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Genetics of a difference in cuticular hydrocarbons between *Drosophila pseudoobscura* and *D. persimilis*

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Summary
We identify a fixed species difference in the relative concentrations of the cuticular hydrocarbons 2-methyl hexacosane and 5,9-pentacosadiene in *Drosophila pseudoobscura* and *D. persimilis*, and determine its genetic basis. In backcross males, this difference is due to genes on both the X and second chromosomes, while the other two major chromosomes have no effect. In backcross females, only the second chromosome has a significant effect on hydrocarbon phenotype, but dominant genes on the X chromosome could also be involved. These results differ in two respects from previous studies of *Drosophila* cuticular hydrocarbons: strong epistasis is observed between the chromosomes that produce the hydrocarbon difference in males, and the difference is apparently unrelated to the strong sexual isolation observed between these species.

1. Introduction

Although species often differ in many easily quantifiable morphological and behavioural characters, genetic analyses of adaptive differences between species are surprisingly rare (see Orr & Coyne, 1992). Genetic investigations of characters that are thought to confer reproductive isolation between sympatric species can elucidate the proximal causes of speciation. If, for example, mating discrimination genetically covaries with a phenotypic character difference, then that character may be a cue that reduces or prevents hybridization in nature.

Insect cuticular hydrocarbons reduce cuticular permeability to water and have been suggested as adaptations to reduce desiccation (Toolson, 1982). In some cases their presence on females induces courtship by conspecific males (e.g. Jallon, 1984). Differences in hydrocarbon profiles between species can also contribute to sexual isolation (Carlson *et al.*, 1971; Grula and Taylor, 1979; Coyne *et al.*, 1994; Coyne & Oyama, 1995). Indeed, in almost every insect species studied, some cuticular hydrocarbons have been found to play a pheromonal role (Cobb & Ferveur, 1996). Because these compounds are easily extracted and quantified by gas chromatography, they are ideal for genetic studies of reproductive isolation. Three previous studies have found that hydrocarbon differences between closely related species were probably caused by only one or a few loci (Grula & Taylor, 1979; Roelofs *et al.*, 1987; Coyne *et al.*, 1994).

Here we identify a difference in the predominant cuticular hydrocarbons of the well-known sibling species *Drosophila pseudoobscura* and *D. persimilis*, analyze its genetic basis, and evaluate its potential effects on reproductive isolation.

D. pseudoobscura ranges across North America from the Pacific coast east to Texas and from British Columbia south to Guatemala. An isolated subspecies of *D. pseudoobscura*, *D. pseudoobscura bogotana*, occurs in the highlands of Colombia. *D. p. bogotana* is given subspecific status because hybrid males are sterile in one of the two reciprocal crosses with North American *D. pseudoobscura* (Ayala & Dobzhansky, 1974). The time of divergence of these subspecies, estimated from DNA sequences, is approximately 150000 years ago (Schaeffer & Miller, 1991).

D. persimilis is completely contained within the range of North American *D. pseudoobscura*, occurring only in the Pacific coastal states. Hybridizations between *D. pseudoobscura* and *D. persimilis* produce sterile male offspring, but the fertility of female hybrids permits genetic analysis through backcrossing.

Blomquist *et al.* (1985) identified the three major cuticular hydrocarbons of *D. pseudoobscura*, all present in both males and females: 2-methyl hexacosane (2-MH), 2-methyloctacosane (2-MO) and 5,9-pentacosadiene (5,9-PD). Together these accounted for 49% by weight of the total long-chain cuticular hydrocarbons. Several other hydrocarbons, such as 2-methyltriacontane, occur in smaller quantities. There have been no reports on the cuticular hydrocarbon composition of *D. persimilis* or the subspecies *D. pseudoobscura bogotana*.

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2. Materials and methods

The wild-type strains of *D. pseudoobscura* and *D. persimilis* used in this study were collected in the summer of 1993 (see Noor, 1995*a*). The *D. pseudoobscura bogotana* strains were obtained from D. Weinreich and were collected from 1 to 6 years before analysis. Although the hydrocarbon composition of *D. pseudoobscura* is known to change through time in laboratory culture (Toolson & Kuper-Simbrón, 1989), the observed difference between the species in their hydrocarbon ratios was several orders of magnitude greater than the differences between strains within either species. Hence, it is unlikely that evolution of these hydrocarbons in the laboratory biased our results. Stocks were kept at 21 °C on cornmeal/Karo syrup/yeast/agar food, and carbon dioxide was used for anaesthetization. After eclosion, flies were held individually for 8 days to reach reproductive maturity.

(i) *Genetics of cuticular hydrocarbon difference*

The reciprocal F_1 hybridizations and backcrosses were made with the *D. persimilis* Mount St Helena strain and a *D. pseudoobscura* strain carrying a recessive mutation on each of the four major chromosomes: yellow (*y* I-75); glass (*gl* II-83); orange (*or* III-0); incomplete (*inc* IV-0) (map locations taken from Anderson & Norman (1977) and Orr (1995)). All backcrosses were done using the fertile F_1 hybrid females. These species differ by large, recombination-inhibiting inversions in the X, second and third chromosomes (Dobzhansky & Epling, 1944), so it was not necessary to use multiple genetic markers on each chromosome. Since F_1 males from both reciprocal crosses had hydrocarbon profiles that resembled *D. persimilis* (see below), genetic analysis was conducted using backcrosses to *D. pseudoobscura*. F_1 females were backcrossed to *y;gl;or;inc* males, yielding 16 identifiable classes of offspring carrying different combinations of the species' chromosomes. All backcross males had a Y chromosome from *D. pseudoobscura*, and all females carried one complete *D. pseudoobscura* X chromosome.

After flies had been aged for 8 days, hydrocarbon extracts were made using the procedure of Ferveur (1991). The dried extract was then redissolved in 5 μl of *n*-hexane, 3 μl of which was injected into a HP 5890 Series II gas chromatograph connected to a HP 3396 Series II integrator. The temperature was increased from 210° to 250 °C at 3 °C/min, each run lasting 18 min. The split ratio was 61:1. Hydrocarbon peaks were identified by previous reports in the literature and by co-migration with peaks from the other species.

Profiles of the two major hydrocarbons are presented as ratios of 2-MH to 5,9-PD (see below). Ratios were used to eliminate the effect of differences in body size. Since the profiles for males and females differed, all results are separated by sex. Five samples were run for each pure-species strain (except *y;gl;or;inc* males) and genotype of F_1 hybrid. All pairwise comparisons were analysed using non-parametric Mann–Whitney *U*-tests, and the backcross data were analysed with ANOVAs. For these latter tests, the hydrocarbon ratios were log-transformed to eliminate the correlation between means and variances (Sokal & Rohlf, 1995). All probabilities presented are two-tailed, and statistical analyses were performed using StatView software.

(ii) *Sexual isolation experiment*

The possible role of the interspecific hydrocarbon difference in sexual isolation was evaluated through mating tests. *D. pseudoobscura* and *D. persimilis* males court heterospecific females as quickly and intensely as they court conspecifics (Noor, 1996), but females reject them. Hence, the interspecific sexual isolation observed in the laboratory appears to be caused by female discrimination against heterospecific males. Therefore, any effect of hydrocarbon differences on sexual isolation must occur through the females' perception of the male phenotype.

Studies of the genetics of sexual isolation of these species are in progress and will be presented in their entirety elsewhere. The strains used for this sexual isolation study were Mount St Helena *D. persimilis* and Flagstaff *D. pseudoobscura*. We made F_1 hybrid males using a cross between *D. pseudoobscura* females and *D. persimilis* males. Single pairs of reproductively mature flies were confined in a food-containing vial, and their courtship was observed for 5 min or until copulation occurred.

3. Results

Fig. 1 shows typical hydrocarbon profiles we observed in *D. pseudoobscura* and *D. persimilis*. The hydrocarbon ratios were consistent across strains within each species, but there was a large interspecific difference in hydrocarbon ratio (see Table 1): the predominant *D. pseudoobscura* hydrocarbon was 2-MH (comprising approximately 60% of the total cuticular hydrocarbon composition), whereas *D. persimilis* flies carried primarily 5,9-PD (approximately 65% of the total hydrocarbon composition). The hydrocarbon profiles of *D. pseudoobscura bogotana* fell within the range of North American *D. pseudoobscura* (Noor, 1995*b*). As previously reported (Toolson & Kuper-Simbrón, 1989) the hydrocarbon profiles in *D. pseudoobscura* were sexually dimorphic, females of both species having a slightly higher proportion of 2-MH, but this difference was much smaller than the difference between species.

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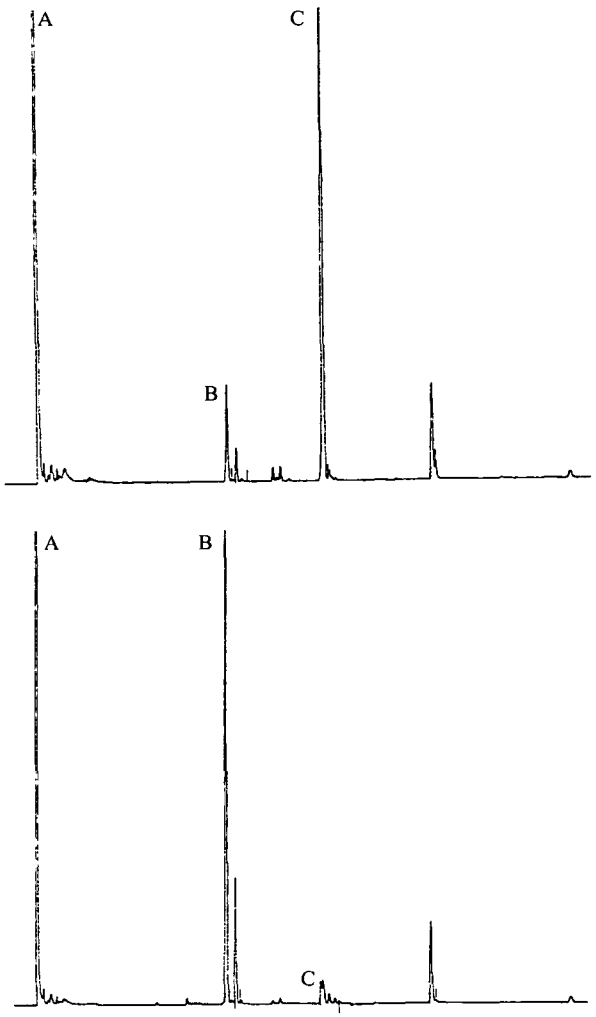

Fig. 1. Typical hydrocarbon profiles of *D. pseudoobscura* (above) and *D. persimilis* (below) obtained from gas chromatography. The major peaks are identified as: A, *n*-hexane; B, 5,9-pentacosadiene; C, 2-methyl hexacosane.

There are some discrepancies between our results and those reported previously (e.g. Toolson, 1982; Blomquist *et al.*, 1985). We did not observe 2-MO in any of the lines we examined, though it was the predominant hydrocarbon observed by Blomquist *et al.* (1985). Some other hydrocarbons identified in those studies were also not detected in our investigation. However, we did observe that the *y;gl;or;inc* mutant line, which had been cultured in the laboratory for several years, had the highest proportion of 5,9-PD of all the *D. pseudoobscura* lines examined (see Table 1). This finding corroborates a previous report that *D. pseudoobscura* hydrocarbons change with time in laboratory culture (Toolson & Kuper-Simbrón, 1989), becoming more similar to *D. persimilis*.

Table 2 gives the hydrocarbon profiles of the F_1 hybrid offspring. Neither class of hybrid male differed significantly in hydrocarbon ratio from pure-species *D. persimilis* males from Mount St Helena. The two classes of F_1 males did, however, differ slightly but significantly ($P = 0{\cdot}016$) from each other: males with a *D. pseudoobscura* X chromosome had a higher ratio of 2-MH to 5,9-PD. This implies a small X-effect in the expected direction (or a cytoplasmic effect, though we consider this less likely). F_1 hybrid females again had a slightly higher proportion of 2-MH to 5,9-PD than the F_1 males, though, unlike the males, they differed from pure-species *D. persimilis* ($P = 0{\cdot}002$). The maternal origin of the hybrid females had no effect on their relative hydrocarbon concentrations ($P = 0{\cdot}46$), implying no cytoplasmic effect.

Table 2. *Ratio of 2-methyl hexacosane to 5,9-pentacosadiene in F_1 hybrids of* D. pseudoobscura *and* D. persimilis

Species of mother	Sex	Ratio	SE
D. persimilis	M	0·035	0·007
	F	0·367	0·108
D. pseudoobscura	M	0·060	0·005
	F	0·261	0·017

Table 1. *Ratio of 2-methyl hexacosane to 5,9-pentacosadiene in population stocks of* D. pseudoobscura *and* D. persimilis.

		Male		Female	
Species	Population	Ratio	SE	Ratio	SE
D. persimilis	Mather, CA	0·042	0·001	0·079	0·004
	Mt San Jacinto, CA	0·040	0·004	0·065	0·004
	Mt St Helena CA	0·050	0·007	0·064	0·006
D. pseudoobscura	Flagstaff, AZ	5·3	2·2	20·4	5·4
	Mather, CA	7·8	3·5	10·6	1·5
	Mt St Helena, CA	11·3	1·9	22·7	2·1
	Provo, UT	1·7	0·2	16·2	1·1
	y;gl;or;inc	3·0[a]	0·3	10·5	1·5
D. pseudoobscura bogotana	Chiquinquira	3·4	0·9	16·2	1·5
	Oicata	2·6	0·5	18·8	1·8

[a] Only 4 males were used for this sample.

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Table 3. *Ratio of 2-methyl hexacosane to 5,9-pentacosadiene in backcross offspring of* D. pseudoobscura *and* D. persimilis

	Male			Female		
Phenotype	No. of samples	Ratio	SE	No. of samples	Ratio	SE
+;+;+;+	19	0·066	0·015	14	0·524	0·245
+;+;+;*inc*	11	0·068	0·005	4	0·365	0·098
+;+;*or*;+	10	0·062	0·007	10	0·227	0·064
+;+;*or*;*inc*	1	0·042	NA	7	1·170	1·029
y;+;+;+	10	0·069	0·006	10	0·485	0·257
y;+;+;*inc*	3	0·082	0·014	5	1·204	0·641
y;+;*or*;+	8	0·060	0·003	2	0·253	0·054
y;+;*or*;*inc*	4	0·055	0·002	1	0·143	NA
+;*gl*;+;+	12	0·078	0·009	17	3·761	1·064
+;*gl*;+;*inc*	6	0·290[a]	0·143	7	5·094	3·688
+;*gl*;*or*;+	10	0·113	0·031	12	4·755	1·698
+;*gl*;*or*;*inc*	1	0·065	NA	3	4·903	0·578
y;*gl*;+;+	10	1·492[a]	0·737	6	4·903	0·578
y;*gl*;+;*inc*	5	0·378	0·076	6	8·911	3·927
y;*gl*;*or*;+	10	0·660	0·228	7	10·348	2·767
y;*gl*;*or*;*inc*	2	0·764	0·701	1	1·596	NA

The statistical analyses reported in the text were performed with log-transformed ratios (see Materials and Methods).

[a] Both these ratios may be misleadingly high because of single outliers.

The hydrocarbon profiles of the backcross males and females are presented in Table 3 and Figs 2 and 3. We obtained rather small samples of some backcross genotypes, particularly those with multiple mutant markers: fewer than 1 fly in 30 possessed more than one marker. Inviability of hybrid or backcross offspring is known in these species (Dobzhansky & Epling 1944; Tan 1946), and Dobzhansky (1951) reported that mutant markers reduce the viability of hybrid flies far more than that of pure-species flies, but our paucity of multiply marked offspring was much more severe than seen in previous work (e.g. Orr, 1987). Nevertheless, the genetic results are clear even with small samples of some genotypes.

The backcross males most similar in hydrocarbon ratio to *D. pseudoobscura* were those having both the X and second chromosome markers from *D. pseudoobscura*. Each of these single-chromosome effects was highly significant ($P < 0{\cdot}0001$), and there was a significant interaction between them ($P_{X.2} = 0{\cdot}0014$). Fig. 2, rows 13–16, illustrates this positive epistasis. No other chromosomes, or interactions between chromosomes, had a significant effect ($P > 0{\cdot}1$) on backcross male hydrocarbon phenotype.

For backcross females, on the other hand, only the second chromosome marker (*gl*) was significantly associated with hydrocarbon ratio ($P < 0{\cdot}0001$). The lack of X-effect in backcross females may be because, unlike the males, they all possess one complete X chromosome from *D. pseudoobscura*. If the *D. pseudoobscura* X-linked factors that contribute to the hydrocarbon phenotype are completely dominant, then no X-effect would be observed. Thus, pooling the results from both backcross males and females, we conclude that the *D. pseudoobscura* hydrocarbon phenotype requires only one X chromosome but two second chromosomes from *D. pseudoobscura*.

Finally, we used mating tests to evaluate the role of the interspecific hydrocarbon difference in sexual isolation. Table 4 summarizes the results of tests of pure-species and F_1 hybrid males presented with pure-species females. Hybrid males with a *D. pseudoobscura* X chromosome are as successful at mating with *D. pseudoobscura* females, and as unsuccessful at mating with *D. persimilis* females, as pure *D. pseudoobscura* males. Hence, F_1 hybrid males behave like *D. pseudoobscura* males in crosses to both species, but they possess a hydrocarbon profile similar to *D. persimilis* males. This contrast provides strong evidence that the difference between the hydrocarbon profiles of these species is not a character used by females of one species to discriminate against males of the other.

4. DISCUSSION

The difference in cuticular hydrocarbon profile between *Drosophila pseudoobscura* and *D. persimilis* seems to be produced as an epistatic effect of factors on the X and second chromosomes. Although only the second chromosome effect was significant in determining the hydrocarbon profiles of backcross females, it is likely that dominant genes on their X chromosomes were also involved. Hence, a *D. pseudoobscura* hydrocarbon phenotype occurs in hybrids possessing both one X chromosome and two

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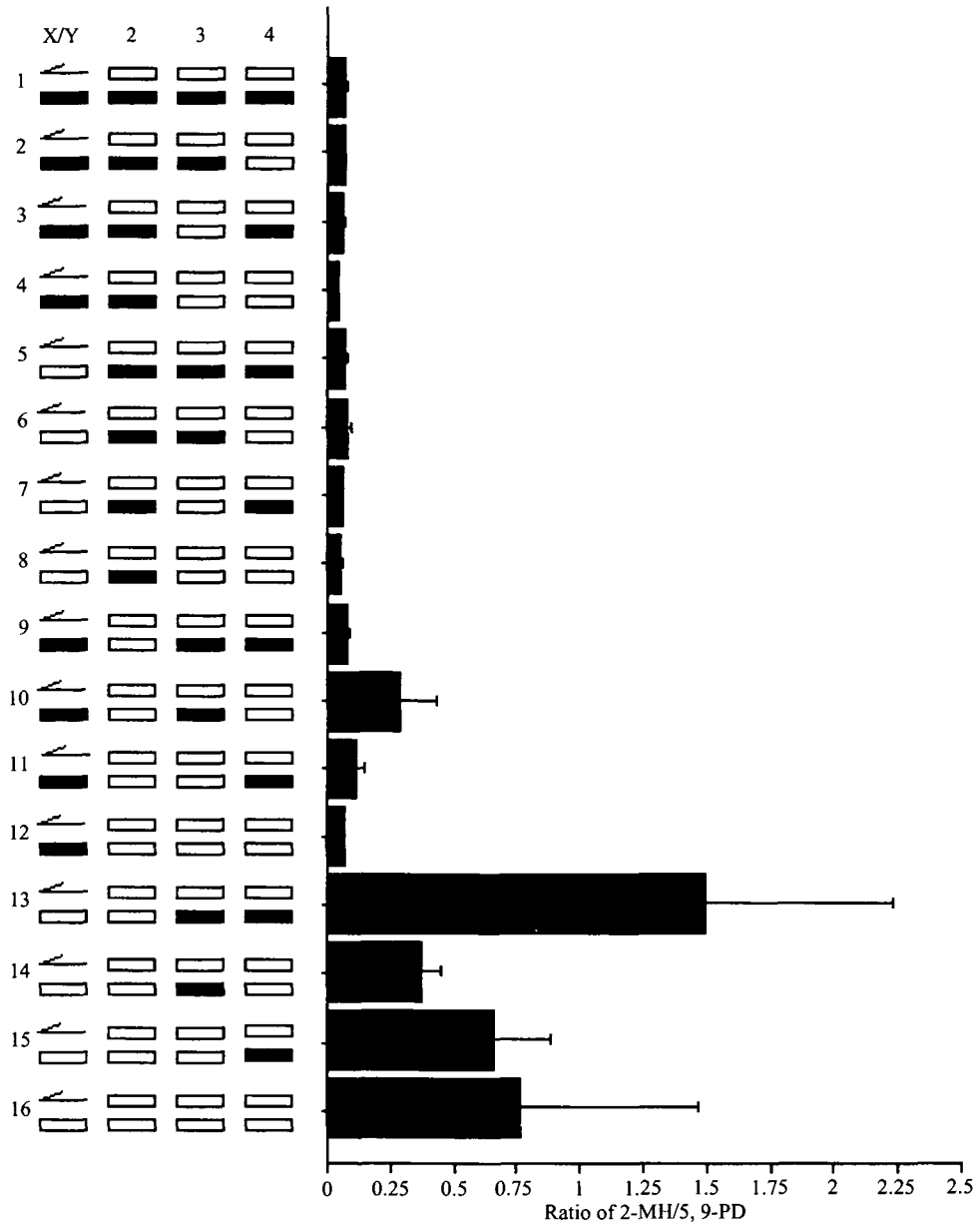

Fig. 2. Ratio of 2-methyl hexacosane (2-MH) to 5,9-pentacosadiene (5,9-PD) in backcross males of *D. pseudoobscura* and *D. persimilis*, grouped by genotype. The backcross genotypes are split, in order, by second, X, third, and fourth chromosomes to best illustrate the epistasis between the X and second chromosomes. All white chromosomes possess a *D. pseudoobscura* recessive mutant marker, and black chromosomes are derived from *D. persimilis*.

second chromosomes from that species. Such strong epistasis has not been observed in any previous studies of cuticular hydrocarbons (e.g. Ferveur, 1991; Coyne *et al.*, 1994).

The exact number of genes responsible for this phenotypic difference cannot be ascertained, as three of the four major chromosomes differ by inversions that prevent recombination in hybrids. However, the lack of any effect of two of the four major autosomes implies that the difference is probably not caused by an accumulation of genes of small effect spread throughout the genome. The presence of an X chromosome effect in this phenotypic difference is not surprising, for the X chromosome comprises nearly half the *D. pseudoobscura* genome (Anderson, 1993). The metabolic mechanisms producing these dominance and epistatic effects on hydrocarbon composition in hybrids are not obvious.

In contrast to findings in the *D. melanogaster* subgroup (Coyne *et al.*, 1994), our results also show that this fixed difference in cuticular hydrocarbon composition plays little or no role in the sexual isolation of these species. However, this hydrocarbon difference may play a role in preventing desiccation. *D. persimilis* is completely contained within the range of *D. pseudoobscura*, but it tends to be more abundant in the cooler and moister environments (Dobzhansky & Powell, 1975; Noor, 1995*c*). Correspondingly, the data of Toolson (1982) and Toolson & Kuper-Simbrón (1989) showed that *D. pseudoobscura* reared at colder

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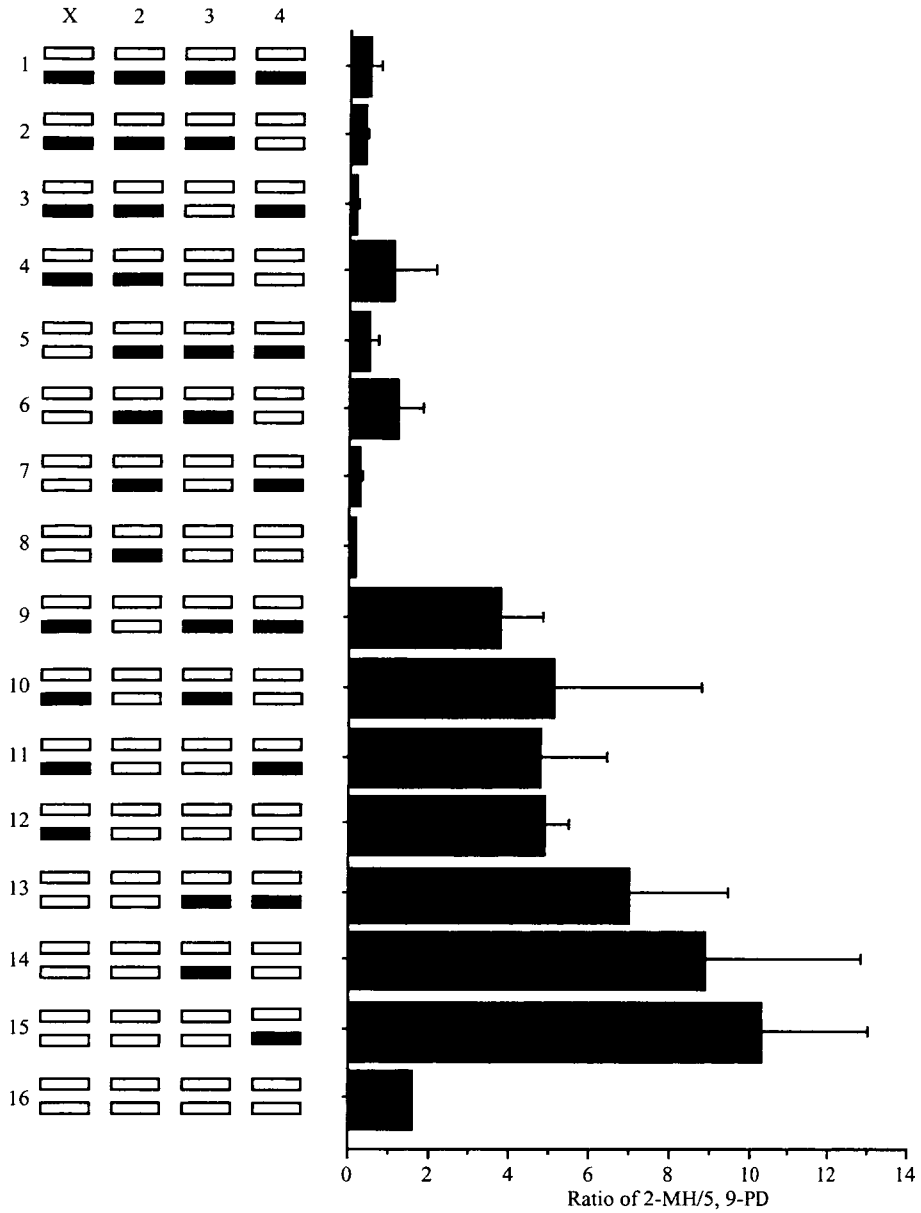

Fig. 3. Ratio of 2-methyl hexacosane (2-MH) to 5,9-pentacosadiene (5,9-PD) in backcross females of *D. pseudoobscura* and *D. persimilis*, grouped by genotype. The backcross genotypes are split, in order, by second, X, third, and fourth chromosomes to best illustrate the strong effect of the second chromosome. All white chromosomes possess a D. pseudoobscura recessive mutant marker, and black chromosomes are derived from D. persimilis.

Table 4. *Copulations in crosses between* D. pseudoobscura, D. persimilis *and hybrid males (with* D. pseudoobscura *mothers)*

	Females	
Males	*D. pseudoobscura*	*D. persimilis*
D. pseudoobscura	25/25	4/29
Hybrid males	25/25	5/25
D. persimilis	6/31	25/26

temperatures produced a higher proportion of 5,9-PD (the predominant *D. persimilis* hydrocarbon) to 2-MH, and that higher cuticular water permeability was correlated with greater proportions of these shorter-chained hydrocarbons. We are currently investigating outgroup species to identify the direction of evolution of these hydrocarbons.

The phenotypic cues that produce the sexual isolation of these species are not known, though male courtship 'song' (wingbeat frequency and pattern) is a possibility. Wingbeat has been clearly implicated as a cause of interspecific sexual isolation in other species of *Drosophila* (e.g. Tomaru *et al.*, 1995), and *D. pseudoobscura* and *D. persimilis* are known to differ in several characters of their male courtship song (Waldron, 1964; Ewing, 1969). Further genetic experiments might help to elucidate the phenotypic cues of the sexual isolation of these and other species.

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