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Levels of Mate Recognition Within and Between Two *Drosophila* Species and Their Hybrids

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ABSTRACT: If sexual selection is to result in speciation, traits involved in mate choice within species need to be capable of producing sexual isolation between species. We investigated the association between mate choice and sexual isolation using interspecific hybrids between two sibling species, *Drosophila serrata* and *Drosophila birchii*. A perfuming experiment demonstrated that olfaction was involved in the sexual isolation between the two species. A quantitative genetic analysis using 30 populations of hybrids between the two species indicated that mating success in hybrid individuals was predominately determined by cuticular hydrocarbons; the average genetic correlation between mating success and cuticular hydrocarbon profile was 0.84, and in some instances exceeded 0.95. Multivariate analysis of the cuticular hydrocarbons of the two species revealed that there were three independent blends of cuticular hydrocarbons that separated three levels of organization: species, sex, and sex within species. The hydrocarbons used by hybrids in mate choice included those that separated the two species, demonstrating that species-specific characters may be used in mate choice within populations. The interspecific reciprocal cross had a major effect on which cuticular hydrocarbons were associated with mating success, indicating that the expression of the cuticular hydrocarbons was strongly sex linked.

Keywords: sexual isolation, mate choice, cuticular hydrocarbons, *Drosophila serrata, Drosophila birchii,* hybrids.

Mate recognition has traditionally been divided into two components: sexual isolation between species and mate choice among individuals within species. Investigations of sexual isolation have been concerned with determining which traits are responsible for sexual isolation between two species and the genetic basis of differences in trait levels. Studies of mate choice within species have focused on determining the relative roles of males and females in mate choice and if and how sexual selection occurs. This dichotomy has been reinforced under some models of the evolution of mate recognition by considering species recognition and sexual selection to be mutually exclusive processes (Paterson 1985). Alternatively, the process of mate recognition may be considered as a continuous scale of assortative mating ranging from that between genotypes within populations, between populations within species, and between species (Spieth and Ringo 1983; Ryan and Rand 1993; Endler and Houde 1995).

Few studies have investigated the association between these levels of recognition (Ryan and Rand 1993). For instance, are traits involved in sexual isolation simply traits that were involved in mate choice within species in the past, where alternate levels have become fixed in different sibling species (Butlin 1995)? This may result in the same mechanism of recognition being used at both levels in extant populations. It is unfortunate that little is known about whether the same mechanisms are involved at both levels of recognition (Wiernasz and Kingsolver 1992; Ryan and Rand 1993). Song in *Drosophila* (Ewing and Miyan 1986), wing melanin pattern in butterflies (Wiernasz and Kingsolver 1992), and calls in frogs (Ryan and Rand 1993) are examples of where a trait used in mate choice within species may also be involved in sexual isolation between closely related species. In contrast, head width in stalk-eyed *Drosophila* is under sexual selection within species but does not appear to contribute to sexual isolation between species (Boake et al. 1997).

We report an investigation of the association between mate choice within species and sexual isolation between two closely related species, *Drosophila serrata* and *Drosophila birchii*. If sexual isolation between two species evolved as a consequence of sexual selection (Lande 1981), traits involved in sexual isolation need to be capable of being used in mate choice within species. We use a series of hybrid lines between the two species to deter-

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mine if species-specific characters, which may be associated with sexual isolation between the two species, are used by the hybrids in mate choice. When using pairs of species, it is difficult to ascertain if the genetic or mechanistic basis of sexual isolation is a consequence of the speciation process or whether they evolved after speciation was effectively completed in the distant past (Coyne 1992; Orr 1995; Wu et al. 1995). Although our experiments contribute to our understanding of the particular speciation event between *D. serrata* and *D. birchii,* our main experimental aim is the broader issue of whether species-level characters can be used within populations in mate choice.

In *Drosophila,* and in particular, members of the *melanogaster* species group, cuticular hydrocarbons have been shown to contribute to mate choice within species (Jallon 1984) and sexual isolation between species (Cobb and Jallon 1990; Coyne et al. 1994; Buckley et al. 1997; Coyne and Charlesworth 1997). We used a perfuming experiment to demonstrate that olfaction was involved in the sexual isolation between *D. serrata* and *D. birchii.* Examination of the cuticular hydrocarbon profiles of *D. serrata* and *D. birchii* by gas chromatography indicated that different hydrocarbons were associated with three levels of organization: species, sex, and sex within species. By generating interspecific hybrids within an isofemale line breeding design, we determined the genetic correlation between mating success and cuticular hydrocarbon profile in F_{11} hybrids. Since 11 generations of recombination had occurred between the genes of the two species before this measure was taken, the high genetic correlation demonstrated that cuticular hydrocarbons were strongly associated with mating success in hybrid individuals. We then show that the hydrocarbons used in mate choice by the hybrids were those that not only distinguished between sexes in the parental species but also included those that were species specific, demonstrating that species-level characters may be involved in mate choice.

Methods

Stocks

Drosophila serrata and *Drosophila birchii* have very different, but overlapping, geographic distributions and habitat preferences along the east coast of Australia (Dobzhansky and Mather 1961; Ayala 1965). Females are morphologically identical, and males can only be reliably distinguished by a single bristle difference on the genital arch (Ayala 1965). They are strongly sexually isolated from each other, with usually $<1\%$ of females capable of being inseminated by males of the other species (Dobzhansky and Mather 1961; Ayala 1965). In contrast, postmating isolation is very weak, as hybrids of both sexes are viable and fertile (Ayala 1965).

The generation of the 30 hybrid isofemale lines used in this study, and their genetic constitution, have been described in detail elsewhere (Blows 1998). Briefly, one successful mating between a *D. serrata* female × *D. birchii* male (S♀ × B♂) and one from the reciprocal cross (B♀ × S♂) were generated. From each female, 15 F_1 female progeny were collected as virgins and sib-mated to a single male. Each pair founded an isofemale line, 30 lines in total, which were maintained in the following generations in one culture bottle each, at $N \approx 100$.

Perfuming Experiment

To demonstrate the contribution of olfaction to mate recognition in this system directly, individuals of both parental species were perfumed (following Coyne et al. 1994) with the other species' attributes by confinement to determine if their transfer increased the frequency of hybridization. The experiment consisted of four treatment-control pairs. First, a single *D. birchii* virgin female was confined in a vial with 10 *D. serrata* virgin females for 3 d. The *D. serrata* females had their wings clipped to allow identification of the two species. The single *D. birchii* female was then removed and confined with five *D. serrata* males, and the presence of larval activity in this vial was scored after a further 3 d. The control for this treatment was 11 *D. birchii* females confined together for 3 d, with one then removed and placed with five *D. serrata* males. This treatment-control pair is referred to as test 1. Test 2 consisted of a single *D. serrata* female confined with 10 *D. birchii* females, and after 3 d, the *D. serrata* female was placed in a vial with five *D. birchii* males. The appropriate control for this treatment was 11 *D. serrata* females. Tests 3 and 4 consisted of male perfuming experiments with the same experimental design. Test 3 had a treatment of a single *D. birchii* male confined with 10 *D. serrata* males, and after 3 d, the *D. birchii* male was placed with five *D. serrata* females, and test 4 confined a single *D. serrata* male with 10 *D. birchii* males. For all four tests, 20 replicates of the treatment and five replicates of the appropriate control were set up, resulting in 100 vials in total.

The entire experiment was subsequently repeated with the change that, instead of confining a single individual with 10 individuals of the other species, 25 individuals were used in an attempt to increase the concentration of pheromones in the vial and, therefore, perhaps increase the treatment effect. Again, 20 replicates of the treatment and five replicates of the appropriate control were set up for each test and are referred to below as tests 5, 6, 7, and

8 (with the same combinations as in tests 1, 2, 3, and 4, respectively).

Gas Chromatography

At the eleventh generation, up to 10 male and 10 female 4-d-old virgin flies from each isofemale line were individually washed in 50 mL of hexane for 4 min, followed by 1 min of agitation on a vortex mixer (following Ferveur 1991). Individual hydrocarbon profiles were determined for nine female and 14 male *D. serrata* and 14 female and 11 male *D. birchii* in the same way. After washing, flies were removed and each sample was evaporated to dryness using nitrogen. Fifteen milliliters of hexane were added to each sample immediately before injection. One milliliter of each sample was injected into a Shimadzu GC17 fitted with a BPX5 glass capillary column, with a 5% phenyl:95% methyl siloxane stationary phase. The temperature program ran from 60° to 300°C at 30°C/min, followed by 1.5°C/min to 330°C. This process resulted in the production of 559 individual hydrocarbon phenotypes.

Statistical Analysis of Cuticular Hydrocarbons

Representative profiles of a male of both species are presented in figure 1. Female traces were qualitatively indistinguishable from the males of their respective species with all peaks in common and are therefore not shown. Ten peaks, representing individual hydrocarbons, consistently appeared in the traces of both species. The hydrocarbon profiles of the hybrid flies invariably resembled those of the *D. serrata* parents in that the two shortest chain peaks that appear in the *D. birchii* parental traces (marked with an asterisk, fig. 1) never appeared in hybrid individuals, and it is therefore not possible to consider them in the analyses below.

A common approach to the analysis of cuticular hydrocarbons is to take the ratio of major-to-minor peaks and use these ratios to investigate the effects of hydrocarbons on behavior. This procedure at best relies on some preexisting knowledge of the biological activity of the chosen peaks and at worst is a selection of peaks based on concentration when it is not clear if concentration and biological activity are related. In this instance, we had no indication of which hydrocarbon, or combination of hydrocarbons, may influence mate choice before the experiment, so a multivariate approach using principal components analysis (PCA) suggested by Neems and Butlin (1995) was followed.

Two PCAs were conducted: one on the 48 individual hydrocarbon profiles from the parental species and one on the 559 individual profiles from hybrid lines. It was

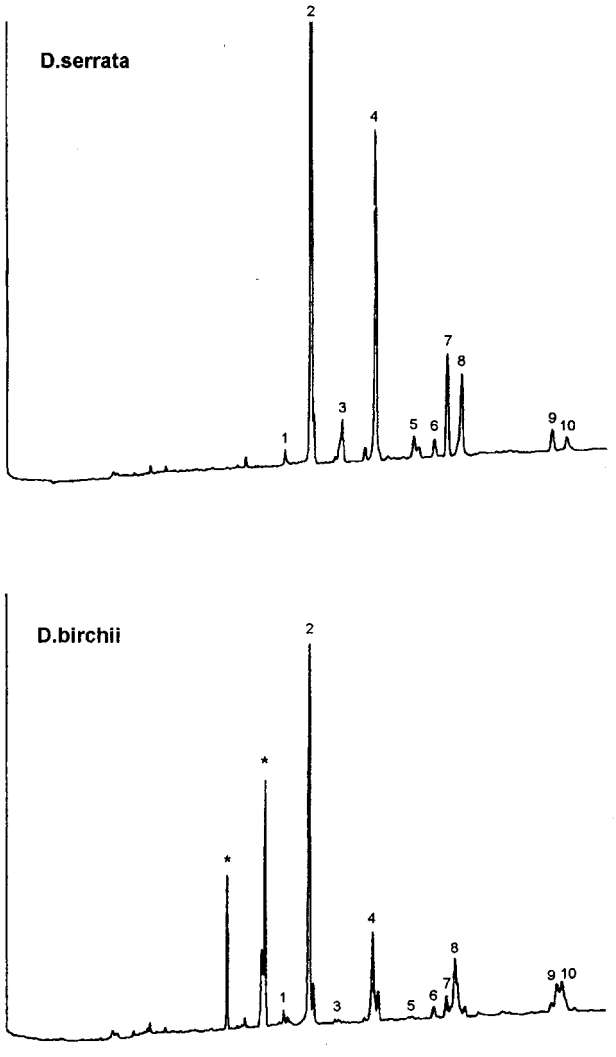


Figure 1: Typical gas chromatographs of a male Drosophila serrata and *Drosophila birchii*. The peaks used in subsequent analyses are numbered 1–10, and their retention times in minutes are 12.8, 13.7, 14.7, 15.8, 17.2, 17.8, 18.2, 18.7, 21.7, and 22.2, respectively.

necessary to separate the analysis of the parentals and the hybrids in this way as the hybrid principal components were used for a genetic analysis below. For both analyses, the area of individual peaks was divided by the total area of all 10 peaks from an individual's profile. In this way, samples of different overall concentration could be compared. Internal standards are often used to control for differences in the quantity of sample injected into the gas chromatography (GC). Dividing by the total area of all peaks accomplished this and, in addition, controlled for any variation in the extraction of the biological sample. The 10 ratios now represented a compositional data set (Aitchison 1986) that was subject to a unit-sum con-

straint (the ratios summed to 1). In response to this constraint, log contrasts were taken (Aitchison 1986), reducing the data to nine variables. Log contrasts were generated by dividing nine of the ratios by the remaining arbitrarily chosen ratio (in this case, that involving peak 8) and, then, taking the log of each of the nine new variables. A high degree of multicollinearity existed between the variables at this stage, so PCA was used to eliminate these correlations, from which nine principal components (PCs) were extracted. Once again, because the proportion of variation explained by a principal component may not be related to its biological activity, all nine PCs are considered in relation to the role of hydrocarbons in mate choice in the analyses below.

Mating Success Experiment

To determine the genetic correlation between cuticular hydrocarbon profile and mating success, we used the measure of mating success from Blows (1998). Briefly, the degree to which each of the hybrid lines could successfully mate with the *D. serrata* parental strain was determined at the fifth generation after hybridization. Mating success was measured as the proportion of five females inseminated by a single male in a vial. So, for each hybrid line, five hybrid females were confined in a vial with a *D. serrata* male, and five *D. serrata* females were confined with a hybrid male. For both treatments, eight replicate vials were set up for each line. The vials were placed at 25°C for 4 d. At the end of this period, each of the 2,400 individual females were placed in a well of a 24-well tissue culture plate that was half-filled with medium. Plates were left at 25°C for a further 3 d and then scored for the presence of larval activity.

This measure of mating success is a "no choice" mating design in that male and female *D. serrata* had only a single type (i.e., hybrid line) to choose from. The confounding influence of male-male competition is not present in this measure (Andersson 1994). This measure does contain, however, a component of larval viability that could potentially confound the interpretation of the experiment. No between-line correlation (approximating a genetic correlation; see below) was found between this measure of mating success and a measure of viability at the eighth generation after hybridization for both treatments (data not presented), excluding this possibility.

The parental *D. serrata* strain was chosen as the reference strain as only one out of 2,400 *D. birchii* females used in the same experimental design was inseminated (Blows 1998). It is possible that this striking dichotomy is a consequence of the two missing peaks in the hybrids from the *D. birchii* parent, but this requires further experiments to be tested. If this is so, the two peaks may be involved in mate recognition in this system, and therefore the following analyses may not include a component of the mechanism involved in mate recognition.

Results

Role of Olfaction in Mate Recognition

The perfuming of individuals of one species using individuals of the same sex from the other species increased the frequency of hybridization between the two species from no successful matings in the controls to 11 successful matings in the treatments across the two experiments (table 1). Both male and female perfuming appeared to increase hybridization. To test for an effect of the perfuming treatment, the eight experimental tests can be considered as eight individual model II 2×2 contingency tables. Of the eight tests, tests 1 and 5 are not informative for the purposes of testing for a treatment effect since no matings occurred in either the treatment or control and are not included in the analysis to follow.

Whether matings occur at the same frequency in treatment and control groups may be tested by determining if the odds ratios, referring to the ratio of the probability of a mating in the control and the probability of a mating in the treatment for each test, is equal to 1 (if it is, there is no treatment effect). The remaining six tests were found to be homogeneous using Zelen's test for homogeneity of odds ratios (exact $P = 1.000$). It was therefore possible to pool the data to test for significance of the common odds ratio across the tests. Since the data were unbalanced to the extent that no matings were recorded in the control groups in any of the tables, the common odds ratio was unable to be calculated and remained undefined. However, it was possible to determine the lower 95% confidence interval for the common odds ratio to determine if it was significantly different from 1 (Cytel

Table 1: Frequency of hybridization between the two parental species after perfuming

	Perfumed		*Control*	
	Mated	*Unmated*	*Mated*	*Unmated*
Test 1	0	20	0	5
Test 2	2	17	0	5
Test 3	1	17	0	5
Test 4	1	19	0	5
Test 5	0	19	0	5
Test 6	3	16	0	5
Test 7	1	19	0	5
Test 8	3	17	0	5

Note: See text for an explanation of the eight experimental tests.

Table 2: Correlations between the relative concentrations of the 10 cuticular hydrocarbon peaks and the nine principal components (PCs) from the principal components analysis on the parental species

	Relative concentration of peaks									
PC	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1	−.773**	−.328*	.882**	.705**	.770**	.147	.934**	−.162	−.680**	−.914**
2	.356*	.824**	.160	−.537**	−.542**	.439**	−.069	−.958**	−.382**	−.133
3	−.122	−.020	−.155	.301*	−.162	−.442**	−.011	−.107	.480**	−.181
4	−.290*	−.270	−.083	.155	−.007	.612**	−.037	.089	.158	.006
5	.129	−.163	.277	−.128	.004	.179	.045	.126	.276	−.097
6	.119	.122	−.102	−.173	.194	−.113	.084	.017	−.038	−.130
7	.259	−.132	.082	.166	.105	.049	−.172	−.024	.006	−.008
8	−.077	−.080	.044	.026	.102	−.021	−.075	.024	.069	.180
9	.163	−.261	.015	.118	−.028	.017	.223	.092	.076	.158

* $P < .05$.
** $P < .01$.
*** $P < .001$.

Software 1992). The mid-*P* corrected lower 95% confidence interval was found to be 1.154, indicating that the treatment of perfuming increased the frequency of hybridization between the two species.

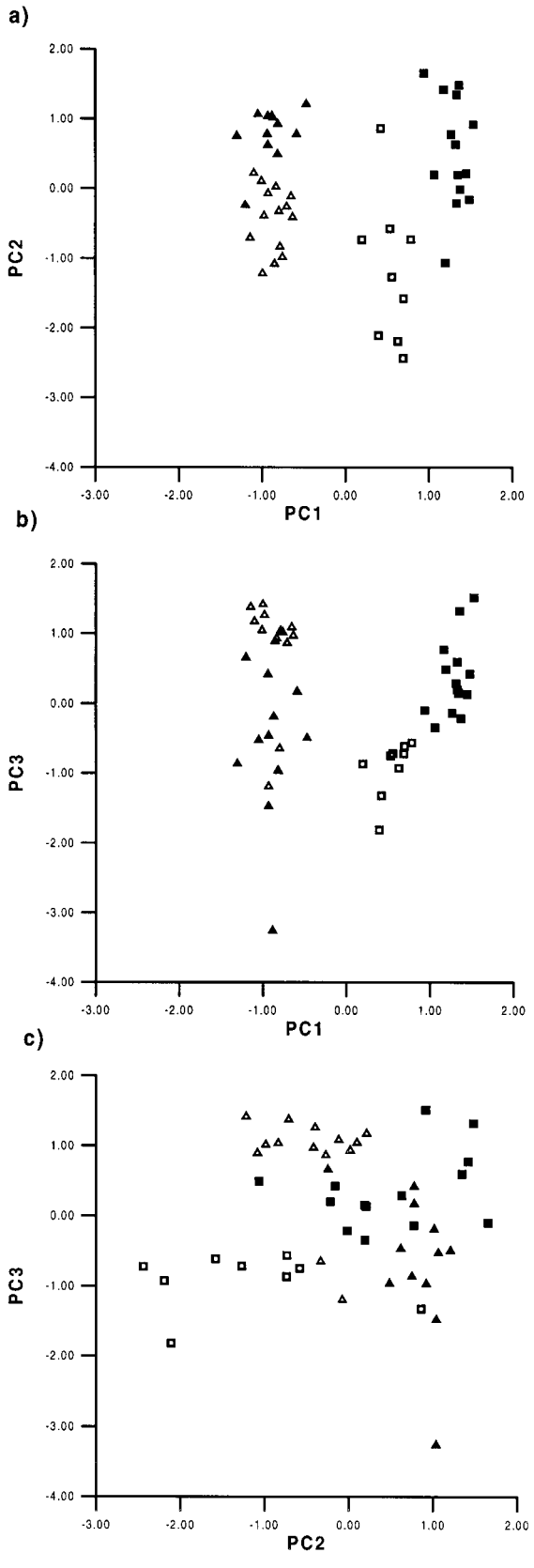
Cuticular Hydrocarbons of Drosophila serrata, Drosophila birchii, *and Their Hybrids*

Correlations between the nine PCs from the PCA involving both sexes of *D. birchii* and *D. serrata* and the relative concentrations of the 10 original peaks are given in table 2. Correlations are presented, rather than the coefficients of the eigenvectors, as they are generally a better indication of the relative importance of the contribution of the original variables to the principal components (Karson 1982). The percentage of the variance explained by each of the nine PCs was 49.1, 32.6, 7.4, 3.7, 2.4, 2.0, 1.2, 0.8, and 0.6, respectively. The biological interpretation of the principal components is best approached by an examination of whether they can discriminate between naturally occurring biological groupings, which in this case were species and sex. MANOVA indicated that the nine PCs were significantly influenced by an interaction between species and sex (table 3). Univariate two-way ANOVAs were then used to determine which PCs were associated with species and sex. The variable PC1 (fig. 2*A*) clearly separated the two species ($F = 1{,}146.4$, df = 1, 45, $P < .001$) and also displayed a significant interaction between species and sex ($F = 53.7$, df = 1, 45, $P < .001$). The variable PC2 separated the sexes, irrespective of species membership (fig. 2*A*); males have higher scores for PC2 than females for both species ($F = 55.2$, df = 1, 45, $P < .001$). The variable PC3 is also involved in separating the sexes (fig. 2*B*), but the pattern of concentration is opposite between the two species, as indicated by the significant interaction ($F = 41.8$, df = 1, 45, $P < .001$). The variables PC2 and PC3 together (fig. 2*C*) again indicate an interaction between species and sex, but on this occasion, the same sex from each species is diagonally opposed.

Table 3: MANOVA testing for an effect of sex and species on the nine principal components of culticular hydrocarbons from the two parental species

Source	*Wilks's* λ	F	*Hypothesis df*	*Error df*	P
Species	.030	131.75	9	18	.040
Sex	.234	13.46	9	18	.203
Interaction	.166	20.70	9	18	.031

Interpreting which original variables contributed to each principal component was conducted using the criterion suggested by Mardia et al. (1979); those variables with correlations above 0.7 times the largest correlation in an eigenvector were considered to have contributed significantly. The hydrocarbons that were different in concentration between the species (i.e., species-specific concentrations) were then those that contributed strongly to PC1 (cutoff point = 0.654). From the correlations in table 2, PC1 is a bipolar principal component that contrasted peaks 1, 9, and 10 with 3, 4, 5, and 7. Those hydrocarbons that were found in different concen-



trations between the sexes of both species (i.e., sex-specific concentrations) were those that contributed strongly to PC2 (cutoff point = 0.669); this was again a bipolar component that contrasted peaks 2 and 8. Finally, those hydrocarbons that were found in different concentrations in the same sex of different species (i.e., sex-specific concentrations within species) contributed to PC3 (cutoff point = 0.336), a bipolar component that contrasted peaks 6 and 9. There was no overlap between the peaks that contributed to the three PCs with the exception of peak 9, which is just over the cutoff for PC1 but is the strongest contributor to PC3. This suggests that there were three independent blends of cuticular hydrocarbons that separated three levels of organization; species (PC1), sex (PC2), and sex within species (PC3).

Correlations between the nine PCs from the PCA on the hybrid hydrocarbon profiles and the relative concentrations of the 10 peaks are given in table 4. The percentage of the variance explained by each of the nine PCs was 62.6, 16.4, 6.7, 5.3, 4.6, 2.2, 1.2, 0.7, and 0.3, respectively. In this instance, there are also two categories to assist in the interpretation of the principal components: sex and reciprocal cross. From figure 3, it is clear that the combination of PC1 and PC2 defines a total separation between the sexes; PC1 (cutoff = 0.683) is a bipolar component contrasting peaks 2 and 3 with 5 and 8, and PC2 (cutoff = 0.583) contrasts peaks 9 and 10 with 4. No principal component was found to be associated with a difference between reciprocal cross.

There was some similarity between the principal component matrices of the parental species (table 2) and the hybrids (table 4). Visual inspection of the two matrices suggests that hybrid PC1 seems to be a combination of the parental PC2 (peaks 2 and 8), and part of the parental PC1 (peaks 3 and 5). The hybrid PC2 represents another part of the parental PC1 (peaks 4, 9, and 10). Therefore, the separation of the sexes in the hybrids by PC1 and PC2 (fig. 3) combines both species-specific and sex-specific components of the parental hydrocarbons.

Genetic Correlation between Hybrid Cuticular Hydrocarbons and Mating Success

The isofemale line experimental design can be used to investigate the importance of cuticular hydrocarbons in

Figure 2: Biological interpretation of the principal components analysis on parental cuticular hydrocarbons. *a,* PC1 versus PC2; *b,* PC1 versus PC3; and *c,* PC2 versus PC3. *Closed symbols,* males; *open symbols,* females; *squares, Drosophila serrata;* and *triangles, Drosophila birchii.*

Table 4: Correlations between the relative concentrations of the 10 cuticular hydrocarbon peaks and the nine principal components (PCs) from the principal components analysis on the interspecific hybrids

	Relative concentration of peaks									
PC	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1	.369***	.899***	.850***	−.176***	−.725***	.119**	−.302***	−.976***	−.522***	−.511***
2	.075	−.047	−.285***	−.833***	−.229***	.125**	.345***	.122**	.727***	.764***
3	.552***	−.187***	−.157***	−.064	.246***	.768***	−.258***	.116**	−.032	.114**
4	−.705***	.003	.040	.038	.041	.508***	.012	.013	.052	.004
5	−.141***	.090*	.100*	−.202***	.388***	−.188***	−.449***	.004	−.073	.243***
6	.061	−.272***	−.216***	.062	.300***	.043	.501***	.130**	.265***	−.064
7	−.008	−.209***	−.086	.282***	.053	.039	.203***	.050	−.125**	.141***
8	.047	−.121**	.061	.262***	.048	.022	−.144***	.024	.196***	.037
9	−.036	.114**	−.292***	−.034	−.063	−.024	−.202***	−.059	−.090*	−.051

* $P < .05$.
** $P < .01$.
*** $P < .001$.

mating success at the genetic level. The genetic correlation between the two traits (see Blows 1998 for the genetic covariance between isofemale lines) may be approximated by the between-line product-moment correlation (Via 1984). Since hydrocarbon profile comprised nine principal components, a multivariate approach was required to determine the association between this trait and mating success. A multiple regression of the mating success score for each line on the means of the nine principal component scores for each line gave an indication of which principal components were involved in mating success, and an estimate of the genetic correlation between these two traits may be gained from the multiple correlation coefficient. Multiple regressions of the mating success of each line, onto the means of the nine PC scores of each line, were conducted separately for the 15 lines from each original interspecific reciprocal species cross. Within each reciprocal cross, a multiple regression was conducted separately for male and female hybrids, resulting in four multiple regressions in total. Separate multiple regressions enabled the effect of sex-linkage to be determined and indicated if male and female hybrids used different hydrocarbons to select mates.

The most appropriate regression model for each of the four multiple regression analyses (table 5) was identified as that which displayed the lowest value of Mallow's *Cp* statistic, which fell below the line $Cp = p$, where $p =$ the

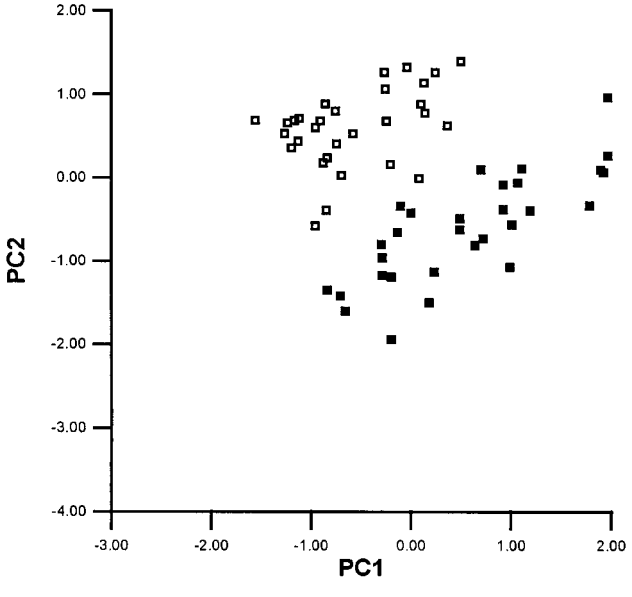


Figure 3: Plot of PC1 versus PC2 from the hybrid PCA displaying the means of the 30 isofemale lines. Female individuals, *open squares;* males, *closed squares.*

Table 5: Multiple regressions of mating success on the nine principal components (PCs) of cuticular hydrocarbon concentration

	S♀ × B♂		*B♀ × S♂*	
	H♀	*H♂*	*H♀*	*H♂*
PCs	1, 3, 4, 6, 7, 8, 9	4, 6	6	1, 2, 4, 7, 8, 9
Multiple r	.951**	.842***	.612*	.957***
Adjusted r^2	.809	.661	.326	.853

Note: Data from hybrid males (H♂) and hybrid females (H♀) from the two original reciprocal crosses between the two parental species (S♀ × B♂ and B♀ × S♂) are analyzed separately. Best regressions were determined by the use of Mallow's *Cp* statistic (see text).

* $P < .05$.
** $P < .01$.
*** $P < .001$.

number of parameters in the model, from among all possible models (Draper and Smith 1981). Cuticular hydrocarbons explained significant levels of the variation in mating success in all four cases. Since these multiple regressions were conducted on the isofemale line means, the multiple correlation coefficients approximated the genetic correlation between mating success and cuticular hydrocarbon profile. The average genetic correlation from these four analyses was 0.841, suggesting that mating success and cuticular hydrocarbons are genetically very similar traits.

The high degree of genetic variation in mating success explained by genetic variation in cuticular hydrocarbons is not simply a consequence of fitting many variables (nine PCs) with a small number of cases ($n = 15$ lines for each regression). A simulation of these data was conducted by regressing, in turn, 1, 2, 6, and 7 normally distributed, randomly generated, variables (i.e., representing the number of PCs from the four models in table 5) onto a tenth randomly generated variable (i.e., mating success), using 15 data points. The simulation was run 100 times for each of the four models. The adjusted r^2 values from the four multiple regressions in table 5 were then compared with the simulated results. This showed that these four results would be expected to appear, if no relationship was present, in less than 6%, 3%, 11%, and 3% of cases, respectively. Combining these conservative probabilities (Sokal and Rohlf 1981, p. 780) indicated that these results would appear in less than 0.5% of cases at random.

Sex-Linkage in the Expression of Cuticular Hydrocarbons

A large sex-linked effect was associated with the hydrocarbon PCs that contributed to mating success (table 5). When the *D. serrata* X chromosome was initially predominant (i.e., in lines from the S♀ × B♂ interspecific cross), hybrid females had seven PCs of hydrocarbons associated with mating success, whereas males had only PC4 and PC6 contributing to success in mating. When the *D. birchii* X chromosome was initially predominant (lines from the B♀ × S♂ interspecific cross), the opposite was true; only hydrocarbon PC6 contributes to mating success in hybrid females, perhaps again in conjunction with PC4 (the regression model with the third lowest *Cp* score for this combination consisted of PC4 and PC6), whereas a complex set of hydrocarbons, similar to that used by hybrid females with the *D. serrata* X chromosome, contributed to male mating success. Since the contribution of hydrocarbons to mating success in hybrid females is dependent on the initial cross, the X chromosome is implicated in its control, but we are unable to distinguish between X or Y chromosome control in the appearance of the hydrocarbon composites in males. Both conclusions assume that maternal cytoplasmic effects are not present after 11 generations.

Table 6: MANCOVAs for hybrid males and females testing for an effect of reciprocal cross on the association between mating success and the nine principal components of cuticular hydrocarbons

Source	*Wilks's* λ	F	*Hypothesis df*	*Error df*	P
Hybrid males:					
Reciprocal cross	.435	2.60	9	18	.040
Mating success	.563	1.55	9	18	.203
Interaction	.418	2.79	9	18	.031
Hybrid females:					
Reciprocal cross	.378	3.29	9	18	.015
Mating success	.881	.27	9	18	.975
Interaction	.442	2.52	9	18	.046

To further investigate the effect of the reciprocal cross on the association between hydrocarbons and mating success, two MANCOVAs (table 6) were conducted across the nine PCs for the hybrid male and hybrid female data (mating success was used as the covariate). The multivariate mating success by reciprocal cross-interaction terms for both MANCOVAs was significant, indicating that the initial cross significantly altered the relationships between the nine PCs and mating success. This suggests that not only are different PCs associated with mating success as a result of the reciprocal cross (multiple regression results), but the concentrations of these PCs that result in mating success changes as a consequence of the reciprocal cross.

To illustrate this result, consider PC6, which contributes to mating success in three of the four models in table 5 (fig. 4). In hybrid females, PC6 displays a significant relationship with mating success, but the initial cross causes this relationship to be in opposite directions; that is, low levels of PC6 increase mating success when hybrid females came from lines where the *D. serrata* X chromosome was predominant initially, but high levels of PC6 increase mating success when hybrid females came from the reciprocal cross. In hybrid males, PC6 displays a significant relationship with mating success when males came from lines that had a *D. serrata* father but no relationship when males came from lines that had a *D. birchii* father, reflecting the absence of PC6 from the model for this last combination in table 5. So, high levels of PC6 increase mating success of both sexes with *D. serrata* in lines from the S♀ × B♂ cross, but low levels of PC6 increase mating success of hybrid females with *D. serrata* in lines from the B♀ × S♂ cross.

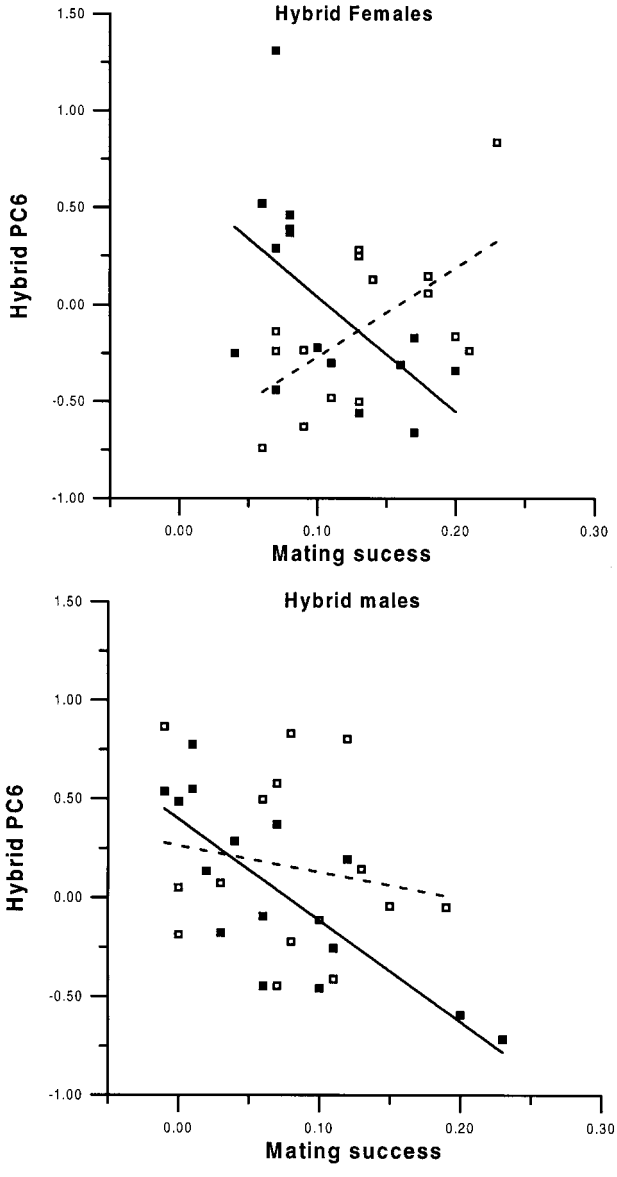


Figure 4: The relationship between mating success and PC6 for hybrid females and hybrid males for the 30 isofemale lines. The initial interspecific cross S♀ × B♂ is represented by closed squares/solid line, and the reciprocal cross B♀ × S♂ by open squares/broken line.

The Role of Species-Specific Cuticular Hydrocarbons in Hybrid Mating Success

The individual hydrocarbon peaks that contributed to the PCs important in hybrid mating success were involved at all three levels of organization in the parental species. Hybrid PC6 and PC4, which were solely associated with mating success in two combinations in table 5, best reflect this. Hybrid PC6 (cutoff = 0.351) represents peak 7, which is the strongest contributor to parental PC1 that distinguishes between species. Hybrid PC4 (cutoff = 0.494) contrasts peak 1 with peak 6; the former is again involved in parental PC1, but the latter is one of two peaks that contribute to parental PC3 that are important in discriminating between the sexes within species. The two remaining, more complex, combinations of hydrocarbons associated with mating success (table 5) also contain species-specific elements. Hybrid PCs 1, 4, 7, 8, and 9, which are all present in both combinations, contain significant contributions (on the basis of Mardia et al.'s [1979] cutoff) from individual peaks that contribute to parental PC1 that separates the two species.

Discussion

Mate Recognition Within and Between Species

The cuticular hydrocarbons of *Drosophila serrata* and *Drosophila birchii* appear to separate three levels of organization: species, sex, and sex within species. Different hydrocarbons were associated with each level of organization. The association of different hydrocarbons with the separation of species and sexes in *D. serrata* and *D. birchii* suggests discrete roles for different pheromones in mate choice within species and sexual isolation between species. Isolation of individual peaks and the application of these hydrocarbons in bioassay experiments would provide a way of testing the association between the hydrocarbons and these putative functions. It should be noted, however, that we may not have described the complete cuticular hydrocarbon system involved in mate recognition in this system; it remains to be seen if the two unique hydrocarbons in the *D. birchii* profiles have a role in either sexual isolation between the two species or mate choice within this species.

In hybrid individuals, the distinction between species-specific and sex-specific hydrocarbons was broken down, with a blend of these two parental composites distinguishing between the sexes. Mate choice in the hybrids involved a mixture of parental species-specific and sex-specific combinations; hydrocarbons that contributed to both putative functions in the parents were important in mating success in the hybrids. This experiment demonstrated that species-specific characters can be associated with mate choice within a group (i.e., the hybrids) and raises the possibility that these characters may have been involved in mate choice before the speciation event.

The Genetic Basis of Mate Recognition

The difficulties associated with the genetic analysis of behavior in studies of mate choice (Bakker and Pomiankowski 1995) and sexual isolation (Wu et al. 1995) has led to single potential mechanisms becoming the focus of

genetic studies in these areas. It is not clear from these types of studies, however, if the genetic basis of a single mechanism is analogous to the genetic basis of mate choice or sexual isolation in toto. Mate choice in *Drosophila* may involve four types of mechanisms: visual cues, acoustic cues, tactile cues, and chemical cues (Spieth and Ringo 1983). Distinguishing the relative importance of these mechanisms has not been resolved (Jallon and David 1987). The high genetic correlations found between cuticular hydrocarbon profile and mating success suggest that mating success is closely associated with chemical cues in this system. In two of the four classes of hybrid individuals, estimates of genetic correlation of >0.95 suggested that mating success and hydrocarbon profile were almost the same trait. The interpretations of genetic correlations are generally subject to the caveat that a third, unmeasured, variable may be responsible for the observed association between the two traits. In this instance, however, the estimates of genetic correlation have been made after 11 generations of recombination. Therefore, for traits other than cuticular hydrocarbon profile to be responsible for the observed genetic correlation with mating success, there would need to be tight physical linkage been the genes controlling both traits. It seems likely, therefore, that mate choice in hybrid individuals is strongly associated with cuticular hydrocarbon profile.

The clear biological relevance of the hydrocarbon principal components is not only demonstrated by their discrimination between species and sex in the parental PCA and their explanatory power in hybrid mating success but is also highlighted by the striking sex-linked effect in their association with hybrid mating success. The control of cuticular hydrocarbons by sex-linked genes has been reported in a number of cases. X-chromosome effects have been identified in male hydrocarbon differences (Averhoff and Richardson 1976; Scott and Richmond 1988), female hydrocarbon differences (Averhoff and Richardson 1976), between strains of *Drosophila melanogaster,* and between mutant strains of *Drosophila simulans* (Ferveur and Jallon 1993). An X-linked effect was found on male cuticular hydrocarbons, but not on female hydrocarbons; between *D. simulans* and a sibling species, *Drosophila sechellia* (Coyne et al. 1994; Coyne 1996); or between *Drosophila mauritiana* and *D. sechellia* (Coyne and Charlesworth 1997). Similarly, sex-linkage does not appear to be a consistent feature of behavioral measures of sexual isolation (Coyne 1989).

Role of Male and Female Hydrocarbons in Mate Choice

Although female cuticular hydrocarbons have frequently been implicated in mate choice in *Drosophila* (Antony and Jallon 1982; Jallon 1984; Coyne et al. 1994), the potential role of male cuticular hydrocarbons has been more controversial (Cobb and Ferveur 1996; Coyne 1996). Both male and female cuticular hydrocarbons were associated with mate choice to a similar extent in this experiment as indicated by the perfuming experiment and the genetic correlation analysis. Males of *Drosophila* species are usually assumed to not discriminate between mates, but a recent review (Noor 1996) suggested that this does occur in about half the cases that have been investigated. In this instance, not only have male *D. serrata* been shown to discriminate on the basis of female cuticular hydrocarbons, but female *D. serrata* were also shown to use male cuticular hydrocarbons in mate choice. This result is consistent with observations of copulatory behavior in *D. serrata* and *D. birchii* that demonstrated that males of both species discriminate between species and sex (A. Hoikkala, S. Crossley, and C. Castillo-Melendez, unpublished manuscript). Male cuticular hydrocarbons have been implicated in mate choice in at least two studies using *D. melanogaster* (Averhoff and Richardson 1976; Scott 1994) and one using *Drosophila mojavensis* (Markow and Toolson 1990), and a small effect was suggested in hybrids between *D. sechellia* and *D. simulans* (Coyne 1996).

Although cuticular hydrocarbons were strongly associated with mate choice in male and female hybrids, the genetic correlation between male and female components of the mate recognition system was relatively small ($r = 0.388$; Blows 1998). This suggested that in spite of males and females using the same trait in mate choice, different genes controlled the expression of the trait in the two sexes to a large extent.

This conclusion is supported by an examination of the genetic correlations between male and female hybrid cuticular hydrocarbon expression (table 7). Of the nine hybrid PCs, five display significant genetic correlations between males and females, but four (all of which are involved in hybrid mate choice) do not.

Conclusions

Species-specific attributes of cuticular hydrocarbons were used by the hybrids in mate choice, suggesting that mate choice within species and sexual isolation between species may be part of the same general process of mate choice (Ryan and Rand 1993; Endler and Houde 1995). If we are to determine whether sexual selection does play a significant role in speciation (Lande 1981), we will need to determine whether traits that have been under sexual selection result in sexual isolation. The perturbation of mate recognition systems by interspecific hybridization provides a convenient way to initiate the evolution of

Table 7: Genetic correlations between male and female expression of cuticular hydrocarbons in the hybrids

PC	*Genetic correlation*
1	.828***
2	.519**
3	.390[a]*
4	.234
5	.765***
6	.504**
7	.192
8	.649***
9	.484[a]**

[a] Indicates not significant after the Bonferroni correction.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

mate choice in subsequent generations under controlled conditions. Tracking the evolution of male and female components of mate recognition and any subsequent sexual isolation between replicate lines, with and without a history of sexual selection, may be one way to achieve this.

The use of fertile interspecific hybrids has been a neglected tool in evolutionary biology (see Wallace et al. 1983 for a notable exception). By adopting a quantitative genetic approach using interspecific hybrids, we have been able to demonstrate that a single mechanism of mate recognition (cuticular hydrocarbons) explains most of the genetic variation that is present between species in mate recognition. If mate recognition is generally determined by a single mechanism, at least under simplified laboratory conditions, the genetic analysis of sexual isolation and mate choice may be reduced to the genetic analysis of these mechanisms and becomes a more manageable task.

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