Genetics of Differences in Pheromonal Hydrocarbons Between *Drosophila melanogaster* and *D. simulans*

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ABSTRACT

Females of *Drosophila melanogaster* and its sibling species *D. simulans* have very different cuticular hydrocarbons, with the former bearing predominantly 7,11-heptacosadiene and the latter 7-tricosene. This difference contributes to reproductive isolation between the species. Genetic analysis shows that this difference maps to only the third chromosome, with the other three chromosomes having no apparent effect. The *D. simulans* alleles on the left arm of chromosome *3* are largely recessive, allowing us to search for the relevant regions using *D. melanogaster* deficiencies. At least four nonoverlapping regions of this arm have large effects on the hydrocarbon profile, implying that several genes on this arm are responsible for the species difference. Because the right arm of chromosome *3* also affects the hydrocarbon profile, a minimum of five genes appear to be involved. The large effect of the third chromosome on hydrocarbons has also been reported in the hybridization between *D. simulans* and its closer relative *D. sechellia,* implying either an evolutionary convergence or the retention in *D. sechellia* of an ancestral sexual dimorphism.

SEVERAL questions have motivated a revival of interest in the genetics of speciation. Foremost among these is the recurring debate about whether genes of large or of small effect are important in speciation and adaptation (ORR and COYNE 1992), a problem that can be settled by only by genetic mapping experiments. This question also bears on particular theories of speciation. One might expect, for example, that runaway sexual selection would produce sexual isolation based on polygenes because of the gradual coadaptation between male trait and female preferences. The particular interactions among loci, also revealed by genetic analysis, may also help one reconstruct the order of evolutionary change (ROELOFS *et al.* 1987; BRADSHAW *et al.* 1995). Moreover, the chromosomal localization of "speciation genes" and the estimation of their effects may reveal generalities that motivate new theories of speciation, such as TURELLI and ORR's (1995) recent explanation of Haldane's rule, the increased severity of postzygotic isolation in hybrids of the heterogametic sex. Finally, the molecular isolation of "speciation genes," essential to a complete understanding of reproductive isolation, requires first that they be mapped to a small region (COYNE 1992; ORR 1992; PEREZ *et al.* 1993).

Most genetic studies of reproductive isolation have been confined to postzygotic isolation: hybrid sterility and inviability. Prezygotic isolation, however, may be a more important cause of speciation. Many species—most notably birds and plants—readily produce fertile hybrids in the laboratory or greenhouse but never hybridize where they exist together in nature. In such cases, sexual isolation must have preceded the evolution of postzygotic isolation.

The paucity of genetic studies of sexual isolation is almost certainly due to the difficulty of this work. Mating behavior is a labile character, easily affected by changes in the laboratory environment, and overcoming this variation demands large samples. In addition, measuring sexual isolation often requires lengthy observations of courtship, making the character much more tedious to score than hybrid inviability or sterility. Finally, the morphological markers usually used for behavioral analyses often affect behavior or vigor, making elaborate controls necessary.

To obviate these problems, we sought a character correlated with sexual isolation, but more easily quantified than behavior and less affected by genetic markers and environmental variation. Pheromones are obvious candidates. Here we report a genetic analysis of a pheromonal difference contributing to sexual isolation between two sibling species, *Drosophila melanogaster* and *D. simulans.*

Drosophila, like other dipterans, have a layer of lipids and hydrocarbons on the cuticle surface that serves the dual function of preventing desiccation and stimulating male courtship (DILLWITH *et al.* 1981; HOWARD and BLOMQUIST 1982; LANGLEY and CARLSON 1983; JALLON 1984; TOOLSON 1988; OGUMA *et al.* 1992). In several species, the pheromonal hydrocarbons are long-chain (20–30-carbon) nonvolatile compounds that the males detect by gustation during courtship (JALLON 1984; OGUMA *et al.* 1992).

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These compounds have been studied most thoroughly in the *D. melanogaster* subgroup, comprising four sibling species of Afrotropical origin. Two of these species, *D. melanogaster* and *D. simulans,* are cosmopolitan human commensals, and the other two, *D. mauritiana* and *D. sechellia,* are endemic to islands in the Indian Ocean (Mauritius and the Seychelles, respectively). Analysis of chromosome banding and DNA sequence shows that *D. simulans* and the two island species form a monophyletic group, while *D. melanogaster* is an outgroup (LEMEUNIER *et al.* 1986). The precise relationships among the three ingroup species are unclear because genetic distances among all three pairs of these species are nearly identical (CARIOU 1988; KLIMAN and HEY 1993). Their divergence is thought to have occurred 1–2 mya, possibly after colonization of the islands by an ancestor of *D. simulans.* (The divergence between *D. melanogaster* and the members of the ingroup occurred ~2–4 mya). There are numerous studies of sexual and postzygotic isolation in these four species and their more distant relatives (see LEMEUNIER *et al.* 1986).

The cuticular hydrocarbons of these four species have been thoroughly characterized (PECHINE *et al.* 1985; JALLON and DAVID; 1987; COBB *et al.* 1989; FERVEUR *et al.* 1994). Individuals of each species have about two dozen different hydrocarbons between 20 and 30 carbons long, but two of these molecules are predominant. Two species, *D. simulans* and *D. mauritiana,* are sexually monomorphic, with both males and females having large quantities (~400 ng/fly, roughly half of all the cuticular hydrocarbons) of the 23-carbon compound *cis* 7-tricosene (henceforth "7-T"). *D. melanogaster* and *D. sechellia,* on the other hand, are sexually dimorphic. Males of these species also have large quantities of 7-T, but females almost completely lack this compound, having instead large amounts (~200 ng/fly) of the 27-carbon compound *cis, cis* 7,11-heptacosadiene (henceforth "7,11-HD"), Some African populations of both *D. simulans* and *D. melanogaster* have high quantities of other hydrocarbons (FERVEUR *et al.* 1994; COBB and FERVEUR 1995). In two species, *D. melanogaster* and *D. simulans,* the predominant female compound has been shown to induce courtship by conspecific males (JALLON 1984).

We recently found that the hydrocarbon *differences* among species contribute to sexual isolation, so that these differences can be considered aspects of reproductive isolation (COYNE *et al.* 1994; COYNE and OYAMA 1995). These observations confirmed a hypothesis of by COBB and JALLON (1990), who noticed a correlation between the hydrocarbon constitution of species in this group and their interspecific courtship behavior. They hypothesized that a male will court a female of another species only if she shares either his predominant hydrocarbon or that present in his conspecific females. In addition, males will not court a female if she carries substantial quantities of a hydrocarbon foreign to his species.

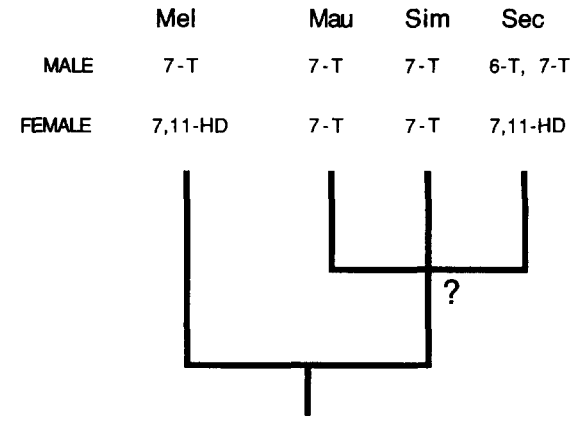

FIGURE 1.—Phylogeny of three species in the *D. melanogaster* subgroup (mel, *melanogaster;* mau, *mauritiana;* sim, *simulans;* and sec, *sechellia*), showing major cuticular hydrocarbons of males and females. The question mark at the divergence between *mauritiana, simulans,* and *sechellia* reflects our ignorance of their relative divergence times (genetic distances between all three possible pairs of these species are nearly identical).

These ideas were supported by experiments that transferred the hydrocarbons among different species (COYNE *et al.* 1994). Our studies showed that *D. simulans* males will not mate with conspecific females if these females carry some 7,11-HD that has been transferred from *D. melanogaster* or *D. sechellia* females. Moreover, *D. simulans* males, who normally do not court *D. melanogaster* or *D. sechellia* females, can be induced to do so if these females carry some 7-T transferred from *D. simulans* females. Studies of the courtship of *D. melanogaster* gynandromorphs by *D. simulans* males provided further evidence that the pheromonal difference between females of these species contribute to sexual isolation (COYNE and OYAMA 1995).

One striking aspect of the phylogenetic distribution of pheromones is that *D. sechellia* has a sexual dimorphism identical to that of the outgroup species *D. melanogaster,* but different from that of its two closer relatives, who are sexually monomorphic (Figure 1). This suggests two possibilities: either the ancestral sexual dimorphism seen in *D. melanogaster* has persisted in *D. sechellia,* with the *D. mauritiana* and *D. simulans* monomorphism representing a shared derived character, or the sexual dimorphism of *D. melanogaster* and *D. sechellia* represents an independent evolutionary convergence.

One way to address these alternatives is through genetic analysis of the difference in female hydrocarbons in two independent hybridizations: *D. simulans/D. sechellia* and *D. simulans/D. melanogaster.* If the *D. sechellia* dimorphism is the retention of an ancestral condition, then the genetic basis of the 7-T/7,11-HD difference should be nearly identical in both hybridizations (Figure 1). If the genetic bases are very different, it is likely that the *D. sechellia* dimorphism is an independently evolved condition.

We have performed the genetic analysis in one of these hybridizations, *D. simulans/D. sechellia* (COYNE *et al.* 1994). Only a single chromosome—the third—is responsible for the 7-T/7,11-HD difference between females of these species. This result implies that the difference is due to evolutionary change at a single locus or several linked loci, and not to many genes spread throughout the genome.

Here we address the questions of gene number and evolutionary convergence through a genetic analysis of hydrocarbons in the other hybridization: *D. melanogaster/D. simulans* females. This analysis is complicated by the complete sterility of the hybrids, which prevents the production of F_2 or backcross progeny. Differences between these two species must hence be studied using genetic trickery, as we have done in our genetic analysis of differences in male genital morphology (COYNE 1983). There are limitations to this method—especially the difficulty of separating the effects of specific chromosomes from their genetic background. On the other hand, *D. melanogaster* has an array of genetic tools, such as deficiency stocks, that facilitate the fine-structure mapping of "pheromone genes." This allows us to determine whether small regions of the genome have large effects on pheromone profile and therefore whether genes of large effect may play a role in reproductive isolation.

MATERIALS AND METHODS

Crosses: The *D. melanogaster/D. simulans* hybridization produces only sterile hybrids of a single sex, that of the *D. melanogaster* parent (STURTEVANT 1920). To obtain females, we usually crossed *D. simulans* males with *D. melanogaster* females, using 10 flies of each sex. The offspring were reared at 24° on agar-yeast-banana food.

The fly stocks used in this analysis are listed below, with each stock given an abbreviation (shown in parentheses) for more concise description in the tables.

***D. melanogaster* stocks:** *Ives (Iv):* A homokaryotypic stock made by combining 200 isofemale lines collected in 1975 in Amherst, MA (see COYNE *et al.* 1991 for further information about the strain).

Davis 3 (D3mel): An isofemale line collected by TIMOTHY PROUT in August, 1991, in Davis, CA.

Mauritius 13 (Mau 13): An isofemale line collected by CATHERINE MONTCHAMP at Port Louis, Mauritius, in August, 1992.

Oregon-R (Or-R): A standard laboratory strain obtained from the National Drosophila Species Resource Center, Bowling Green, OH.

Valparaiso (Valmel): An isofemale line collected by Mohamed Noor at Anderson Orchards, Valparaiso, IN, in September, 1994.

4-sim/ci^D: A stock of *D. melanogaster* containing fourth chromosomes of *D. simulans*. These foreign chromosomes were originally introduced into *D. melanogaster* by PONTECORVO (1943). The *D. simulans* fourth chromosome is homozygous-sterile in males, and *ci^D* [a dominant allele of the fourth-chromosome gene *cubitus-interruptus* (4-0)] is homozygous lethal in both sexes. The presence of the *D. simulans* fourth chromosome in this stock was confirmed genetically and cytologically by ORR (1992); we also checked it by crossing the wild-type males appearing in the stock (putatively homozygous for 4-sim) to *D. melanogaster* Ives females. No progeny were ever produced, confirming the presence of the foreign chromosome.

w;4-sim/ey^D: A stock similar to the above, but containing the *X*-linked mutation *white* and the fourth-chromosome dominant allele of *eyeless* (4-2.0; also homozygous lethal). This stock was constructed by ORR (1992), who verified the presence of the *D. simulans* fourth chromosome.

ebony (e mel): A strain containing the third-chromosome mutation *ebony* (*e*, 3-70.7). To place the mutation on a noninbred genetic background, males from an *ebony* strain were outcrossed to Ives females and the mutant re-extracted in the F_2. This procedure was repeated three times. The outcrossed stock was used in deficiency-mapping (see below).

Compound-arm stocks: For mapping pheromone loci to autosomal arms, we used five compound-arm stocks covering both major autosomes: *C(2L)RM, dp/F(2R)bw* [abbreviated *C(2L)*]; *C(2R)RM, cn/F(2L)dp* [abbreviated *C(2R)*]; *C(3L)RM h^2/F(3R) e* [abbreviated *C(3L)A*]; *y;C(3L)RM, h^2/F(3R) e(s) ro ca* [abbreviated C(3L)B]; and *C(3R)RM, sr/F(3L)h^2* [abbreviated C(3R)].

Deficiencies: To localize regions on the left arm of chromosome 3 affecting the interspecific difference in hydrocarbons, we crossed *D. simulans* males to each stock in the "3L deficiency kit" obtained from the stock center at Bloomington, IN (see RESULTS). This kit contains 32 balanced deficiencies covering ~86% of the chromosome arm. As described below, only 14 of these interspecific crosses produced the two classes of offspring necessary to determine whether the deficiency affected the hydrocarbon profile. These 14 stocks are listed below, along with the cytological position of the deficiency. An abbreviation of each stock, for use in the text and tables, is given in parentheses, *Df(3L)R-G5 ve/TM6* (*RG5*): 62A10-B0, 62C04-D01; *Df(3L)R-G7 ve/TM6B, Tb$^+$* (*RG7*): 62B08–09, 62F02–05; *Df(3L)HR232/TM6B* (*HR232*): 63C0, 63D03; *Df(3L)HR119/TM6B* (*HR119*): 63C06, 63E; *Df(3L)pblX1/TM6B* (*pblX1*): 65F03, 66B10; *Df(3L)66C-G28/TM3;y w spl* (*66C*): 66B08–09, 66C09–10; *Df(3L)lxd6/y$^+$ TM3, Sb e Ser* (*lxd6*: 67E01–02, 68C01–02; *Df(3L)Ly, mwh/TM1, jv* (*Ly*): 70A02–03, 70A05–06; *Df(3L)fzGF3b/TM6B* (*fzGF3b*): 70C01–02, 70D04–05; *Df(3L)fzM21/TM6* (*FzM21*): 70D02–03, 71E04–05; *Df(3L)th102, h ri e^s/TM6C, cu Sb e ca* (*th102*): 71F3–5, 72D12; *Df(3L)st^4, gt^2 e^4/TM6* (*st4*: 72D10, 73C01; *Df(3L)in61j/TM1* (*in61j*): 76F, 77D; *Df(3L)1-16/TM3, Ser*(*1-16*): 80F a-g,h.

***D. simulans* stocks:** *Davis 2 (D2sim):* An isofemale line collected by TIMOTHY PROUT in August, 1991, in Davis, CA.

ebony (e sim): A mutant stock provided by JEAN DAVID and used in deficiency mapping (see RESULTS).

Florida City (FC): An isofemale line collected in Florida City, FL, in June, 1985.

Valparaiso (Valsim): An isofemale line collected by MOHAMED NOOR at Anderson Orchards, Valparaiso, IN, in September, 1994.

scarlet (st): A mutant stock used for deficiency-mapping.

C(1)RM, yw females, + males. [*C(1)RM*]: A stock of *D. simulans* with females having attached-*X* chromosomes that carry the mutations *yellow* (1-0.0) and *white* (1-1.5). The free *X* chromosome in males carries the wild-type alleles at both loci.

detached-X, yw[*C(1)RM-det*]: A stock derived from one vial of the attached-*X* stock of *D. simulans* (see above) in which the fused *X* chromosomes became detached, producing wild-type females and *y, w* males. These genotypes were crossed to each other and, in the next two generations, a pure *y, w* stock extracted. Females of this stock have a genetic background and markers identical to the attached-*X* stock, but their *X* chromosomes were free. The stock was used in a cross testing the effect of the *X* chromosome on hydrocarbon profiles (see RESULTS).

Y-autosome translocation stocks: Two *D. simulans* stocks, *T(Y;2)3 + /nt bk* [abbreviated *T(Y;2)*], and *T(Y;3)1, Ubx*^{m}*/ cutsy ca*^{2} *f* [abbreviated T*(Y;3)*] were used in crosses to the *D. melanogaster* compound-autosome stocks. In each of these stocks, one of the autosomes has been broken at the centromere and the *Y* chromosome translocated to one of the free arms, so that males are heterozygous for one intact metacentric autosome and two free autosomal arms, one of which carries the *Y*. As described below, these stocks were used to produce F_1 female hybrids that are homospecific for the *D. melanogaster* compound-arm but heterospecific for all other chromosome arms (GRELL 1976; OHNISHI *et al.* 1982)

Gas chromatography: Extraction of cuticular hydrocarbons and gas chromatography of these extracts were performed as previously described (FERVEUR 1991; COYNE *et al.* 1994). Hydrocarbon peaks were identified by their comigration with known standards, and absolute quantities of the two hydrocarbons of interest (7-T and 7,11-HD) estimated by comparing their peak areas with that of an internal n-hexacosane standard added to each sample (peak areas are directly proportional to hydrocarbon quantity). Flies were usually analyzed individually, but on a few occasions up to four flies per sample were used. All data are presented as hydrocarbons per individual fly.

Statistics: Individuals were characterized by the ratio of the main hydrocarbons from each species (see RESULTS). For comparing these ratios between genotypes, we used the nonparametric Mann-Whitney *U* test. One-tailed probabilities were calculated when we were testing the *a priori* hypothesis that the 7,11-HD/7-T ratio would be higher in the genotype having more genome from *D. melanogaster*. When multiple comparisons were used, significance levels were adjusted using the sequential Bonferroni test (RICE 1989).

RESULTS

Table 1 gives the pheromonal constitution of females from the two species and various F_1 hybrids, presented not only as the absolute amounts of the two major hydrocarbons, but also as their ratio (7,11-HD/7-T), a figure that should be unaffected by the size of the fly. Higher values of this ratio indicate more *melanogaster*-like hydrocarbon profiles.

As described previously (JALLON and DAVID 1987; COBB and FERVEUR 1995), we find that females from non-African strains of *D. melanogaster* have large amounts of 7,11-HD and very little 7-T, while *D. simulans* females have high levels of 7-T and no 7,11-HD at all. In both species, there is clearly variation among strains in the absolute amounts of the major hydrocarbons. Moreover, *D. melanogaster* strains also exhibit variation of the 7,11-HD/7-T ratio, which ranges between 4.6 and 22.4. In *D. simulans*, however, these values are always zero, so there is no overlap between the species.

Regular F_1 females (Table 1, crosses 1–4) have hydrocarbon ratios intermediate to the two parental species. Ranging between 1.0 and 4.7, the F_1 values slightly overlap the lowest ratios seen in pure *D. melanogaster* strains. Considering the absolute amounts of hydrocarbons, the alleles from *D. melanogaster* are dominant for 7,11-HD (hybrids have as much of this compound as do their pure *D. melanogaster* mothers), but semidominant for 7-T (hybrids have only half as much tricosene as do pure *D. simulans* females).

Effect of the *X* chromosome: To estimate the effect of the *X* chromosome on hydrocarbon profile, we produced hybrids differing from normal F_1 females only by having both *X* chromosomes from *D. simulans*. This genotype was made by crossing attached-*X D. simulans* females to males from the Ives strain of *D. melanogaster*, producing *XXY* hybrid females (cross 5 in Table 1). If the *X* chromosome carries genes affecting the interspecific difference in hydrocarbons, we would expect these F_1 females to have a lower 7,11-HD/7-T ratio than normal F_1 hybrids. For comparison, we made two crosses that produced "normal" F_1 females with one *X* chromosome from each species, but with the rest of the genetic background similar to that of the attached-*X* F_1s. The first such cross involved males from the *D. simulans* attached-*X* stock and females from to *D. melanogaster* Ives females (Table 1, cross 6). The second cross involved males from the "detached" attached-*X* stock and *D. melanogaster* Ives females (Table 1, cross 7). Both of these crosses also produce offspring with cytoplasm from *D. melanogaster*, while the attached-*X* hybrids have cytoplasm from *D. simulans*.

Comparing genotype 5 with genotypes 6 and 7, one sees that neither the *X* chromosome nor cytoplasm have a perceptible effect on the hydrocarbon ratio. The attached-*X* genotype has, in fact, a slightly *higher* 7,11-HD/7-T ratio than the control F_1s, a difference that is not in the expected direction and hence is clearly not significant under a one-tailed test. There is therefore no evidence that the *X* chromosome carries genes affecting the pheromonal ratio. We cannot, however, rule out the possibility that there are recessively acting *D. melanogaster* alleles that could only be detected in a female having both *X* chromosomes from this species.

Effects of the second and third chromosome: The effects of the two major autosomes were estimated using a method devised by GRELL (1976) to map allozyme differences between these species. This method, diagrammed by OHNISHI *et al.* (1982), involves crossing females from compound-autosome-arm stocks of *D. melanogaster* to males of *D. simulans Y*-autosome translocation stocks. The F_1 hybrid females get both attached autosomal arms from *D. melanogaster*, while the free arms of the same chromosome (and the rest of the chromosomes) come equally from each species. If the compound arm carries loci responsible for the species difference, these unbalanced hybrids should have a more *melanogaster*-like hydrocarbon profile than normal F_1 hybrids. Unlike the *X*-chromosome crosses described above, we could not produce control hybrids with genetic backgrounds similar to the unbalanced F_1. We therefore determined whether the hydrocarbon ratios of these unbalanced hybrids fell above the range of ratios seen among "normal" F_1 hybrids having a completely balanced genome (crosses 1–4, 6 and 7 in Table 1).

Considering the second chromosome, neither the left or the right arms (crosses 8 and 9 in Table 1) affect the hydrocarbon ratios: the values for the offspring of

TABLE 1

Major hydrocarbons of female *D. melanogaster*, *D. simulans*, and their hybrids

	Mean hydrocarbons (ng/fly)			
	N	7-T	7.11-HD	7,11-HD/7-T
D. melanogaster				
D3 mel	18	19.8 (0.8)	130.1 (5.3)	6.65 (0.27)
e mel	18	14.0 (0.6)	311.0 (12.8)	22.35 (0.50)
Iv	19	10.5 (1.4)	187.4 (11.9)	21.32 (1.74)
Mau13	17	19.4 (0.9)	228.1 (9.2)	11.96 (0.49)
OR-R	15	32.6 (2.3)	147.6 (11.2)	4.62 (0.29)
C(2L)	15	25.5 (1.0)	191.9 (6.7)	7.58 (0.23)
C(2R)	18	31.9 (2.1)	192.1 (15.8)	6.36 (0.54)
C(3L)A	13	13.0 (0.8)	154.2 (7.0)	12.27 (0.52)
C(3R)	16	22.1 (1.2)	284.6 (9.0)	13.30 (0.65)
Valmel	15	21.8 (1.7)	401.4 (21.8)	19.26 (1.18)
D. simulans				
D2 sim	16	506.5 (27.8)	0	0
e sim	17	369.2 (29.6)	0	0
FC	20	282.1 (15.9)	0	0
C(*1*)RM	16	378.7 (17.5)	0	0
T(*Y;3*)	16	408.52 (52.4)	0	0
T(*Y;2*)	15	412.5 (14.0)	0	0
Valsim	16	653.1 (40.7)	0	0
F_1 (♂ × ♀)				
Cross				
1. FC × Iv	21	67.7 (6.8)	306.1 (36.1)	4.64 (0.12)
2. D2 sim × Iv	16	197.8 (13.4)	196.5 (12.2)	1.01 (0.04)
3. *e* sim × Mau 13	16	333.4 (63.4)	453.7 (64.0)	2.02 (0.32)
4. Valsim × Valmel	15	166.1 (19.4)	506.8 (62.1)	3.15 (0.26)
5. Iv × C(*1*)RM	23	57.0 (2.9)	297.6 (10.4)	5.32 (0.14)
6. ♂ C(*1*)RM × Iv	16	238.5 (20.0)	866.5 (46.2)	3.83 (0.21)
7. C(*1*)RMdet × Iv	4	177.5 (56.4)	406.6 (145.7)	2.18 (0.31)
8. T(*Y;2*) × C(2L)	16	103.8 (6.9)	289.5 (16.2)	2.86 (0.13)
9. T(*Y;2*) × C(2R)	17	87.1 (8.5)	112.4 (13.6)	1.35 (0.10)
10. T(*Y;3*) × C(3R)	18	39.6 (1.3)	379.2 (12.2)	9.65 (0.27)
11. T(*Y;3*) × C(3L)A	27	651.6 (32.1)	2.3 (1.1)	0.004 (0.002)
12. T(*Y;3*) × C(3L)B	4	797.2 (17.5)	0	0

Absolute quantities of 7-tricosene (7-T) and 7,11-heptacosadiene (7,11-HD) are given, along with their ratios; the standard errors of all three quantities are given in parentheses. Abbreviations for the genotypes are given in METHODS AND MATERIALS. See RESULTS for descriptions of crosses.

these crosses (2.86 and 1.35, respectively) are well within the range seen among "normal" F_1 offspring (1.01–4.64).

The right arm of third chromosome, however, does have an effect, (Table 1, cross 10): the 7,11-HD/7-T ratio, 9.65, is more than twice as high as any value seen among the normal F_1 offspring. This constitutes fairly strong evidence that the 3R arm of *D. melanogaster* carries at least one gene having semidominant effects on the hydrocarbon ratio.

The crosses involving the left arm of chromosome 3 (Table 1, cross 11) gave a peculiar result. Instead of producing the expected F_1 females having two 3L arms from *D. melanogaster* (and showing the h^2 marker), the cross produced hybrids lacking the h^2 marker or any other marker—including the dominant Ubx^m mutant on the free 3R of *D. simulans*. (We determined in other crosses that Ubx^m from *D. simulans* is indeed expressed in species hybrids when it is present.) These anomalous flies were clearly species hybrids: all were female, and a sample tested for fertility produced no offspring when crossed to either *D. simulans* or *D. melanogaster* males. These hybrids must therefore be the reciprocal unbalanced genotype missing in the progeny of the other compound arm-crosses; in this case, the hybrids have an entire intact third chromosome and one free 3L from *D. simulans*, with the other free 3R coming from *D. melanogaster*. (The appearance of this genotype in the 3L cross, and the absence of the corresponding genotypes the other crosses, must reflect the effects of these chromosome arms on hybrid viability). These offspring thus differ from normal F_1 hybrids by possessing an extra 3L from *D. simulans*. We thus expect that, if 3L carries genes contributing to the species difference in hydrocarbons, the 7,11-HD/7-T ratio of these hybrids will be below that of the normal, balanced F_1 females.

The results are striking (Table 1, cross 11): these

TABLE 2

Three tests of the effects of the fourth chromosome on female hydrocarbon profiles

Comparison	Genotype	N	Mean hydrocarbons (ng/fly) 7-T	7,11-HD	7,11-HD/7-T
1	*4-mel/4-mel*	16	18.06 (1.65)	221.86 (8.63)	13.53 (1.06)
	4-mel/4-sim	16	17.74 (0.69)	282.34 (12.63)	16.26 (0.97)
2	*4-mel/4-sim*	16	29.79 (2.51)	203.00 (6.53)	7.72 (0.75)
	4-sim/4-sim	16	32.40 (2.97)	192.36 (12.44)	6.76 (0.74)
3	*4-mel/4-sim*	15	20.81 (1.87)	290.96 (18.77)	14.46 (0.58)
	4-sim/4-sim	15	13.36 (1.02)	224.50 (21.27)	16.85 (0.82)

Each comparison tests the effect of a fourth chromosome substitution in a controlled genetic background. Values in parentheses are standard errors. See RESULTS for descriptions of the three comparisons.

hybrids have not only a very low 7,11-HD/7-T ratio, but one that is nearly zero (0.004)—the *D. melanogaster* hydrocarbon is almost completely absent. The *D. simulans* chromosome arm 3L obviously carries genes that, when homozygous, produce a hydrocarbon profile nearly identical to that of pure *D. simulans* females, regardless of the hybrid nature of the rest of the genome.

We checked this result by making a similar cross using a different compound-3L strain from *D. melanogaster* (there is only one *Y;3* translocation stock of *D. simulans*). Although this cross (Table 1, cross 12) produced only four offspring, the results were similar to those described above. In fact, these hybrid offspring completely lacked 7,11-HD, so their hydrocarbon profile was identical to that of pure *D. simulans* females. The recessively acting *D. simulans* "hydrocarbon alleles" (as well as viability alleles) on 3L are therefore species-specific and not just strain-specific.

In sum, the second chromosome has no discernible effect on hydrocarbon profile, while 3R has a moderate effect and 3L a very large effect. We cannot, of course, determine whether the second chromosome or the right arm of the third chromosome contain recessively acting *D. simulans* alleles similar to those on 3L, as the relevant genotypes cannot be produced. (Such genes can, however, be ruled out for the *X* chromosome, as evidenced by the F_1-like hydrocarbon profile of hybrids having both *X* chromosomes from *D. simulans*).

The recessivity of *D. simulans* "hydrocarbon genes" on 3L allows us to localize the relevant regions of this arm using deficiencies from *D. melanogaster*. We describe this analysis below.

Effects of the fourth chromosome: The fourth chromosome is very small, comprising only 2% of the genome, and we did not expect it to affect the hydrocarbon ratio. This expectation was confirmed by the three crosses presented in Table 2. Each cross produced two genotypes of offspring, differing on average only by the species composition of the fourth chromosome. All of these crosses used stocks of *D. melanogaster* containing *D. simulans* chromosomes produced by the hybridization experiments of PONTECORVO (1943).

In comparison 1 (Table 2), males of the *4-sim/ci*^{D} stock were crossed to *D. melanogaster* Ives females. This cross produced two genotypes of offspring, those showing the *ci*^{D} marker, and hence homozygous for fourth chromosomes from *D. melanogaster*, and those with wild-type wings, which have one fourth chromosome from each species. These two classes did not differ in hydrocarbon ratio: a one-tailed Mann-Whitney *U* test gives a probability of 0.89.

Comparison 2 (Table 2) examined possible recessive effects of *D. simulans* fourth chromosomes by comparing the two genotypes of females segregating within the *4-sim/ci*^{D} stock: those having the *ci*^{D} marker (and hence heterospecific for fourth chromosomes) and those lacking the marker (homozygous for *D. simulans* fourth chromosomes). Again, there was no significant difference between these two genotypes (one-tailed Mann-Whitney *U* test gives a probability of 0.25). A similar comparison (Table 2, comparison 3) of the two genotypes of females segregating in the *w;4-sim/ey*^{D} stock showed no significant difference in the hydrocarbon ratio of homozygous four-sim chromosomes *vs.* the heterospecific genotype ($P = 0.95$). There is hence no perceptible effect of the fourth chromosome on hydrocarbon ratio.

Deficiency-mapping of regions on chromosome 3L: The largely recessive effect of the *D. simulans* "hydrocarbon alleles" on chromosome 3L allows us to localize the important regions more accurately by deficiency mapping. To do this, we crossed *D. simulans* males to females from a variety of *D. melanogaster* stocks containing deficiencies on this arm. The pheromonal constitution of these hybrids is then compared with control F_1 hybrids not containing the deficiency. If the deficiency heterozygotes have a significantly lower 7,11-HD/7-T ratio than controls, the region of the deficiency is assumed to contain a gene influencing hydrocarbon profile.

Because most of the deficiencies are balanced against various TM (third-multiple) chromosomes that carry the *ebony* allele, we crossed females from the balanced deficiency stocks to males from a *D. simulans* stock homozygous for *ebony*. This cross should produce two ge-

TABLE 3

Tests of the effects of deficiencies on female hydrocarbon profiles

Cross	Deficiency	Genotype	*N*	7,11-HD/7-T	*P*
1	*RG5*	*Df*/+	14	0.407 (0.023)	<0.0001[a]
		+/+	17	1.701 (0.129)	
2	*RG7*	*Df*/+	4	0.358 (0.157)	0.0007[a]
		+/+	28	3.991 (0.019)	
3	*HR232*	*Df*/+	16	1.566 (0.112)	<0.0001[a]
		+/+	15	4.899 (0.265)	
4	*HR119*	*Df*/+	3	1.101 (0.485)	0.0026[a]
		+/+	26	4.540 (0.293)	
5	*pblx1*	*Df*/+	13	1.200 (0.099)	<0.0001[a]
		+/+	9	4.614 (0.216)	
6	*66C*	*Df*/+	22	0.923 (0.071)	0.993
		+/+	2	0.600 (0.031)	
7	*lxd6*	*Df*/+	12	1.706 (1.194)	0.0156[a]
		+/+	7	4.751 (2.91)	
8	*Ly*	*Df*/+	4	0.872 (0.055)	0.0653
		+/+	7	1.501 (0.208)	
9	*fzGF3b*	*Df*/+	32	1.163 (0.055)	0.0042[a]
		+/+	26	1.670 (0.135)	
10	*FzM21*	*Df*/+	9	3.904 (0.323)	0.9831
		+/+	2	1.076 (0.027)	
11	*th102*	*Df*/+	7	0.186 (0.051)	0.00045[a]
		+/+	9	3.872 (0.382)	
12	*st4*	*Df*/+	8	3.470 (0.258)	0.9985
		+/+	7	1.949 (0.155)	
13	*in6lj*	*Df*/+	2	0.374 (0.097)	0.0633
		+/+	3	1.406 (0.445)	
14	*1-16*	*Df*/+	15	0.521 (0.022)	0.9983
		+/+	15	0.410 (0.015)	

All offspring are F_1 female hybrids from crosses of *D. simulans* males to *D. melanogaster* females heterozygous for deficiencies on chromosome 3L. For each deficiency cross, the hydrocarbon ratio is given for the two classes of offspring; values in parentheses are standard errors. Probabilities are determined by comparing the ratios for the two genotypes using the nonparametric Mann-Whitney *U* test. All probabilities are one-tailed, and significance levels were determined by the modified Bonferroni test.

[a] Significant after Bonferroni correction.

notypes of offspring: those heterozygous for the deficiency (with wild-type body color) and those carrying the nondeficient TM chromosome (ebony body color). One deficiency chromosome, *Df(3L)st*^{4}, *gl*^{2} *e*^{4}, also carried *ebony*, so the requisite two classes were produced by crossing females from this strain to *D. simulans* males homozygous for *scarlet*.

Only 14 of the 32 *D. melanogaster* deficiencies covering 3L produced the two classes of offspring necessary to make this comparison. The remaining deficiency stocks either did not cross successfully to *D. simulans* or produced only wild-type offspring. Even the successful crosses, however, often produced only a small number of offspring (Table 3). The absence of ebony offspring in some crosses indicates that the balancer chromosome, which carries dominant alleles, is lethal in species hybrids. We have observed in other studies that dominant alleles of *D. simulans* are sometimes lethal in hybrids with *D. melanogaster*, and the same might be expected for dominant alleles from *D. melanogaster*.

Surprisingly, eight of these 14 crosses showed a significant hydrocarbon difference in the expected direction between the two genotypes of offspring. This difference between genotypes is not due to an effect of the *ebony* allele on cuticular hydrocarbons, because intraspecific crosses within both *D. simulans* and *D. melanogaster* showed no effect of the allele on the absolute or relative amounts of the compounds (Table 4). Moreover, four of the other six deficiencies, which were balanced against an ebony-containing chromosome, showed effects in the opposite direction.

The eight deficiencies with effects in the expected direction merited further investigation as regions possibly containing hydrocarbon genes. To determine whether these effects represented interspecific effects of the deficient region and not simply intraspecific genetic differences between the balancer and the deficiency chromosome (which may nevertheless indicate that the deficiency contains genes affecting hydrocarbons), we made intraspecific control crosses within *D. melanogaster*. In these crosses, females from the deficiency stocks were crossed to *D. melanogaster* males homozygous for *ebony*, producing two pure *D. melanogaster* genotypes, one carrying and one lacking the deficiency. Table 5 gives the results of these eight control crosses. In all cases, the two genotypes of offspring had hydro-

TABLE 4

Test of the effect of the *ebony* allele on hydrocarbon profiles within each species

Species	Genotype	*N*	Mean hydrocarbons (ng/fly) 7-T	7,11-HD	7,11-HD/7-T
melanogaster	*e/e*	16	15.1 (0.9)	331.6 (21.3)	22.7 (1.5)
	e/+	16	13.4 (0.4)	338.7 (16.2)	24.9 (1.0)
simulans	*e/e*	15	500.2 (28.5)	0	0
	e/+	15	548.8 (29.7)	0	0

For each set of crosses, homozygous *ebony* males of a species were crossed to wild-type females from that same species (the Ives stock for *D. melanogaster* and the FC stock for *D. simulans*). The F_1 females were then backcrossed to homozygous *ebony* males and the two classes of offspring analyzed for hydrocarbons. None of the differences in either absolute amounts of hydrocarbons or their ratios were significant using either *t*-tests or nonparametric Mann-Whitney *U* test. Values in parentheses are standard errors.

carbon differences similar in direction to those in the interspecific crosses; but the effect of the deficiency on hydrocarbon quantity was usually much smaller in the intraspecific than in the interspecific cross (see below). This is, in fact, the result expected when the *D. simulans* alleles are, like those shown for 3L, largely recessive in hybrids and the *D. melanogaster* alleles are not completely dominant.

It is nevertheless surprising that so many tested deficiencies affected the hydrocarbon profile. To localize those regions with only very large effects, we narrowed our search to those deficiencies producing much larger effects in the interspecific than in the intraspecific cross. This requires comparing the relative magnitudes of two differences, a comparison necessitating parametric statistics. We did this by comparing not the ratios of the two hydrocarbons (statistics that do not follow a normal distribution) but the absolute *difference* between their nanogram quantities: 7,11-HD − 7-T (COYNE and OYAMA 1995). For each genotype, the magnitude of this difference expresses the "*melanogaster*-likeness" of the hydrocarbon profile. This quantity was then calculated for each of the two genotypes (*Df/+* and *+/+* in both the interspecific experimental and the intraspecific control cross. Within each cross, the overall effect of the deficiency can be seen by comparing the mean (7,11-HD − 7-T) difference between the two genotypes.

Table 6 shows that deficiencies usually have very large effects in the interspecific crosses, often affecting the pheromonal difference by several hundred (≤900) nanograms. In contrast, the effect of the deficiencies in the intraspecific control crosses are much smaller—usually <100 ng.

We then compared the relative effects of the deficiency in the interspecific *vs.* control cross using a *t*-test of the "difference of the differences" (SNEDECOR and COCHRAN 1967). This was done by first calculating for the interspecific cross the quantity (7,11-HD − 7-T) for each of the two genotypes (*Df/+* and *+/+*). The difference between these two quantities was taken as

TABLE 5

Hydrocarbons of females from control crosses all within *D. melanogaster*

Deficiency	Genotype	*N*	7,11-HD/7-T	*P*
RG5	*Df/+*	15	9.867 (0.776)	
	+/+	15	15.331 (0.512)	<0.0001
RG7	*Df/+*	20	10.426 (0.526)	
	+/+	20	18.032 (0.496)	<0.0001
HR232	*Df/+*	15	12.988 (0.700)	
	+/+	15	18.707 (0.800)	<0.0001
HR119	*Df/+*	15	11.466 (0.540)	
	+/+	15	14.183 (0.588)	0.0015
pblx1	*Df/+*	39	14.240 (0.560)	
	+/+	39	19.032 (10.857)	<0.0001
lxd6	*Df/+*	17	9.872 (0.600)	
	+/+	17	13.455 (0.414)	0.0002
fzGF3b	*Df/+*	15	12.540 (0.672)	
	+/+	15	15.924 (1.170)	0.0084
th102	*Df/+*	15	13.084 (0.776)	
	+/+	15	21.008 (0.615)	<0.0001

Hydrocarbon ratios of the two classes were compared using the Mann-Whitney *U* test and one-tailed probabilities with standard errors in parentheses. All values were significant using the modified Bonferroni test. Intraspecific crosses were made for all deficiency stocks sharing a significant effect in Table 3.

TABLE 6

Overall tests of the effects of deficiencies on hydrocarbon profiles

Deficiency	Control or experiment	Genotype	7,11-HD/7-T (ng/fly)[a]	*t* (C *vs.* E)	*P*
RG5	E	*Df*/+	−481.85 ± 51.65	10.81	<0.0001[b]
		+/+	282.51 ± 36.62		
	C	*Df*/+	295.29 ± 18.91		
		+/+	327.52 ± 14.85		
RG7	E	*Df*/+	−326.67 ± 66.27	9.78	<0.0001[b]
		+/+	540.50 ± 34.55		
	C	*Df*/+	323.01 ± 13.91		
		+/+	412.55 ± 23.25		
HR232	E	*Df*/+	61.21 ± 14.50	3.70	<0.0001[b]
		+/+	431.52 ± 84.38		
	C	*Df*/+	229.16 ± 17.96		
		+/+	267.76 ± 19.35		
HR119	E	*Df*/+	105.67 ± 132.37	3.44	0.0003[b]
		+/+	441.32 ± 28.65		
	C	*Df*/+	398.30 ± 32.17		
		+/+	251.53 ± 13.65		
pblx1	E	*Df*/+	73.49 ± 35.79	9.09	<0.0001[b]
		+/+	601.08 ± 39.36		
	C	*Df*/+	365.81 ± 11.43		
		+/+	386.12 ± 12.43		
lxd6	E	*Df*/+	−360.30 ± 102.19	0.85	0.20
		+/+	−167.59 ± 173.64		
	C	*Df*/+	329.23 ± 29.08		
		+/+	347.69 ± 23.83		
FzGF3b	E	*Df*/+	11.73 ± 17.85	1.01	0.16
		+/+	184.07 ± 47.67		
	C	*Df*/+	267.11 ± 24.28		
		+/+	369.35 ± 39.63		
th102	E	*Df*/+	−600.05 ± 90.75	9.15	<0.0001[b]
		+/+	389.04 ± 49.12		
	C	*Df*/+	507.84 ± 40.45		
		+/+	435.45 ± 34.11		

For each deficiency tested, the effect of the deficiency in the experimental (E) interspecific cross was compared with its effect in the control (C) cross with *D. melanogaster* (see RESULTS for description of statistical test). The *t* value and probability values given are for comparison of control *vs.* experimental, using one-tailed tests, with threshold significance levels determined by the modified Bonferroni test.

[a] Values are means ± SE.

[b] Significant after Bonferroni correction.

the effect of the deficiency on the hydrocarbon profile, and its variance taken as the sum of variances of the two quantities. In the same way, the effect of the deficiency was determined for the control intraspecific cross. These two effects were then compared by subtracting them and dividing this difference by an estimate of its standard error [the square root of the sum of the four variances of (7,11-HD − 7-T)]. Under the null hypothesis of no difference, this statistic follows a *t* distribution with $N - 4$ degrees of freedom, where N is the total number of individuals involved in the comparisons. Our *a priori* hypothesis was that the effect of the deficiency would be larger in the interspecific than in the intraspecific cross, so we used one-tailed probability values and adjusted the threshold significance levels using the modified Bonferroni correction.

Table 6 gives the results of this final comparison. Of eight deficiencies having a significant effect on hydrocarbon profile in the interspecific cross, six had a significantly smaller effect in the intraspecific control cross. In all six cases, this difference between the intra- and interpecific cross is very large and highly significant.

Two pairs of these six deficiencies are overlapping (*RG5* with *RG7* and *HR119* with *HR232*), so we can say only that at least four regions of 3L appear to be involved in the hydrocarbon difference. For two reasons this underestimates the true number of loci causing the species difference: we could examine only a portion of this chromosome arm and some deficiencies could include more than one gene.

All six chromosomes showing significant effects were balanced against either *TM6, TM6B,* or *TM6C,* which are related balancers. This fact, plus the consistent differences in hydrocarbon ratio between balancer and deficiency-bearing chromosomes in the six control

crosses, raises the possibility that *TM6* balancers and not deficiencies are somehow responsible for the large interspecific differences. This cannot be a general explanation of the results, because in one interspecific cross (involving the deficiency *FzM21*), the difference between balancer and deficiency heterozygote is in the direction opposite to that of the others.

It may still be possible, however, to explain our results by positing that TM6 balancers raise the 7,11-HD/7-T ratio in only some *D. melanogaster* genetic backgrounds and that in hybrids with *D. simulans,* these effects are highly exaggerated, accounting for the significant difference between the intraspecific control crosses and the experimental interspecific crosses.

While this is a formal possibility, the data show that it is unlikely. First, this "balancer-effect" hypothesis posits that, for those deficiencies showing the largest effect in the interspecific crosses, the difference in 7,11-HD/7-T ratios between deficiency and balancer heterozygotes is not due to a lowering of this ratio by the deficiency chromosome, but to its elevation by the balancer chromosome (*i.e.,* there are no "hydrocarbon genes" in the deficiency). Under this hypothesis, one would expect to see that the interspecific individuals heterozygous for the deficiency are similar in 7,11-HD/7-T ratios to normal F_1 hybrids, while heterozygotes for the balancer would have ratios higher than those of the F_1 hybrids. However, this is clearly not the case. The interspecific balancer heterozygotes have ratios close to those of the normal F_1 females, while ratios of deficiency 7,11-HD/7-T heterozygotes are much lower. Seven of the deficiency stocks shown in Tables 5 and 6 are balanced against *TM6*-containing chromosomes. When crossed to *D. simulans* stocks, the mean 7,11-HD/7-T ratio of deficiency heterozygotes was 0.854 (range 0.186–1.56; data taken from Table 3). For these same stocks and crosses, the mean ratio for the balancer heterozygotes was 3.61 (range 1.67–4.89). The nondeficiency heterozygotes have ratios much closer to those of the pure F_1 hybrids (genotypes 1–7 in Table 1), whose average ratio is 3.16 (range 1.01–5.32). These results suggest that the deficiency heterozygotes strongly depress the hydrocarbon ratios, as one expects if the missing regions contain "hydrocarbon genes."

Second, although the controls show differences in the same direction as the experimentals when one considers hydrocarbon ratios, the same is not true for the hydrocarbon *differences* (7,11-HD − 7-T) that were used to compare the intraspecific and interspecific effects of deficiencies. For two of the six *TM6*-containing stocks showing significant effects in Table 6 (*HR119* and *th102*), the differences between the deficiency and balancer heterozygotes were in opposite directions in the intra- and interspecific crosses. Thus, in this comparison of results from the two crosses, the significant effects of certain deficiencies cannot be artifacts of the balancer chromosome.

Nevertheless, although hypothesis of background-specific effects of the balancer chromosome seems unlikely, it is difficult to produce proper controls for the interspecific crosses, particularly because regions containing genes affecting the hydrocarbon ratios should, if not completely recessive, also show some effects in intraspecific control crosses. Pending a more thorough testing of the deficiencies of interest, we prefer to regard the present results not as definitive mapping experiments, but as suggestions of where "pheromone genes" might be located on chromosome *3*.

In sum, the hydrocarbon difference between the species appears to involve a minimum of five chromosome regions, four on *3*L and at least one on *3*R. The *X*, second, and fourth chromosomes have no perceptible effect, although for the second chromosome, we are unable to detect any recessively acting loci producing the *D. simulans* hydrocarbon or any *X*-linked recessive alleles producing the *D. melanogaster* hydrocarbon.

DISCUSSION

The species difference in major female hydrocarbons appears to be caused by evolutionary changes at a minimum of five loci, at least one on the right arm and four on the left arm of the third chromosome. The other 60% of the genome, represented by the *X*, second, and fourth chromosomes, has no apparent effect. The lack of obvious effects of two of the three major chromosomes suggests that the pheromone difference is not caused by a large number of genes spread throughout the genome.

A striking result, identical to that seen in the *D. simulans/D. sechellia* hybridization (Coyne *et al.* 1994), is that the only chromosome affecting the 7,11-HD/7-T ratio is the third. (We did not study the small fourth chromosome in our earlier work). We do not yet know whether this parallel reflects the involvement of the same chromosome regions in the two hybridizations. In the *D. simulans/D. sechellia* hybridization, the absence of available markers prevented us from determining whether the third-chromosome effect was due to one or to several genes; we know only that the effect is linked to the *Hairless* locus, at position 61 near the middle of the chromosome (Sturtevant 1929).

The similarity of chromosomal effects between these two hybridizations implies two possibilities. First, the possession of 7,11-HD by *D. sechellia* females could be a remnant of an ancestral condition present in the outgroup *D. melanogaster,* so that the large quantities of 7-T in *D. simulans* and *D. mauritiana* females represent shared derived characters. Under this possibility, the genetic analyses of the *D. simulans/D. sechellia* and *D. simulans/D. melanogaster* hydrocarbon differences are simply two studies of the same evolutionary event, and so should give similar results. (One would also, then, expect similar results in a *D. mauritiana/D. sechellia* or *D. mauritiana/D. melanogaster* hybridizations).

Alternatively, the hydrocarbon differences in these

two hybridizations could represent two independent evolutionary events. In this case, the parallel third-chromosome effects would be a "genetic convergence," indicating either that genes affecting female hydrocarbons happen to be concentrated on the third chromosome or that, for some reason, third-chromosome genes are more susceptible to evolutionary change.

The limited data available do not suggest that hydrocarbon genes are concentrated on the third chromosome. Although the polymorphism in *D. melanogaster* for the two isomers of heptacosadiene (7,11-HD and 5,9-HD) is apparently due solely to genes on 3R (FERVEUR *et al.* 1994; C. WICKER, personal communication), the *D. simulans* polymorphism between 7-T and 7-pentacosene in females is caused largely by a single locus, *Ngbo,* at map position 66.3 on the second chromosome (FERVEUR 1991). Chemical mutagenesis in *D. simulans* also produced an *X*-linked mutant allele, *kété,* that lowers the amount of 7-T in both males and females (FERVEUR and JALLON 1993). These latter two genes may, of course, affect pathways different from those involving the 7-T/7,11-HD alternative. Possible sites for such changes can be identified in the metabolic scheme proposed by JALLON (1984).

Although we cannot distinguish between these two possibilities, we have failed to falsify the idea that the sexual dimorphism for hydrocarbons in *D. sechellia* is an ancestral trait that has persisted through two speciation events. Ultimately, the putative identity of genetic change in these two hybridizations will require fine-structure mapping, isolation of the relevant genes, and DNA sequencing to determine the exact locus of such change.

We regard our deficiency-mapping of "hydrocarbon genes" as a preliminary result that should be confirmed by further mapping. Of six such regions that we identified, two pair were overlapping, so we are justified in estimating no more than four possible genes on 3L. It is somewhat reassuring that of all the deficiencies producing the requisite two classes of offspring, only two pair overlapped broadly (*RG5* with *RG7,* and *HR232* with *HR119*); and both deficiencies of each pair appeared to have a large effect on hydrocarbon profile. Moreover, the magnitude of effects of both deficiencies in an overlapping pair are similar (Table 6), so we can have some confidence that there are indeed species-specific genes in the regions of overlap (62B8–9; 62C4-D1 and 63C6; 63D3, respectively).

For several reasons, our figure of five genes probably underestimates the true evolutionary divergence affecting the 7-T/7,11-HD difference. First, we could deficiency-map only a small portion— ~25%—of the left arm of chromosome *3.* The remainder of this arm was either not covered by available deficiencies (~14% of the arm), produced only one of the two classes of hybrid offspring necessary for a proper comparison, or produced no offspring at all. In addition, we did not deficiency-map the right arm of chromosome *3.* Although the whole-arm substitution clearly indicates that 3R affects the hydrocarbon profile, the effect could be due to more than one gene. Finally, although the other chromosomes and arms had no apparent effect, our analysis was necessarily crude and incapable of detecting either small effects or partially recessive alleles such as those seen on chromosome 3L.

These regions identified by deficiency mapping appear to have large effects on the quantities of hydrocarbons. Each of the two overlapping *RG* deficiencies, for example, raises the quantity of 7-T by 400 ng and lowers 7,11-HD by a similar amount. [All of the deficiencies have opposite effects on the amount of these two compounds. As we have noted before (COYNE *et al.* 1994)], this implies that these hydrocarbons are made by either the same or interacting metabolic pathways). These large effects of small regions imply, though they do not prove, that genes of large effect contribute to the species difference in hydrocarbons. In the absence of fine-structure mapping, this conclusion must remain tentative. Moreover, without additional work we are even less justified in assuming that the sexual isolation between these species is due to genes of large effects. Although we know that cuticular hydrocarbons contribute to sexual isolation, we do not know how much of the sexual isolation in existence at the time of speciation was due to hydrocarbon differences, and how much to other factors such as differences in courtship behavior and wingbeat "song." Finally, we do not yet know whether large differences in cuticular hydrocarbons translate into large differences in sexual isolation. In the transfer experiments of COYNE *et al.* (1994), however, reducing the quantity of 7-T on *D. simulans* females by only 200 ng and increasing the quantity of 7,11-HD by 85 ng strongly reduced their attractiveness to *D. simulans* males. These hydrocarbon differences are smaller than many of those produced by our *D. melanogaster* deficiencies.

It is clearly of interest to determine whether any genes known to reside within the six regions of 3L might affect the female pheromones. (We do not expect, however, that most genes affecting cuticular hydrocarbons have already been identified. Unless they have some pleiotropic effect on morphology or viability, they would be detectable only by gas chromatography). The compilation of LINDSLEY and ZIM (1992) shows only one gene in these regions with a possible connection to cuticular hydrocarbons: *ecdysoneless* (*ecd*). Lying in cytological region 62D1–62D5, the temperature-sensitive allele of this locus ($ecd\text{-}1^{ts}$) produces a 50% reduction in 7,11-HD levels in *D. melanogaster* females, and a smaller increase in the amount of 7-heptacosene when females are raised at the restrictive temperature 29° (JALLON *et al.* 1982; FERVEUR *et al.* 1994). Unfortunately, this locus lies within *Df(3L)R-G5ve* but not within *Df(3L)R-G7ve* (LINDSLEY and ZIMM 1992), an overlapping deficiency that also has large effects on the inter-

specific hydrocarbon profile. Further use of such deficiencies will narrow down the regions thought to contain genes affecting pheromones.

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