Genetics of a Pheromonal Difference Affecting Sexual Isolation Between *Drosophila mauritiana* and *D. sechellia*

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Manuscript received September 3, 1996
Accepted for publication December 30, 1996

ABSTRACT

Females of the sibling species *Drosophila sechellia* and *D. mauritiana* differ in their cuticular hydrocarbons: the predominant compound in *D. sechellia* is 7,11-heptacosadiene (7,11-HD), while that in *D. mauritiana* is 7-tricosene (7-T). We investigate the genetic basis of this difference and its involvement in reproductive isolation between the species. Behavioral studies involving hydrocarbon transfer suggest that these compounds play a large role in the sexual isolation between *D. mauritiana* males and *D. sechellia* females, while sexual isolation in the reciprocal hybridization results more from differences in female behavior than hydrocarbons. This interspecific difference in hydrocarbon profile is due to evolutionary change at a minimum of six loci, all on the third chromosome. The localization of evolutionary change to the third chromosome has been seen in every other genetic analysis of female hydrocarbon differences in the *D. melanogaster* group. We suggest that the high 7,11-HD phenotype seen in two species evolved twice independently from ancestors having the high 7-T phenotype, and that the recurrent third-chromosome effects are evolutionary convergences that may be due to a concentration of "hydrocarbon genes" on that chromosome.

SEXUAL isolation is probably one of the primary causes of speciation in animals, as it may result from the ubiquitous process of sexual selection (ENDLER and HOUDE 1995; IWASA and POMIANKOWSKI 1995). In birds, for example, taxa with more extreme sexual dimorphism or more polygamous mating systems seem to speciate more frequently (BARRACLOUGH *et al.* 1995; MITRA *et al.* 1996). This suggests that reproductive isolation may be an important byproduct of sexual selection. Moreover, natural selection may increase sexual isolation through "reinforcement," a process that reduces maladaptive hybridization between incipient species (DOBZHANSKY 1935). Reinforcement may explain why sexual isolation in Drosophila is much higher between sympatric than between allopatric species of similar divergence time (COYNE and ORR 1989, 1996), although the frequency of reinforcement is a controversial issue (RICE and HOSTERT 1993; BUTLIN 1995).

Genetic studies of sexual isolation have, however, lagged far behind those of *postzygotic* isolation (sterility and inviability of hybrids), undoubtedly because it is hard to do genetic analysis of behaviors, particularly those involving interaction between the sexes. We have dealt with this problem by studying an easily measured character affecting sexual isolation in Drosophila: pheromonal cuticular hydrocarbons.

Like all insects examined to date, Drosophila carry a layer of long-chain hydrocarbons on the surface of the cuticle (HOWARD and BLOMQUIST 1982). These compounds protect the animal against desiccation and act in some species of Lepidoptera, Diptera, and Coleoptera as female pheromones that stimulate courtship by conspecific males (CARLSON *et al.* 1971; UEBEL *et al.* 1975; GRULA *et al.* 1980; HOWARD and BLOMQUIST 1982; JALLON 1984; PESCHKE 1987; OGUMA *et al.* 1992).

Most of the work on Drosophila hydrocarbons has concentrated on the *D. melanogaster* subgroup, which comprises eight closely related species. Four of these (*D. yakuba, D. orena, D. erecta,* and *D. tessieri*) are restricted to Africa, two (*D. melanogaster* and *D. simulans*) are cosmopolitan human commensals, and two (*D. sechellia* and *D. mauritiana*) are endemic to islands in the Indian Ocean. The phylogeny of this group has been well characterized using chromosomal, morphological, and molecular traits (*e.g.*, LEMEUNIER *et al.* 1986; CACCONE *et al.* 1996), and the cuticular hydrocarbons were described by JALLON and DAVID (1987). Males of all species have similar hydrocarbon profiles, with large quantities of *cis*-7-tricosene (henceforth called 7-T), although *D. sechellia* males also carry large amounts of the isomer 6-tricosene. Considering female hydrocarbons, one can divide the species into three groups: (1) those whose females resemble males in having large amounts of 7-T (*D. orena, D. tessieri, D. yakuba, D. simulans,* and *D. mauritiana*), (2) those whose females have very little 7-T but high levels of *cis,cis*-7,11-heptacosadiene (henceforth 7,11-HD; these species include *D. sechellia* and *D. melanogaster*), and (3) the anomalous *D. erecta,* whose females carry an array of long-chain hydrocarbons not found in the other species.

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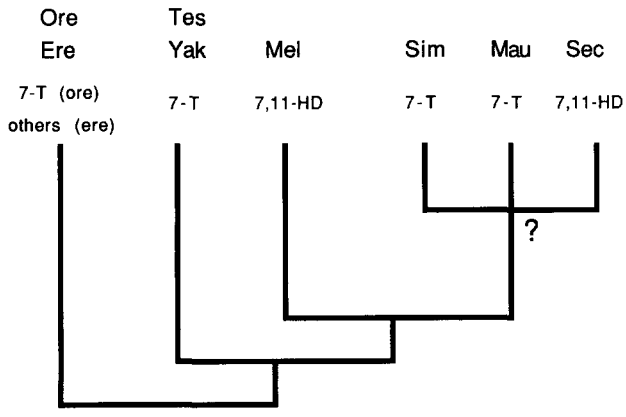

FIGURE 1.—Phylogeny of *D. melanogaster* group, showing proposed relationships among the eight species and the predominant hydrocarbon found in females of each species. ere, *D. erecta;* mau, *D. mauritiana;* mel, *D. melanogaster;* ore, *D. orena;* sec, *D. sechellia;* sim, *D. simulans;* tes, *D. tessieri;* yak, *D. yakuba.* Phylogeny shows only the order of branching; lengths of lines do not indicate relative divergence times. The question mark at the common ancestor of *D. simulans, D. mauritiana,* and *D. sechellia* indicates that the branching order of these three taxa are not resolved. Data taken from LACHAISE *et al.* (1988), HILTON *et al.* 1995, and CACCONE *et al.* (1996).

Figure 1 shows the consensus phylogeny of this group determined by DNA sequencing (KLIMAN and HEY 1993; HILTON *et al.* 1995; CACCONE *et al.* 1996); superimposed on this are the predominant cuticular hydrocarbons of females. Not shown is the polymorphism for hydrocarbons seen in some African populations of both *D. melanogaster* and *D. simulans* (JALLON 1984; JALLON and DAVID 1987; COBB and JALLON 1990; FERVEUR *et al.* 1994).

In *D. melanogaster* and *D. simulans,* the predominant female hydrocarbon stimulates courtship behavior by conspecific males (JALLON 1984; the function of hydrocarbons in other species of the group have not been studied). Moreover, hydrocarbon *differences* among species of this group apparently contribute to sexual isolation. This possibility was originally suggested by COBB and JALLON (1990) who noticed a correlation between interspecific courtship behavior and pheromones in the *D. melanogaster* subgroup. Males of the two species sexually dimorphic for hydrocarbons (males with 7-T and females with 7,11-HD) court females of all species in the subgroup much more readily than do males of the five sexually monomorphic species (males and females both having 7-T), who usually court females only from other 7-T-monomorphic species. COBB and JALLON concluded that males from dimorphic species respond to a wider range of hydrocarbons than do males from monomorphic species.

COBB and JALLON's hypothesis was supported by experiments in *D. melanogaster, D. simulans,* and *D. sechellia* involving transfer of hydrocarbons among females of different species. These transfers affected their attractiveness to males in exactly the predicted direction (COYNE *et al.* 1994; COYNE and OYAMA 1995); for example, when some 7,11-HD is added to a *D. simulans* female, she becomes much less attractive to conspecific males. Such experiments imply that differences in female pheromones contribute to sexual isolation, although males of different species must also differ in how they perceive or respond to these compounds. Moreover, other characters must be responsible for sexual isolation between species whose hydrocarbon profiles are nearly identical (COYNE 1989, 1996b).

We previously studied the genetic basis of female hydrocarbon differences contributing to reproductive isolation in two hybridizations: *D. sechellia/D. simulans* and *D. simulans/D. melanogaster* (COYNE *et al.* 1994; COYNE 1996a). In both cases, the hydrocarbon difference (7-T *vs.* 7,11-HD) mapped to only one of the four chromosomes, the third. In the former hybridization, we were unable to determine how many third-chromosome genes caused the hydrocarbon difference, but in the *D. melanogaster/D. simulans* hybridization at least five regions of this chromosome were involved, with at least one gene on right arm and four on the left.

Because the phylogeny of the three species in the *D. simulans* clade is unresolved, it is useful to perform the third possible genetic analysis of hydrocarbon differences between species of this group, the *D. sechellia/D. mauritiana* hybridization. CACCONE *et al.* (1996) suggest that *D. sechellia* and *D. mauritiana* are the most closely related species of the trio, but this conclusion is controversial (HILTON *et al.* 1995): the recency of divergence between these species, coupled with their shared polymorphisms, may make it impossible to ever resolve this trichotomy. If such a resolution does become possible, however, the presence of genetic analyses in all three hybridizations will help determine the evolutionary direction of hydrocarbon evolution. Moreover, the development of new genetic tools in *D. mauritiana* allows examination of many regions of the genome, a technique not previously possible given the dearth of mutant markers in these species. These tools allow a rough estimate of the number of genes causing the hydrocarbon difference, and of the general effects of different chromosome regions on the character.

Here we report this genetic analysis, accompanying it with behavioral experiments to determine whether sexual isolation between *D. mauritiana* and *D. simulans* is based at least partially, as it is in other species in the group, on differences in female hydrocarbons. To this end, we first describe the sexual isolation between these species, and then determine whether it can be altered by interspecific transfer of hydrocarbons (COYNE *et al.* 1994).

MATERIALS AND METHODS

***D. sechellia* stocks:** *line 1:* This isofemale line is derived from the first female of this species ever collected, described by TSACAS and BÄCHLI (1981).

white (w): Stock is homozygous for the *w* allele [cytological location 3C2 in *D. melanogaster* and map position 1-4.1 in the sibling species *D. simulans* (STURTEVANT 1929; LINDSLEY and ZIMM 1992)]. This mutation, provided by I. HIGA and Y. FUYAMA, appeared in a strain collected in 1986 at Praslin, Seychelles.

jaunty, cinnabar (j cn): This line contains the two second-chromosome mutations *jaunty* (wings upturned) and *cinnabar* (bright red eyes), both of which arose in *D. sechellia* line 1. In *D. melanogaster, jaunty* is in cytological region 34E2 (map position 48.7) and *cinnabar* at 43E3-14 (map position 57.5), so in that species the genes are roughly 9 cM apart. This stock was used for comparing rates of recombination in *D. sechellia* and *D. mauritiana*/*D. sechellia* hybrids with those observed in *D. mauritiana, D. simulans,* and *D. melanogaster* (TRUE *et al.* 1996a).

***D. mauritiana* stocks:** *Synthetic:* A mixture of six isofemale lines was collected on Mauritius in 1981 and combined in 1983. This stock was used in our earlier studies of sexual isolation in the *D. simulans* subgroup (COYNE 1989, 1993).

jaunty, cinnabar (j cn): *j cn* is a stock with mutations identical to those in the *D. sechellia j cn* stock. This was used for determining third-chromosome recombination rates within *D. mauritiana.*

Insert lines: TRUE *et al.* (1996b) described the nature, construction, and mapping of the P-element transformed lines of *D. mauritiana.* Each line consists of individuals homozygous for the *X*-linked *white* mutation and also for a wild-type allele of *white* inserted into a known chromosomal location using a P-*lacZ* vector (BIER *et al.* 1989). The inserted w^+ allele does not restore wild-type eye color because of position effects: each stock has a characteristic pattern of pigmentation ranging from striped to solid, and a characteristic color ranging from very pale yellow to dark orange.

We used 15 insertion lines for mapping the pheromone difference between *D. sechellia* and *D. mauritiana,* choosing markers spaced fairly evenly to maximize our chances of detecting "hydrocarbon genes." These strains include three with inserts on the *X* chromosome (we give in parentheses the cytological position of the insertion based on the *D. melanogaster* map, and the cumulative recombination fraction in centimorgans along the *D. mauritiana* chromosome; all data provided by J. TRUE and C. LAURIE): NENEH2 (1EF, 0), 2A1 (10EF, 60.8), and 3X1 (19BC, 114.2); three with inserts on the second chromosome: 2V1 (24CD, 1.5), V1 (42B, 71.1) and GINA1 (58A, 140.5); eight with inserts on the third chromosome: L1B (61CD, 0), 4N1 (65A, 25.1), 4F1 (69D, 57.7), 3J1 (78CD, 91.1), 2K3 (82A, 94.9), AMY1 (91BC, 118.6), 4C1 (85BC, 167.3), and 2Y1 (99A, 208.6); and one with an insert on the fourth chromosome: 4M1 (102A). *D. melanogaster* differs from *D. sechellia, D. mauritiana,* and *D. simulans* by a paracentric inversion on 3R [84F-93F], so that the AMY1 and 4C1 insertions have recombination fractions discordant with their band positions). We used extra markers on the third chromosome because preliminary work showed it to be the only chromosome with a major effect on the hydrocarbon difference. Because there is no recombination on the tiny fourth chromosome, the 4M1 marker is associated with all potential "hydrocarbon genes" on that chromosome.

***D. melanogaster* stocks:** *Ives:* A homokaryotypic stock was made by combining 21 isofemale lines collected in 1977 in Amherst, Massachusetts (CHARLESWORTH and CHARLESWORTH 1985).

Crosses: To obtain F_1 hybrids for our genetic analysis, we crossed *D. mauritiana* males from the insert lines to *D. sechellia white* females, using 10–15 flies of each sex per vial (Because of strong sexual isolation, the reciprocal cross is almost never successful). For backcrosses, five F_1 females were crossed to five *D. sechellia white* males per vial. The offspring of this cross have two distinguishable genotypes: one with yellow or orange eyes (thus carrying the w^+ insert and a linked genomic segment from *D. mauritiana*), and the other with white eyes (hence carrying the corresponding segment from *D. sechellia*). Depending on its location, each insert (or, in the white-eyed flies, the lack of a w^+ insert) is nonrandomly associated with between 50 and 80 cM of genome (NAVEIRA and BARBADILLA 1992).

For each backcross, we determined the hydrocarbon profile of 20 females of each of the two genotypes. All flies were reared at 24° on agar-yeast-banana food. Other crosses are described in the RESULTS section.

Gas chromatography: Extraction of cuticular hydrocarbons and gas chromatography of the extracts were performed as described previously (FERVEUR 1991; COYNE *et al.* 1994). Flies were analyzed individually, and all data are presented as mean quantities of hydrocarbons per fly.

Statistics: The tricosene profile of each female was characterized by the ratio of the quantities of predominant hydrocarbons from each species: 7,11-HD/7-T, higher values of this ratio indicate a more *D. sechellia*-like profile. Among individuals within a strain or strains within a species, this ratio is far less variable than are the absolute quantities of each hydrocarbon, probably because absolute amounts are strongly affected by body size.

Hydrocarbon transfer experiments: In a previous study (COYNE *et al.* 1994), we found that a target female crowded for several days with females of a different species (the "donor" species) acquire substantial quantities of hydrocarbons from the donor; these compounds are undoubtedly transferred by direct contact between flies. Here we used this method to change the hydrocarbon profile of both *D. sechellia* and *D. mauritiana* females. We crowded five females of the target species (marked by wing clipping) with 50 females of the donor species in ~6 cm^3 of space in a food-containing vial (see COYNE *et al.* 1994). After 4 days, target females were collected under light CO_2 anesthesia and immediately used for either gas chromatography or behavioral observations. (Females were allowed to recover for 10 min before either procedure.) Each behavioral experiment also included control females who, like experimental females, had their wings clipped, but were crowded instead among *conspecific* females. The stocks used in these studies were strain 1 of *D. sechellia,* the synthetic strain of *D. mauritiana,* and the Ives strain of *D. melanogaster.*

Observations of courtship and copulation: We conducted two sets of "no-choice" behavioral observations. The first compared conspecific with heterospecific pairings to determine the nature and degree of sexual isolation between these species. The second compared the courtship of males toward either unaltered conspecific females or conspecific females whose hydrocarbon profiles had been altered by transfer experiments.

In each set of observations, one male was paired with one female in a food-containing vial (both flies introduced without anesthesia), and two such vials were watched simultaneously for 20 min, each by a different observer. The vials were coded and the observer did not know the identity of the flies. As each study involved two types of females and two observers, the females were alternated among observers to cancel out any systematic differences in how behaviors were scored. A total of four or five such pairs of vials were scored each day, after which the code was broken and the data recorded.

In some experiments we used dead instead of live females, a procedure designed to separate behavioral from chemical inducers of male courtship. Females were killed by flash-freezing in liquid nitrogen [this treatment has no detectable effect

on the quantity or ratios of the cuticular hydrocarbons (COYNE *et al.* 1994)] and immediately were used for behavioral experiments. Females were placed on the edge of the food in a vial, live males were introduced, and male behavior scored as in the studies with live females.

Male courtship behaviors were defined and scored as described by COBB *et al.* 1989 and COYNE *et al.* 1994). These behaviors included the following: courtship latency, courtship duration, copulation attempts, copulation latency, and successful copulation. We also scored courtship intensity, which was defined in two ways. For pairs involving live individuals of both sexes, courtship intensity was defined as the total courtship duration divided by the difference between the courtship latency and the copulation latency. This represents the proportion of time between the first courtship and copulation that is occupied by courtship behavior. (This statistic is hence not calculated for males who do not copulate). For pairs involving live males and dead females, courtship intensity was defined as the proportion of time between the first courtship and the end of the 20-min observation period that was occupied by courtship.

For analyzing pheromonal transfer experiments, our hypothesis was that if sexual isolation was affected by the female's pheromonal profile, a male encountering a conspecific female carrying some hydrocarbons from the other species would be less likely to court or mate than he would with a normal, unaltered conspecific female. Because of this expectation, behaviors were compared using a one-tailed statistical tests. Other comparisons of courtship behaviors, using unaltered males and females, were analyzed with two-tailed tests.

RESULTS

Genetic analysis

Pure species and F_1's: Table 1 shows the hydrocarbon profiles of females from the 15 insert lines of *D. mauritiana*, four strains of *D. sechellia*, and interspecific F_1 female hybrids between males from five of the insert lines and *white D. sechellia* females. The species differ markedly in this profile: a *D. mauritiana* female carries 400–600 ng of 7-T and no 7,11-HD, while a *D. sechellia* female carries only 6–12 ng of 7-T but 200–300 ng of 7,11-HD. Within each species there are also interstrain differences in the quantities of the predominant compound (these may be due to differences in body size), but the 7,11-HD / 7-T ratios do not overlap between the species, being 0 for all *D. mauritiana* strains and ranging from 27 to 40 among *D. sechellia* strains.

F_1 hybrid females have roughly 60–98% of the 7-T of *D. mauritiana* parent but <25% of the 7,11-HD of the *D. sechellia* parent. The 7,11-HD / 7-T ratios range from 0.065 to 0.114, hence showing dominance for the *D. mauritiana* phenotype. Because dominance is in this direction, the best resolution for genetic analysis is obtained by backcrossing F_1 hybrid females to *D. sechellia* males.

Backcrosses: Table 2 summarizes the hydrocarbon ratios in backcrosses involving each of the 15 insert lines (F_1 hybrid females crossed to *D. sechellia white* males). If an insert is linked to a species-differentiating "hydrocarbon gene," we expect the 7,11-HD / 7-T ratio to be higher for the w/w than for the w/w^+ genotype. An analysis of transformed ratios, described below, shows

TABLE 1

Predominant hydrocarbons and their ratios in pure-species and F_1 females

Strain	7-T, ng	7,11-HD, ng	7,11HD/7-T
D. mauritiana			
NENEH 2 (X,1EF)	500 (26)	0	0
2A1 (2,10EF)	588 (83)	0	0
3X1 (2,19BC)	495 (37)	0	0
2V1 (2,24CD)	519 (62)	0	0
V1 (3,42B)	386 (23)	0	0
GINA1 (2,58A)	433 (30)	0	0
L1B (3,61CD)	615 (59)	0	0
4N1 (3,65A)	589 (27)	0	0
4F1 (3,69D)	529 (31)	0	0
3J1 (3,78CD)	662 (70)	0	0
2K3 (3,82A)	587 (54)	0	0
4C1 (3,85BC)	430 (49)	0	0
AMY 1 (3,91BC)	434 (30)	0	0
2Y1 (3,99C)	635 (57)	0	0
4M1 (4,102A)	499 (11)	0	0
D. sechellia			
w	6 (1)	216 (16)	38.09 (2.99)
1	7 (1)	269 (11)	44.51 (4.56)
77	12 (1)	300 (10)	28.25 (2.93)
81	8 (2)	214 (7)	27.06 (1.80)
F_1			
NENEH2 X w	397 (22)	43 (2)	0.110 (0.006)
3X1 X w	484 (62)	52 (10)	0.105 (0.012)
GINA1 X w	366 (28)	23 (2)	0.065 (0.008)
3J1 X w	401 (44)	42 (5)	0.110 (0.016)
4M1X w	440 (44)	50 (11)	0.114 (0.023)

All values are the means among females; sample sizes are six for *D. mauritiana*, 15 for *D. sechellia*, and six for F_1 females. SEs are shown for all statistics in parentheses. *D. mauritiana* strains also give the chromosomal location and cytological position of the insert. All F_1 hybrids are obtained by crossing *D. mauritiana* insert-line males to *D. sechellia white* females.

that none of the seven *X*-, second-, and fourth-chromosome inserts has a significant effect on the character. In contrast, six of the eight third-chromosome inserts showed a significant difference between genotypes. Each of these six regions exerts its effects by changing the absolute quantities of 7,11-HD and 7-T in opposite directions, suggesting that both compounds are part of same biosynthetic pathway.

It is clear that genes with a detectable effect on the hydrocarbon ratio reside only on the third chromosome. Moreover, because we did not observe *any* backcross offspring having hydrocarbon ratios similar to those of the pure *D. sechellia* parent, the effect of this chromosome must be due to more than one gene. However, because the third-chromosome markers are linked, their effects may not be independent, and so we cannot automatically equate the number of marker effects with the number of hydrocarbon genes. We present below a statistical analysis of this problem, which first requires us to estimate levels of recombination in hybrids compared to the parental species.

TABLE 2

Predominant hydrocarbons and their ratios in backcrosses involving each of the fifteen insert lines of *D. mauritiana*

Strain and genotypes	Chromosome	7-T, ng	7, 11-HD, ng	7,11-HD/7-T		*P*
				Untransformed	Transformed	
NENEH 2 (1EF)	*X*					
w/w^+		133 (24)	125 (10)	1.73 (0.33)	−0.271 (0.244)	0.144
w/w		194 (23)	129 (16)	1.33 (0.38)	0.210 (0.211)	
2A1 (10EF)	*X*					
w/w^+		190 (20)	121 (18)	1.04 (0.25)	−0.105 (0.236)	0.449
w/w		178 (20)	157 (29)	1.49 (0.36)	−0.350 (0.216)	
3X1 (19BC)	*X*					
w/w^+		185 (17)	119 (11)	1.13 (0.31)	0.246 (0.199)	0.046
w/w		208 (69)	190 (38)	1.96 (0.35)	−0.292 (0.171)	
2V1 (24CD)	*2*					
w/w^+		102 (16)	100 (13)	3.45 (1.76)	0.306 (0.296)	0.822
w/w		95 (15)	86 (9)	3.37 (1.36)	0.210 (0.306)	
V1 (42B)	*2*					
w/w^+		203 (30)	148 (14)	1.17 (0.24)	0.151 (0.196)	0.323
w/w		162 (20)	167 (17)	1.73 (0.41)	−0.118 (0.184)	
GINA1 (58A)	*2*					
w/w^+		91 (12)	176 (21)	2.72 (0.59)	0.757 (0.211)	0.416
w/w		125 (18)	173 (20)	3.36 (0.79)	0.495 (0.239)	
L1B (61CD)	*3*					
w/w^+		144 (17)	44 (7)	0.85 (0.40)	−0.347 (0.230)	0.094
w/w		131 (15)	75 (8)	1.25 (0.44)	−0.920 (0.242)	
4N1 (65A)	*3*					
w/w^+		230 (29)	110 (16)	0.88 (0.24)	1.060 (0.274)	<0.0001
w/w		89 (15)	206 (23)	5.02 (1.03)	−0.608 (0.231)	
4F1 (69D)	*3*					
w/w^+		290 (31)	114 (16)	0.63 (0.18)	0.728 (0.204)	<0.0001
w/w		137 (18)	238 (23)	3.10 (0.71)	−0.764 (0.180)	
3J1 (78CD)	*3*					
w/w^+		171 (13)	50 (7)	0.35 (0.06)	0.485 (0.264)	<0.0001
w/w		97 (18)	116 (18)	2.89 (0.74)	−1.138 (0.144)	
2K3 (82BC)	*3*					
w/w^+		158 (16)	149 (14)	1.42 (0.34)	1.295 (0.259)	0.0003
w/w		84 (14)	234 (23)	6.17 (1.32)	0.046 (0.177)	
AMY 1 (91BC)	*3*					
w/w^+		211 (23)	108 (15)	0.76 (0.17)	0.849 (0.251)	<0.0001
w/w		99 (18)	187 (25)	4.17 (1.10)	−0.572 (0.190)	
4C1 (85BC)	*3*					
w/w^+		227 (19)	73 (7)	0.44 (0.12)	0.843 (0.262)	<0.0001
w/w		132 (26)	199 (15)	3.98 (0.83)	−0.970 (0.137)	
2Y1 (99C)	*3*					
w/w^+		161 (18)	153 (22)	1.70 (0.44)	1.034 (0.282)	0.0071
w/w		95 (17)	172 (11)	6.21 (1.85)	0.015 (0.223)	
4M1 (102A)	*4*					
w/w^+		125 (20)	205 (25)	3.73 (0.81)	0.603 (0.221)	0.746
w/w		110 (13)	175 (17)	2.93 (0.78)	0.717 (0.272)	

For each insert, 20 individuals were analyzed for each of the two backcross genotypes. Mean values are given for both the untransformed and transformed hydrocarbon ratios; standard errors are given in parentheses. For each insert, the transformed ratios were compared using a *t* test; two-tailed probabilities are given for each such comparison.

Estimating recombination in hybrids: TRUE *et al.* (1996a) report that recombinational map distances on the third chromosome of *D. mauritiana* are nearly twice as large as those seen in *D. melanogaster,* with *D. simulans* being roughly intermediate. Moreover, unlike *D. melanogaster, D. mauritiana* and *D. simulans* do not show reduced recombination near the centromere. Because of a lack of mutant markers, however, there were no corresponding data from *D. sechellia.* We have now obtained two markers on the third chromosome of *D. sechellia* that allow us to compare their map distances (and those of *D. sechellia*/*D. mauritiana* hybrids) to those of other species.

We crossed *D. sechellia j cn* females to *D. mauritiana*

TABLE 3

Recombination between *j* and *cn* in intraspecific crosses (*D. sechellia* and *D. mauritiana*) and interspecific backcrosses (*D. sechellia*/*D. mauritiana*)

Genotype	*N* (*D. sechellia*)	*N* (interspecific)	*N* (*D. mauritiana*)
j cn	428	464	499
j	100	89	101
cn	107	232	103
Wild-type	527	802	515
Total	1162	1587	1218
Actual recombination (*j* + *cn*/Total)	0.178	0.202	0.167

Map distances estimated from backcrosses using *j* and *cn* markers on the second chromosome. See text for details of crosses.

syn males, and backcrossed the F_1 hybrid females to *D. sechellia j cn* males. We made two intraspecific controls, one in *D. sechellia* (strains 1 and *j cn*) and one in *D. mauritiana* (strains syn and *j cn*). As shown in Table 3, recombination is near 0.2 for all of these crosses, and the proportion of recombinants is not heterogeneous among them ($G_2 = 5.91$, $P = 0.052$). The estimated map distances between these loci (calculated using the mapping function of FOSS *et al.* (1993) with $m = 4$ and standard errors from STAHL and LANDE (1995) are nearly equal to the observed proportion of recombinants; in hybrids, for example, this map distance is 0.205 ± 0.011, while the observed proportion of recombinants is 0.202. [We note that recombination between *jaunty* and *cinnabar* in all of these crosses is roughly twice its reported value in *D. melanogaster* (9 cM), but considerably smaller than the 31 cM separating these loci in *D. mauritiana* (recombination calculated from data provided by J. TRUE]. There is, however, a significant difference among crosses in the *proportion* of the recovered genotypes, with a relative deficit of the *jaunty* and *jaunty, cinnabar* classes in the interspecific cross compared to the intraspecific crosses ($G_6 = 78.6$, $P < 0.001$). This may reflect viability effects of either the markers themselves or linked loci. Nevertheless, it appears that levels of recombination in the hybrids are fairly similar to those of *D. mauritiana*, and so in the following analysis we will assume no difference in recombination rates (TRUE *et al.* 1996a). However, a moderate discrepancy in map distances among these crosses has little effect on our genetic analysis (see APPENDIX).

Preliminary biometrical analysis of gene number: For convenience in the following discussion, we call the *D. sechellia* hydrocarbon phenotype "H" (standing for a high 7,11-HD/7-T ratio), the *D. mauritiana phenotype*, "L", and the species-specific alleles contributing to this difference *H* alleles and *L* alleles, respectively. It is apparent from inspecting the data on the parental strains and F_1 females that there is considerable nonnormality of the within-strain distribution of the ratio of 7,11-HD/7-T hydrocarbons, with a strong dependence of the variance on the mean. In addition, the means for the backcrosses to the *sechellia w* strain are much closer to the F_1 means than to the means of the corresponding parental and F_1 values (Table 2), indicating strong interactions among the genes underlying this trait, such that a high 7,11/7-T phenotype requires several *H* alleles from *D. sechellia* (see below). Since only the F_1 and *D. sechellia w* strain values are relevant to the analysis of the backcross data, the following analyses will be concerned with only these and the backcrosses.

We sought a suitable scale transformation that would produce approximate normality of hydrocarbon ratios within strains, thus enabling the use of normal-based statistical tests (WRIGHT 1968, Chap. 10). Trial and error suggested the variable $z = \ln(0.05 + x)$, where x is the value of the 7,11-HD/7-T ratio. This transformation produced distributions in the *D. sechellia w* parental strain, F_1 females, and backcrosses whose skewness and kurtosis were not significantly different from zero. Moreover, the observed ranges of the transformed statistic in the *D. sechellia w* parental and pooled F_1 strains are in good agreement with those expected from the corresponding standard deviations under normality (PEARSON and HARTLEY 1954), and the difference in within-strain variances between the *D. sechellia w* parent and the pooled F_1 strains is not significant (analysis not shown).

Tests for epistasis and estimation of numbers of genes: Having produced a satisfactory scale for use in further analysis, we first demonstrate that there is still substantial epistasis on this scale, which may bias the estimate of the number of loci (WRIGHT 1952, 1968, Chap. 15; MATHER and JINKS 1982). In the absence of epistasis (but regardless of linkage and dominance), the backcross mean is expected to satisfy the relation

$$\overline{BC} = \tfrac{1}{2}(\overline{F_1} + \bar{P}). \qquad (1)$$

With large sample sizes, the difference between a backcross mean and the mean of the corresponding F_1 and parental means, relative to its standard error, provides a normal-deviate test for epistasis (MATHER 1949; WRIGHT 1952).

The evidence described below suggests that chromosome *3* carries most or all of the genes responsible for the difference in trait value. We shall therefore consider in detail only the results of the tests for epistasis on the backcrosses with inserts on other chromosomes, and for which F_1 data are available. These crosses are least likely to violate the assumptions of the biometrical methods since no prior selection of marker genotypes linked to the trait loci was practiced. Table 4 shows the results of normal-deviate tests for interactions for each of these backcrosses separately. In all cases, there was

TABLE 4

Tests of epistasis on backcrosses of inserts on chromosomes other than *3*

Insert	$\frac{1}{2}(\bar{F}_1 + \bar{P}) - \overline{BC}$
1EF	2.74 ± 0.080
19BC	2.72 ± 0.080
58A	2.89 ± 0.072
4M1	2.73 ± 0.080

See text for details. All tests are significant ($P < 0.001$). Values are ± SE.

highly significant evidence for epistasis, such that the backcross mean of *z* was far smaller than expected without epistasis.

It will also be noticed that the backcross variances are always much larger than the F_1 or parental variances, indicating the segregation of a relatively small number of loci affecting the trait. WRIGHT's "Segregation Index," *S* (WRIGHT 1952, 1968, Chap. 15), can be used to obtain a lower bound to the number of segregating loci in a backcross, from the formula

$$S = \frac{(\bar{P} - \bar{F}_1)^2}{4s_s^2}, \qquad (2)$$

where s_s^2 is the segregational variance, given by the difference between the within-backcross variance and the nongenetic component of variance (WRIGHT 1968, p. 394).

The nongenetic component of variance is most accurately estimated by pooling the estimates of within-strain sums of squares for all the F_1 strains and the *sechellia w* strain, and is equal to 0.066 (42 d.f.). The pooled within-backcross sums of squares give an estimate of the backcross variance of 1.075 (168 d.f.) for the four crosses in Table 4. This is highly significantly different from the estimate of the nongenetic variance ($F_{168,42} = 16.3$). We thus obtain $s_s^2 = 1.009$.

Pooling all four relevant F_1 means (including that for GINA), we have the gene number $S = 7.68$. The approximate sampling standard deviation of *S* can be calculated using the "delta" method, modifying the procedure of LANDE (1981) to take account of the fact that we have a backcross to only one parent. This standard deviation is 0.91 for the full data set. ZENG *et al.* (1990) found in simulating F_2 populations that 90% of the estimates of *S* lie within approximately two estimated standard deviations of the true value, so that we can with some confidence take the number of segregating loci to be least six. The method yields an underestimate if there is epistasis, which we have shown to exist. Linkage and unequal effects of segregating loci, both of which also seem likely to apply to the present case, also contribute to a downward bias in the estimate of the number of loci (WRIGHT 1968; MATHER and JINKS 1982; ZENG 1992). We can therefore conclude that there are at least six genes controlling the hydrocarbon difference between *D. sechellia* and *D. mauritiana*.

Analysis of third-chromosome effects: Further analysis requires the use of information on the phenotypic differences associated with the segregation of *w* and w^+ in the backcrosses, where w^+ acts as a marker for a heterozygous chromosomal segment derived from *D. mauritiana*. The means and standard errors of the *w* and w^+ individuals on the *z* scale for the various backcrosses are given in Table 2. It is evident that there are no individually significant effects of inserts on chromosomes other than *3*, and that most of the chromosome *3* inserts have significantly higher values for w^+ than for *w*, although the effects of the two markers at the ends of the chromosome (inserts L1B and 2Y1) are not significant under RICE's (1989) sequential Bonferroni test (the initial significance level for $n = 15$ comparisons is $P = 0.003$). Even if the data for *all* the *X*- and second-chromosome inserts are pooled, there is no significant difference between means for the w^+ and *w* genotypes (pooled difference in means is 0.18 ± 0.13).

This suggests that all of the loci detected in the biometrical analysis are on chromosome *3*, with the possible exception of some genes with very minor effects. We further estimate the number of genes on this chromosome (and their approximate positions) using a Mendelian / statistical analysis of each insert. This analysis, given in the APPENDIX, suggests that chromosome *3* harbors at least five hydrocarbon loci, concentrated more toward the middle than the end of the chromosome. This estimate is consistent with the biometrical estimate of at least six loci in the entire genome and with the observation of no effects of the *X*, second, and fourth chromosomes. Given the conservative nature of our assumptions, we believe that at least six genes on this chromosome contribute to the hydrocarbon difference between *D. mauritiana* and *D. sechellia*.

Behavioral analysis

Pure species: Table 5 shows the degree and nature of sexual isolation between the pure species, measured in four separate "no-choice" tests, each accompanied by a simultaneously observed no-choice intraspecific control. Test 1 shows that *D. mauritiana* males display almost no courtship toward *D. sechellia* females: courtship duration, copulation attempts, and number of copulations were significantly lower with *D. sechellia* than with *D. mauritiana* females. This form of sexual isolation differs from that seen between *D. simulans* males and *D. mauritiana* females, in which the males court ardently but their overtures are always rejected (COYNE 1993). Because *D. mauritiana* and *D. sechellia* females appear morphologically identical, at least to the human observer, this isolation is probably due to either chemical differences among the females, behavioral differences that affect male courtship, or a combination of these factors.

This sexual isolation persists, however, when these

TABLE 5

Sexual isolation between *D. mauritiana* and *D. sechellia*

Test	Male	Female	♀ live (L) or dead (D)	Courtship				Copulation		
				N	Latency (sec)	Duration (sec)	Intensity	Attempts/vial	Total	Latency (sec)
1	mau	mau	L	20	168 (37)*	210 (50)***	0.862 (0.093)	6.05 (1.91)***	15***	413 (86)
		sec	L	16	359 (74)	5 (74)	—	0	0	—
2	mau	mau	D	19***	174 (42)**	310 (62)***	0.32 (0.06)***	1.70 (.61)**	—	—
		sec	D	9	314 (64)	14 (8)	0.03 (0.02)	0	—	—
3	sec	mau	L	17	387 (60)***	34 (14)***	—	0***	0***	—
		sec	L	19	76 (12)	394 (71)	0.74 (0.05)	2.10 (0.77)	11	568 (73)
4	sec	mau	D	20	445 (131)	11 (6)*	0.04 (0.02)	0	—	—
		sec	D	20	382 (79)	84 (39)	0.18 (0.08)	0.20 (0.16)	—	—

Table gives mean values of courtship parameters out of 20 observations; standard errors are in parentheses. Observations were done in pairs, as indicated by the test numbers (see text for details). Twenty replicate observations were done for each combination of male and female. All statistical comparisons were done on courtship parameters between species of female within a test. Mann Whitney *U* tests were used to compare all courtship and copulatory behaviors except for the number of courtships or copulations, which were compared within each pair of females using Fisher's exact test. All probabilities given are two-tailed. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

experiments are repeated using dead instead of live females (Table 5, test 2): *D. mauritiana* males court dead conspecific females nearly as intensely as they do live ones, but remain reluctant to court dead *D. sechellia* females. This suggests that sexual isolation is mediated by chemical and not behavioral cues, an idea supported by the hydrocarbon transfer studies described below.

The nature of interspecific courtship is somewhat different in tests using *D. sechellia* males. These males court and copulate freely with live conspecific females, but, like *D. mauritiana* males, court heterospecific females only infrequently and weakly (Table 5, test 3). In this direction of the cross, however, the use of dead females largely abolishes *both* conspecific and heterospecific courtship (Table 5, test 4), so that no sexual isolation can be seen (the only significant difference between the courtship of dead *D. simulans* and dead *D. mauritiana* females is in the lowered duration of courtship toward the latter). These observations suggest that female cuticular hydrocarbons play a relatively small role in intraspecific sexual behavior of *D. sechellia,* and hence that the compounds are not strongly involved in sexual isolation between *D. sechellia* males and *D. mauritiana* females. We suspect that this sexual isolation involves an interspecific difference in female behavior. These conclusions are supported by the hydrocarbon transfer experiments described below.

Hydrocarbon transfer experiments: To study the role of pheromonal differences in sexual isolation, we transferred hydrocarbons between *D. mauritiana* and *D. simulans* females and observed how males courted these modified females.

We first determined the effect of crowding on hydrocarbon profile by performing three interspecific crowding experiments, each employing a control in which wing-clipped females were crowded with conspecific females (Table 6). In test 1, *D. mauritiana* females crowded among *D. sechellia* females received an average of 57 ng of 7,11-HD, and their 7-T was reduced from 700 to ~250 ng. These transfers raised the 7,11-HD/7-T ratio of the target females from 0 to 0.239.

We made a similar test using *D. melanogaster* females from the Ives strain in place of *D. sechellia* females (Table 6, test 2). Because *D. melanogaster* females also carry high levels of 7,11-HD (JALLON and DAVID 1987; COYNE 1996), we predicted that their use as donor females would alter the behavior of *D. sechellia* males in a manner similar to that effected by *D. sechellia* donor females. In fact, *D. melanogaster* females transferred even larger amounts of 7,11-HD (probably because these flies are very active), raising 7,11-HD/7-T ratio of the *D. mauritiana* females from 0 to 0.78 (Table 6).

Finally, *D. sechellia* females crowded with many *D. mauri-*

TABLE 6

Hydrocarbon transfer in "ruboff" experiments

Test	Target	Donor	Hydrocarbons of target female after crowding		
			ng 7-T	ng 7,11-HD	7,11-HD/7-T
1	mau	mau	708 (43)	0 (0)	0 (0)
	mau	sec	243 (8)	57 (3)	0.239 (0.014)
2	mau	mau	691 (47)	0 (0)	0 (0)
	mau	mel	329 (16)	251 (14)	0.781 (0.050)
3	sec	sec	7 (0.4)	126 (6)	20.13 (1.72)
	sec	mau	319 (24)	99 (7)	0.32 (0.02)

Hydrocarbon quantities and ratios present on target females who were crowded with large numbers of either conspecific or heterospecific females ("donors"). The table shows mean values of a sample of 15 target females subjected to each treatment with SE in parentheses.

TABLE 7

Sexual isolation in pheromone ruboff experiments

Test	Male	Female[a]	Live or dead	Courtship				Copulations		
				N	Latency (sec)	Duration (sec)	Intensity	Attempts/vial	Total	Latency
1	mau	mau (mau)	L	42	87 (16)*	172 (25)**	0.790 (.046)**	3.74 (0.74)**	27***	236 (42)
		mau (sec)	L	38	157 (33)	89 (14)	0.552 (0.087)	1.61 (0.45)	9	286 (65)
2	mau	mau (mau)	D	34*	267 (38)	297 (48)***	0.398 (0.051)***	0.88 (0.38)**	—	—
		mau (sec)	D	24	340 (54)	72 (27)	0.156 (0.056)	0.14 (0.10)	—	—
3	mau	mau (mau)	L	42	72 (9)	233 (26)***	0.794 (0.057)	4.71 (0.84)***	27***	302 (38)
		mau (mel)	L	39	143 (38)	87 (19)	0.744 (0.099)	1.02 (0.31)	8	359 (131)
4	mau	mau (mau)	D	38*	169 (22)	236 (41)***	0.259 (0.044)***	0.62 (0.21)**	—	—
		mau (mel)	D	30	251 (48)	40 (10)	0.054 (0.011)	0 (0)	—	—
5	sec	sec (sec)	L	41	147 (31)	266 (37)	0.867 (0.043)	2.12 (0.60)	30	420 (50)
		sec (mau)	L	41	143 (25)	238 (31)	0.901 (0.035)	1.88 (0.65)	28	392 (54)
6	sec	sec (sec)	D	13	349 (59)	14 (8)	0.055 (0.036)	0 (0)	—	—
		sec (mau)	D	21	398 (52)	18 (5)	0.053 (0.021)	0.36 (0.21)	—	—

Standard errors of mean courtship and copulation parameters are in parentheses; 42 replicate observations were done for each combination of male and female. Mann-Whitney *U* tests were used to compare all courtship and copulatory behaviors between members of a pair except for the number of courtships or copulations, which were compared using Fisher's exact test. All comparisons were done among members of a pair, and all probabilities are one-tailed. Species abbreviations: mau, *D. mauritiana;* sec, *D. sechellia;* mel, *D. melanogaster.* $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

[a] For each test, males of one species were tested against conspecific females who were crowded with either other conspecific females or females of another species. The females used to crowd the target females are given in parentheses.

tiana females (Table 6, comparison 3) experience a sizable increase in 7-T (from 7 to 319 ng/fly), their 7,11-HD is reduced from 126 to 99 ng, and the 7,11-HD/7T ratio drops from the control value of 20.1 to 0.3.

Behavioral effects of hydrocarbon transfer: Table 7 summarizes the effects of hydrocarbon transfers on the courtship behavior of males. In all of these tests, males are exposed to conspecific females previously crowded with either heterospecific or conspecific females. Each test involved 42 replicate observations of both control and experimental flies.

As test 1 (Table 7) indicates, rearing *D. mauritiana* females with *D. sechellia* females severely lowers their attractiveness to *D. mauritiana* males, significantly reducing every aspect of courtship and copulation. The significant differences in courtship and attempted copulation remain when this experiment is repeated with dead females (Table 7, test 2), indicating that the inhibition of courtship produced by heterospecific crowding is due not to a change in female behavior, but more probably to a change in their chemical profile. This could involve the addition of 7,11-HD, the reduction of 7-T, a combination of both, or conceivably some other substance transferred by crowding that is not detected by gas chromatography.

If sexual isolation is caused by either a change in 7,11-HD/7T ratio or simply an increase in the absolute amount of 7,11-HD, we predict that crowding *D. mauritiana* females with *D. melanogaster* females (which, like *D. sechellia females,* have large amounts of 7,11-HD) would reduce their subsequent attractiveness to *D. mauritiana* males. Tests 3 and 4 in Table 7 show that this prediction is correct, and is obeyed whether the females are alive (test 3) or dead (test 4). Note that although both the absolute amount of 7,11-HD and the 7,11-HD/7T ratio of *D. mauritiana* females is increased more strongly by crowding with *D. melanogaster* than with *D. sechellia* females, the effect on courtship of *D. mauritiana* males is about the same. This implies that changes in male courtship are not directly proportional to either hydrocarbon ratios or absolute amounts of foreign hydrocarbons. Perhaps the inhibition of male courtship by foreign hydrocarbons acts in a threshold manner.

In contrast to the above results, transferring foreign hydrocarbons onto *D. sechellia* females has no noticeable effect on the courtship behavior of *D. sechellia* males, regardless of whether females are tested alive (Table 7, test 5) or dead (Table 7, test 6). (It should be noted, as documented in Table 6, that the hydrocarbon ratios of these females are changed much more radically than in tests 1–4 on *D. mauritiana* females). Moreover, unlike *D. mauritiana* males, *D. sechellia* males almost completely lose interest in both "perfumed" and normal conspecific females when these females are dead; these males perform very few courtships, and even these are very short (Table 6, test 6). These data, like those given in Table 4 (test 3 and 4), suggest that *D. sechellia* males are much more dependent than *D. mauritiana* males on behavioral inducers of courtship, and also that sexual isolation between *D. sechellia* males and *D. mauritiana* females results more from differences in female behavior than from differences in female pheromones.

DISCUSSION

Genetic analysis: We have shown that the difference in hydrocarbon profiles between *D. mauritiana* and *D. sechellia* is probably a significant component of sexual isolation between these species, and is due to evolutionary change at a minimum of six genes, all on the third chromosome. This aspect of reproductive isolation thus has a polygenic basis, although most third-chromosome regions have large effects on the character, and those from *D. sechellia* interact epistatically. Our estimate of gene number is of course tentative, but the congruence between estimates derived from the biometrical and Mendelian analyses, as well as the conservative nature of our assumptions, give us some confidence that the actual number of genes causing the character difference is even larger.

It is useful to compare these results to the two previous genetic analyses of 7,11-HD/7-T hydrocarbon ratios in interspecific hybridizations: *D. sechellia*/*D. simulans* (COYNE *et al.* 1994) and *D. melanogaster*/*D. simulans* (COYNE 1996a). The most striking result is that in all three studies the character difference is affected only by genes on the third chromosome. As the *D. simulans*/*D. sechellia* analysis used only one marker on that chromosome, we cannot estimate the number of linked genes involved, but there are probably several because no parental phenotypes were recovered in the backcross to *D. sechellia*. In *D. melanogaster*/*D. simulans* hybrids, genes affecting the hydrocarbon ratio are located on both the left and right arms of the third chromosome. There is at least one gene on 3R (only the effect of the entire arm could be estimated), but deficiency-mapping of 3L revealed at least four regions affecting the species difference. Hence at least five genes were involved, an estimate similar to that of the present study.

On the other hand, the nature of dominance in hybrids differs among these hybrids. Both the *D. sechellia*/*D. simulans* and *D. sechellia*/*D. mauritiana* hybridizations show dominance for the high-7T (*D. simulans*-like) phenotype: in both cases the 7,11-HD/7T ratio is 0 in *D. simulans*, 30–40 in the other species, and roughly 0.1 in F_1 hybrid females, which have very little 7,11-HD but nearly as much 7-T as the *D. simulans* parent (COYNE *et al.* 1994). In the *D. melanogaster*/*D. simulans* cross, on the other hand, the hybrids have much more 7,11-HD: the 7,11-HD/7-T ratio ranges from 4 to 23 in *D. melanogaster* (depending on the strain), 0 in *D. simulans*, and between 1 and 5 in the F_1 hybrid females, which have nearly 10 times the nanogram quantity of 7,11-HD as do *D. simulans*/*D. sechellia* or *D. simulans*/*D. mauritiana* hybrids (COYNE 1996a). These differences in dominance relationships imply that either different genes are involved in the hydrocarbon differences, or there are differences in modifier genes that incidentally affect dominance in hybrids.

The present localization of genetic differences to the third chromosome is probably not accidental because the chance that all five gene segments of large effect would be located there is roughly $(0.4)^5$, or 0.01. This, plus the presence of a third-chromosome effect in the other two hybridizations, may reflect a special role of this chromosome in evolutionary change. Alternatively, female hydrocarbon genes may be preferentially located on this chromosome, as suggested by COBB and FERVEUR (1995).

Other studies of hydrocarbon polymorphisms or species differences have not found a consistent third-chromosome effect. While the polymorphism between 5,9-HD in African *D. melanogaster* and 7,11-HD in other strains apparently resides on the third chromosome (FERVEUR *et al.* 1994), variation in *male* hydrocarbons between African and non-African populations of the same species involves at least two genes on the second chromosome (FERVEUR and JALLON 1996). A polymorphism for male hydrocarbons in *D. simulans* is due to alleles of a single locus (*Ngbo*) on the second chromosome (FERVEUR 1991) and the difference between *D. simulans* and *D. sechellia* males in the proportions of 6- and 7-tricosene is due to at least five genes scattered among all chromosome arms (COYNE 1996c). None of these other differences are known to affect sexual isolation, although some male hydrocarbons may act as antiaphrodesiacs within species, inhibiting males from courting already-mated females (SCOTT and JACKSON 1988). As the male-specific hydrocarbons are all monoenes while those in females are dienes, genes for inserting the second double bond may reside solely on chromosome three (COBB and FERVEUR 1995).

Evolutionary pathway of hydrocarbon evolution: Can we use these results to interpret the direction of hydrocarbon evolution in this group? The discovery of strong third-chromosome effects in three distinct hybridizations raises the question of whether these represent three analyses of the same evolutionary transition, or whether they represent at least two independent transitions. Although we cannot definitively answer this question, we can give some suggestions.

Assuming that the phylogeny shown in Figure 1 is correct, we may assume from the outgroup state that the ancestral condition of the *melanogaster*/*sechellia*/*simulans*/*mauritiana* clade is sexual monomorphism for 7-T. The presence of two species with high-7,11-HD females (*D. melanogaster* and *D. sechellia*) could have two explanations: (1) the high-7,11-HD condition evolved independently in the two lineages leading to *D. melanogaster* and *D. sechellia*, or (2) the high-7,11-HD state evolved in the common ancestor of all four species in this clade after it separated from the ancestor of the other four species, and then the high 7-T female phenotype re-evolved in the lineage(s) leading to *D. simulans* and to *D. mauritiana*.

This question can be resolved by either a knowledge

of the specific loci and alleles involved in the evolution of new hydrocarbons, or by an accurate knowledge of the phylogeny of the entire group. However, one crucial aspect of the phylogeny shown in Figure 1 is still unknown: the relative branching order of *D. simulans, D. mauritiana,* and *D. sechellia.* Although CACCONE *et al.* (1996) place *D. mauritiana* and *D. sechellia* as sister species, with *D. simulans* the outgroup, other workers have reached different conclusions (*e.g.,* COYNE and KREITMAN 1986; KLIMAN and HEY 1993; HILTON *et al.* 1995). Analyses of different loci give different results, largely because species in this group share polymorphisms. It does seem difficult to regard *D. mauritiana* and *D. sechellia* as sister species because both are endemic to small and distant oceanic islands; a double colonization from the mainland ancestor of *D. simulans* would seem more reasonable. Nevertheless, *all* authors agree that the two branching events producing these three species occurred within a fairly short period of time.

Of all possible phylogenetic arrangements of these three species, only one would allow our three genetic analyses to represent repeated studies of a single evolutionary transition. This is the phylogeny having *D. sechellia* as an outgroup to the sister species *D. simulans* and *D. mauritiana.* If (1) this is the true phylogeny, (2) the high 7,11-HD condition arose once in the common ancestor of *D. melanogaster, D. sechellia, D. mauritiana,* and *D. simulans,* and (3) this condition was retained in *D. melanogaster* and *D. sechellia* while the common ancestor of *D. simulans* and *D. mauritiana* re-evolved the high 7-T phenotype, then our genetic analyses of *D. melanogaster/D. simulans, D. sechellia/D. simulans* and *D. mauritiana/D. simulans* would all be samples of the single evolutionary transition from 7,11-HD to 7-T in the joint ancestor of *D. simulans* and *D. mauritiana.*

The genetic evidence, however, supports *multiple* origins of the high 7,11-HD phenotype. As noted above, the dominance relationship in the *D. melanogaster/D. simulans* hybridization differs from those in the *D. simulans/D. sechellia* and *D. mauritiana/D. sechellia* hybridizations. This suggests the involvement of different gene substitutions, unless dominance was modified during or after these substitutions. The latter seems unlikely since there is no selection pressure to modify the dominance of alleles that have already been fixed by selection. The former also seems unlikely, as the pressure of selection for dominance modification during gene substitution is in the direction of increased dominance of the favorable alleles (HALDANE 1956). It is therefore quite difficult to explain the evolution of different directions of dominance by this mechanism. HALDANE (1956) also gives a quantitative argument that dominance modification during the course of a gene substitution is unlikely. The only possibility is that dominance has been changed as an indirect consequence of some other selection pressure on hydrocarbon content, at other loci in the genome. This obviously cannot be ruled out, but lacks an obvious biological justification.

The other relevant evidence is that the locations of the genes causing the *D. mauritiana/D. sechellia* difference are not completely coincident with those involved in the *D. melanogaster/D. simulans* difference. The study of the latter hybridization shows a large effect of the 62A-62C region (COYNE 1996a), whereas no significant effect of the nearby 61CD region is found in the present comparison between *D. mauritiana* and *D. sechellia* (see APPENDIX). At least one locus, then, has evolved independently in the two cases.

From these considerations, we propose that the common ancestor of *D. simulans, D. mauritiana, D. sechellia,* and *D. melanogaster* was fixed for the high 7-T phenotype in females, and that acquisition of high levels of 7-11 HD evolved independently in the lineages leading to *D. melanogaster* and to *D. sechellia.* This scenario explains why the dominance relationships are identical in the *D. simulans/D. sechellia* and *D. mauritiana/D. sechellia* hybridizations (they would represent duplicate analyses of one evolutionary transition), but differ from that of the *D. melanogaster/D. simulans* hybridization (an independent evolutionary transition). Moreover, the hypothesis is consistent with the observation that the outgroup species have the high-7-T female phenotype, and is also parsimonious in the sense of invoking an ancestral state of sexual monomorphism instead of dimorphism. The test of this hypothesis, however, awaits either a definitive resolution of the phylogeny, or the identification of the responsible genes using DNA analyses or fine-scale mapping.

Our proposal of two independent derivations of the high 7,11-HD phenotype raises two problems. The first is how a such a phenotype, based on largely recessive alleles, could evolve by selection in the ancestors of *D. sechellia,* for HALDANE's sieve (TURNER 1977) predicts that recessive autosomal alleles are unlikely to be fixed by selection in large random-mating populations. This problem is compounded by strong epistasis in *D. mauritiana/D. sechellia* hybridization, so that a substantially high 7,11-HD phenotype apparently requires the cooperation of several high-7,11-HD alleles from *D. sechellia.* (The evolution of the high-7,11 HD in *D. melanogaster* is not a problem as it is semidominant, at least in hybrids with *D. simulans*).

There are two possible solutions to this problem. One is that recessivity and epistasis are not complete, so that some departure from the ancestral phenotype is caused by a single novel heterozygous allele. If true, this implies the possibility of some degree of (albeit rather weak) selection for a new mutation in a large population. Indeed, the *D. sechellia* alleles do not appear to be completely recessive, though we have no definite information on single genes. The other solution is to invoke restricted population size, which greatly enhances the fixation probability of a rare recessive autosomal muta-

tion (KIMURA 1962). This is consistent with the evidence that *D. sechellia* has a remarkably low degree of allozyme, intron, and silent-site diversity (CARIOU *et al.* 1990; KLIMAN and HEY 1993; J. A. COYNE and M. KREITMAN, unpublished data), suggesting that the species has undergone a recent bottleneck in population size.

The remaining evolutionary problem is to explain the evolution of sexual dimorphism for pheromones under the assumption that the ancestral condition was sexual monomorphism for 7-T, as suggested by the phenotypes of outgroup species. If males of sexually monomorphic species discriminate against females carrying 7,11-HD, how could the latter phenotype ever evolve? The problem may be resolved by realizing that we are seeing the result of an evolutionary process that involved coadaptation between a female phenotype and male preference. It is conceivable that the male preferences changed before the female phenotype. Moreover, other selective advantages (such as selection for desiccation resistance) may have outweighed any negative effects on mating success.

Sexual behavior and sexual isolation: Our most novel observation about sexual isolation is that *D. mauritiana* females may be isolated from *D. sechellia* males because of behavioral differences between females of these species. This is suggested by two observations. First, although *D. sechellia* males do not readily court or copulate with *D. mauritiana* females, they are not deterred from courting *D. sechellia* females carrying substantial amounts of the foreign hydrocarbon 7-T. Second, the differential attractiveness of females of the two species to *D. sechellia* males is largely abolished if dead females are used: in this case females of both species become equally unattractive. This in turn suggests that *D. sechellia* males are attracted to conspecific females by their behavior rather than by their hydrocarbon content.

We therefore propose that some subtle behavioral difference between females of the two species influences their attractiveness to *D. sechellia* males. If true, this would to be one of the few cases in Drosophila in which specific female behaviors are required to stimulate male courtship. In other species of the *D. melanogaster* group, males readily court dead conspecific females, and sexual isolation is due to either males' refusal to court females of another species (as *D. mauritiana* males *vs. D. sechellia* females), or to females' refusal to accept the persistent courtship of males from another species (*e.g., D. simulans* males and *D. mauritiana* females). The neglect of female behaviors beyond simple refusal of male courtship may stem from a common idea that female Drosophila are passive consumers of male courtship, and to the absence of obvious and elaborate courtship "dances" in females. (There are certainly no obvious differences between the behavior of courted *D. mauritiana* and *D. sechellia* females). This area is obviously ripe for further work.

Finally, we note that in our genetic analysis, all of the chromosome segments from one species affect the hydrocarbon ratio in the same direction, which suggests that hydrocarbon differences arose from selection and not genetic drift (see COYNE 1996c). Either natural or sexual selection could be involved, but we cannot go further without knowing the effects of different hydrocarbons on fitness. As always, it is much easier to show that differences among species cause reproductive isolation than to understand the evolutionary forces that produced those differences.

We thank KATHLEEN KYLE and PEGGY ROONEY for technical assistance, JOHN TRUE and CATHY LAURIE for providing the *D. mauritiana* insertion lines, MICHELE KALL for typing the tables, RUSS LANDE for help with the mapping functions, and MATTHEW COBB, JEAN-FRANÇOIS FERVEUR, and two anonymous reviewers for their comments on the manuscript. This work was supported by National Institutes of Health grant GM-50355.

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Communicating editor: A. G. CLARK

APPENDIX

A Mendelian analysis of gene number on chromosome *3*: A rough estimate of the number of third-chromosome hydrocarbon genes causing the species difference can be pursued by considering the effects of each insert, moving from left to right along the chromosome as follows. Although the 61CD insert has a positive effect, this is not significant (Table 2), so we can conclude that there is at best only loose linkage of any major factors to this locus. The 65A insert is associated with a significant effect, which is about 30% of the total *D. sechellia* $w - F_1$ difference of 5.46, estimated using all the F_1 means except for the anomalous GINA insert (see text). This locus is therefore linked to one or more genes with relatively large effects on the trait. The following argument shows that there must be more than one such gene. In a sample of 20 from a normal distribution, there is a probability of 95% that the most extreme high and low values will be $\leq$2.8 SD from the mean (PEARSON and HARTLEY 1954). Using the joint

estimate of $\sqrt{0.066} = 0.26$ for the nongenetic standard deviation of z (see above), and the value 3.60 for the w parental mean, any w individual whose value is below 2.87 is unlikely to belong to the parental distribution. Since there is uncertainty in the estimates of both mean and variance, we will conservatively use the value 2.75 as the cut-off for classification of a phenotype as parental (H). According to this criterion, only one out of the twenty w backcross individuals can be H (with a value of 2.78), which is inconsistent with even a loosely-linked single gene associated with 65A.

A similar argument also excludes the possibility of two genes. The frequency of the H phenotype appearing among the w class is <0.25 on a two-gene model, implying that the binomial probability of recovering only one member of this class is <0.024. The observed w class distribution is thus unlikely, but not impossible, on this hypothesis. The maximum frequency of the H phenotype compatible with the w^+ data at the 1% level is 0.21, which equals the maximum underlying frequency of w^+ gametes that have obtained H alleles at both loci by crossing over. This is the same as the expected frequency of w individuals having *D. mauritiana* L alleles at both loci, which is less than or equal to the frequency of w individuals with the F_1 (L) phenotype. The estimated F_1 mean is -1.86, so that the upper 95% limit among 20 L individuals is -1.13. To be conservative, we set the cut-off for the L class as -1.00. None of the 20 w individuals fall below -0.92, so that under this hypothesis there is a probability ≤ 0.01 of obtaining the w distribution. The possibility that both the w and the w^+ distributions are produced with probabilities ≤ 0.01 can be safely rejected.

A minimum of three genes is therefore required to explain the data on the 65A insert alone. Moving from left to right on the chromosome, let the H alleles at these three hydrocarbon loci be H_1, H_2, and H_3, and the corresponding L alleles be L_1, L_2, and L_3. To account for the significant association of the L phenotype with the 65A insert, at least one locus must show linkage to 65A. But the low frequency of the H phenotype among the w class and the high frequency of non-L individuals among the w^+ class (11/20) suggest that at least one of these loci must be loosely linked to 65A, or that there are additional, more distant, genes. Since we are trying to determine the minimum number of hydrocarbon genes, we will adopt the three-gene model as a working hypothesis. Given the fact that 65A is <25 cM from the tip of the chromosome, it follows that gene 3 must be the most remote and to the right of 65A on this hypothesis; the arrangement of the other two genes is not obvious. It is further necessary to postulate that the full H phenotype is recovered only in $H_1H_2H_3$ individuals.

The three-gene model can thus explain the fact that only one H individual is recovered in the w class, provided that the total expected frequency of non-$H_1H_2H_3$ w gametes is ≥ 0.75, which obviously requires very loose linkage of genes 2 and 3 to 65A. If the order of the genes is 65A-1-2-3, and if gene 3 is loosely linked to gene 2, the frequency of the $H_1H_2H_3$ genotype among the w^+ class is less than or equal to one-half the map distance between 65A and gene 1 (c_1), neglecting quadruple crossovers. Since no such recombinants were found, the maximum value of c_1 that is consistent with the w^+ data at the 5% probability level is 0.28 (this is found by solving $[1 - 0.5\ c_1]^{20} = 0.05$). Alternatively, gene 1 could be to the left of 65A, in which case a double crossover (one in each of the intervals adjacent to 65A) is needed to produce the w^+ $H_1H_2H_3$ genotype, which has a low probability if linkage is tight. The frequency of doubles on this model must be ≤ 0.14 to be compatible with the data at the 5% probability level. On this ordering, fairly close linkage of gene 1 to 65A is needed to explain our failure to detect an effect of the 61CD insert, given that 61CD and 65A are ~25 cM apart.

Some further information about the properties of these genes can be obtained as follows. The disparity between the numbers of the apparently nonrecombinant phenotypes in the w^+ and w classes (9 and 1 respectively) has a probability of about 0.01 of occurring on the null hypothesis of equality of parental class frequencies (continuity-corrected $\chi^2 = 6.53$). Some of the w^+ recombinants must therefore overlap the L phenotype distribution. If the order is 65A-1-2-3, the most frequent w^+ recombinant classes are $L_1L_2H_3$, $L_1H_2H_3$, and $L_1H_2L_3$, at least some members of which must be L in phenotype. But none of the w class overlap the F_1 distribution, so that the common w recombinants $H_1H_2L_3$, $H_1L_2L_3$ and $H_1L_2H_3$ must have a low chance of displaying the L phenotype. This suggests that L_1 has a major effect of reducing the trait value, and that H_1 is required to produce a non-L phenotype. The absence of L phenotypes among the w class also suggests that gene 1 is closely linked to 65A. Alternatively, the order could be 1-65A-2-3, with fairly close linkage of gene 1 to 65A, as argued above. Again, the presence of H_1 would seem to be needed to produce a non-L phenotype. The data do not allow discrimination between the two alternative orderings.

The insert at 69D (~32 cM from 65A) is also associated with a significant phenotypic effect (~27% of the total *H-L* difference). At least one hydrocarbon gene must thus be linked to 69D. This raises the question of whether a further gene must be postulated in addition to the three linked to 65A. This can be examined as follows. In this cross, nine out of 20 individuals in the w^+ class have the L phenotype. This is consistent with the effect of the L_1 allele postulated above; if gene 1 is loosely linked to 69D, about half the w^+ recombinants

would be L in phenotype as a result of receiving L_1. But this is difficult to reconcile with the absence of individuals in the w class that overlap the L phenotype; the frequency of L in this class should be similar to the frequency of non-L in the w^+ class if $L_1H_2H_3$ etc. are L in phenotype. Fisher's exact test gives a probability of <0.001 assuming equality of these two frequencies, showing that this interpretation is incorrect. One possibility is that H_3 also confers a high probability of having a non-L phenotype, and that gene 3 is quite closely linked to 69D. The common w recombinant class $L_1L_2H_3$ would then have a high chance of being both non-L and non-H.

On this basis, very loose linkage of genes 1 and 2 to 69D is needed to explain the lack of the nonrecombinant H phenotype in the w class: the maximum frequency of nonrecombinants that is compatible with the w data even at the 1% probability level is 0.21. But this is inconsistent with the map distance of only 32 cM between 65A and 69D, and the requirement that genes 1 and 3 be closely linked to 65A and 69D, respectively. This inconsistency indicates the presence of yet another hydrocarbon gene, 4, to the right of 69D and gene 3, such that $H_1H_2H_3L_4$ is likely to be non-H in phenotype. The existence of such a gene slightly relaxes the requirement for gene 3 to be tightly linked to 69D, if either $H_1L_2L_3L_4$ or $L_1L_2L_3H_4$ have a high chance of being non-L. It is unclear whether gene 3 is to the left or right of 69D.

We next consider the evidence from the 78CD insert (34 cM from 69D), which again is associated with a highly significant phenotypic effect (30% of the overall difference). Once again, there are no H phenotypes among the w class. This reinforces the evidence for the existence of gene 4, on the same reasoning as for 69D. The existence (4/20) of L phenotypes among the w class suggests fairly loose linkage between 78CD and genes 2 and 4, so that there is a good chance that w recombinants such as $L_1L_2H_3L_4$ occur. This is also consistent with the detection of non-L phenotypes (6/20) among the w^+ class, which must represent recombinants that have acquired several H alleles. There is no firm evidence concerning the location of gene 4 in relation to 78CD.

The 82A insert will not be considered here, since it is closely linked (3 cM) to 78CD. In addition, the overall mean (0.67) for this backcross is anomalously high, presumably because of an environmental effect, which makes it impossible to classify backcross individuals reliably. The next insert is at 91BC, 27 map units from 78D. The effect of this insert is again highly significant (26% of the total effect). This time, there are now two w individuals that overlap the H class, suggesting that linkage to gene 4 is sufficiently tight that $H_1H_2H_3H_4$ nonrecombinants have been detected. There is no difficulty in accounting for the lack of w individuals with L phenotypes if H_4 confers a non-L phenotype on most genotypes (see above). Given that genes 1 and 4 must recombine freely, and the evidence that L_1 confers the L phenotype with high probability, the maximum frequency of recombination between gene 4 and 91BC that is compatible with the w data at the 5% probability level is 0.28 (see the above method for estimating c_1). The observation of nearly equal numbers of H and L phenotypes among the w^+ class (11 and nine, respectively) is consistent with this model, on similar logic to that applied to the 78CD insert. Given that gene 3 is likely to be closely linked to 69D (see above), which is 41 cM from 91BC, the major part of the association between the 91BC insert and the hydrocarbon phenotype must to be due to linkage of gene 4 to 91BC, but the order of these loci is unclear from these data, although it must be to the right of 78CD to account for the relatively close linkage of gene 4 to 91BC and looseness of its linkage to 78CD.

The insert at 85BC is 49 map units away from 91BC and has a highly significant effect (33% of the total). If this effect is due to gene 4, it must therefore be to the right of 91BC, consistent with the above conclusion. Gene 4 would therefore be ~21 cM to the left of 85BC. Arguments similar to those used previously show that this model is consistent with the fact that all the w individuals have the non-L phenotype but none have the H phenotype, and that 14 out of 20 individuals in the w^+ class have the L phenotype and none have the H phenotype. A model in which gene 4 is located between 78CD and 91BC, and there is an additional gene 5 between 91BC and 85BC, is also consistent with the results.

The 99A insert also has an apparently significant effect (19% of the total). Since 99A is 41 cM from 85BC, it is impossible to attribute this effect to gene 4, given its location to the left of 85BC. There must therefore be a further gene, 5, probably located between 85BC and 99A. The generally rather high phenotypic values for both the w and w^+ classes, suggesting a common environmental effect, makes it unsafe to pursue the analysis further. It should be noted that this insert has the least significant of the seven chromosome *3* effects we have analyzed in detail ($P < 0.005$ under a one-tailed test). The probability on the null hypothesis of obtaining at least one significant result at this level out of seven is 0.034, so that it seems reasonable to accept this as truly significant.

In conclusion, the data suggest that the species difference is due to at least five hydrocarbon loci scattered along chromosome *3*. This estimate is fairly consistent with the biometrical estimate of at least six genes and the lack of evidence for effects of the other chromosomes. Given the conservative nature of the assumptions that we have made, there are probably at least six hydrocarbon genes on this chro-

mosome. The data suggest that the leftmost locus, gene 1, has a large effect, in the sense of the *H* allele at this locus being required for a non-F_1 phenotype. This gene is probably quite close to 65A, but could be either to the right or left of that position. Gene 2 is probably located roughly midway between 65A and 69D, and gene 3 is fairly close to 69D, either to the right or left. Gene 4 is probably roughly midway between 78CD and 91BC; there may be another gene between 91BC and 85BC, and there is a gene 5 or 6 between 91BC and 99A. Gene 4 also appears to have a relatively large effect, in the sense that its *H* allele appears to confer a non-F_1 phenotype with high probability when homozygous.