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Genetics of sexual isolation in females of the *Drosophila simulans* species complex

JERRY A. COYNE
Department of Ecology and Evolution, The University of Chicago, 1101 E. 57th St, Chicago, IL 60637 USA

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Summary

Genetic analysis of hybrids between *Drosophila simulans* and *D. sechellia* shows that sexual isolation in females is caused by at least two genes, one on each major autosome, while the X chromosome has no effect. These results are similar to those of a previous study of hybrids between *D. simulans* and another sibling species, *D. mauritiana*. In this latter hybridization, each arm of the second chromosome carries genes causing sexual isolation in females, implying a total divergence of at least three loci. The genetic similarity between the *D. simulans*/*D. mauritiana* and *D. simulans*/*D. sechellia* hybridizations probably results from independent evolution and not phylogenetic artifacts, because the dominance relationships and behavioural interactions differ between the two hybridizations. The lack of an X-chromosome effect on sexual isolation contrasts with genetic studies of post-zygotic reproductive isolation, which invariably show strong effects of this chromosome.

1. Introduction

Sexual isolation is probably one of the most important causes of speciation in animals, but, like all forms of reproductive isolation, its genetic basis is largely unknown. Does reproductive isolation have a simple genetic basis, or, as neo-Darwinians postulate, are many genes involved? Are 'speciation genes' spread evenly throughout the genome, or concentrated in a few places? [The X chromosome, for example, nearly always has the largest effect on hybrid sterility and inviability (Coyne & Orr, 1989)]. Because sexual isolation must result from evolutionary changes in both sexes, are the same genes involved in males and females?

The only way to answer these questions is through direct genetic analysis: crossing closely-related but sexually-isolated species and observing the Mendelian segregation of mate discrimination or its linkage to mapped morphological or biochemical markers. Only five such studies have been done: four in *Drosophila* (Tan, 1946; Ehrman, 1961; Zouros, 1981; Coyne, 1989) and one in corn borers (Roelofs *et al.* 1987). Here I report a genetic analysis of mate discrimination in female hybrids between two closely-related species, *Drosophila simulans* and *D. sechellia*, and an extension of a previous study of hybrids between *D. simulans* and *D. mauritiana* (Coyne 1989).

D. simulans, like its relative *D. melanogaster*, is a cosmopolitan human commensal. Their two sibling species, *D. mauritiana* and *D. sechellia*, are endemic to the Indian Ocean islands of Mauritius and the Seychelles. *D. simulans*, *D. mauritiana* and *D. sechellia* form a monophyletic lineage whose outgroup is *D. melanogaster* (Lemeunier *et al.* 1986). The phylogeny of the three ingroup species is unclear, but their geographic distribution implies independent colonizations of the two widely-separated islands by the ancestor of the three species. *D. simulans*, *D. mauritiana* and *D. sechellia* have identical polytene chromosome banding patterns and nearly identical morphologies, differing mainly in the shape of the male genital arch. Crosses among all three species yield fertile females and sterile males. These crosses also show strongly asymmetrical sexual isolation, so that in each species pair one reciprocal mating occurs much more readily than the other. Males from either *D. sechellia* or *D. mauritiana*, for example, cross readily to *D. simulans* females, but the reciprocal crosses are very difficult (Robertson, 1983; Lachaise *et al.* 1986; Coyne, 1989).

A previous study (Coyne, 1989) analysed the genetic basis of sexual isolation in *D. simulans*/*D. mauritiana* hybrid females, quantified as insemination of these hybrids by *D. simulans* males. The genes for 'discrimination' were dominant, so that F_1 females readily

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accepted *D. simulans* males. In backcrosses to *D. mauritiana*, each of the two major autosomes had a significant effect on female mating propensity, while the X chromosome had little or no effect. Behavioural observations indicated that sexual isolation was caused not by the failure of *D. simulans* males to court foreign females, but by the females' unwillingness to mate when courted.

Genetic and behavioural analyses of sexual isolation between *D. simulans* and *D. sechellia*, which we describe here, afford an interesting comparison to the *D. simulans*/*D. mauritiana* study, as the two hybridizations may represent independent evolutionary divergences. Moreover, because additional genetic markers have become available in *D. mauritiana*, we report a genetic dissection of the effect of the second chromosome on sexual isolation with *D. simulans*.

2. Methods and materials

(1) *Fly stocks*

D. simulans FC. An isofemale line collected in Florida City, Florida on June 1, 1985 and maintained in large numbers. This line was used in the earlier genetic analysis of sexual isolation with *D. mauritiana* (Coyne, 1989).

D. simulans Bx. Stock homozygous for the dominant X-linked wing mutation *Beadex* (1-56), obtained from T. Watanabe.

D. simulans H/+. Stock segregating for the third-chromosome dominant bristle mutation *Hairless* (3–56·1), obtained from T. Watanabe. *Hairless* is lethal when homozygous, and the stock is maintained by selection.

D. sechellia IF. The first collected isofemale line of this species, obtained in 1981 and described by Tsacas & Bächli (1981).

D. sechellia cn. Stock homozygous for the second-chromosome eye-colour mutation *cinnabar*. This marker is located at 2–57·5 in *D. melanogaster*, and should, according to the map of the *D. simulans* second chromosome (Sturtevant, 1929), occur at a similar position in *D. sechellia*.

D. mauritiana syn. Wild-type stock made by combining six isofemale lines collected on Mauritius by O. Kitagawa in 1981. This stock was used in the earlier genetic analysis of sexual isolation from *D. simulans* (Coyne, 1989).

D. mauritiana j, cn, bw; *irr*. Stock containing the three recessive second chromosome mutations *jaunty*, *cinnabar* and *brown* (map positions in *D. melanogaster* 2–48·7, 2–57·5 and 2–104·5 respectively) and the recessive third-chromosome mutation *irregular*, which has not been precisely mapped. This stock was used to dissect the second chromosome into two non-overlapping segments (*j-cn* and *cn-bw*).

(ii) *Genetic crosses*

The effects of marked chromosome segments on female mating propensity were assessed using backcrosses between *D. simulans* and the two island species. These backcrosses provided pairs of hybrid genotypes differing by only one marked chromosomal segment. (The tiny fourth chromosome, comprising about 2% of the genome, was not studied.) An equal number of offspring of the two genotypic classes were collected daily and tested simultaneously with *D. simulans* males (see below).

Control experiments were performed to examine any effects of the marker alleles themselves on insemination frequency. In these controls, intraspecific backcrosses produced two genotypes containing gentic background from a single species and differing only by whether they were homozygous or heterozygous for the marker alleles used in interspecific crosses. Females of these two genotypes were confined with males of the other species.

(iii) *Mating experiments*

As in Coyne's (1985) study of *D. simulans*/*D. mauritiana*, sexual isolation was quantified using 'no choice' mating experiments, in which hybrid females were presented with males of a single species. Three 3-day-old virgin males were introduced without anesthesia into 8-dram food vials containing three 3-day-old virgin females. After 24 h of confinement, these females were dissected and their spermathecae and seminal receptacles examined for sperm. Any vials containing individuals that died during the confinement period were discarded. As described below, crosses showing little or no insemination of females also showed few or no matings in direct observations, so the lack of insemination almost certainly reflects a lack of copulation and not simply a lack of sperm transfer during mating.

(iii) *Behavioural observations of* D. simulans *and* D. sechellia

These observations were made to determine the behavioural reasons for the lack of insemination of *D. sechellia* females by *D. simulans* males. Three-day-old virgin females were stored individually in 8-dram food-containing vials. Two 3-day-old virgin males were aspirated into each vial and the trio of flies observed for 45 min. We recorded the time to first male courtship ('courtship latency', defined as wing vibration, circling, or genital licking) and the time to copulation ('mating latency'). Ten females were observed daily for ten observation periods, and simultaneous control observations were made on vials containing conspecific *D. simulans* males and females. These studies were done at room temperature, which ranged between 20·5 and 22 °C.

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Flies for all the above experiments were reared on cornmeal-corn syrup-agar food at 24 °C at 12: 12 light–dark cycle. Mating experiments always began 30 min after the beginning of the light cycle.

3. Results

(i) *Genetic analysis of* D. simulans/D. sechellia

Nearly all females of both species were inseminated after 24 h of confinement with conspecific males (Table 1). Interspecific confinements, however, give asymmetrical results: most *D. simulans* females were inseminated by *D. sechellia* males, but the reciprocal cross yielded almost no inseminations. This asymmetry, also seen in hybridizations between *D. simulans* and *D. mauritiana* (Cobb *et al.* 1988, Coyne, 1989), is a common feature of sexual isolation in *Drosophila* (Giddings & Templeton, 1983; Robertson, 1983; Coyne, 1989). The F_1 hybrid females with *D. simulans* mothers give intermediate results, with 49% of them mating with *D. simulans* males. [This additivity was not seen in the earlier study of *D. mauritiana*/*D. simulans*: F_1 hybrid females were completely inseminated after 24 h of confinement with *D. simulans* males (Coyne, 1989)]. We were not able to investigate cytoplasmic effects on sexual isolation because of the difficulty of obtaining reciprocal F_1 hybrid females from *D. sechellia* mothers.

The effects of individual chromosomes on mating discrimination were determined in backcrosses. Preliminary tests showed that F_1 hybrid females backcrossed to *D. simulans* males produced female offspring that were fully inseminated by *D. simulans* males (94·4%, $N = 144$); this high level of insemination was unexpected because the intermediate insemination frequency of F_1 females implied that only about 75% of these backcross females would be inseminated.

Table 1. *Proportion of females mated after 24 h of confinement with males. In crosses, the female parent is described first*

Female	Male	Mated	Total	Proportion mated
Pure species and F_1s				
sim	sim	197	201	0·98
sec	sec	199	201	0·99
sim	sec	147	201	0·73
sec	sim	1	201	0·01
F_1 (sim × sec)	sim	107	219	0·49
Backcross female genotypes (F_1 [sim × sec] × sec)				
+/+ (Chrom. 1)	sim	65	270	0·24
Bx/+	sim	64	270	0·24
cn/*cn* (Chrom. 2)	sim	39	348	0·11*
cn/+	sim	70	348	0·20
+/+ (Chrom. 3)	sim	15	252	0·06*
H/+	sim	68	252	0·27
Intraspecific controls for marker effects				
+/+ (sim)	sim	135	135	1·00
Bx/+ (sim)	sim	134	135	0·99
cn/*cn* (sec)	sim	1	249	0·004
cn/+ (sec)	sim	1	249	0·004
+/+ (sim)	sim	179	180	0·99
H/+ (sim)	sim	180	180	1·00

sim, *D. simulans* FC; sec, *D. sechellia IF.*
* $P < 0·001$ using the one-tailed Fisher's exact test.

The complete insemination means that there is no possibility of obtaining information about the genetics of sexual isolation from a backcross to *D. simulans*. We were thus constrained to backcross the F_1 females to *D. sechellia* males. This, in turn, mandated the use of either dominant mutations in *D. simulans* or recessive mutations in *D. sechellia*. Only a few genetic markers were available (see Methods and materials): *Beadex* on the *D. simulans* X chromosome, *Hairless* on the *D. simulans* third chromosome, and *cinnabar* on the *D. sechellia* second chromosome. Each autosomal marker is located near the middle of its metacentric chromosome (each roughly 100 cM long), so in backcrosses each marker will be nonrandomly associated with most of its species' autosome. *Beadex* is on the base of the acrocentric X (about 67 map units long in *D. simulans*), so this marker will be nonrandomly associated in backcrosses with most of the *D. simulans* X chromosome. As we were forced to use mutations from both species (and because the simultaneous presence of two dominant alleles drastically reduces viability), we studied a single chromosome in each of three different backcrosses. Our results are thus limited to the main effects of chromosomes against a segregating genetic background, precluding information about chromosome interactions.

To study the X and third chromosomes, *Beadex* or *Hairless* females of *D. simulans* were crossed to *D. sechellia* males, and F_1 females carrying the dominant marker were backcrossed to *D. sechellia* males. The third chromozome was analysed by crossing *D. sechellia* males homozygous for *cinnabar* to *D. simulans* females, and backcrossing the F_1 females to *D. sechellia cinnabar* males.

As Table 1 indicates, the X chromosome has no significant effect on mate discrimination, with the two genotypes differing in insemination frequency by less than one percent (one-tailed $P = 0·50$ with Fisher's exact test). Both the second and third chromozomes do, however, have highly significant effects in the expected direction, with females homozygous for *D. sechellia* autosomes being inseminated less often than females heterozygous for one autosome from each species (effect of second chromosome: one-tailed $P = 0·0008$; effect of third chromosome, one-tailed $P = 1·2 \times 10^{-10}$). The third chromosome, which reduces insemination frequency by about 20%, has about twice the effect of the second. The 95% confidence limits for the effect of the X chromosome, about 7%, do not overlap the effects of either autosome.

The control experiments (Table 1) show that the mutant markers do not affect insemination frequency

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Table 2. *Proportion of females mated after 24 h of confinement with males. In crosses, the female parent is described first*

Female	Male	Mated	Total	Proportion mated
Pure species and F_1s				
mau *j cn bw*; *irr*	mau	101	102	0·99
Mau *j cn bw*; *irr*	sim	0	102	0·00
F_1 (*j cn bw*; *irr* × sim)	sim	100	102	0·98
Backcross genotypes (F_1 [*j cn bw*; *irr* × sim] × *j cn bw*; *irr*)				
j cn bw; *irr*	sim	14	201	0·07
j; *irr*	sim	31	120	0·26
bw; *irr*	sim	63	201	0·31
irr	sim	105	201	0·52
Intraspecific controls for markers (F_1 [*j cn bw*; *irr* × mau] × *j cn bw*; *irr*)				
j cn bw; *irr*	sim	6	120	0·05
irr	sim	4	120	0·03
j cn bw; *irr*	mau	120	120	1·00
irr	mau	119	120	0·99

sim, *D. simulans* FC; mau, *D. mauritiana* syn.

when segregating within a single species, so the effect of the marked chromosomes is caused by genes producing sexual isolation. This isolation in females therefore involves evolutionary divergence at a minimum of two loci.

It is of interest to know whether the lack of mating between *D. simulans* males and *D. sechellia* females results from the females' refusal to copulate with these males [as is the case with *D. mauritiana* females (Coyne, 1989)], or simply from the males' unwillingness to court females of another species. These possibilities can be studied by direct observation of courtship. Of 100 *D. simulans* females presented with conspecific males, there were 77 courtships and 62 matings in the 45-min observation period. The mean courtship latency (time to first courtship) was 12·6 min (S.E. 1·1 min), and the mean mating latency of successful males was 20·8 min (S.E. 1·4 min). When 100 *D. sechellia* females were presented with *D. simulans* males, however, there were only 10 courtships and no copulations, both of which differ significantly from the data from *D. simulans* females (*G* tests under expectation of equal numbers of courtships and copulations both have probabilities of less than 0·0001). The proportion of courtships leading to copulations also differed significantly (2-tailed Fisher's exact test, $P = 8 \times 10^{-7}$). However, the courtship latency of males with *D. sechellia* females, 16·6 min (S.E. 2·9 min), did not differ significantly from that with *D. simulans* females (using the log-transformed data, $t_{85} = 1{\cdot}68$, $P = 0{\cdot}1$). Observations revealed that *D. simulans* males do approach *D. sechellia* females, but usually wander away without courtship and do not subsequently approach them. On the rare occasions when the males do court, they are rebuffed. The lack of courtship suggests that some aspect of the female, possibly olfactory, plays a role in the mating asymmetry (see below). This result differs from observations of *D. mauritiana*: *D. simulans* males court *D. mauritiana* females as vigorously as they do their own females, but the females always reject their approaches (Coyne, 1989). This difference in behaviour between the two hybridizations is a real among-species phenomenon and not limited to only our strains, as this same difference was observed by Cobb & Jallon (1990) in other strains.

(ii) D. mauritiana/D. simulans *analysis*

As observed previously (Robertson, 1983; Cobb *et al.* 1988; Coyne, 1989), *D. mauritiana* males mate readily with *D. simulans* females, but the reciprocal cross rarely occurs. We confirm these observations using our new marker stock (Table 2). F_1 hybrid females also mate readily with *D. simulans* males, so genetic analysis of discrimination must involve backcrossing these females to *D. mauritiana* males. Using multiply-marked stocks of *D. mauritiana*, Coyne (1989) found that each of the two autosomes had a large effect on mating discrimination, but that the X had little or no effect. To dissect the effect of the second chromosome, we used the *j cn bw*; *irr* stock, and tested four of the sixteen backcross genotypes produced when F_1 females heterozygous for these markers were backcrossed to *D. mauritiana j cn bw*; *irr* males. The third-chromosome *irregular* mutation was used to reduce background variation in insemination frequency; we did not control the X chromosome because Coyne (1989) found that it did not affect insemination frequency.

The four backcross genotypes (Table 2) allow an independent comparison of the effect of each arm of the second chromosome on insemination frequency. The effect of the left arm (the region from 0–49) can be seen by comparing *j cn bw*; *irr* females with *j*; *irr*

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females; the latter must have undergone recombination between *j* and *cn*. This segment has a highly significant effect on insemination frequenty ($P = 3{\cdot}8 \times 10^{-6}$ according to a one-tailed Fisher's exact test). The effect of the right arm (the region from 57 to 104) can be seen by comparing insemination of *j cn bw*; *irr* females with that of *bw*; *irr* females; the latter must have undergone recombination between *cn* and *bw*. This effect is again highly significant ($P = 1{\cdot}8 \times 10^{-10}$.

The effects of these chromosome segments are not due to the mutant markers themselves. Control crosses, in which the markers were segregating within a pure *D. mauritiana* genome, showed that the chromosome carrying *j*, *cn* and *bw* had no effect on the frequency of insemination of females by either conspecific males or *D. simulans* males (Table 2). The effects of the chromosome arms on insemination of hybrid females must thus be due to linked loci causing sexual isolation.

We conclude that each arm of the second chromosome contains a gene reducing the propensity of *D. mauritiana* females to mate with *D. simulans* males. These two segments appear to act additively; the sum of the effects of the two arms on insemination frequency (0·19 and 0·24 respectively) almost equals the effect of the entire chromosome (0·45) seen by comparing the *j cn bw*; *irr* with the *irr* genotype.

4. Discussion

The power of our analysis is limited by the paucity of genetic markers in these species. Nevertheless, at least two genes have diverged in females to cause mating discrimination between *D. simulans* and *D. sechellia* and three genes between *D. simulans* and *D. mauritiana*. In both cases the largest effects are on the autosomes, with the X chromosome having little or no effect. This is a minimum estimate of the number of genes in females, and there could be many more, but we lack morphological markers for a finer genetic analysis (molecular markers, such as RFLPs are one possible solution). Sexual isolation must of course involve genetic change in males as well, because few matings occur between *D. sechellia* females and *D. simulans* males. Estimates of the total number of genes causing sexual isolation must therefore include non-homologous genes that have changed in the two sexes.

One should also note that we have examined only a few strains of each species, and that the genetic architecture of reproductive isolation may be different in other strains, or in studies conducted under different conditions. Pheromone polymorphisms have, for example, been described in *D. simulans* (see below). We cannot say with certainty that we have revealed the genetics of interspecific reproductive isolation until similar studies are conducted with other strains.

The lack of X-chromosome effects in both the *simulans/mauritiana* and *simulans/sechellia* hybridizations implies either that the number of genes causing isolation in females is not large, or that there is a higher concentration of polygenes on the autosomes than X chromosomes (this would itself require an evolutionary or genetic explanation). The lack of an X-chromosome effect contrasts with other studies of postzygotic isolation in *Drosophila* and other species, which invariably show that this chromosome has largest effect on hybrid sterility and inviability in both sexes (Coyne & Orr, 1989). This dichotomy suggests in turn that the evolutionary forces or types of genes causing postzygotic isolation differ from those causing other forms of reproductive isolation (Coyne, 1991). The large effect of the X chromosome on sterility and inviability has been attributed to its faster accumulation of advantageous recessive or underdominant alleles that produce deleterious effects in hybrids (Charlesworth *et al.* 1987; Coyne & Orr 1989). This explanation would not apply to genes with female-limited effects, because in females the X chromosome is not hemizygous.

The rough similarity of genetic patterns of mating discrimination in two hybridizations may imply that identical gene substitutions are responsible for both. If, for example, *D. sechellia* and *D. mauritiana* were more closely related to each other than to *D. simulans*, evolutionary change in their common ancestor could cause sexual isolation between females of both island species and *D. simulans* males. The similar genetic architectures would then reflect not two independent events, but two non-independent samples of the same evolutionary event. This scenario, however, is unlikely. First, *D. sechellia* and *D. mauritiana* show strong sexual isolation against each other (unpublished observations), so even if they are sister species, additional sexual isolation evolved after they diverged from each other. Second, the effects of the autosomes are not identical: in the present study the third chromosome has a larger effect than the second, while in the *D. simulans/D. mauritiana* hybridization the second has a larger effect than the third. Third, it seems unlikely that *D. simulans* and *D. mauritiana* are indeed sister species, as this would require that species from one small island colonized another nearly 1000 km away. A more likely occurrence is independent colonizations of these islands from the mainland by *D. simulans* or its ancestor. Unfortunately, molecular phylogenies are inconclusive, showing an unresolved trichotomy that implies nearly simultaneous divergence of all three species (Coyne & Kreitman, 1986, unpublished observations; Cariou, 1987). Fourth, the genes causing sexual isolation in *D. sechellia/D. simulans* females, which are partially dominant, behave differently from those in *D. mauritiana/D. simulans* females, which are completely recessive. Finally, the behavioural manifestation of sexual isolation differs in the two hybridizations. *D. simulans* males court *D. mauritiana* females as vigorously as they court their own females, but nearly always fail to

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court *D. sechellia* females. Thus, genes causing sexual isolation of *D. mauritiana* females increase their rejection of *D. simulans* males, while in *D. sechellia* females the genes also discourage male courtship.

If the evolutionary divergences are independent, they could result from either changes at different loci in the two pairs, or from fixation of different alleles at the same loci. This question will not be resolved without fine-structure mapping of the genes affecting courtship.

The discouraging of male courtship in the latter case may involve either female behaviour, female pheromones, or both. Pheromones are a likely candidate because of the observed difference among these species in female cuticular hydrocarbons, which apparently play a pheronomonal role in courtship (Jallon 1984, Cobb & Jallon 1990 and references therein). The main cuticular hydrocarbon in *D. mauritiana* males and females is 7-Tricosene (7-T). *D. simulans* is polymorphic, with 7-T being the predominant hydrocarbon in males and females of most strains but those from Central Africa having a predominance of 7-pentacosene in both sexes. [The intraspecific polymorphism is apparently due to single gene on the second chromosome (Ferveur, 1991)]. Given the biogeography of this polymorphism, our *D. simulans* strain probably has a predominance of 7-T, making it similar to *D. mauritiana*. *D. sechellia* is sexually dimorphic for cuticular hydrocarbons, with females predominantly possessing 7,11-heptacosadiene and males 6-tricosene. If males court females more readily when they share pheromones, these profiles may explain why our *D. simulans* males court *D. mauritiana* but not *D. sechellia* females. Genetic analysis will show whether the difference in females pheromones between *D. simulans* and *D. sechellia* map, like the sexual isolation, to the two autosomes.

It is difficult to pinpoint the behavioural differences that might cause sexual isolation. Male and female mating behaviour in this group has been extensively described by Cobb *et al.* (1985, 1989). *D. simulans*, *D. mauritiana* and *D. sechellia* differ in both the repertoire of courtship elements and in the frequency with which shared elements are displayed. In these three species, the only behaviour unique to *D. sechellia* females is abdominal extrusion (Cobb *et al.* 1989), but this cannot explain their lack of courtship by *D. simulans* males, as we did not observe extrusion.

Because of the lack of an X-effect in both of these hybridizations, it is possible that sexual isolation has involved only a few genetic changes in females. (If sexual isolation were caused by many genes spread through the genome, each chromosome should be involved). A more extensive genetic analysis is required to answer this question. It is not inconceivable, however, that sexual isolation may have a simple genetic basis. In two races of *Ostrinia nubilalis*, the European corn borer, a single gene causes the difference in female pheromones that differentially attracts males (Roelofs *et al.* 1987; the difference in male response to these pheromones also involves a single locus). In Zouros's (1981) study of *Drosophila mojavensis/D. arizonensis* hybrids, only two of the four chromosomes examined affected sexual isolation in females, again implying the involvement of relatively few genes. Other adaptations not involving sexual selection may also be based on one or a few genes of large effect (Orr & Coyne, 1991). Of course, a complete analysis of sexual isolation in these species must include a genetic study of males and of the interaction between genetic changes in the two sexes.

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