their own males (Fig. 3), this suggests at least some of the genetic basis of discrimination between and within species is shared or linked. Further support for an association of discrimination phenotypes is that females use epicuticular hydrocarbons to discriminate against both conspecific and heterospecific males (Dyer et al. 2014). However, while these results are suggestive, genetic mapping of both behaviors is necessary before any firm conclusions can be made.

Alternatively, selection on these traits may be independent, but clines are coupled through means other than reinforcement of mate discrimination. With the exception of the mtDNA, all of our cline centers fall within the Rocky Mountain range. It is possible that the physical barrier of the mountain range is recruiting clines to it, which acts to sharpen clines and enhance barriers to gene flow (Barton and Hewitt 1985; Bierne et al. 2011; Abbott et al. 2013). This can allow the build up of reproductive barriers (Felsenstein 1981; Barton and de Cara 2009). On the other hand, if a single physical barrier to dispersal is coupling otherwise independent clines, we would predict the shapes and phenotypic distribution models to be the same across clines. Instead, we see that some traits move across the physical barrier somewhat easily (i.e., discrimination against sympatric and inland allopatric males), while others appear to be maintained by selection and not solely by reduced dispersal (i.e., discrimination against coastal and *D. recens* males).

In conclusion, cascade reinforcement involves the interaction of selection, migration, and the genetic relationship between phenotypes, whose relative roles will ultimately determine the outcome of incipient speciation. Our results suggest that genetic variation in mate discrimination within populations can be high in certain circumstances, and reinforcing selection can be strong enough to drive phenotypes to fixation in sympatry. Allopatric females will accept the male signal trait from both allopatric and sympatric populations of *D. subquinaria*, and many will accept heterospecific *D. recens* males as well. In contrast, sympatric *D. subquinaria* females have narrowed the range in the male signals they find acceptable to only include the conspecific males they co-occur with. This asymmetry could have significant consequences for the direction of gene flow among populations and the potential for divergence within *D. subquinaria*. Because behavioral isolation among conspecific populations is asymmetric and gene flow occurs among populations, this may slow the process of reproductive isolation to maintain the tension zone indefinitely. On the other hand, the coupling of clines and genetic association of discrimination traits may allow for genetic divergence to accumulate across the genome and thus promote eventual complete isolation even in the face of ongoing gene flow. Ultimately, to distinguish whether these clines have been coupled by exogenous factors or are acted upon by the same forces of selection, we need to uncover the genetic basis of the within- and between-species

female discrimination traits as well as of the male signal trait. This, in combination with identifying the pattern of fine-scale genomic differentiation both among populations and between species, will provide a robust understanding of the mechanisms of ongoing divergence.

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#### DATA ARCHIVING

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## Supporting Information

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

**Figure S1.** Minimum spanning tree for *Drosophila subquinaria* mtDNA *COI*, using samples collected in 2010–2011.

**Figure S2.** Results from structure showing differentiation of sympatric and allopatric populations using the microsatellite markers.

**Figure S3.** For each isofemale line, the mean proportion of pairs mated in 2 h against each male type.

**Table S1.** Primers used to amplify microsatellite loci.

**Table S2.** Variability measures for each of the eight microsatellites within each population and across all populations.

**Table S3.** Molecular analysis of variance (AMOVA) of the mtDNA *COI* locus and eight microsatellites using different groupings of *D. subquinaria* populations.

**Table S4.** Estimates of pairwise population differentiation at the mtDNA *COI* locus of *D. subquinaria*.

**Table S5.** Pairwise population differentiation at the eight microsatellite loci using  $F_{ST}$ .

**Table S6.** Results of the structure analyses to infer the most likely number of genetic clusters ( $K$ ).

**Table S7.** Proportion of membership of each population into each cluster, as defined by the structure analyses.

**Table S8.** Comparison of three models to describe each phenotypic and genotypic cline.

**Table S9.** Constraints between clines in discrimination against *D. recens* and coastal allopatric males.