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Evolution

Genetic basis of male sterility in hybrids between two closely related species of *Drosophila*

(evolution/hybridization/reproductive isolation/speciation)

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ABSTRACT **_Drosophila simulans_ and _Drosophila mauritiana_ are closely related sibling species, the former cosmopolitan and the latter restricted to the small oceanic island of Mauritius. Genetic analysis of male sterility in hybrids between these species shows that at least five loci (one on each chromosome arm) are responsible for this reproductive isolation. This is the most loci that could have been detected with the techniques used and implies that the true genetic divergence for sterility is even greater. The effects of chromosome segments on the character are roughly additive, with the X-linked segment making the largest contribution to sterility. The large effect of X chromosomes on male-limited reproductive isolation and the frequent limitation of hybrid sterility to males may be attributable to fertility interactions between X and Y chromosomes. These results parallel what has been found in other _Drosophila_ species and relate to recent theories of how reproductive isolation evolves in small founder populations.**

The origin of species has been discussed since the time of Darwin, but only recently have evolutionary biologists tried to understand the process by using principles of population genetics (1, 2). These early models demonstrate that the genetic basis of differences among species—especially those resulting in reproductive isolation—may give clues to the process of speciation itself. Particular interest has been devoted to species whose origin involved founder events (e.g., island colonization). The possibility of random genetic drift in such small populations may make the rate and genetic basis of speciation different from that occurring in larger populations (3–6).

The fundamental data for comparing these theories to what actually happens in nature must include descriptions of the genetic basis of reproductive isolation between related species. Because congeneric species often cannot form hybrids, only a few of these studies exist (see ref. 7 for references). Most, however, report genetic differences among species that have been separated for a long time. Such differences may have occurred after speciation and thus may not represent what happens during the actual evolution of reproductive isolation.

This study reports the genetic basis of sterility in male hybrids between two very closely related species of *Drosophila*: *Drosophila simulans* and *Drosophila mauritiana*. These species are morphologically identical except for the shape of the male genitalia (7, 8). Analysis of the chromosome banding pattern (identical in the two species) and of protein polymorphism by standard and two-dimensional electrophoresis shows that the two species are very recently separated and are among the most closely related species of *Drosophila* (9–11). In addition, the divergence between them probably involved a founder event. *D. simulans*, like its relative *Drosophila melanogaster*, is a worldwide human commensal, while *D. mauritiana* is found only on the 2040-km^2 island of Mauritius, 960 km east of Madagascar. Neither *D. melanogaster* nor *D. simulans* occurs on Mauritius, and it is likely that *D. mauritiana* evolved from a small population of *D. simulans* colonizing the island (12).

D. simulans and *D. mauritiana* can be crossed, and although male hybrids are sterile, females are fertile and can be backcrossed to either species (13). A small proportion of the backcross males are fertile (14). The fertility of the F_1 females permits analysis of the numbers, effects, and interactions of genes contributing to the sterility of backcross males and thus allows a study of the genetic basis of reproductive isolation between two closely related species.

METHODS AND MATERIALS

The method of analysis is similar to that used by Dobzhansky in his classic analysis of testis size in *Drosophila pseudoobscura/Drosophila persimilis* hybrids (15). Flies of one species having each of the five major chromosome arms marked with a recessive mutation are crossed to the other species. Hybrid females are backcrossed to the marked parental species. The presence or absence of the recessive markers in backcross males indicates the specific identity of each chromosome segment, and this can be correlated with the male's fertility. (The tiny fourth chromosome, constituting 1–3% of the genome, was not studied.)

The parental *D. simulans* stock was homozygous for the recessive mutants f^2; *nt pm*; *st e*, with forked-2 on the X chromosome (f^2: I-60), net and plum on the two arms of the second chromosome (*nt*: II-0, 2L; *pm*: II-103, 2R), and scarlet and ebony on the two arms of the third chromosome (*st*: III-44, 3L; *e*: III-71, 3R). Females from this stock were crossed to males of a *D. mauritiana* stock from the Bowling Green Stock Center, and the F_1 females were backcrossed to the multiply marked stock of *D. simulans*. Fourteen of the 32 possible backcross classes were chosen for analysis of male fertility. The choice was made in such a way that each chromosome or chromosome arm could be tested in at least two independent comparisons between pairs of genotypes (see ref. 7 for a further description of the stocks and crossing scheme).

Analysis of the backcross to *D. mauritiana* is more problematic since only two recessive genes have been described in this species: burgundy (*bg*) on the X chromosome and upturned (*upt*) on an unidentified autosome. Male *bg*; *upt D. mauritiana* were crossed to a *D. simulans* stock from Oxnard, California, and the F_1 females were backcrossed to the *bg*; *upt* stock. All four phenotypic classes of backcross males were analyzed for fertility.

Because it is an all-or-none property of individuals, the fertility of males in a genotypic class was defined as the *proportion* of males in that class having motile sperm. Testes and seminal vesicles were dissected from 4-day-old virgin males, crushed in physiological saline, and examined under a compound microscope. If a male had one or more motile sperm, he was scored as fertile. Sterile males were those

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with either no sperm or wholly nonmotile sperm.

Independent assessments of the fertility of males in the backcross to *D. simulans* were made by placing males from all 32 backcross classes together into bottles with virgin f^2; *nt pm*; *st e D. simulans* females; the genotypes of the few eclosing offspring in each bottle indicate which genotypes successfully mated. In the backcross to *D. mauritiana*, separate fertility tests were made of each of the four genotypes of backcross males by crossing them to virgin *bg*; *upt D. mauritiana* females.

All flies were raised at 23°C.

RESULTS

Table 1 shows the genotypes analyzed in the backcross to *D. simulans*, the number and proportion of fertile males in each class, and an indication (with asterisks) of those genotypes yielding progeny in the crossing tests. Fig. 1 gives these results graphically, indicating the average chromosomal constitution of each backcross class along with its fertility. All genotypes in Table 1 have a Y chromosome from *D. simulans*.

Both parental species show high fertility and, as reported previously (12), hybrid males are completely sterile. This sterility is due to failed spermatogenesis and not testicular atrophy, since the hybrid males have normal-sized testes but no visible sperm.

Table 1 immediately shows that the male sterility has at least a partial genetic basis, since there is significant heterogeneity among the backcross classes in the proportion of fertile males ($G_{13} = 249.5$, $P < 0.001$). Nongenetic factors such as cytoplasmic incompatibility, infectious agents, or poor meiotic pairing of chromosomes (the species are homosequential) are therefore not the sole determinants of male sterility. Some cytoplasmic interaction with nuclear genes may be indicated by the low fertility of the f^2; *nt pm*; *st e* backcross class, which has all of the markers present in the parental *D. simulans* stock but cytoplasm from a hybrid mother. The reduced fertility could, however, be due to undetected crossing-over.

The effects of individual chromosomes and chromosome arms on male fertility can be gauged by comparing pairs of genotypes differing only in the relevant recessive markers. The effect of the X chromosome can, for instance, be seen by comparing genotypes 1 versus 2 (from Table 1) or 13 versus 14, both pairs differing only by the X-linked marker f^2. Similarly, the effect of the left arm of chromosome 2 can be seen by comparing genotypes 1 versus 4, 3 versus 7, and 9 versus 13. The effect of each whole chromosome can thus be tested in two independent comparisons, and that of each arm in three comparisons. To avoid using excessive degrees of freedom, I combined all tests of each chromosome or arm into a single comparison of main effects. For example, the effect of segment 2L was tested by comparing the summed fertility of genotypes 1, 3, and 9 with that of genotypes 4, 7, and 13. These tests have one degree of freedom.

Table 1. Male fertility of stocks of both species, their hybrids, and male offspring of the cross between female hybrids and *D. simulans* f^2; *nt pm*; *st e* males

Genotype	Total no.	Proportion with motile sperm
D. simulans f^2; *nt pm*; *st e*	100	0.760
D. mauritiana Bowling Green	100	0.830
F_1 hybrid of above (*D. simulans* ♀ × *D. mauritiana* ♂)	200	0.000
Backcross males		
*1. f^2; *nt pm*; *st e*	239	0.297
2. *nt pm*; *st e*	187	0.000
*3. f^2; *nt*; *st e*	163	0.172
*4. f^2; *pm*; *st e*	212	0.146
5. f^2; *nt pm*; *st*	51	0.118
*6. f^2; *nt pm*; *e*	66	0.212
*7. f^2; *st e*	221	0.118
*8. f^2; *nt pm*	222	0.086
*9. f^2; *nt*	174	0.040
10. f^2; *pm*	156	0.006
11. f^2; *st*	67	0.030
12. f^2; *e*	76	0.013
*13. f^2;	222	0.014
14. ± (wild type)	200	0.000

Asterisks show which backcross males produced offspring in the crossing tests.

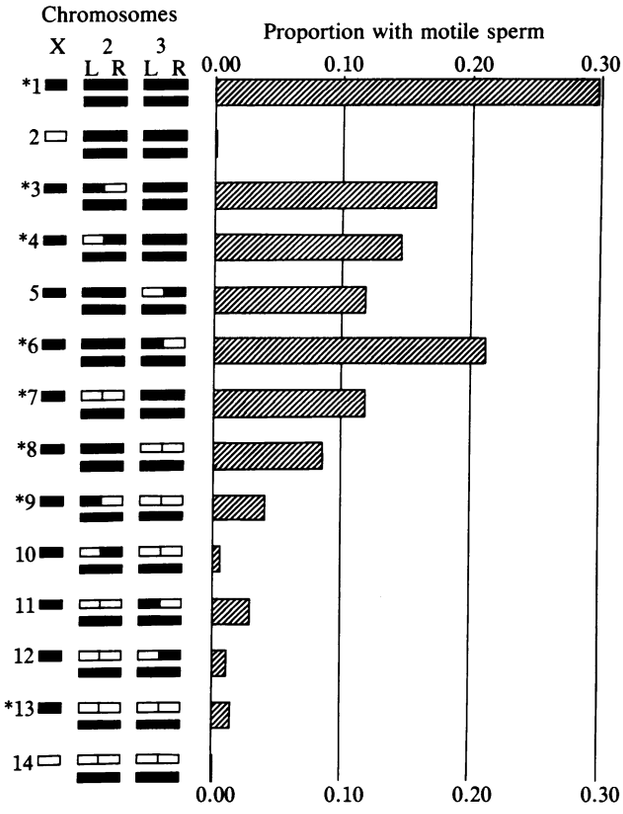

FIG. 1. Graphic depiction of the effect of chromosome arms on the sterility of male hybrids in the backcross to *D. simulans* (data taken from Table 1). Segments of chromosome from *D. simulans* are shown in black, those from *D. mauritiana* in white. Asterisks show which genotypes produced progeny in the crossing tests. All genotypes have a *D. simulans* Y chromosome.

This analysis shows that all three major chromosomes have significant effects on male fertility, so that substitution of each chromosome from *D. simulans* into the backcross males increases their average fertility (X chromosome: $G_1 = 96.1$; second chromosome: $G_1 = 34.8$; third chromosome: $G_1 = 55.2$; all Ps < 0.001). Similarly, each of the four autosomal arms has a significant effect on male fertility (2L: $G_1 = 28.9$; 2R: $G_1 = 19.6$; 3L: $G_1 = 40.4$; 3R: $G_1 = 51.1$; all Ps < 0.001). The male sterility in this interspecific cross is caused by evolutionary divergence at a minimum of five loci, with at least one occupying each of the five chromosome arms. This is the greatest genetic difference that could have been detected with the methods used, and implies that there are additional undetected loci contributing to hybrid sterility.

The most dramatic reduction of fertility is caused by the X chromosome (Table 1 and Fig. 1). In this backcross the presence of *D. mauritiana* X-linked segment (seen as a nonforked phenotype) renders hybrid males completely sterile.

This effect is especially striking in the comparison of genotypes 1 and 2 in Table 1. Since the f^2 marker is near the base of the X chromosome (which is more than 60 map units long), at least half of the genotypic class *nt pm*; *st e* carries a substantial portion of *D. simulans* X chromosome that has recombined away from the f^2 marker. The fact that this class is nevertheless completely sterile implies that the forked locus is closely linked to one or more loci having large effects on hybrid fertility. It is worth noting that all backcross males carry a *D. simulans* Y chromosome and that fertility is only possible when the forked segment of the X chromosome is from the same species.

In general, the effects of autosomal segments on male sterility are roughly additive: each additional segment of *D. mauritiana* genome leads to increased sterility. This is evident when reading Fig. 1 from the top down. The average fertility of backcross males with a *D. simulans* X chromosome and all four autosomal arms (f^2; *nt pm*; *st e* genotype) is 0.297. This is reduced to 0.161 with only three autosomal arms, 0.101 with two autosomal arms, 0.023 with one autosomal arm, and 0.014 with no autosomal arms (f^2 genotype).

The results of the mating tests confirm the fertility judgments based on presence or absence of motile sperm. Except for the four infrequent genotypes 5, 10, 11, and 12 (Table 1), flies of all genotypes with motile sperm were able to produce offspring. The lack of successful matings of these four classes almost certainly reflects their rarity in the competitive mating tests and not their innate sterility. What is more important is that *no other genotypes* of the 32 tested produced offspring in this test, including the 18 not examined for motile sperm. In particular, although roughly half of the backcross males tested lacked the segment of the *D. simulans* X chromosome marked by f^2, they produced no progeny because no non-forked females were seen among 1566 offspring. This confirms the absolute sterility of all genotypes missing this X-linked segment from *D. simulans*.

The backcross to *D. mauritiana* is less informative because only two mutant markers segregated among the progeny. Table 2 gives the male fertilities of both parental stocks, the F_1 hybrid, and the four backcross classes as well as their actual production of progeny (indicated by asterisks) in the mating tests. In this backcross, the *bg* marker is associated with the X chromosome and *upt* with an unidentified autosome.

As in the backcross to *D. simulans*, there is a significant effect of genotype on the proportion of fertile males (G_3 = 31.9, $P < 0.001$), indicating again an influence of genes on sterility (the overall fertility is somewhat lower). The cause of the heterogeneity, however, rests entirely on the higher fertility of the *bg*; *upt* genotype. If this class is excluded there is no significant heterogeneity among fertilities of the remaining three genotypes (G_2 = 1.2).

Table 2. Male fertility of stocks of both species, their hybrids, and male offspring of the cross between female hybrids and *D. mauritiana bg*; *upt* males

Genotype	Total no.	Proportion with motile sperm
D. simulans Oxnard, CA	100	0.910
D. mauritiana bg; *upt*	100	0.800
F_1 hybrid of above (*D. simulans* ♀ × *D. mauritiana* ♂)	100	0.000
Backcross males		
*1. *bg*; *upt*	334	0.054
*2. *upt*	351	0.006
*3. *bg*	419	0.002
4. ± (wild type)	393	0.008

Asterisks show which backcross genotypes produced offspring in the crossing tests.

The effect of each marker on fertility can be tested in the same way as in the backcross to *D. simulans*. The test for the X-chromosomal *bg* segment (genotypes 1 + 3 versus 2 + 4 from Table 2) shows a significant effect on fertility (G_1 = 14.8, $P < 0.001$), as does the autosomal *upt* segment (genotypes 1 + 2 versus 3 + 4; G_1 = 8.7, $P < 0.01$). The effects are again due entirely to the excessive fertility of the *bg*; *upt* genotype. There appears to be an epistatic effect of both chromosome segments, so that their simultaneous presence is necessary for higher fertility, while the absence of either or both markers confers equally low fertility.

Because both genotypes lacking the *bg* marker showed some fertility, this segment does not have the all-or-none sterility effect of the f^2 segment of the *D. simulans* X chromosome. Since the location of *bg* on the X chromosome is unknown, this does not necessarily indicate a fundamental difference between the reciprocal backcrosses in the effect of this chromosome.

The tests of mating ability showed that all genotypes except for wild type (lacking both markers) produced progeny. The lack of offspring of males from this latter class probably reflects the rarity of fertile males rather than absolute sterility of the genotype.

DISCUSSION

This analysis demonstrates that all chromosome segments tested in both backcrosses carry genes reducing the fertility of backcross males. The backcross to *D. simulans* shows in addition one or more loci near the base of the X chromosome having a large effect on hybrid male fertility. It is possible that other chromosome segments have equally large effects that were not seen because of undetected crossing-over. For example, the tip of the X chromosome may also carry genes with large effects on fertility. Their weak linkage to the f^2 marker could account for the much diminished fertility of the f^2; *nt pm*; *st e* backcross class compared to the pure *D. simulans* stock having the same markers. What is clear is that at least five loci are responsible for the lowered fertility of the backcross males, and there are undoubtedly more.

The cross to *D. mauritiana* also reveals the maximal possible number of detectable sterility factors, which in this case is only two. A possible epistatic interaction is seen in this cross; Wu and Beckenbach (16) discuss why one might often expect extreme epistatic interactions among genes causing reproductive isolation.

The results of this analysis are qualitatively similar to those of other *Drosophila* species, including those whose divergence may not have involved founder events. Dobzhansky (15) showed that all seven chromosome segments of *D. pseudoobscura* and *D. persimilis* carried genes reducing testis size in backcross hybrids but that the largest effect was attributable to the X chromosome. Similar effects of the X chromosome on hybrid male sterility were found in Dobzhansky's analysis of hybrids between North American populations of *D. pseudoobscura* and an isolated subspecific population from Bogota, Colombia (17). Other analyses of lowered viability and male mating success in *Drosophila* hybrids likewise reveal polygenic architectures, frequently with large effects of the X chromosome (18–22).

Why should this chromosome be of such importance in the development of male-limited reproductive isolation? This cannot be attributed to its harboring more genes of all types, since in the *D. melanogaster* group it is the shortest major chromosome, smaller than any of the four autosomal arms. Another explanation is the demonstration by Berg (23) that the X chromosome carries the largest *proportion* of loci affecting male fertility as determined by x-ray induction of fertility mutants. Why such loci should predominate on the smallest major chromosome is an unanswered evolutionary

question. It is possible that there is some evolutionary reason for the localization of genes for male-limited characters on the X chromosome, but genetic analysis of male genital morphology in these species does not support this hypothesis (7).

Another possibility is that the unique nature of the sex chromosomes makes them likely candidates for mutations reducing fertility of hybrid males. In *Drosophila* and many other animals, two nonhomologous chromosomes (the X and Y) contribute to male fertility. In the sibling species *D. melanogaster*, males lacking a Y chromosome (X0) are phenotypically normal but unable to produce motile sperm (24). Males with an extra Y chromosome (XYY) are often sterile or weakly fertile (25). The Y chromosome in this species carries at least seven loci affecting male fertility but appears to have no other effect on the phenotype (26). Because X0 males produce all components of sperm but cannot assemble them into a motile product, it has been suggested that the Y chromosome carries genetic information regulating sperm assembly (27).

It is thus possible that in *Drosophila* and some other animals male fertility is determined by an interaction of the X and Y chromosomes. In hybrids between species, such interactions may be unsuccessful because of divergent evolution of the nonhomologous sex chromosomes. Females, on the other hand, contain homologous sex chromosomes. Because these produce similar proteins, evolutionary divergence between them may not have such deleterious effects on fertility.

The interaction of X and Y chromosomes from different species may be one explanation for "Haldane's Rule": the generalization that in crosses between species the heterogametic sex is the one most frequently sterile or absent (28). Haldane and others (28, 29) explain this sterility by assuming that a genic *balance* between X chromosomes and autosomes is necessary for fertility of hybrids. Female hybrids have a conspecific X chromosome for every autosome set, while males lack an X corresponding to one species' autosomes. This explanation is not as likely as the X–Y interaction hypothesis for the data presented here, because some of the backcross males (in classes 3–13) are fertile despite an imbalance between X chromosomes and autosomes, while *no* hybrid males are fertile if the marked segment of the X does not correspond in species origin to the Y from *D. simulans*.

A minor reason for the concentration of genes leading to reproductive isolation on the X chromosome is that their fixation may be enhanced by genetic drift (2). Sex chromosomes are more subject to drift than autosomes because their population size is only 75% as large, and this figure is even smaller in populations founded by multiply inseminated females.

Because of the primitive nature of speciation theory as well as of a genetic analysis framed in terms of chromosome segments, it is premature to decide whether the genetic basis of reproductive isolation found here supports one or another model of speciation. Templeton (1) has divided the genetic architecture of species differences into three categories: (*i*) many genes of small effect, (*ii*) a few major loci with minor modifiers, and (*iii*) complementary or duplicate pairs of loci. He postulates that the classical theories of allopatric speciation by adaptive divergence would favor types *i* and *ii*, while special processes of speciation involving genetic drift would favor types *ii* and *iii*. Wright (6) also theorizes that type *iii* monogenic architectures would be associated with the occupation of new niches (as might occur during island colonizations), and advocates of the theory of punctuated equilibrium have suggested that single mutations of large effect may occasionally lead to evolutionary novelties (30, 31). This study clearly rules out a type *iii* genetic architecture of male sterility in hybrids between *D. simulans* and *D. mauritiana*. When combined with previous studies of the genetic basis of differences between this and other species pairs (see ref. 7), the data offer no support for the assertion that reproductive and morphological differences among species may often be caused by single loci of large effect.

Since the effect of the X-linked segment containing the f^2 locus may be due to either one or several loci, my analysis cannot distinguish between type *i* or type *ii* architectures of hybrid sterility. There is some similarity between my results and those taken from other species of *Drosophila*: male hybrid sterility usually involves several or many loci with a particularly strong effect of the X chromosome. Since the few species tested are of different evolutionary relatedness, this may indicate that reproductive isolation often evolves in similar ways among diverse species. In addition, nothing unusual is seen in genetic analysis of differences between species arising via colonizations (e.g., *D. mauritiana* or *D. pseudoobscura bogotana*) and their progenitors. Speculations about unorthodox genetic processes occurring in colonizing species may thus be premature. Further studies of *D. mauritiana* and *D. simulans* should show if any unusual genetic architecture underlies this founder-related speciation event.

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