Genetics of Reproductive Isolation in the *Drosophila simulans* Clade: Complex Epistasis Underlying Hybrid Male Sterility

Eric L. Cabot, Andrew W. Davis, Norman A. Johnson and Chung-I Wu[1]

Department of Ecology and Evolution, University of Chicago, Chicago Illinois 60637

Manuscript received August 17, 1993
Accepted for publication January 28, 1994

ABSTRACT

We have analyzed the sterility associated with introgressions of the distal one-fourth of the *X* chromosome from either *Drosophila mauritiana* or *Drosophila sechellia* into the genome of *Drosophila simulans* using a series of visible and DNA markers. Because in Drosophila hybrids, male sterility is usually complete and is often tightly linked with each of several markers used in crosses, a simple genetic basis has generally been assumed. In our low resolution mapping experiment, we were not able to reject the null hypothesis that a single gene, introgressed from either *D. mauritiana* or *D. sechellia*, is the cause of male sterility. High resolution mapping, however, reveals a much more complex picture. At least three distinct factors from *D. mauritiana*, or two from *D. sechellia*, were identified that need to be jointly present to confer full sterility. Each individual factor by itself is relatively ineffective in causing sterility, or even a partial spermatogenic defect. Moreover, there appear to be more sterility factors on comparable introgressions from *D. mauritiana* than from *D. sechellia*. On the basis of these observations, we propose a model which suggests that multilocus weak allele interactions are a very common cause of reproductive incompatibility between closely related species. We also present theoretical argument and empirical evidence against extrapolating the results of within-species analysis to interpret the genetic basis of species differences. The implications of this model on the theories of evolution of species differences and the attempt to understand the mechanisms of hybrid sterility/inviability at the molecular level are discussed.

The nature of genetic differences between species is an important topic in evolutionary biology (MAYR 1963; DOBZHANSKY 1970). It is of special interest to understand the genetic basis of biological traits that define species, be they morphological, developmental, biochemical or behavioral differences. There is already a wealth of information on the level of genetic differentiation between populations or species based on protein or DNA data (LEWONTIN 1974; NEI 1975; SELANDER *et al.* 1991). However, very few of these observations at the molecular level can be directly related to biological traits that define species. We have therefore concentrated on the genetics of hybrid male sterility in a series of studies on *Drosophila simulans* and its sibling species, *Drosophila mauritiana* and *Drosophila sechellia* (WU *et al.* 1993; JOHNSON *et al.* 1992, 1993; PEREZ *et al.* 1993).

As discussed in WU and DAVIS (1993), sterility in F_1 hybrids or backcross F_2 hybrids is not a tractable genetic problem. Instead, our approach is to introgress a small piece of chromosome from one species into the genetic background of another species (*e.g.*, PEREZ *et al.* 1993). Even between very closely related species of Drosophila, there are usually many chromosomal segments that can cause male sterility upon introgression (WU and BECKENBACH 1983; NAVEIRA and FONTDEVILA 1986; COYNE and CHARLESWORTH 1986; ZOUROS *et al.* 1988). The two main questions are thus (i) how many hybrid sterility genes are contained in an introgressed segment and (ii) how do these genes interact to cause sterility?

What answers have evolutionary genetic theories furnished to the two simple but fundamental questions posed above? For simplicity, we shall follow the diagram WRIGHT (1982; his Figure 1) presented to illustrate three possible relationships of genotype to phenotype: (1) there is a single gene within the introgression that is solely responsible for the sterility phenotype, (2) the sterility phenotype is largely determined by the additive effect of many genes within the introgression and (3) complex gene interactions are the primary cause of sterility. In this view, there are multiple components within the introgression but their joint effect on male fertility is much greater than the sum of each individual effect (*i.e.*, epistasis). "Single gene" in this report always refers to the relationship (1) above. While the two latter views both invoke multigenes, they differ in the assumed relative importance of the additive component and epistatic component of hybrid fertility. Quantitatively, fertility is defined as the percentage of males of a given genotype that produce motile sperm (see MATERIALS AND METHODS for details). It is also important to note that we analyze only genes within the introgressed segment but not those in the genetic background of the host with which the introgression is incompatible.

Previous genetic analyses of hybrid sterility generally did not have the resolution to provide unequivocal answers. While most authors assume that genes of major

[1] To whom correspondence should be addressed.

effect are responsible for the sterility they observed (*e.g.*, HENNIG 1977; WU and BECKENBACH 1983; COYNE and CHARLESWORTH 1986, 1989; ORR 1992; PEREZ *et al.* 1993; PANTAZIDIS *et al.* 1993; ZENG and SINGH 1993), others interpret their results, sometimes from the same chromosome of the same species, as due to polygenic influence (NAVEIRA and FONTDEVILA 1986, 1991; NAVEIRA 1992). The experiments in most cases are indeed compatible with all three possible relationships given above.

To resolve the issue, it is best to study incipient, or at least recently diverged, species. The three species we have been studying, *D. simulans, D. mauritiana* and *D. sechellia*, produce fertile females and sterile males *inter se*, have homosequential chromosomes, and share DNA polymorphisms extensively (HEY and KLIMAN 1993). All these suggest recent divergence. Their basic biology is described in LACHAISE *et al.* (1988).

In a previous analysis, we examined sterility associated with the introgression of the proximal end of the *X* chromosome from *D. mauritiana* into *D. simulans* (PEREZ *et al.* 1993). The evidence appeared to fit the "single gene" interpretation (see DISCUSSION). In this report, we analyze the distal one-fourth of the *X* chromosome introgressed from both *D. mauritiana* and *D. sechellia*. The results of this analysis and the accompanying studies (DAVIS *et al.* 1994) show a general pattern of complex epistasis underlying hybrid sterility between these three species. In no region were we able to localize a single gene capable of causing hybrid sterility by itself in a foreign background. We discuss the implications of our observations for understanding the genetics of species differences from both an evolutionary and molecular/mechanistic perspective.

The genetic analysis of hybrid sterility should also shed some light on how such seemingly maladaptive traits as sterility or inviability could evolve. Clearly, sterility or inviability could not have been manifested within species at any time. How evolution could have circumvented such difficulties has always been an intriguing question. Our results provide some interesting insights into how that could have been achieved.

MATERIALS AND METHODS

Strains and mutants: The single *D. mauritiana* and *D. sechellia* lines as well as many *D. simulans* strains used have been described in PEREZ *et al.* (1993). Only additions and modifications of *D. simulans* mutant strains are given below. All visible and molecular markers used are *X*-linked and their positions are shown in Figures 2, 5 and 6. (1) *In (1) f*^{66} (Stock IU 1099 of Indiana University Stock Center)–*In (1)* has breakpoints at 2B and 8B of the polytene chromosome. (2) *y v f* (Stock IU 1089)–this stock carries *yellow* (1B), *vermilion* (10A) and *forked* (15F). (3) *y w* (Stock IU 928)–*w* (*white*) is at 3C. (4) *In (1) v f*–derived from *In (1) f*^{66} and *y v f*. (5) *In (1) y v f*–derived from a recombinant between *In (1) v f* and *y w*. The recombination rate between *y* and *In (1)* is less than 0.001 (3 out of over 4000). (6) *rb–ruby* is at 9.7 cM or 4C. (7) *np–nipped* is at 21.0 cM and roughly 7AB. The *np* phenotype is fully penetrant only at ≥25°. Stocks (6) and (7) and the map positions of *rb* and *np* were kindly provided to us by J. COYNE. (8) *y np v*–essentially a double recombinant between the *np* and the *y v f* stock. The actual construction involved a series of steps that yielded other stocks and will not be described further. (9) *y rb*–derived from a single recombinant between the *rb* and the *y v f* stock.

Fertility measurement: Operationally, an introgression was classified as fertile if an attached-*X* line could be established. An introgression that enables males to produce a few progeny but reduces their fertility below a level necessary for sustainable culture with attached-*X* females was classified as quasi-sterile. Sterile males are those that never produce any progeny. Whenever possible, fertility measurement was done on multiple males of an identical genotype. For sterile and quasi-sterile genotypes, the introgressions were propagated through females and always from a single female initially. The quantitative definition of fertility is the percentage of males of a given genotype with motile sperm in their seminal vesicles. Except the quasi-sterile class, most genotypes are >90% or <1% fertile.

Since the presence of motile sperm in the seminal vesicles is an insufficient description of spermatogenic development and male fertility, a detailed analysis of the key genotypes was performed. In that analysis, male fertility was determined by both mating and phenotypic analysis. Two 1–2-day-old introgressed males were mated to virgin *D. simulans* attached-*X* females for 5–7 days. After the mating, the spermatogenic phenotypes of males were determined by phase contrast microscopy of testes squashed in Drosophila Ringer's solution. The number of motile sperm contained in a male's seminal vesicles is classified into four arbitrary classes: no motile sperm, fewer than 25, 25–100, and more than 100 motile sperm. Production of more than 100 motile sperm was taken to be equivalent to that of pure species males.

Fly culture: Fly stocks were maintained at 22–23° in non-crowded conditions within shell vials containing standard corn meal-yeast-agar medium. In the case of male-sterile lines, females with introgressed chromosomes and their pure *D. simulans* brothers had to be collected for crossing each generation. The fertile lines had to be checked every few generations for the presence of white-eyed males or wild-type females that occasionally arose through the detachment of the individual arms of the attached-*X* chromosome.

Probes: Probes for Southern blotting were labeled with [^{32}P]dATP by the random hexamer primer method (FEINBERG and VOGELSTEIN 1983). The probes used consisted of plasmid clones from the loci *norpA* (BLOOMQUIST *et al.* 1988) and *swallow* (ZALOKAR *et al.* 1975) and were gifts from W. PAK (Purdue University) and E. STEPHENSON (University of Alabama), respectively.

SSCP/PCR analysis: In the fine-scale mapping experiments, introgression extents were determined on the basis of the species-specific pattern at the loci *norpA*, *actin5C* (FYRBERG *et al.* 1980; VIGOREAUX and TOBIN 1987), *ovo* (MOHLER 1977), and *swallow*, using the polymerase chain reaction (PCR) and a modified version of the single strand conformation polymorphism (SSCP) procedure of ORITA (1989). PCRs were performed in 15-μl volumes with of 5 μCi of [^{32}P]dATP included. The annealing temperature for all PCR reactions was 61°. For *actin5C*, SSCP was performed by denaturing 1–3 μl of PCR product in 9 μl of denaturing buffer (95% formamide, 10 mM NaOH, 0.5% bromphenol blue, 0.5% xylene cyanol) at 94° for 2–3 min, followed by chilling on ice for at least 5 min prior to electrophoresis of the entire preparation on polyacrylamide under non-denaturing conditions. With *norpA*, *ovo* and *swallow*, denaturation was preceded by digesting 5–8 μl of PCR product with restriction endonucleases *Hae*III, *Taq*I and *Rsa*I, respectively, for 2 h in 10-μl volumes. Aliquots of 1–3 μl were

used for SSCP analysis of digested DNA. Two kinds of polyacrylamide with low bis:acrylamide ratios were used: 2% MDE™ gel (AT Biochem, catalog no. 500) or 6% Protogel™ (National Diagnostics, catalog no. EC-890). Gels were made with 0.6 × TBE (54 mM Tris-base, 54 mM boric acid, 1.2 mM EDTA, pH 8.0) which was also used for electrophoresis buffer. Samples were electrophoresed at low temperature (350 V at room temperature or 800 V at 4°), dried on a sequencing gel drier and autoradiographed for 2–48 hr on Xomat AR™ film.

Primers: The *norpA* primers, which were devised on the basis of the published coding sequence from *D. melanogaster* (GenBank accession no. J03138) and information on the sizes and locations of introns that was provided by W. PAK, amplified a 1.5-kb fragment that spanned the 861-bp intron IV and the 604-bp intron V. The *actin5C* primers were based on the *D. melanogaster* sequence from VIGOREAUX and TOBIN (1987) (GenBank accession nos. X06382 and X06383) and amplified a 1.1-kb fragment extending from the 5′ end of exon I to the middle of exon II. Oligonucleotide primers pairs for *ovo* and *swallow* were a gifts of A. MAHOWALD (University of Chicago) and E. STEPHENSON (University of Alabama), respectively. The *ovo* and *swallow* primers, respectively, produced 1.2- and 2.3-kb fragments from the three species of this study. The respective 5′ and 3′ primer sequences are:

norpA (GATAAGGTGACGAAGAAGAACGG
and GCGGTTATTATGCGTGATCAGAC),

actin5C (TACTCCTTCCCGACACAAAGCCG
and CGCACGGTTTGAAAGGAATGAC),

ovo (GCAACAGTCCGCTCCTAGATGCAAA
and GGATTGCTGCTGTTGCACCGAC),

swallow (CCGCTCCAATTGGAATTCGCGTG
and GTGACGAATTCTGAAGCTCTGC).

RESULTS

We attempt to characterize the sterility associated with the tip of the *X* chromosome that has been introgressed from *D. mauritiana* or *D. sechellia* into *D. simulans*. The advantage of using the *yellow* marker at the tip of the *X* chromosome is that the sterility factor(s) can only be on one side of it. The results will be presented in two phases–a low resolution and a high resolution phase of mapping. In the low resolution phase, we relied on *y* (*yellow*) and *v* (*vermilion*) and then refined the mapping in the high resolution phase by using a series of visible and molecular markers whose positions are given in Figure 2 and Figures 5–6.

Low resolution mapping: The mating scheme is given in Figure 1. In F_1 and subsequent generations, the *D. simulans y v f* chromosome bears *In(1)*, which suppresses recombination between *y* and *v*. Thus, the introgressed segment can remain intact during backcrosses.

Recombination mapping: In stage I of Figure 1, a segment bearing $[y^+]$ from *D. mauritiana* or *D. sechellia* that contains male sterility factors was introgressed ([ ] denotes introgressed materials). At this stage, 12 *D. mauritiana*, introgression lines were generated, two of which were sterile. We then used the flanking markers, *y* and *v*, to carry out recombination analysis on one of the two sterile *D. mauritiana* introgression lines in stage II. In total, 52 $[y^+]$ v^+ recombinants were recovered in females; in males, 32 $[y^+]$ v^+ and 31 *y v* recombinants were recovered. Each recombinant genotype was scored for fertility as described in MATERIALS AND METHODS.

The proportions of fertile introgressions obtained from $[y^+]$ v^+ and *y [ ] v* recombinants are given in Table 1. If there exists a single discrete gene associated with $[y^+]$ that causes male sterility, we expect the proportions of fertile males among $[y^+]$ v^+ and *y [ ] v* types to be complementary (*i.e.*, to add up to 100%). However, if sterile recombinants, which have longer introgressed segments than fertile ones, are less viable, such complementarity will not be observed. For that reason, *X*-linked recombinants recovered in females are less biased toward fertility than those recovered in males, as seen in the difference between the first two rows of Table 1. Ideally, we should rely only on $[y^+]$ v^+ and *y [ ] v* recombinants recovered in females. Unfortunately, the *y [ ] v* recombinant chromosomes carry the same markers as their homolog in *y [ ] v/In(1) y v* females and, therefore, are not readily distinguished in the fertility test.

When we compare the $[y^+]$ v^+ recombinants recovered in females with the *y [ ] v* recombinants recovered in males, as shown in Table 1, the proportions that are fertile are nearly complementary (65% + 42% = 107%). At this level of resolution, we cannot reject the null hypothesis that a single gene is responsible for the male sterility caused by the introgression of the distal end of the *D. mauritiana X* into *D. simulans*. (Low resolution recombination mapping was not performed with *D. sechellia* introgressions.)

DNA marker-assisted mapping: From the results of Table 1, we expect a sterility factor to be mappable to the cytological interval 4–5 on polytene chromosomes corresponding roughly to recombination map position 10–15. We thus selected two DNA markers, *norpA* at 4B6-C1 (BLOOMQUIST *et al.* 1988) and *swallow* (*sww*) at 5E6-7 (STEPHENSON and MAHOWALD 1987), to analyze the recombinants of the first two rows of Table 1. Fertile introgressions were propagated by mating males to attached-*X* females.

The results summarized in Figure 2 indicate that fertile introgressions can pass the *norpA* locus at 4B/C but sterile introgressions do not have to pass *sww* at 5E. These two markers thus delimit the interval containing the sterility factors from both *D. mauritiana* and *D. sechellia*. (Not all lines were probed with both markers; but see high resolution mapping below.) Because less than 10% of the fertile introgressions examined extend beyond *norpA*, we estimate the *D. mauritiana* sterility factor(s) to be closer to 4C within the cytological interval 4C and 5E. The results of Figure 2 also position the *D. sechellia* sterility factor(s) within the same interval.

It seems reasonable to conclude tentatively that there is a major sterility gene in the interval 4C-5E from either *D. mauritiana* or *D. sechellia*. These mapping results

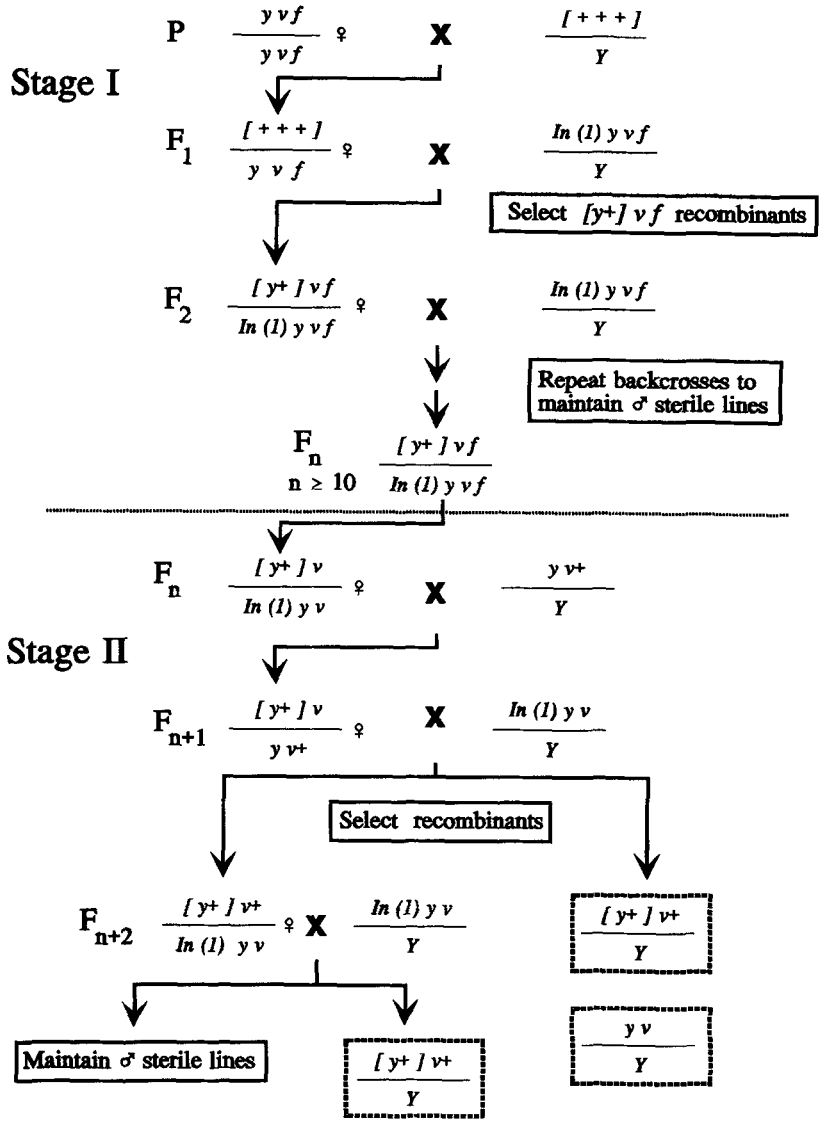

FIGURE 1.—Crossing scheme for low resolution mapping. The symbol, [ ], denotes an introgressed segment from either *D. mauritiana* or *D. sechellia*. All other notations designate *D. simulans* genes. In stage II, the *f* marker was no longer monitored because it is too far from the region of interest. *In(1)* suppresses recombination between *y* and *v* (see MATERIALS AND METHODS). Recombinants were selected as single females. Individual males tested for fertility are shown in boxes at the lower right.

are at least comparable in resolution with several prior studies that suggested the presence of a single sterility factor in Drosophila hybridizations (WU and BECKENBACH 1983; COYNE and CHARLESWORTH 1986; PANTAZIDIS *et al.* 1993). We tested this hypothesis by high resolution mapping described below.

High resolution mapping: We performed higher resolution mapping of the factor(s) in 4C-5E using the crossing scheme outlined in Figure 3. The high resolution mapping utilized the *D. simulans* morphological marker *nipped* (*np*) and *ruby* (*rb*) and four DNA markers in between (see Figure 5). The first step was to construct fresh $[y^+\ np^+\ v^+]$ introgressions from both *D. sechellia* and *D. mauritiana*. The sterile $[y^+\ np^+\ v^+]$ introgressions were then used to generate smaller introgressions by recombination. Recombinants were recovered in both males and females in generation G_2 of stage II. It is easier to test the G_2 male recombinants but only those that are fertile enough to yield a culture with attached-*X* females can be propagated for molecular and phenotypic analysis. Such a bias should be greatly reduced among female recombinants. We will discuss male and female recombinants separately.

Analysis of G_2 males: Recombination and DNA mapping will be discussed.

Recombination mapping The pervasive sterility of the last three genotypes in the G_2 section of Table 1, *i.e.*, $[y^+\ np^+]\ v$ and $y\ np\ [v^+]$ and $y\ [np^+\ v^+]$, suggests the presence of two sterility factors. One is distal to *np* as shown in Figure 2 and the other proximal to *np*, close to the *v* marker (see also WU *et al.* 1993). This is true for introgressions from both species. We will not pursue this second factor(s) between *np* and *v*.

As shown in Table 1, 18 out of 34 *D. mauritiana* $[y^+]$ *np v* males (53%) were fertile in test-crosses, enabling us to map the sterility factor in the *y-np* interval approximately to map position 11.1. This position is close to the marker ruby at 9.7, which was used in later rounds of mapping. In the case of the *D. sechellia* introgressions, 24 out of 38 (63%) were fertile indicat-

TABLE 1

Recombinational analysis of male sterility

Mapping		*D. mauritiana* Fertile	Sterile	Percent fertile	Map position (cM)	*D. sechellia* Fertile	Sterile	Percent fertile	Map position (cM)
Low resolution mapping[a]									
$[y^+]v^+$	♂	21	11	66					
$[y^+]v^+/In\ (1)\ y\ v$	♀	22	30	42	13.9				
$y[\ ]v$	♂	20	11	65					
High resolution mapping at G_2[b]									
$[y^+]\ np\ v$	♂	18	16	52.9	11.1	24	14	63.2	13.3
$[y^+]\ np\ v/In\ (1)\ y\ v$	♀	28	18	60.8	12.8	39	13	75	15.8
$y[np^+\ v^+]$	♂	0	>20	0		0	>20	0	
$y\ np[v^+]$	♂	1	10	9.1		0	12	0	
$[y^+\ np^+]v$	♂	0	>20	0		0	>20	0	
High resolution mapping at H_2[c]									
$[y^+]\ rb\ np^+\ v^+$		16	0	100		25	0	100	
$[y^+\ rb^+]\ np^+\ v^+$		1	40	2.4	10.0	11	35	23.9	12.4
$y\ [rb^+]\ np\ v$		1	21	4.5		9	11	45	

[a] F_{n+2} recombinants from $[y^+]v/y\ v^+$ ♀♀; see Figure 1, stage II.
[b] G_2 recombinants from $[y^+v^+]/y\ np\ v$ ♀♀; see Figure 3, stage II.
[c] H_2 recombinants from $[y^+rb^+]np\ v/y\ rb\ np^+v^+$ ♀♀; see Figure 3, stage III.

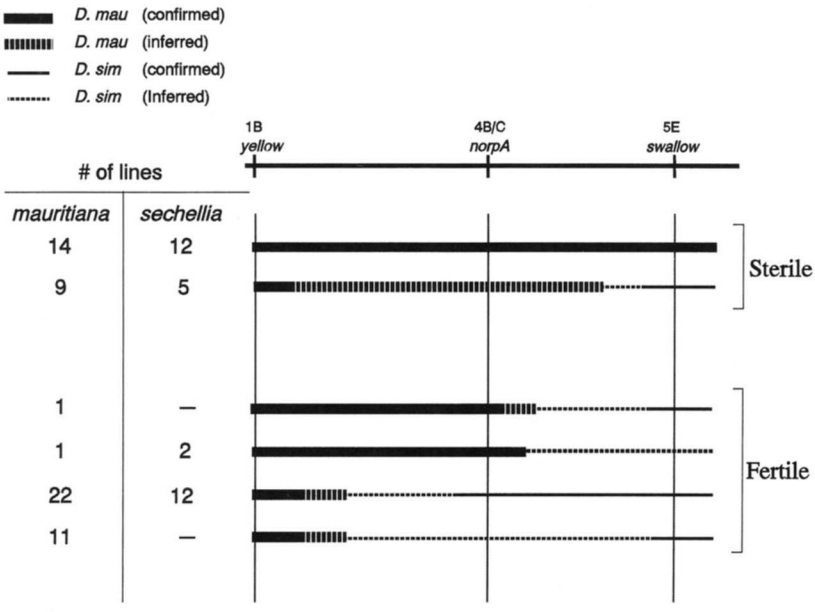

FIGURE 2.—Results from the low resolution mapping. Thick bar (solid and hatched) represents introgressed segment from *D. mauritiana* or *D. sechellia* and thin line (solid or dotted) represents *D. simulans* material. Solid bar is used at the marker site and between two flanking markers when both markers exhibit the *D. mauritiana* (or *D. sechellia*) patterns. Solid line is used at the marker site and between two flanking markers when both markers exhibit the *D. simulans* patterns. Hatched bar and dotted line represent the inferred patterns on the assumption that sterile introgressions are longer than fertile ones. Often sterile lines were not probed at *norpA* because even fertile introgressions can pass that marker. Sterile introgressions were thus inferred to pass it. Likewise, fertile introgressions may not be probed at *swallow* because many sterile lines do not pass that marker; thus the fertile ones were inferred to be short of that marker. Both inferences have since been confirmed in Figures 5 and 6. Approximate cytological positions are given above the names of the relevant probes and markers. The number of lines for each introgression class is shown on the left.

ing that the *D. sechellia* factor is 13.3 cM from *yellow*. These positions provided a rough map for selecting DNA markers.

DNA marker-assisted mapping: The fertile recombinants that were propagated with attached-*X* females, were genotyped by using the PCR/SSCP analysis with primer pairs specific to *norpA* (4B6), *ovo* (4E2), *actin5C* (5C2-5) and *swallow* (5E6). An example is given in Figure 4. Since fertile introgressions can extend beyond *norpA*, we probed 12 fertile *D. mauritiana* fertile G_2 $[y^+]\ np\ v$ lines with *ovo* at 4E (see Figure 5, G_2 column). None of them had introgressions that passed the *ovo* marker. The *D. sechellia* results are given in the G_2 column of Figure 6. In contrast with *D. mauritiana* mapping, the *D. sechellia* sterility factor apparently maps proximal to *ovo*.

Analysis of lines derived from G_2 females: Since sterile introgressions cannot be propagated by G_2 males, $[y^+]\ np$ and $[y^+\ np^+]$ introgressions were analyzed by examining the sons of individual G_2 $[y^+]$ females (see Figure 3, stage II). All the $[y^+\ np^+]\ v$ sons from all lines (9 *D. mauritiana* lines and 5 *D. sechellia* lines, respectively) were sterile by mating. The remaining sections will thus deal with the $[y^+]\ np\ v$ recombinants.

Recombination mapping between y and np: For *D. mauritiana*, 46 $[y^+]\ np\ v/In(1)\ y\ v$ lines derived from single G_2 females were established. Among them, 28 were fertile and 18 were sterile by the criteria of Mate-

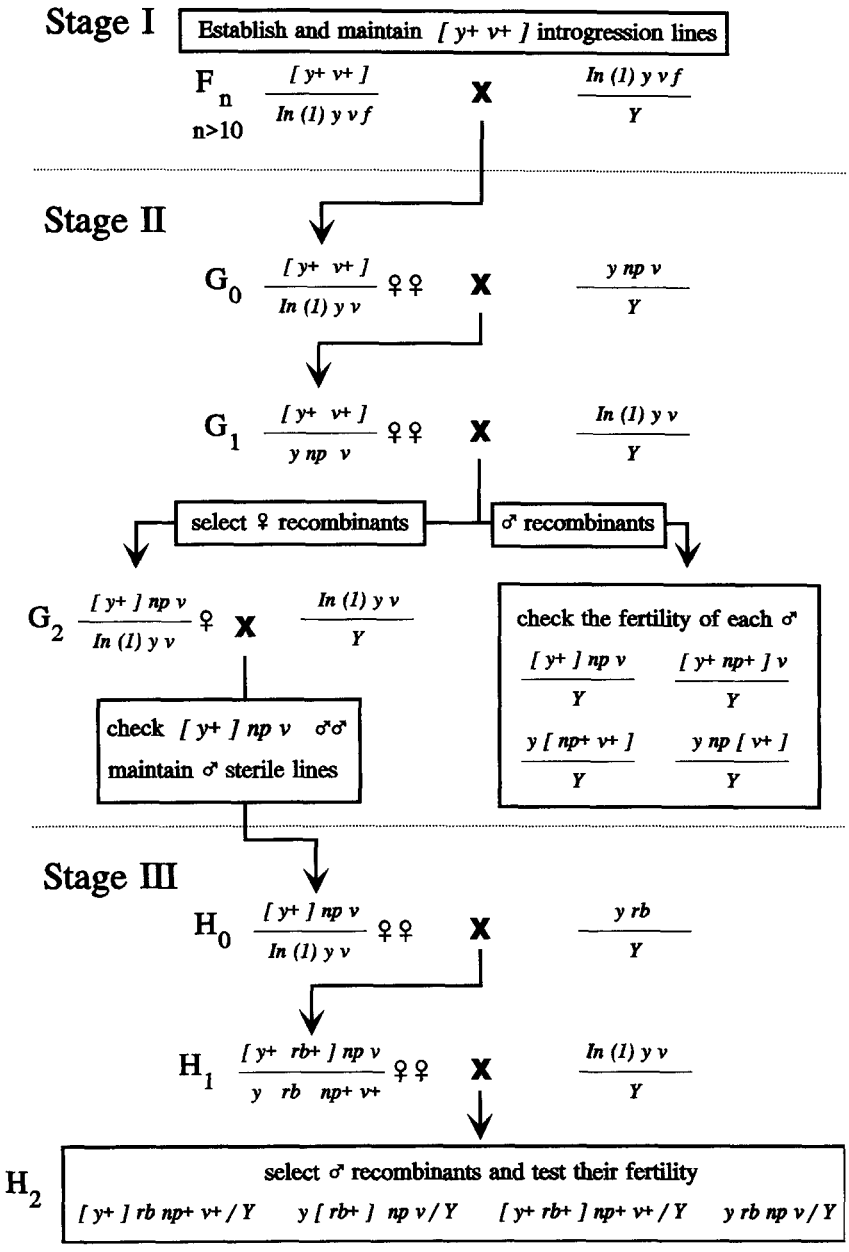

FIGURE 3.—Crossing scheme for high resolution mapping. The symbol, [ ], again denotes segments introgressed from either *D. mauritiana* or *D. sechellia*. Other symbols also follow the legend of Figure 1.

rials and Methods. We thus map the sterility factor to 21 cM × (28/46) = 12.8 cM, which is greater than, but close to, the 11.1 cM distance estimated from G_2 males. Both estimates are somewhat larger than the 9.7 cM obtained by COYNE and CHARLESWORTH (1989). For *D. sechellia*, 52 lines were established–39 of them were fertile and 13 were sterile. The estimated recombination distance for the putative *D. sechellia* factor is 21 cM × 39/52 = 15.8 cM. Equivalent numbers of $[y^+]$ recombinants consistently yielded higher proportions of sterility in the *D. mauritiana* introgressions than in the *D. sechellia* introgressions.

Interestingly, hybrid male sterility in the *y-np* region is associated with two distinct phenotypes. While males from most sterile lines have no motile sperm as their spermatogenic development is arrested before sperm individualization, $[y^+]$ *np v* males from three sterile lines have motile sperm but are not sufficiently fertile to establish a patrilineal line with attached-*X* females. This quasi-sterility correlates with the physical mapping presented below.

DNA marker-assisted mapping: For *D. mauritiana*, the results of Figure 5 (combining G_2 males and females) show that only 1 out 33 fertile recombinants have introgressions extending beyond *norpA* while none passes *ovo*. This strongly suggests that the factor is close to *norpA*, most likely in 4C/D. The three quasi-sterile lines provide direct support for this assignment as all three of them have a crossover point between *norpA* and *ovo*. Clearly, *D. mauritiana* introgressions carry a factor causing quasi-sterility in this interval.

To account for the difference between quasi-sterile and sterile lines, we note that all 13 sterile introgressions

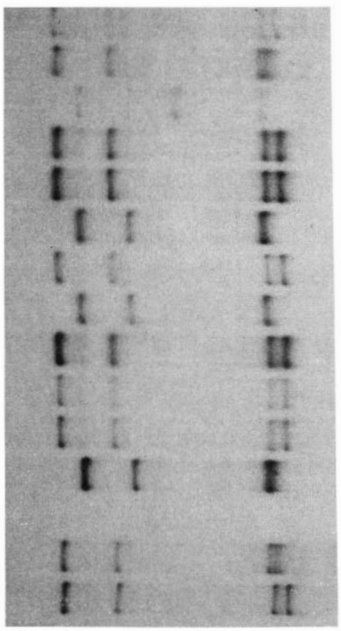

FIGURE 4.—SSCP/PCR results for the *ovo* locus demonstrating that fertile *[y⁺]* introgressions from *D. sechellia* can extend beyond *ovo* (sf3, sf27, sf49), while those from *D. mauritiana* cannot. Of the four *D. mauritiana* introgressed lines shown, only the sterile line ms18 displayed the *D. mauritiana* pattern and, therefore, passed *ovo*. The introgressions of semi-sterile strains ms17 and ms23 (see Table 2) did not extend beyond the *ovo* locus. Strain designations: ms, *mauritiana* sterile; mf, *mauritiana* fertile; sf, *sechellia* fertile.

pass *ovo*. (Males from the quasi-sterile lines fail to produce more than one progeny per male but often have motile sperm; complete sterility means the absence of motile sperm in all males examined.) Since 8 of the 13 sterile introgressions do not pass *actin5C*, the difference between quasi-sterility and complete sterility probably resides in the interval between *ovo* and *actin5C*. Thus, the *D. mauritiana* introgressions contain two distinct hybrid sterility factors: one likely between *ovo* and *actin5C* (*fixA*, for factor A on the introgressed *X*), and the other, between *norpA* and *ovo* (*fixB*). We shall designate the alleles from *D. mauritiana* as $fixA^{mau}$ and $fixB^{mau}$. The factor $fixB^{mau}$, while effectively sterilizing the male, still allows many sperm to develop into maturity. With the addition of $fixA^{mau}$, no motile sperm is produced.

The results for *D. sechellia* are shown in Figure 6. In total, 7 fertile introgressions pass *ovo* at 4E. Apparently, $fixB^{sec}$ is not functionally equivalent to $fixB^{mau}$ because the former does not cause sterility upon introgression into *D. simulans*. Figure 6 suggests the presence of a factor, *fixD*, between *ovo* and *actin5C*. It is quite possible that *fixD* is the same locus as *fixA* but this cannot be determined without finer physical demarcation. (In that case, $fixA^{mau} = fixA^{sec} \neq fixA^{sim}$).

Analysis of H_2 males: Because the mapping suggested the presence of factors causing sterility close to *ruby* (cytological position 4C6), we used this marker in another round of mapping as shown in stage III of Figure 3. The results are given in the last three lines (H_2) of Table 1. Sixteen H_2 *[y⁺] rb np⁺ v⁺* males were recovered and they were all very fertile, with an abundance of motile sperm. In contrast, only one out of 41 H_2 *[y⁺ rb⁺] np⁺ v⁺* males is fertile. This fertile line has an introgression that does not pass *ovo* (Figure 5). These results confirm the existence of a factor proximal but close to *rb*, which we have named *fixB*.

A most surprising observation of Table 1 is that one of the 22 *y [rb⁺] np v* recombinants was actually fertile. A stock with attached-*X* females was established. Molecular probing of these males is shown in the last line (introgression type VII) of Figure 5. These males are fertile despite the fact that they carry the *D. mauritiana* segment containing the sterility factors *fixA* and *fixB*. It is thus necessary to postulate an additional locus, distal to *rb*, which is required for the manifestation of the sterility effect. We shall refer to this hypothetical factor as *fixC*. If recombination data can serve as a guide, *fixC* must be distal but very close to *rb*. The simultaneous requirement of more than one introgressed factor to confer sterility in *D. simulans* can also be demonstrated in the *D. sechellia* introgressions described below.

As shown in Table 1 all 25 H_2 *[y⁺] rb np⁺ v⁺* males recovered were fertile and 11 out of 46 H_2 *[y⁺ rb⁺] np⁺ v⁺* lines were fertile. The higher proportion of fertile H_2 *[y⁺ rb⁺] np⁺ v⁺* males in *D. sechellia* introgression lines than in *D. mauritiana* lines is consistent with the absence of a sterility factor (*fixB*) between *norpA* and *ovo*. Seven of the 11 fertile *D. sechellia [y⁺ rb⁺] np⁺ v⁺* lines were also mapped by DNA markers as shown in Figure 6.

Like the *D. mauritiana* introgressions, H_2 *y [rb⁺] np v* males were not expected to be fertile because all of them carried the $fixD^{sec}$ factor. However, a very high proportion (9 out of 20, Table 1) of H_2 *y [rb⁺] np v* recombinant males obtained through one of the sterile G_3 lines (SS50) were fertile. Molecular probing of males from six lines is shown in the last line (introgression type VII) of Figure 6. Clearly, the $fixD^{sec}$ factor is insufficient by itself to cause sterility in *D. simulans*. An additional introgressed element, designated *fixE*, distal to *rb* is also needed to confer sterility. Based on the recombination data alone, we estimate *fixE* to be far distal to *fixD*. To conclude, both *D. sechellia* and *D. mauritiana* introgressions contain factors between *yellow* and *ruby* that must be present in order to permit other, more proximal, factors to cause sterility.

Spermatogenic defects: In this section we shall describe the spermatogenic defects associated with each genotype listed in Table 2. These genotypes correspond with the Roman numeral designation in Figures 5 and 6. In most cases, the sample sizes were larger than those shown, which were obtained under a standardized condition (see MATERIALS AND METHODS) to corroborate the analyses done over an extended period of time. Detailed

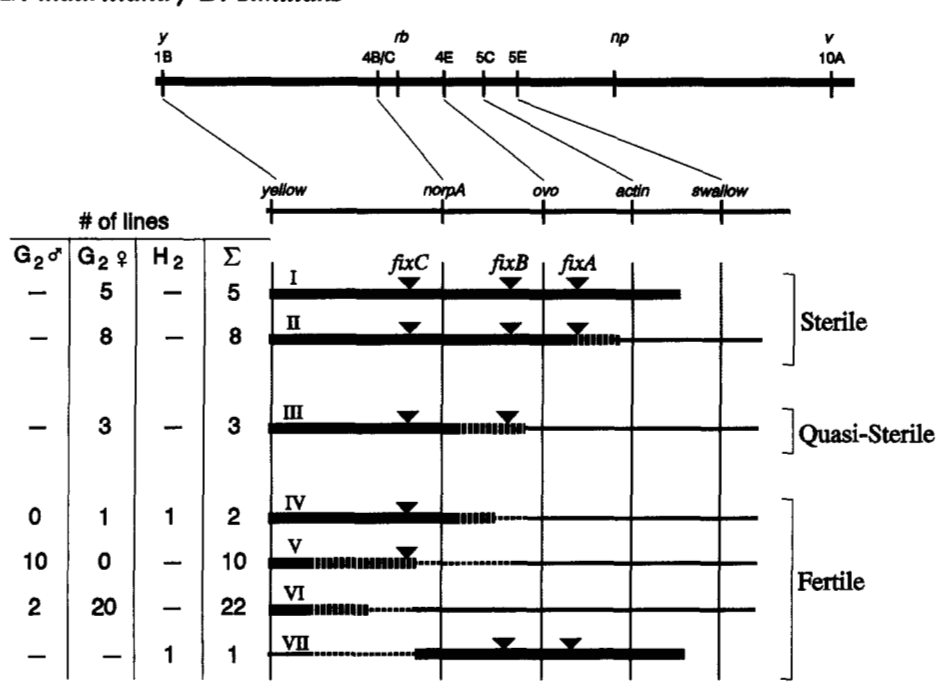

FIGURE 5.—High resolution mapping of *D. mauritiana* introgressions. Thick bar (solid and hatched) represents introgressed segment from *D. mauritiana* and thin line (solid or dotted) represents *D. simulans* material. Solid bar is used at the marker site and between two flanking markers when both markers exhibit the *D. mauritiana* patterns. Solid line is used at the marker site and between two flanking markers when both markers exhibit the *D. simulans* patterns. Hatched bar and dotted line are used when the two adjacent markers exhibit different species patterns; hence, the extent of introgression cannot be determined between these two markers. In those cases, we position the breakpoint of the introgression according to both the fertility/sterility of the genotype, assuming that the sterile introgressions are longer, and the relative number of the fertile *vs.* sterile introgressions. A schematic representation of the distal half of the *X* chromosome is given above, showing the approximate cytological locations of the relevant markers. The number of recombinant lines and the method by which these recombinants were obtained is shown on the left (see Figure 3 designations). Roman numerals are used to classify each introgression type, as used in Table 2. The notations, *fixA*, *fixB* and *fixC* denote the approximate locations of the putative hybrid sterility factors.

descriptions of normal spermatogenesis can be found in LINDSLEY and TOKUYASU (1980), KEMPHUES *et al.* (1982) and FULLER (1993). Below we shall describe *D. mauritiana* introgressions of Table 2 first.

M1-4 and M3-3 lines: These are males carrying complete $[y^+\ np^+\ v^+]$ introgressions. In these males, spermatogenesis only proceeded as far as the early primary spermatocyte stage and no cell types characteristic of spermiogenesis were observed. Only a few mature primary spermatocytes were identified in the testes of these males and their testes were filled with debris (Figure 7A). An example of the spermatogenic phenotype is shown in Figure 7B. The phenotype is roughly equivalent to that of *D. sechellia* $[y^+]$-introgression reported in JOHNSON *et al.* (1992).

Type I and II (3 sterile lines): The spermatogenic phenotypes were not as severe as those observed for $[y^+\ np^+\ v^+]$ males of the M1-4 and M3-3 lines. In their testes, spermatogenesis appeared essentially normal until the elongation stage and, in their seminal vesicles, only coiled bundles and debris were observed (Figure 7, C–E). Nevertheless, 10 out of 14 males examined from these 3 lines contained cysts with defective onion cells. The defects observed included onion cells with too many nuclei or mitochondrial derivatives and cells with a gross asymmetry in size between the nucleus and the mitochondrial derivative. Only a few cells per cyst showed these defects.

Type III (2 quasi-sterile lines): Only some of the matings by these males produced a few progeny and, in those cases, a sustained culture with attached-*X* females could not be established in several attempts. Spermatogenesis showed no sign of arrest but motile sperm production was clearly reduced in these males. This result also illustrates that progeny production and motile sperm production are not always equivalent measures of male fertility.

Type IV (2 fertile lines): All matings by males carrying either introgression produced progeny and the introgression could be maintained as a stock with attached-*X* females. These males did show a reduction in the production of motile sperm. The long fertile introgressions of type IV are in fact distinguishable from normal fertile introgressions on one hand and quasi-sterile introgressions on the other. Normal fertile males with an introgression not reaching *norpA* usually have a sperm-production profile resembling type VII (see Table 2).

TABLE 2

Phenotypic analysis of representative hybrid genotypes

Introgression type	Line	Test crosses[a] Total no. matings	No. matings w/Larvae	Total no. males	Dissections: Spermatocyte morphology Normal	Spermatocyte morphology Defective	Onion cell morphology Normal	Onion cell morphology Defective	Motile sperm estimate 0	<25	25–100	>100	Comments
D. mauritiana introgressions													
	M1-4[b]			7	0	7	0	7	7	0	0	0	No stages identified past apolar spermatocyte stage
	M3-3[b]			6	0	6	0	6	6	0	0	0	No stages identified past apolar spermatocyte stage
I	MS-47[b]			3	0	3	0	3	3	0	0	0	Males with coiled bundles only. No individualized sperm were observed
II	MS-40[b]			6	6	0	4	1	6	0	0	0	Onion cells only checked for 5 males
II	MS-24	3	0	7	7	0	0	6	7	0	0	0	All males with a few individualized sperm and many coiled bundles
III	MS-17	13	9	28	25	1	25	1	14	12	0	0	All males had few if any individualized sperm. One mating produced larvae, but both males produced no motile sperm
III	MS-23	11	3	24	15	0	13	2	12	5	1	1	All males with few individualized sperm; 19 males were checked for motile sperm but only 15 of these were analyzed for spermatogenic defects.
IV	MF-38	7	7	14	14	0	14	0	4	6	4	0	All males with debris in seminal vesicle
IV	Mrb-1	13	13	27	25	1	20	4	10	11	5	0	Sterile males with coiled bundles only
VII	Myrb-1	10	10	19	17	0	17	0	1	5	8	3	All crosses with good progeny production
D. sechellia introgressions													
III	Srb-3	8	8	16	16	0	15	0	4	9	3	0	
IV	Srb-4	10	10	20	17	0	17	0	3	8	4	2	
VII	Syrb-5	7	7	15	13	1	12	1	1	1	1	11	Male with abnormal onion cells had tumorous testes
VII	Syrb-1	7	7	14	12	1	12	1	6	7	0	0	Spermatocyte nuclei looked somewhat "grainy" with many phase dense particles. Male with abnormal spermatocytes and onion cells had tumorous testes
	SS-50	2	0	4	4	0	4	0	4	0	0	0	
	SF-63	2	0	4	0	4	0	4	0	1	1	1	Very few progeny were produced

[a] Two 1–2-day-old males were mated with virgin attached-*X* females for 5–7 days.
[b] Lines known to be completely sterile were not used for test crosses.

D. sechellia / D. simulans

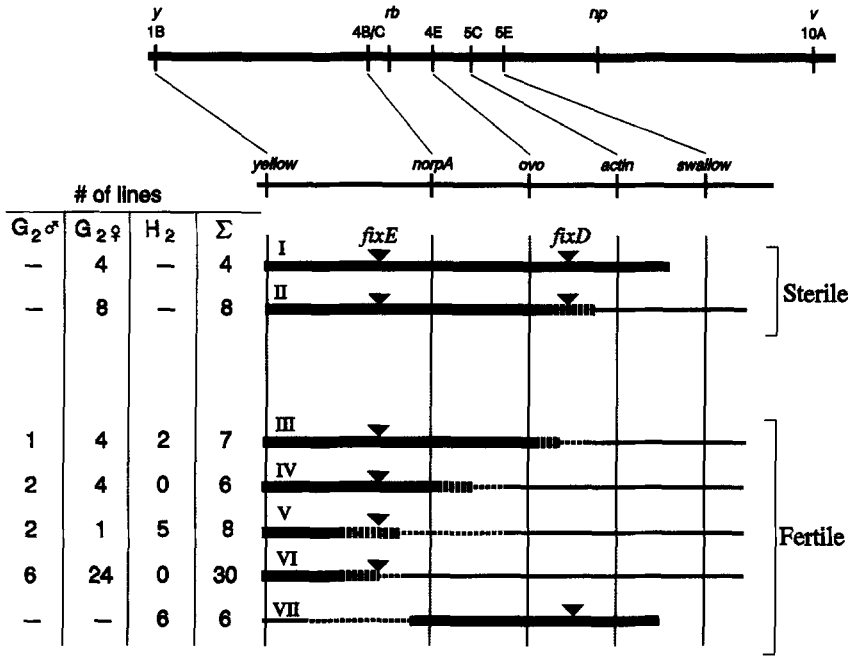

FIGURE 6.—High resolution mapping of *D. sechellia* introgressions. See legends of Figure 6 for detail (substituting *D. sechellia* for *D. mauritiana*).

The presence of two classes of fertile introgressions could be due to $fixC^{mau}$.

Type VII (one fertile line): While these males carry $fixA^{mau}$ and $fixB^{mau}$, their fertility is in fact very close to that of pure species males. All matings produced healthy cultures. Sperm counts in these males were nearly normal. No abnormalities in spermatogenesis were observed.

D. sechellia introgressions of Table 2: Males with *D. sechellia* introgression type II of Figure 6 usually exhibit some (postmeiotic) spermiogenic activities with elongated sperm bundle and sometimes produce a few motile sperm. These males are not fertile by mating. Comparisons between one line each of genotype III (the longest fertile introgression in our collection) and IV of Figure 6, respectively, reveal no difference in fertility (Table 2). Males from the Syrb-5 line of genotype VII that carry the $fixD^{sec}$ sterility factor are also fully fertile by both sperm count and the mating test. Males from another line of genotype VII, Syrb-1, are fertile by the mating test but have low sperm counts. It is not clear why these counts were so low as to contradict the mating test (both measures were carried out on the same males with dissection done 5–7 days after the mating test started). Both the age and the experimental conditions may contribute to the anomaly. Nevertheless, it is unambiguous that $fixD^{sec}$ by itself does not cause male sterility in a *D. simulans* background.

DISCUSSION

In this study, a combination of genetic and molecular mapping techniques was used to examine hybrid male sterility caused by factors introduced from either *D. mauritiana* or *D. sechellia* into *D. simulans*. The findings are quite clear: the observed sterility associated with the distal one-fourth of the *X* chromosome is due to the replacement of *D. simulans* factors by at least two (in the case of *D. sechellia*) or three interacting genetic elements (*D. mauritiana*). Our analysis of the wide ranging spermatogenic defects associated with introgressions of various length also underscores the complexity of hybrid male sterility. None of these factors, when introgressed alone, causes sterility. Since the introgressed factors must interact with other genes in the *D. simulans* background, the number of interacting genes causing sterility in these instances must be greater than two or three. The loci which jointly cause sterility are not necessarily the same when genes from different species are introgressed. For example, no element with the same effect and location as the *D. mauritiana* factor *fixB* could be identified on *D. sechellia* introgressions. Similar observations have been made on the *Ods* factor identified by PEREZ *et al.* (1993). The *Ods* allele from *D. mauritiana*, but not from *D. sechellia*, caused sterility when introgressed into *D. simulans*.

The unexpected observations on the genetics of hybrid sterility are: (i) extensive differentiation even between very closely related species and (ii) strong epistasis between tightly clustered genes. Do these observations represent a general pattern of reproductive isolation?

Prior studies: The contrast between the low resolution and the high resolution mapping serves as a caveat against the interpretation of a simple genetic basis for hybrid sterility, which many previous studies made (*e.g.*,

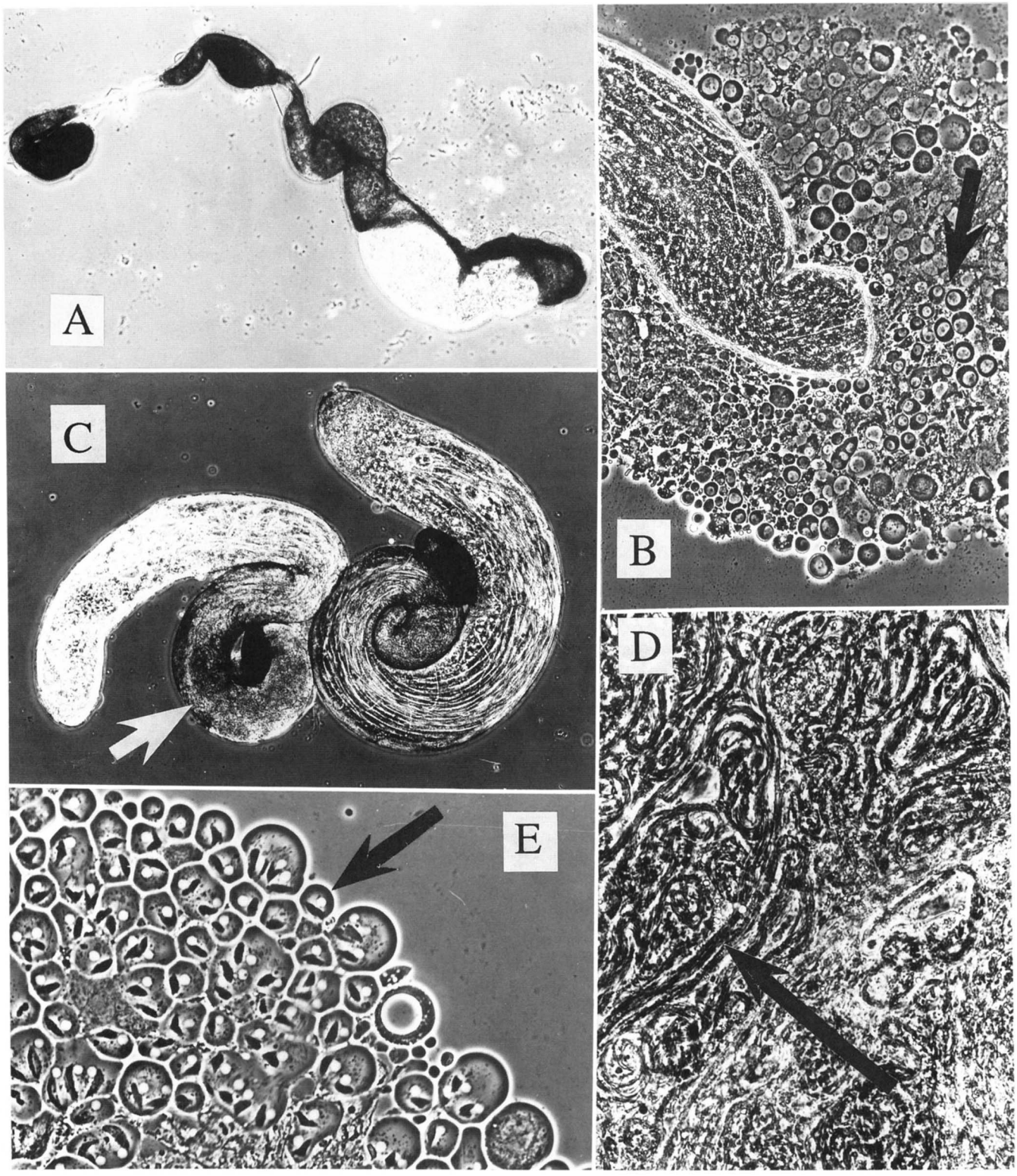

FIGURE 7.—Testis morphology (A) and defective spermatocytes (B) in sterile males with a introgression extending from *y* to *v* (*cf.* JOHNSON *et al.* 1992). Note the drastically reduced testis size and the mature spermatocytes (arrow in B) that fail to enter or complete meiosis. Testis morphology (C), sperm bundles (D) and spermatids (E) of sterile males with genotype II of Figure 5. In (C), a whole testis of the sterile male is placed next to a normal testis from a fertile male. There is only a slight reduction in size. The arrow in (C) points to sperm bundles in the sterile testis; the arrow in (D) is a magnified view of those bundles. The arrow in (E) points to the product of normal meiosis with eleongated mitochondria derivatives (*cf.* PEREZ *et al.* 1993). The spermatogenic defects are detectable cytologically only after elongation when aberrantly coiled sperm bundles are visible.

HENNIG 1977; WU and BECKENBACH 1983; COYNE and CHARLESWORTH 1986; ZENG and SINGH 1993; PANTAZIDIS *et al.* 1993; JOHNSON *et al.* 1993). Because few of them had a resolution beyond what is accomplished by our low resolution mapping, it is prudent not to draw the conclusion, however tentatively, that a single gene within the introgression causes hybrid sterility.

Two studies of higher resolution are discussed below. PEREZ *et al.* (1993) suggested that the *Ods* gene of *D. mauritiana* may be a single gene of complete sterility effect in the *D. simulans* background based on three criteria–the existence of two discrete phenotypic classes, recombination mapping and physical demarcation. Despite its fulfillment of these criteria, we have recently obtained direct evidence that *Ods* requires the joint presence of another *D. mauritiana* factor(s) within the same small introgression for full sterility (D. E. PEREZ and C.-I WU, unpublished results). Indeed, the identification of a single component causing sterility in the context of an entire introgressed chromosomal segment does not mean the gene is the sole determinant of that phenotype. Such a caveat applies to other systems like the fourth chromosome of *D. simulans* (ORR 1992) as well.

Another line of evidence for single genes causing species incompatibility is the mutations that rescue inviable hybrids between *D. melanogaster* and *D. simulans* (WATANABE 1979; HUTTER and ASHBURNER 1987; HUTTER *et al.* 1990; SAWAMURA *et al.* 1993a,b; SAWAMURA and YAMAMOTO 1993). While there is good evidence that these mutations are single discrete genes, their presence by no means implies a simple genetic basis for hybrid inviability. The rescue mutations could be second site suppressors that by-pass the genetic control. An analogy can be drawn from the segregation distorter (SD) system, which is a complex of strongly interacting genes [see LYTTLE (1991) and WU and HAMMER (1991) for recent reviews], and yet single mutations that can suppress the distorting phenotype are quite common. The presence of single genes suppressing SD does not imply SD is a one-locus system.

Multigenic basis for hybrid male sterility has been emphasized by NAVEIRA and FONTDEVILA (1986, 1991) and NAVEIRA (1992). In our companion study, DAVIS *et al.* (1994) showed that hybrid female sterility in the *D. simulans* clade is also caused by complex epistatic interactions. The extent of genetic differentiation, the complexity of interactions and the evolutionary implications of hybrid sterility between *D. simulans* and *D. mauritiana* will be further explored by M. F. PALOPOLI and C.-I WU (unpublished results).

Relationships between intraspecific variations and interspecific differences: For any phenotypic difference between species it is often possible to find mutations within a species that result in similar phenotypes. Because single genes of a major effect on phenotypes are the usual subjects of genetic analysis, and because male sterility mutations are very common (LINDSLEY and TOKUYASU 1980; CASTRILLON *et al.* 1993), it seems most parsimonious to assume a comparable genetic basis for hybrid sterility, as has usually been done. There are, however, considerations that caution against such extrapolations. First, interspecific differences represent a very special spectrum of mutations, *i.e.*, those that could eventually become fixed in the species, whereas mutational analyses include predominantly those that have a substantial phenotypic effect. Many evolutionary genetic models tacitly assume that evolution proceeds by imperceptibly small changes (*e.g.*, FISHER 1930). These changes are precisely the kind least likely to be studied in mutational analyses. Neither are there compelling reasons to believe within-species variations recovered from natural populations fairly represent interspecific differences (WU and DAVIS 1993). The latter should include many products of positive Darwinian selection whereas within-species variations are probably mostly maintained by a balance between negative selection and mutation or, occasionally, between counteracting selective forces in a vast background of neutral changes.

Second, for discrete phenotypes such as sterility or inviability, mutation analysis has conventionally been biased toward single genes. Multiple loci that have to act in concert to cause a discrete phenotype are difficult to identify. One well known example is that of synthetic lethals and synthetic steriles, which require multiple interacting loci for inviability or sterility to occur (DOBZHANSKY 1970). The search for synthetic lethals, however, has not always been successful (THOMPSON 1986). Such genetic systems may indeed be uncommon within a species because they require polymorphism at multiple loci, which may be difficult to maintain in the presence of genetic recombination. The situation is quite different in interspecific comparisons because different species do accumulate many different changes.

Thus the conventional analysis of mutations of major effect offers only partial clues to the nature of hybrid sterility. Several recent mutational analyses of weak alleles that interact along biochemical pathways may provide some of the missing clues (*e.g.*, SIMON *et al* 1991). The central idea is to look for weak alleles that, singly, do not have a noticeable effect on the phenotype. These alleles at many different loci could be recovered because they jointly affect the phenotype of interest. When the mutations are screened in the presence of weak mutations of an interacting locus, the signaling along the pathway may be barely adequate and so another weak allele, even a recessive loss-of-function mutation can, in some cases, give rise to a dominant strong phenotype in such a "sensitized" genetic background.

An interesting case of interacting male-sterile mutations is in the double heterozygotes $hay^{nc}/+$ and $B2t^{0}/+$ of *D. melanogaster* (REGAN and FULLER 1988, 1990; MOUNKES *et al.* 1992). Either heterozygote is male fertile

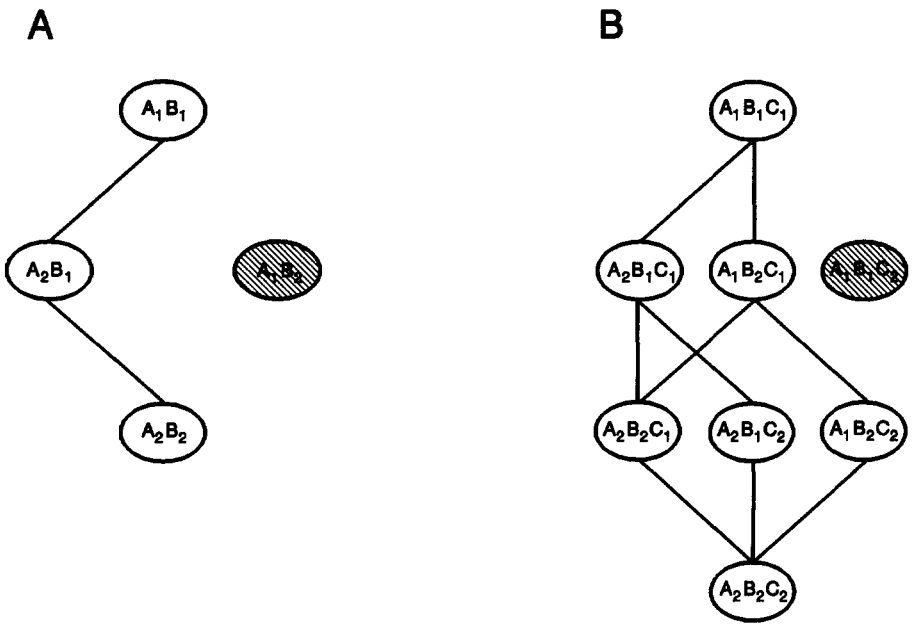

FIGURE 8.—A model for the evolution of hybrid sterility. (A) Single gene from each species. Hybrid sterility results from the interaction between locus A of species 1, A_1, and locus B of species 2, B_2, as shown in shade. Note that there is only one pathway to connect the two extant states, A_1B_1 and A_2B_2, for species 1 and 2, respectively. (B) Two loci for one species. Locus A and B of species 1 interact with locus C of species 2, causing hybrid sterility, also shown in shade. With slightly more complex interactions, there are now four pathways to connect the extant species, $A_1B_1C_1$ and $A_2B_2C_2$.

by itself but the double heterozygote is completely male sterile. The *hay* locus is known to be a member of the DNA helicase family that performs a very general function (MOUNKES *et al.* 1992) and thus is unlikely to be specific to the spermatogenic pathway where the *B2t* locus plays a central role (KEMPHUES *et al.* 1982). Weak alleles of many loci that jointly result in male sterility but do not necessarily interact in any specific manner (for example, along the same biochemical pathway) may turn out to be a common genetic basis for hybrid sterility.

Genetic differences beyond the incipient stage of speciation: The genetic architecture underlying species differences is likely to depend on the stage of divergence. Genes of major effect on hybrid sterility or inviability are probably more common between species that have diverged far beyond the incipient stage. When molecular introgression by means of gene transformation was carried out between divergent species, such as *D. melanogaster* and *Drosophila pseudoobscura*, many genes exhibited strong effects on viability and gene expression. SEEGER and KAUFMAN (1990) showed that the *D. pseudoobscura bicoid* gene, which is necessary during embryonic development, cannot be substituted for its homolog in *D. melanogaster*. In another example, BRADY and RICHMOND (1990) demonstrated that the *Est-5* gene of *D. pseudoobscura* failed to express in the reproductive tract of *D. melanogaster*, whereas its expression pattern appeared normal in other tissues. Single genes of major effect on fertility and viability could be very common after the species have diverged beyond the incipient stage of speciation, but in our analysis of closely related species, no such gene is evident.

Evolution of postmating reproductive isolation: The evolution of hybrid sterility and inviability is intriguing because there must be strong selection against the expression of sterility or inviability within species. Thus one of the central questions of the evolution of postmating reproductive isolation is: How did the underlying genetic architecture diverge without manifesting inviability or sterility during the course of evolution?

The multilocus weak allele interactions envisaged here may alleviate some of the difficulties in explaining the evolution of hybrid sterility and inviability. Most models require at least one different locus from each species as illustrated in Figure 8A. (To simplify the presentation, we assume that all genes are co-dominant although the actual relationship does not affect the conclusion.) In that example, the introgression of the A allele from species 1 into species 2 causes sterility due to the incompatibility between A_1 and B_2. The reciprocal introgression, A_2B_1, is usually fertile (WU and BECKENBACH 1983; VIGNEAULT and ZOUROS 1986) because it must have represented the evolutionary link. In such a simple system of two loci, there is only one pathway connecting the two extant species. Assuming that A_2B_1 is the ancestral state which evolves to A_1B_1 and A_2B_2 in species 1 and 2, respectively, it is apparent that neither step could have taken place if A_2B_1 has a higher fitness than either derived state.

The restrictiveness of the model of Figure 8A is relaxed as the number of interacting loci increases. We shall consider the next simplest case of three loci where the alleles from two loci, A and B, of species 1 interact with the C locus of species 2 to cause male sterility (Figure 8B). This is equivalent to the case of *D. sechellia* introgression depicted in Figure 6, provided that a single C locus from species 2 (*D. simulans*) is sufficient for the sterility interaction. In this model, there are six pathways connecting the two extant species. The sterility of $A_1B_1C_2$ makes one-third of the pathways unpassable but evolution could still proceed via any of the four remaining

links. In general, if there are n interacting loci with i of them from one species and $(n\text{-}i)$ from the other, then the number of passable pathways connecting the two extant species is $n!\ [1 - i!(n\text{-}i)!/n!]$. Thus, in the example of *D. sechellia* introgression, if two loci from *D. simulans* are involved in the sterility interactions, the number of open pathways increases to 20. In the case of *D. mauritiana* introgressions of Figure 5 where three loci are needed, the number of passable pathways may increase to 108 if two loci from *D. simulans* are also necessary for the sterility interaction. Of course, many of the pathways would encounter semisterility and are therefore not passable; but the general picture is that the number of pathways connecting two species increases as the number of interacting loci increases. Thus, multilocus weak allele interactions between closely related species are not unexpected as such a system may offer more opportunities for hybrid sterility to evolve.

Conclusion and implication: Recent evidence has increasingly favored the view that hybrid sterility between incipient species is largely due to strong epistasis between genes of minor or no effect individually. There is, to date, no conclusive evidence that a single gene could cause complete sterility when introduced from one Drosophila species into another, closely related species. The new evidence is compatible with the concept of universal epistasis in highly integrated genetic systems (WRIGHT 1977, 1982; MAYR 1963). An implication of the epistatic view is that the number of genes involved in hybrid sterility between closely related species could be quite large. Many of them would lead to sterility only when a particular combination of introgressions is made. Between *D. mauritiana* and *D. simulans*, we estimate 30 differences contributing to male sterility on the *X* chromosome alone (M. F. PALOPOLI and C.-I WU, submitted for publication).

Any attempt at studying reproductive isolation at the molecular level needs to heed the results of the genetic analysis. Sterile mutations can only provide a very incomplete, if not biased, guide to understanding hybrid sterility and species differences. If hybrid sterility between closely related species most often entails several co-introgressed genes, then a direct assault by molecular means, such as germ line transformation of one male sterility gene at a time, is not likely to reveal very much about the essence of the genetic basis of speciation. Careful genetic analysis will remain the prerequisite for molecular biological work.

We are grateful to MAO-LIEN WU for technical assistance and MIKE PALOPOLI for stimulating discussions and criticisms of various drafts of this manuscipt. We thank J. COYNE for fly strains and E. STEPHENSON, M. GARFINKEL, A. MAHOWALD and W. PAK for DNA clones and/or primers. Comments from H. HOLLOCHER, M. WADE and C. LAURIE are greatly appreciated. E.L.C. was supported by a postdoctoral fellowship from the Alfred P. Sloan Foundation and C.I.W. is supported by National Institutes of Health grants (GM39902 and HG00005) and a National Down Syndrome Society Fellowship.

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Communicating editor: C. C. LAURIE