Genetics of Reproductive Isolation in the *Drosophila simulans* Clade: DNA Marker-Assisted Mapping and Characterization of a Hybrid-Male Sterility Gene, *Odysseus* (*Ods*)

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ABSTRACT

In this study, we address the question of whether there exist major genes that cause complete male sterility in the interspecific hybrids of Drosophila and, if they do, how these genes may be characterized at the molecular level. Our approach is to introgress small segments of the *X* chromosome from *Drosophila mauritiana* (or *Drosophila sechellia*) into *Drosophila simulans* by repeated backcrosses for more than 20 generations. The introgressions are monitored by both visible mutations and a series of DNA markers. We compare the extent of introgressions that cause male sterility with those that do not. If a major sterility factor exists, there should be a sharp boundary between these two classes of introgressions and their breakpoints should demarcate such a gene. Furthermore, if male sterility is the only major fitness effect associated with the introgression, recombination analysis should yield a pattern predicted by the classical three-point cross. Both the genetic and molecular analyses suggest the presence of a major sterility factor from *D. mauritiana*, which we named *Odysseus* (*Ods*), in the cytological interval of 16D. We thus formalize three criteria for inferring the existence of a major gene within an introgression: (1) complete penetrance of sterility, (2) complementarity in recombination analysis, and (3) physical demarcation. Introgressions of *Ods* from *D. sechellia* do not cause sterility. Twenty-two introgressions in our collection have breakpoints in this interval of about 500 kb, making it possible to delineate *Ods* more precisely for molecular identification. The recombination analysis also reveals the complexity of the introgressed segments—even relatively short ones may contain a second male sterility factor and partial viability genes and may also interfere with crossovers. The spermatogenic defects associated with *Ods* and/or a second factor were characterized by phase-contrast microscopy.

THE evolution of reproductive isolation is undoubtedly one of the central issues in evolutionary biology (DARWIN 1859; DOBZHANSKY 1970). Our understanding of the genetic basis of this important phenomenon, unfortunately, has remained primitive in an era when large strides have already been made on many difficult biological questions, such as morphogenesis and sex determination (*e.g.*, HODGKIN 1990). In this report, we wish to provide a framework of analysis that attempts genetic fine-mapping and characterization of genes involved in reproductive isolation by means of DNA markers. We are optimistic that this approach, in conjunction with others (WATANABE 1979; PANTAZIDIS and ZOUROS 1988; HUTTER, ROOTE and ASHBURNER 1990; JEAN-FRANCOIS 1991; PALUMBI 1992; ORR 1992; SAWAMURA, TAIRA and WATANABE 1993), may eventually lead to an understanding of reproductive isolation at the molecular level.

Among the different aspects of reproductive isolation, hybrid male sterility is of particular interest for several reasons. First, in animal species whose males are heterogametic (such as mammals and Drosophila), hybrid male sterility appears very quickly after species divergence as is evidenced by a large number of interspecific crosses (WU 1992). In fact, the rapid appearance of hybrid male sterility accounts for the majority of cases in Drosophila and mammals that follow Haldane's rule (1922), which states that if only one of the two sexes in the F_1 hybrids is inviable or sterile, it is the heterogametic sex. Inviability in hybrid males, in contrast, is relatively infrequent between incipient species despite a much greater mutagenic potential for inviability than for sterility (WU and DAVIS 1993). Second, hybrid male sterility represents a well-defined developmental system for genetic analysis, namely spermatogenesis. Studies have shown that hybrid sterility in Drosophila is germ-cell autonomous (DOBZHANSKY and BEADLE 1936) and that some hybrid sterility factors may have no detectable effects on viability (JOHNSON and WU 1993). It is possible to view hybrid male sterility as a pure developmental genetic question where the spermatogenic "mutants" are evolutionarily successful variants of another species. De-

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spite their crucial role in postmating reproductive isolation, spermatogenic defects have been characterized cytologically in only a few hybridization studies (*e.g.*, SCHAEFER 1978; NAVEIRA and FONTDEVILA 1991; PANTAZIDIS *et al.* 1993). Third, traits of postmating reproductive isolation *per se* (hybrid inviability and sterility) are apparently "maladaptive." Thus, the evolution of these traits is a perplexing problem.

A central question about the genetic basis of hybrid sterility is whether there are a small number of discrete factors, each with a complete (or at least major) effect on male fertility or, alternatively, a large number of genes, each having only minor effects. An understanding of reproductive isolation at the molecular level is immensely more achievable if the former is true. Although many publications have already suggested the existence of major effect genes (*e.g.*, ZOUROS 1981; WU and BECKENBACH 1983; COYNE and CHARLESWORTH 1986), in almost all cases the observations are also compatible with the alternative interpretation that many genes of minor effect are responsible. Some authors indeed consistently favor the latter view (NAVEIRA and FONTDEVILA 1986, 1991; NAVEIRA 1992). The contrasting interpretations of essentially the same type of data clearly point out a need for more rigorous criteria.

In this report, we propose three criteria for inferring the existence of a major effect gene. First, under the major gene hypothesis, penetrance of sterility for any genotype should be (nearly) complete. It is important to note that sterility or fertility is the property of an introgression genotype and this property should be deduced from a population of genotypically identical individuals. Second, recombination analysis by markers flanking the putative sterility factor should map the factor to the same location from both ends (complementarity). Third, the putative major factor should be assignable to an ever more refined interval demarcated by a series of DNA markers. In DISCUSSION, we will review briefly the quest to identify major genes in light of these criteria.

Physical demarcation of hybrid sterility genes can now be attempted thanks to many recent developments in DNA technology. In theory, if two species have diverged by 1%, one expects two chromosomes to have one base pair (bp) different out of 100 bp, which is the theoretical limit of marker density. In practice, the resolution of mapping depends on the number of recombinant lines that can be generated and analyzed for their DNA markers in stage II of Figure 1. Thus, the practical limit is determined by the biology of the species chosen (such as their chromosomal constitution and the genetic tools available in the species), the crossing scheme employed and the available molecular techniques for detecting nucleotide differences at specific chromosomal locations.

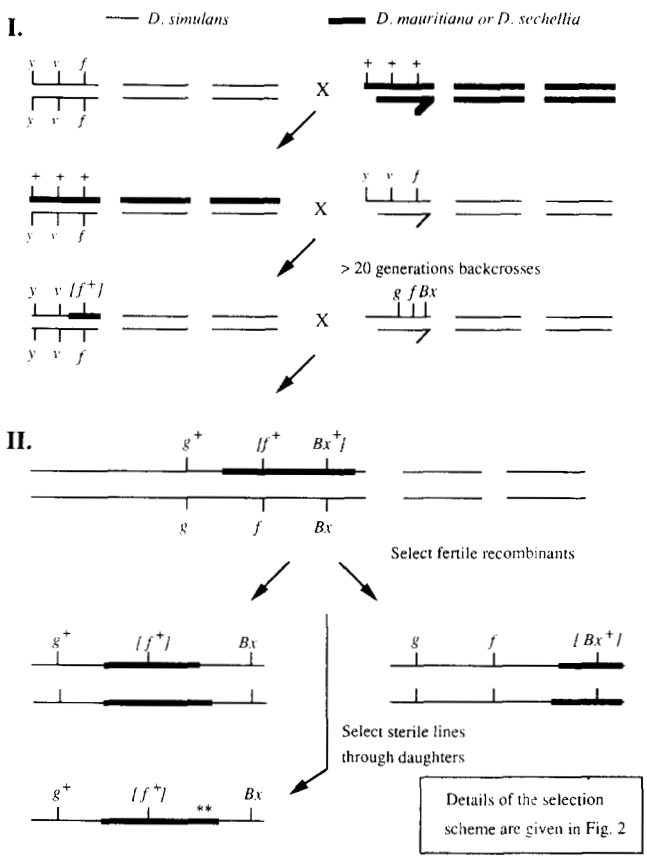


FIGURE 1.—The mating scheme to create *X*-linked introgressions. In stage I, only one marker, *f*, was used to keep track of the introgression. Fertile lines were selected in this stage for coarse molecular mapping of Figure 4. In stage II, the flanking markers *g* and *Bx* were introduced into several sterile lines for recombination analysis and fine molecular mapping (detailed in Figure 2). In scale the *X* chromosome is only about half as long as either major autosome; ** denotes a putative sterility gene.

These molecular techniques range from the identification of restriction fragment length polymorphisms (RFLP) to the detection of 1 bp difference in a given DNA fragment (ORITA *et al.* 1989; NICKERSON 1991). A prerequisite for molecular mapping is a comprehensive cytological or linkage map of DNA clones. Such a map has recently become available in human, mouse (DIETRICH *et al.* 1992), Drosophila (MERRIAM *et al.* 1991), and several other species. These methodological considerations are crucial if the goal is to characterize hybrid sterility genes at the molecular level.

We study reproductive isolation in the *D. simulans* clade, which includes *D. simulans*, *D. mauritiana* and *D. sechellia*; their basic biology is described in LACHAISE *et al.* (1988) and ASHBURNER (1989). These three species are the closest relatives of *D. melanogaster*. The *X* chromosome is homosequential between *D. melanogaster* and these three species, except a very small inversion (LEMEUNIER and ASHBURNER 1976). The level of DNA divergence is 4–8% between *D. melanogaster* and *D. simulans* (COYNE and KREITMAN 1986; CACCONE *et al.* 1988); thus, DNA clones and

sequence information from *D. melanogaster* are directly applicable to this clade. None of the three species produces fertile F_1 progeny with *D. melanogaster* (LACHAISE *et al.* 1988). Within the *D. simulans* clade, fertile females and sterile males are produced *inter se*. Their chromosomes are entirely homosequential, making the introgression study feasible for the whole genome.

Like hybridizations documented previously for other species (WU and BECKENBACH 1983; NAVEIRA and FONTDEVILA 1986), male sterility in the hybrids between *D. simulans* and its two sibling species is associated with all three different regions on the *X* chromosome that have been analyzed (COYNE and CHARLESWORTH 1986, 1989). A more detailed characterization reveals an even higher density of sterility factors (WU *et al.* 1993). In this study, we analyze the hybrid male sterility that was shown to be closely associated with the *forked* (*f*) marker in the introgression from *D. mauritiana* into *D. simulans* (COYNE and CHARLESWORTH 1986). COYNE and CHARLESWORTH (1989) later reported a loose association between *f* and male sterility in the introgression from *D. sechellia*, which is also included in our analysis to infer the evolutionary history of this sterility. We present evidence that all three criteria for a discrete major effect gene are fulfilled and map this major factor to the cytological interval 16D of *D. mauritiana*. The cytological location agrees with the linkage analysis of COYNE and CHARLESWORTH (1986). The interval where this sterility gene is located can be incrementally narrowed to facilitate molecular cloning. We also characterize the spermatogenic defects in sterile males of different genotypes cytologically. Furthermore, the possibility of many minor genes affecting, to a variable extent, viability, fertility, recombination frequency and other subtle traits in the hybrids will be discussed. Such minor-effect genes should be heeded in the pursuit of major genes for methodological reasons, but they may later become subjects of interest in their own right.

MATERIALS AND METHODS

Strains and mutants: We used one strain each of *D. mauritiana* (TSACAS and DAVID 1974) and *D. sechellia* (TSACAS and BACHLI 1981) provided to us by J. COYNE. The strains of *D. simulans* used carry combinations of these visible markers: *y* (*yellow* 1B, 1–0.0), *v* (*vermilion* 10A, 1–33.0), *f* (*forked* 15F, 1–56.7), *g* (*garnet* 12B, 1–44.4) and *Bx*: (*Beadex* 17A, 1–59.4). The cytological locations given (1B, 10A etc.) are the polytene chromosome bands. The crossover distances given are those of *D. melanogaster*, which differ slightly from those of *D. simulans* but the linear order is the same (STURTEVANT 1929; LEMEUNIER and ASHBURNER 1976). The basic stocks are: stock 1089 (carrying *y v f*), 919 (*g*) and 1084 (*f*) from the Indiana University Stock Center; a *Bx* and a *C(1) y w* stock from J. COYNE; and a *D. simulans* wild-type strain from the University of Wisconsin collection. Other strains bearing multiple markers were assembled by recombination from these basic stocks. In the *C(1) y w* stock, females carry attached-*X*s (compound 1), which are homozygous for *y* and *w*. Males mating to these females transmit the paternal *X* to all their sons while their daughters inherit the maternal attached-*X*s. Detachment of the compound chromosomes can be detected by inspecting these visible markers. Unless otherwise noted, all fly cultures were maintained at 22–23° and reared on cornmeal medium.

Introgression scheme: Repeated backcrosses permit the introgression of marked chromosome segments from one species into the genome of another. We introgressed *D. mauritiana* and *D. sechellia X* chromosome segments into the *forked* region of the multiply marked *y v f D. simulans* strain. Our initial experiments relied on the scheme shown in the stage I of Figure 1. The purpose of this stage is twofold: (1) to generate male-sterile and male-fertile introgressions for the coarse physical mapping of the sterility factor(s); (2) to prepare females carrying male-sterile introgression for the recombination analysis of stage II. Without a coarse physical map, we would not know if the sterility is caused by several factors on both sides of the *f* marker or, if by a single factor, which side of the marker it is on. A prerequisite for the recombination analysis of stage II of Figure 1 is that a sterility factor or factors exist on only one side of the marker. In other words, we need to know the interval with which sterility is associated and select flanking markers accordingly. If there are factors on both sides of *f*, recombination between *f* and *Bx* will not yield fertile males, creating a false impression of a very tight linkage between a major factor and the marker, *f*.

Stage I of Figure 1 is detailed below. From F_2 on, 20 independent lines are maintained. In each generation, some $[f^+]$ males in some lines will become fertile because of crossover between *f* and the putative sterility factor(s). (The brackets, [ ], denote introgressed material from *D. mauritiana* or *D. sechellia* into *D. simulans*.) Such fertility restoration is not detectable in the early backcross generations because the mixed genetic backgrounds engender sterility on their own. Between F_1 and F_7, virgin $[f^+]/f$ females are backcrossed to *y v f*/*Y* males of *D. simulans*. From F_8 and on when the background is sufficiently pure, two sets of crosses are done each generation using the four genotypes produced: *f*/*f*, $[f^+]/f$, *f*/*Y* and $[f^+]/Y$. (A) The maintenance cross: $[f^+]/f$ females are mated to *f*/*Y* males as shown in Figure 1. (B) The test cross for $[f^+]/Y$ fertility: *f*/*f* females are mated to $[f^+]/Y$ males. If some of these males are fertile, they would produce $[f^+]/f$ daughters. Their $[f^+]$ grandsons were then mated to females carrying the attached-*X* chromosomes; thus, the fertile introgression is established as a (paternal transmission) line for molecular analysis. The maintenance cross where the introgression is transmitted maternally is discontinued when the paternal transmission line is established (*i.e.*, the introgression is male-fertile). Another male-sterile line is then split into two to keep the total number of male sterile lines around 20. In each generation, on average, one to two lines of the 20 maintenance lines become fertile due to crossover. Forty fertile lines with *D. mauritiana* introgression and nine with *D. sechellia* introgression were established and subsequently mapped with molecular markers.

The test cross is a more efficient way of detecting the presence of fertile *y v* $[f^+]/Y$ males than directly mating single males to virgin females carrying attached-*X*s. Sometimes, a single weakly fertile male failed to produce sons when mated to attached-*X* females. By passing the fertile introgression through females once, enough males can usually be obtained to establish a line with attached-*X* females. More importantly, the scheme does not require the collec-

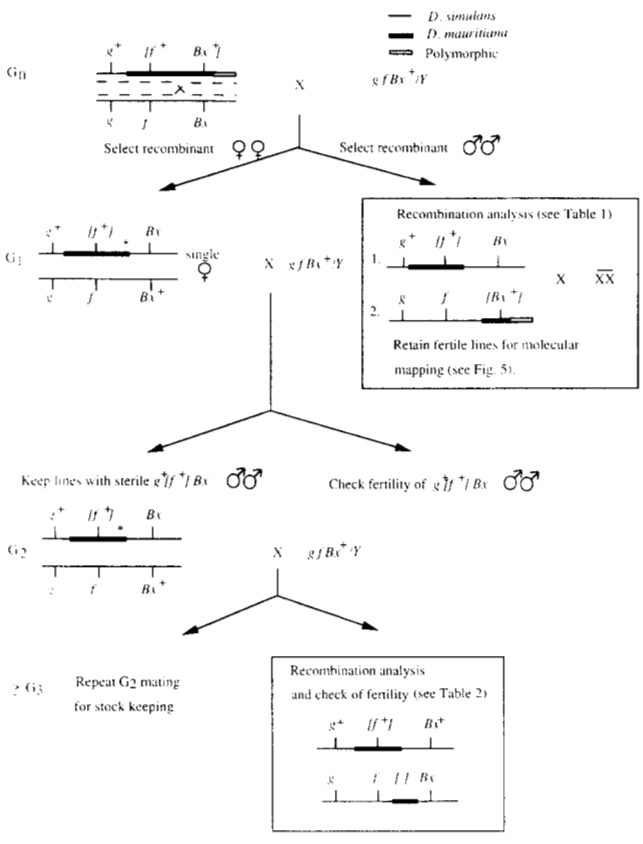


FIGURE 2.—Mating scheme for the recombination and molecular analysis of hybrid sterility, corresponding to a detailed mating scheme for stage II of Figure 1. The females of G_2 are from a sterile line L2-5D. The distal (left) end of the introgression is between 13F and 14B, while the proximal side extends to *Bx* at 17C, but is somewhat heterogeneous beyond *Bx* (because of the absence of markers) as shown by stipples; * denotes a putative sterility gene. *D. mauritiana* segments are always enclosed with [ ] and indicated by a thick line. All others come from *D. simulans*.

tion of virgin females for either cross A or B, an important feature when a large number of lines need to be maintained for a long period of time. In cross B, *f/f* females could have mated to *f/Y* males but the daughters are *f/f*, distinguishable from the desired *[f⁺]/f* genotype. In cross A, females are usually mated to their *y v f/Y* brothers after F_8 but *y v f/Y* males from the pure species stock are also supplemented. This procedure slowed down the purification of the background somewhat but made the stock keeping more manageable.

Recombination analysis: Two different experiments were done to map the sterility factor by recombination analysis as shown in Figure 2—one on long introgressions as shown in the box of G_1 and the other on short introgressions as shown in G_3. These introgressions have been previously shown to contain sterility factors only to the right of *f* (Figure 4). In the first experiment, recombinants *[f⁺] Bx* and *f [Bx⁺]*, are selected. Each independent recombinant male has a specific introgression length. The proportion of fertile males for each recombinant type is scored as described below and more than 60 male fertile lines were established with attached-*X* females for later molecular mapping. In the second mapping experiment, sterile lines with shorter introgressions are obtained by selecting female recombinants at G_1, where the introgression does not include *Bx*. If their sons carrying the introgression are sterile, the line is kept as a stock by repeating the mating. For the recombination mapping, *g⁺[f⁺] Bx⁺* and *g f [ ] Bx* sons are continually collected from the stocks and scored for fertility.

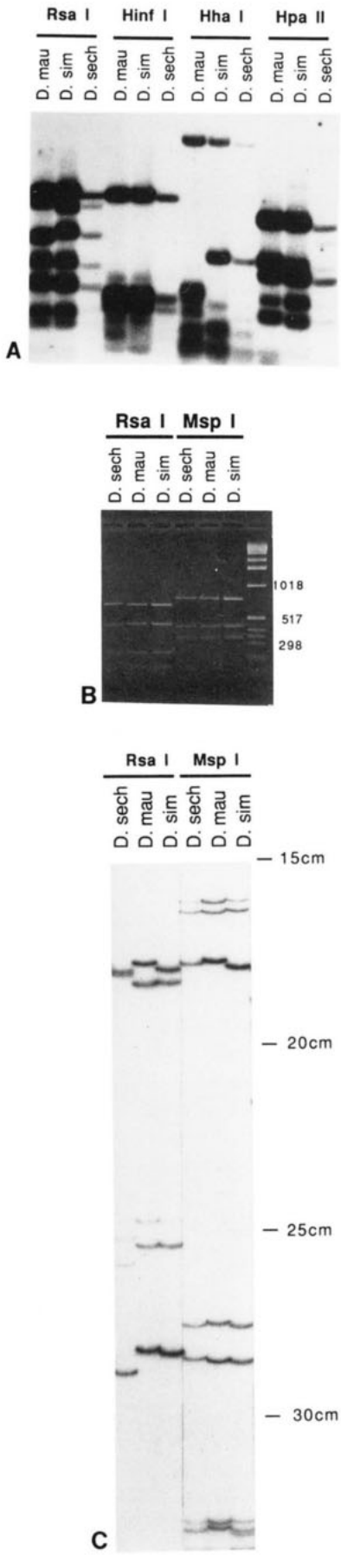
General considerations of molecular mapping: A large number of well-mapped *D. melanogaster* clones are available for the analysis of restriction fragment length differences (RFLP) at numerous genomic sites (KAFATOS *et al.* 1991; MERRIAM *et al.* 1991). Sequence information from *D. melanogaster* can also be used for PCR-based DNA diagnostics. The level of nucleotide sequence divergence between randomly chosen genes from each of the three species in the *D. simulans* clade is about 1–2% (COYNE and KREITMAN 1986; CACCONE *et al.* 1988), sufficient for identifying the species origin of chromosome segments at the DNA level. For example, any phage clone of about 15 kb can be used to detect RFLP differences for most 4-bp restriction enzymes. Furthermore, any DNA fragments of more than 100 bp are expected to be informative of their species origin when analyzed by methods that can detect a 1-bp change (ORITA *et al.* 1989; NICKERSON *et al.* 1991).

Finally, since the level of DNA polymorphism in *D. simulans* is quite high (AQUADRO, LADO and NOON 1987), we have kept track of each *D. simulans* stock used in the introgression experiments and always used the same stock in the molecular analysis. The potential within-stock polymorphism has also been eliminated by mating a single *g f Bx* or *g f Bx⁺* male to attached-*X* females to reestablish the stock. We have never observed within-stock polymorphisms in their DNA diagnostic patterns, including the *y v f* strain.

RFLP analysis: A total of eight *D. melanogaster* DNA clones were used to map the extent of the introgressions by their RFLP patterns at each genomic location. These clones and their cytological locations are: *sd* located at 13F (CAMPBELL and CHOVNICK, personal communication), G2 also at 13F (SULLIVAN *et al.* 1985), IM75 at 14B (UNDERWOOD and LENGYEL 1988), *r* at 15A (SEGRAVES *et al.* 1984), KBA at 16C (BYERS *et al.* 1989), *Sh*A at 16F (KAMB, IVERSON and TANOUYE 1987), *fu* at 17C (KALFAYAN, personal communication) and A57 at 18CD (STEPHENSON, personal communication). These are either phage or plasmid clones with inserts ranging from 3 to more than 20 kb. Genomic DNA was digested with *Rsa*I, *Hae*III or *Hha*I and probed with these clones. The RFLP analysis was done using the standard Southern blotting technique as described in MANIATIS, FRITSCH and SAMBROOK (1989). The extent of each introgression was determined by comparing its RFLP patterns at several DNA sites with those of the appropriate strains from each species. The hybrid male sterility factor can be located to the segment not contained in the longest fertile introgression but present in the shortest sterile introgression.

To find suitable restriction enzymes that yield species-diagnostic patterns, we first made a "tester blot" of genomic DNAs from the appropriate strains of each species digested with a panel of 4-bp restriction enzymes. This tester blot was then hybridized to a number of clones in sequence. The restriction enzyme of choice was the one showing distinct RFLPs for several DNA clones. By doing this, we minimized the number of blots that need to be prepared when each introgression line is checked with many clones. Figure 3A shows an example of the tester blot hybridized to the KBA (16C) clone. In this case RFLP differences are seen between *D. simulans* and *D. mauritiana* for every enzyme, but the clearest distinction is shown by the enzyme *Hha*I. Most molecular mapping in this paper was done with the RFLP analysis. Some introgression lines were also mapped by the SSCP analysis at the *Sh* locus, as described below.

SSCP analysis: The SSCP method (single stranded con-



formation polymorphism; ORITA *et al.* 1989) detects the differential migration of single strand DNA of the same length on a neutral polyacrylamide gel. Apparently, two single-stranded DNA molecules of a few hundred bp can assume different conformations in a neutral gel even with only 1 bp difference between them. Such conformations affect migration. The SSCP analysis of short stretches of DNA amplified by PCR (polymerase chain reaction) greatly increases the efficiency and resolution of molecular mapping. In this study we applied the SSCP technique only at the *Sh* locus.

PCR amplification: A 1.5-kb fragment within the *Shaker* gene was PCR-amplified from the three species and from the introgression lines for SSCP analysis. Primer A: 5′-ggt caa tgt ccc ttt aga cgt a-3′ in exon 9; primer B: 5′-gga aga aag gat ctg tga tgt c-3′ in exon 11. The primers, chosen from the sequence of PONGS *et al.* (1988), were designed to amplify a fragment spanning two introns, which presumably have high substitution rates. ^{32}P-labeled DNA amplifications were performed using a reaction mixture of 1–2 ng of genomic DNA, 20 μM of each primer, 10 mM of each deoxyribonucleotide plus 1 μl of ^{32}P-dATP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°), 0.1% Triton X-100, and 2.5 units of *Taq* polymerase I. The samples were overlaid with 40 μl of mineral oil and subjected to 30 amplification cycles (program A: 1′ 94°, 1′ 45° and 3′ 72° (1×); program B: 15″ 92°, 1′ 48° and 3′ 72° (29×) in a MJ Research PTC-100 thermal cycler. PCR products were checked with 0.8% agarose gel.

Restriction enzyme digestion: 1–2 μg of ^{32}P-labeled PCR products were digested with *Rsa*I and *Msp*I, respectively. After digestion (2 hr at 37°), a fraction of the digestion mixtures was run on a 3% NuSieve GTG gel (Figure 3B). The digestion mixtures were then purified as follows: extract once in phenol:$CHCl_3$, once in $CHCl_3$ and then add 1/10 volume of 3 M sodium acetate to the aqueous phase; precipitate in 2.5 volume of cold 95% EtOH for 30 min at −80°. Spin down the DNA for 20 min at 4°, drain and dry for 30 min at room temperature and then resuspend the DNA in 4 μl of 0.05 M EDTA.

Gel electrophoresis: MDE™ gels (mutation detection enhancement gel, AT Biochem, Inc.) were used although 6% polyacrylamide gels with 10% glycerol gave comparable results. 0.5 × MDE gels were prepared according to the protocol supplied. Gels were prerun for 30 min at 8 W; 1 μl of digested and purified ^{32}P-labeled PCR products were added to 9 μl of sequencing stop solution (95% formamide, 10 mM NaOH and tracking dyes), heated at 94° for 2 min, chilled directly on ice for several minutes and then loaded onto the gel. Running conditions were 0.6 × TBE buffer at 8 W constant power for 16 hr at room temperature. A pair of aluminum plates were clamped onto the glass plates. After electrophoresis, the gel was dried and autoradiographed, as shown in Figure 3C.

A comparison between Figures 3B and 3C reveals the resolution of the SSCP analysis. We detected no RFLP among the three strains, each from a different species, on the PCR-amplified products. The differences are clearly shown by the SSCP analysis for both enzymes (Figure 3C).

FIGURE 3.—(A) An example of a tester blot showing RFLPs for the KBA(16C) chromosome site among the three species. (B) PCR products of the *Sh* locus from the three species, which are indistinguishable on an agarose gel. (C) On the SSCP gel, the same, 3 PCR-amplified products yield species-specific patterns for both restriction enzymes.

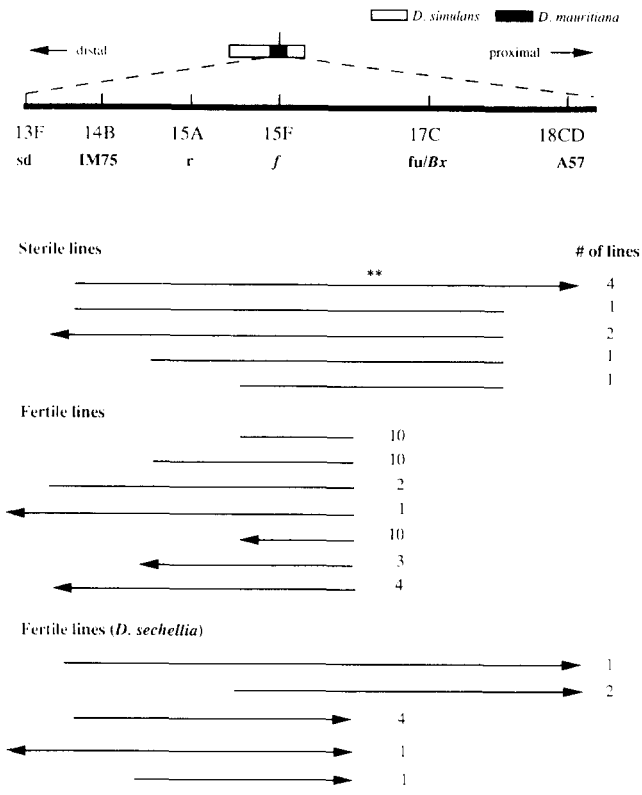


FIGURE 4.—Coarse molecular mapping of the introgression lines generated in stage I of Figure 1. The arrowhead indicates that the introgression is beyond the particular DNA marker. (We did not proceed to determine the locations of all breakpoints unless they would be informative about the sterility factor.) The end without an arrowhead indicates that the line does not pass the next molecular marker. *D. sechellia* fertile introgressions are also presented; ** denotes a putative sterility factor from *D. mauritiana*, but not from *D. sechellia*.

This is true for all other DNA fragments we have examined so far.

Criteria of fertility/sterility: The criteria we used for male fertility are (1) the presence of motile sperm in the seminal vesicle and (2) the actual production of progeny. In our experience the two criteria are very well correlated and can be used interchangeably. It is important to emphasize that the fertility or sterility in Figures 4 and 5 is the property of an introgression *genotype*, of which many genetically identical males have been examined. In these two figures, sterility means no males of a particular genotype were ever found to be fertile whereas fertility means most males (usually >90%) of a particular genotype are fertile. The fertility/sterility designation for the recombinants in Tables 1 and 2, however, is based on *individual* males, each representing a slightly different introgression genotype.

Light microscopy of spermatogenic defects: Several 1- to 2-day-old males of each sterile genotype were further examined cytologically according to the description of KEMPHUES *et al.* (1982). Testes were individually dissected in a drop of Drosophila Ringer's solution and gently squashed under a coverslip. Cells were drawn out of the testis lumen by gently absorbing some of the solution under the coverslip with strips of tissue paper. All preparations were immediately examined under a phase-contrast microscope.

Nomenclature: The major gene that we tentatively mapped to 16DE is, by metaphor, named *Ods* for *Odysseus* who, in the well known epic, was the major figure hidden in the Trojan horse that in the end caused complete destruction of the foreign land it was brought into. Likewise, genes of reproductive isolation manifest their sterility or inviability effect when brought into a foreign genome by either natural or artificial means. The convention for allelic designation thus needs to be modified in the study of species differences by introgression. Clearly, the wild-type alleles from different species are not functionally equivalent. We suggest substituting the species designation for the "+" notation. Thus, the wild-type alleles of *Ods* from each of these species are Ods^{sim}, Ods^{sec} and Ods^{mau}. Functionally, $Ods^{sim} = Ods^{sec} \neq Ods^{mau}$ as shown in RESULTS. There will be a need for a nomenclature system as the genetic studies of hybrid inviability or sterility intensify (WATANABE 1979; HUTTER and ASHBURNER 1987; SAWAMURA, TAIRA and WATANABE 1992; SAWAMURA, YAMAMOTO and WATANABE 1992; PANTAZIDIS and ZOUROS 1989; WU *et al.* 1993).

RESULTS

Physical demarcation I. (Coarse mapping): The first step of our analysis is on $[f^+]$-introgression lines generated in stage I of Figure 1 (see MATERIALS AND METHODS; introgression scheme). We compare the extent of fertile introgressions with that of sterile introgressions by examining their RFLP patterns at a series of chromosomal locations. A summary of the results is shown in Figure 4. In the *D. mauritiana/D. simulans* hybridization, the data show that the lines containing the sterile introgressions cover at least from the polytene chromosome band 15F to 17C. In contrast, the extent of the fertile introgressions is mostly restricted to the distal (left hand) side of *forked*, extending in some cases from 13F to beyond *f* (15F). This pattern suggests that the sterility factor is probably located to the right side of *f* between 15F and 17C. At the same time these results also demonstrate that no sterility factor exists between 13F and 15F. For the *D. sechellia/D. simulans* hybridization the fertile introgressions can extend from 13F to beyond 18C. Our analysis of the *forked* region in the *D. sechellia/D. simulans* hybridization is limited to verifying the absence of a sterility effect in this region, in contrast with the strong effect of the *D. mauritiana* introgression (see also JOHNSON 1992).

Recombination analysis on long introgressions: The observation that in the *D. mauritiana/D. simulans* hybridization the factor is located to the right side of the *forked* marker led to an attempt to map this factor by recombination between visible markers. We selected a sterile line (L2-5D; one of the four sterile lines at the top in Figure 4) whose introgressed segment does not extend beyond 13F and, hence, does not carry a sterility factor on the distal (left) side of the *forked* (15F) marker. Two other visible markers, *garnet* (*g*) and *Beadex* (*Bx*) were then introduced into this line as shown in Figure 2. The recombination analysis was done between *f* and *Bx* where a sterility factor (or factors) has been tentatively mapped in Figure 4. The *g* marker was used to ensure that all

TABLE 1

Recombination analysis of hybrid sterility on long introgressions

Genotypes	No. of males			% Fertility
	Fertile	Sterile	Total	
fBx	103	4	107	96.3
[f⁺]Bx	134	183	317	42.2
f[Bx⁺]	27	162	189	14.3
[f⁺Bx⁺]	0	97	97	0.0

The analysis was done on the L2-5D line with an introgression from 14B at the distal end to beyond 17C; the proximal end is heterogeneous as shown in G_0 of Figure 2.

the *[f⁺] Bx* recombinants have the same distal breakpoints; hence, the difference in fertility/sterility should result strictly from the difference in the proximal end of the introgression. Although the 13F–15F interval by itself is insufficient for sterility, it may still contain a locus that interacts with the *[f⁺]*-proximal region (see DISCUSSION). It is thus prudent to keep the distal breakpoint constant.

The proportions of fertile recombinant males *[f⁺] Bx* and *f [Bx⁺]*, obtained as shown in the first box of Figure 2, are given in Table 1, together with those of the nonrecombinant types, *[f⁺ Bx⁺]* and *f Bx*. All males carrying the *[f⁺ Bx⁺]* introgression are sterile, while the *f Bx* (pure *D. simulans*) males are almost completely fertile. This result shows the complete association between hybrid male sterility and this segment. The proportion of fertile *[f⁺] Bx* males may suggest that a major factor is located at about 42% of the distance from *f* to *Bx*. Surprisingly, the reciprocal recombinants *f [Bx⁺]* are only 14% fertile, substantially lower than the expected value of 58%, if the introgression of L2-5D (*i.e.*, G_0 of Figure 2) differs from its *D. simulans* homolog by only a single sterility gene. Both frequencies of fertility were relatively constant throughout the sampling period of several weeks. Thus, the simplest assumption that the introgressed segment contains a single male sterility gene without any other factors of partial sterility or inviability, is untenable. In fact, these data alone do not automatically suggest the existence of discrete major sterility factors. To demonstrate their existence, it is necessary to map these recombinants with DNA markers, as will be shown in the next section.

There are at least two possibilities for the noncomplementarity of the observations of Table 1. First, there may exist more than one major sterility factor in the interval between *f* and *Bx*—one is 42% of the distance from *f* to *Bx* and the other is 14% of the same distance from *Bx* to *f*. This hypothesis was tested by the molecular mapping shown in Figure 5. Second, within the *f-Bx* interval, only one major factor is present but a second major factor exists proximal to the *Bx* marker. In that case, one should not expect *f [Bx⁺]* males to be fertile. However, since the introgression has no visible marker proximal to *Bx*, its breakpoint on that end is variable. Our RFLP analysis of the L2-5D line at 18CD confirms that assumption. Thus, some chromosomes in the L2-5D line could have lost this second factor and the 14% fertility among *f [Bx⁺]* males is due to its polymorphism in the L2-5D line as shown in stipples in Figure 2. (A variation of this second explanation is that the factor is not polymorphic in L2-5D but is incompletely penetrant, allowing 14% of its carriers to be fertile. This possibility can be ruled out because the established *f [Bx⁺]* lines are >90% fertile.) We suspect that only 1/4 of the *[f⁺ Bx⁺]* chromosomes at that time of analysis had lost this second factor, resulting in the 14% fertility among the *f [Bx⁺]* males (= 58% × 1/4). In the later section on spermatogenic defects, we will describe two distinctive sterility phenotypes associated with *[f⁺ Bx⁺]* (Table 3 and Figure 7), an observation consistent with the hypothesis for the existence of two separate sterility factors. If this second hypothesis is correct, one would expect to observe complementarity when the putative second factor is eliminated (as shown later in Table 2).

In addition, there may be other factors of lesser significance that could still have cumulative effects on the fertility or viability. For example, some of the *[f⁺] Bx* recombinants from G_1 of Figure 2 are between 80–90% fertile after being propagated with attached-*X* females (data not shown). We also noticed that the total number of *f [Bx⁺]* males recovered are far fewer than the reciprocal kind, *[f⁺] Bx* (The two recombinant types examined in Table 1 are in proportion with the total number recovered.) Such inviability may again be due to introgressed factors proximal to *Bx*. Inviability associated with introgression has been reported in hybridizations where F_1 males are fully viable (HENNIG 1977; NAVEIRA and FONTDEVILA 1986; WU *et al.* 1993). Since such inviability may not be correlated with the size of introgression, its genetic basis remains to be explored. The results presented so far demonstrate the complexity of the genetic make up within the introgressions, which should be seriously heeded in any attempt at genetic mapping of hybrid sterility.

Physical demarcation II. (Fine mapping): Molecular mapping was done on a number of fertile *[f⁺] Bx* and *f [Bx⁺]* recombinant lines, each established from a single male as shown in the box at G_1 of Figure 2. Both the RFLP and SSCP analyses were carried out during this round of fine mapping. The selection of sterile recombinants took one more generation as shown in G_2 of Figure 2. It is important to note that the fertility or sterility is determined not from a single fly but from a population of flies with an identical genotype; therefore, our results are not affected by

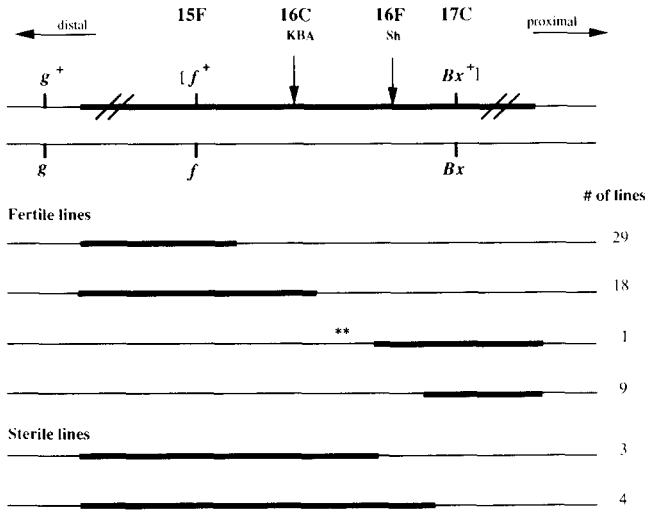


FIGURE 5.—Fine molecular mapping of the introgression lines generated in Figure 2; ** denotes a putative sterility factor.

incomplete penetrance or partial sterility factors. A fertile genotype is one that can be propagated with attached-*X* females and maintained as a stock (>90% fertile) and a sterile genotype is one that is less than 1% fertile.

A compilation of the molecular mapping results of the fertile and sterile lines are given in Figure 5. A total of 47 *[f⁺] Bx* fertile lines were examined. Only 18 of these have breakpoints beyond the 16C (KBA) DNA marker and none of these passes 16F. From the proximal side, of 10 *f [Bx⁺]* fertile introgressions examined only one passes the *Sh* marker and none extends all the way to 16C. This indicates that the factor is located in the interval between KBA and *Sh*. Fertile introgressions covering the distal side (*[f⁺] Bx*) can extend from 14A to beyond 16C. Fertile introgressions on the proximal side can extend from 16F to 18CD. Data on the sterile lines further strengthen this conclusion: three of seven sterile lines tested had introgressions that do not extend to 16F. We thus restrict the factor(s) to an interval between 16C and 16F, and most likely within the polytene band 16D or 16E (see below).

While it is still plausible that there are multiple minor genes within the interval 16DE, the data strongly support the existence of a major gene: the sterile introgressions are completely sterile and the fertile introgressions are on average more than 90% fertile. There does not appear to be a gradual decline in fertility as a function of the size of introgression. We thus name this putative major gene *Ods* (for *Odysseus*; see NOMENCLATURE in MATERIALS AND METHODS). Further refinement in molecular localization of *Ods* is currently underway. It is in fact possible to infer the location of *Ods* from the distribution of recombination breakpoints. On the distal side, 29 of the 47 breakpoints fall between 15F and 16C while 18 of them fall between 16C and *Ods*, suggesting its location approximately at 16D. From the proximal side, 14 breakpoints (including four sterile and 10 fertile recombinants) fall between 16F and 17C while four of them (three sterile and one fertile) are between *Ods* and 16F, suggesting, again, 16D. A crude estimate of the region between KBA at 16C and *Sh* at 16F is 500 kb (MERRIAM *et al.* 1991), within which we have so far 22 breakpoints. This gives an average of 22 kb between these breakpoints. It is also feasible to select for more recombinants in this interval.

Results from the molecular mapping also help explain the observations of Table 1. The map does not support the proposal for the existence of a second major sterility factor *within* the *f-Bx* interval (at 14% of the distance from *f* to *Bx*) to account for the low percent fertility in *f [Bx⁺]* males. On the other hand, the possibility of a second putative factor proximal to *Bx* does not contradict the molecular results. If it exists, it must be proximal to 18CD because some of the fertile *f [Bx⁺]* males carry the *D. mauritiana* introgression up to the 18CD marker (data not shown).

Recombination analysis on short introgressions: If there exists one and only one major sterility factor between *f* and *Bx*, recombination analysis of Table 1 does not provide the rigorous proof, mainly because the long introgressed segment appears to contain other factors of sterility or partial viability. In this second round of analysis, we created a set of five sterile lines with shorter introgressions by recombining off the proximal side of the original L2-5D introgression (see G_2 and G_3 of Figure 2). Because of their smaller sizes, these introgressions are less likely to harbor other factors that interfere with the recombination analysis. Stock keeping and the collection of recombinant males are also simpler as described in MATERIALS AND METHODS.

Mapping carried out on these five sterile lines with introgressions short of *Bx* did provide complementary frequencies of fertility for the recombinant genotypes. The results, shown in Table 2, indicate that the *g f [ ] Bx* recombinant type is 78% fertile, while the *g⁺[f⁺] Bx⁺* genotype is 24% fertile, summing up to 102%. There is some variation among the lines but the overall pattern is close to the expected complementarity. An interesting comparison between the results of Table 2 and Table 1 is that the proportion of fertile *g⁺[f⁺] Bx⁺* males of Table 2 (23.8%) is lower than that of fertile *g⁺[f⁺] Bx* males of Table 1 (42.2%), even though the recombinant products are genotypically comparable (except for the *Bx* marker, which has no apparent effect on male fertility). A most likely explanation is that the distribution of breakpoints in the G_2 females, *g⁺[f⁺] Bx/g f Bx⁺*, is uneven on both sides of *Ods*. These breakpoints may be more likely to fall between *Ods* and *Bx* where the chromatids are both of the *D. simulans* origin than between *f* and

TABLE 2

Recombination analysis on short introgressions

Genotypes	Sterile lines					Average fertility
	I	II	V	VIII	XV	
gf[ ]Bx	0.84 (n = 100)	0.80 (n = 99)	0.66 (n = 100)	0.83 (n = 42)	0.8 (n = 97)	78.5 (n = 438)
$g^+[f^+]Bx^+$	0.17 (n = 100)	0.31 (n = 100)	0.13 (n = 87)	0.33 (n = 46)	0.28 (n = 100)	23.8 (n = 433)

The numbers are the proportions of fertile males among those examined (n). The five sterile lines were derived from L2-5D (see G_1 and G_2 of Figure 2). These lines have introgressions extending from around 14B to around 16F, but not 17C.

Ods, where the chromatids are heterospecific. The result will be a decrease in the proportion of fertile $g^+[f^+]\ Bx^+$ males and an increase in that of fertile *g f [ ] Bx* males. This possibility will have to be explored in the future. (Note that the complementarity is expected even if the breakpoints are not uniformly distributed.)

The cytology of sterility: The sterility in these hybrids is strictly a germ-cell phenomenon, not one of somatic weakness. DOBZHANSKY and BEADLE (1936) have shown by pole cell transplantation that the male sterility in the F_1 hybrids between these species is germ cell-autonomous. JOHNSON and WU (1993) further demonstrated that the sterility caused by the introgression of the *[f⁺]*-region from *D. mauritiana* into *D. simulans* is not responsible for viability differences. Our cytological analysis of male sterility thus focuses on spermatogenic defects, especially on two clearly discernible stages of spermatogenesis in *Drosophila*—the early spermatid ("onion cell") and the sperm bundle stage (LINSDLEY and TOKUYASU 1980; KEMPHUES *et al.* 1982; HOYLE and RAFF 1990). In normal fertile males of *D. melanogaster*, early spermatids are usually made of two smooth and round bodies of similar size, a mitochondrial derivative (black in phase contrast microscopy) and a white nucleus (KEMPHUES *et al.* 1982). The presence of this structure, *i.e.*, the onion cells as shown in Figure 6A, is indicative of the completion of meiosis. Normal sperm bundles are smooth, elongated sacs where the sperm is packed in tight parallel arrangement until its individualization (Figure 6B). Previous studies of male-sterile mutations of *D. melanogaster* have found that spermatogenic defects often lack a definitive stage that can be specifically attributed to the action of the mutations (LINDSLEY and TOKUYASU 1980; FULLER 1993). A majority of them appear to arrest spermatogenesis at a relatively late stage, *i.e.*, after meiosis during spermiogenesis. This may be related to the absence of transcription regulation during spermiogenesis when all cellular components are self-assembled. In general, we found that spermatogenic defects in hybrid males, either F_1 or those carrying introgressions, often parallel the phenotypes of sterile mutations of *D. melanogaster* in their late action during sperm maturation. Some other sterile introgressions, however, appear to manifest their effects at an earlier stage (*e.g.*, JOHNSON *et al.* 1992 on the introgression of the distal end of the *X* chromosome). The following is a description of the spermatogenic development in each type of sterile males.

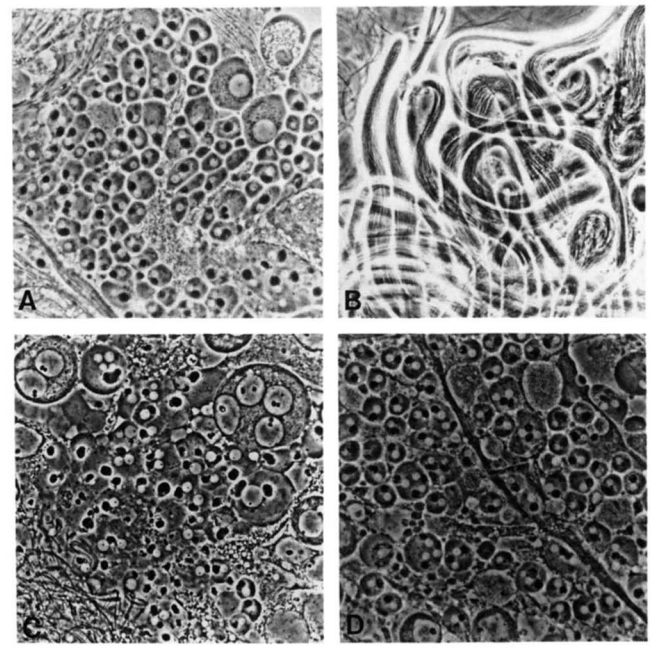


FIGURE 6.—The spermatogenic phenotypes of wild-type and F_1 hybrid males. (A) Cyst of normal onion cells in wild-type males. (B) Wild-type sperm bundles before individualization, typically smooth with tightly packed sperm. (C) Onion cyst in the F_1 hybrid males between *D. simulans* and *D. mauritiana*. Some onion cells show a disparity in size between the mitochondrial derivative (MD) and the nucleus. (D) Normal onion cyst in F_1 hybrid males between *D. simulans* and *D. sechellia*. Occasionally several nuclei may attach to an enlarged MD, likely a result of cytokinesis failure.

F_1 hybrid males: Due to the complex array of possible interactions, the sterility in F_1 hybrids is nearly impossible to assign to the action of any specific genes. Nevertheless, its phenotypic defects can serve as a useful comparison. The following is a synopsis from the observations made on 26 F_1 hybrid males from the cross between *D. simulans* females × *D. mauritiana* males. These males are always sterile but they go through the initial stages of spermatogenesis with no

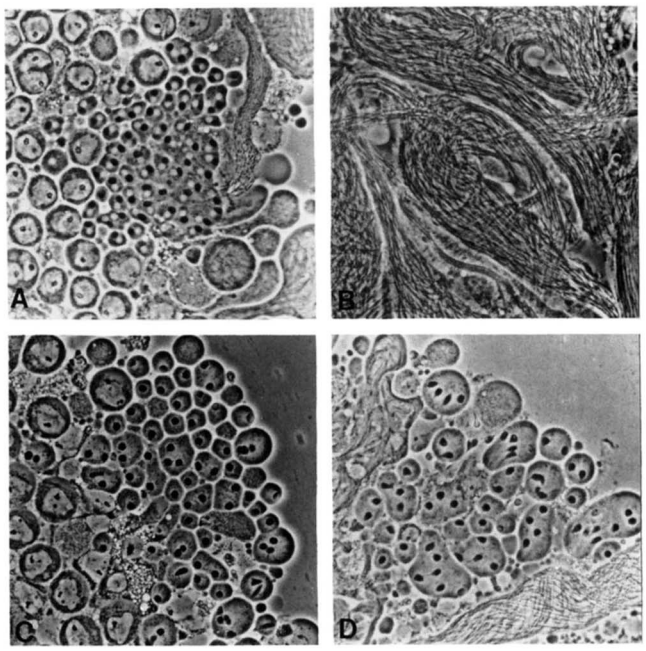


FIGURE 7.—The spermatogenic defects in males with a sterile introgression. (A)*[f⁺] Bx* sterile males have normal onion cells in their testes, but (B) their sperm bundles are disheveled. (C) Cysts of "anucleated" onion cells in *[f⁺ Bx⁺]* males. Nuclei are not apparent as in the wild-type males. (D) *f [Bx⁺]* sterile males' onion cells have the same "anucleated" appearance.

discernible defects. Inside their testes, cysts of growing and mature spermatocytes are abundant. Normally one nucleolar body per primary spermatocyte is observed but in some males spermatocytes having a disrupted nucleolus or several nucleolar bodies are occasionally seen. The early spermatids, or onion cells, are also mostly normal (Figure 6C), sometimes showing mitochondrial derivatives or nuclei of unequal size. Two nuclei attached to a larger mitochondrial derivative produced by the fusion of two normal ones were present sporadically. Nevertheless, these phenotypes are infrequent and do not represent a specific and consistent spermatogenic defect of these sterile males. The general impression with light microscopy is that of normal development until at least the completion of meiosis. The first consistent defect is observed rather late, at the elongation stage of spermiogenesis, where the sperm bundles fail to develop normally. The resultant disheveled bundles of sperm are never motile.

We also examined the F_1 hybrid males from the cross, *D. simulans* females × *D. sechellia* males. These hybrids are also always sterile. Cytological observations on a sample of 15 males reveal that the testis size, early spermatogenic development and even onion cysts usually appear normal (Figure 6D). Some males were found to have many abnormal onion cells where a fused and enlarged mitochondrial derivative is surrounded by two to four nuclei.

The effect of Ods in sterile [f⁺] Bx and f [ ] Bx males: The sterile *[f⁺] Bx* males of Figure 2 presumably carry only the *Ods* factor. Similar to F_1 males, the testes of these males are mostly of normal appearance in all the initial spermatogenic stages. Onion cells also appear normal (Figure 7A). At the sperm bundle stage, their sperm are in characteristic disarray (Figure 7B). After individualization the sperm often appear to be tangled or even broken. The disheveled sperm bundles rarely get into the seminal vesicle, which is instead filled with cellular debris. The agglomeration of disheveled bundles and degraded sperm are observed in the testis lumen. *Ods* thus resembles the majority of *D. melanogaster* male sterile mutations in its phenotypic effects (LINSDLEY and TOKUYASU 1980). Table 3 summarizes the observations on the sterile phenotype of *[f⁺] Bx* and *f [ ] Bx* males. The latter are presumably sterile for the same reason as the former.

TABLE 3

Spermatogenic development of various introgression genotypes

Sterility factors	Onion cell phenotype	
	"Anucleated"	Normal
Ods		
[f⁺]Bx	0	69
f[ ]Bx	0	16
Ods and the *Bx*-proximal factor		
[f⁺Bx⁺]		
G_{25}–G_{32}[a]	55	3
G_{38}	6	4
L2-5D.2[b]	6	0
L2-5D.1 and D.3[b]	0	10
f[Bx⁺]		
G_{32}–G_{38}	16	0

The number of sterile males examined that have either "anucleated" or normal onion cells are given.

[a] G_{25} denotes backcross generation 25. Because no marker proximal to *Bx* was used, the proximal ends of introgressions were gradually recombined off.

[b] Single females were bred at G_{42} and their sons examined at G_{43}.

Throughout this study fly cultures were normally maintained at 22–23°. To assess whether temperature influences the sterility or spermatogenesis of *[f⁺] Bx* males, two groups of females from the L2-5D.1 sterile line were raised at 18 and 28°. Samples of 10 *[f⁺] Bx* sons from each group were cytologically analyzed: changes in temperature did not revert the sterility associated with introgressions and no apparent differences were observed in their spermatogenic phenotype. Only the testis size of males raised at 18° showed an overall increase, but this may be a general physiological response to larvae's slower development in cooler temperature.

The joint effect of Ods and the Bx-proximal introgression in [f⁺ Bx⁺] and f [Bx⁺] males: Here we describe the phenotypes of *sterile [f⁺ Bx⁺] and f [Bx⁺]* males. Their testes are slightly smaller than those of wild-type

males. Most of the *[f⁺Bx⁺]* males in the L2-5D line examined (Table 3) showed an interesting phenotype up to the 40th generation. Cysts of onion cells with "anucleated" mitochondrial derivatives were found (Figure 7C), whereas the white nuclear body was barely showing or clearly missing. This phenotype was also observed in the *[f⁺ Bx⁺]* males from other lines, which should have a comparable *D. mauritiana* introgression. The mitochondrial derivatives of these "anucleated" onions were not structurally normal since they characteristically had a distorted shape and appeared less compact. Sperm bundles in these males are also defective, much like the disheveled bundles of F_1 and *[f⁺] Bx* sterile males. Particularly relevant was that the "anucleated" phenotype of *[f⁺Bx⁺]* males was shared by the *f [Bx⁺]* sterile males (Figure 7D), while the sterile *[f⁺] Bx* males only showed normal looking onion cells (Figure 7A). A possible explanation for this new phenotype is that other factors located in the introgression, but beyond the *Bx* marker, can influence spermatogenesis. We have reasoned from the observations of Table 1 that a sterility factor probably exists proximal to *Bx*. It is tantalizing to speculate that the "anucleated" phenotype represents the cytological effect of that second factor. The gradual disappearance of this "anucleated" phenotype in *[f⁺Bx⁺]* males indicates that recombination beyond the *Bx* marker may have caused its loss. The small amount of variation in the "anucleated" phenotype in earlier generations (see Table 3) is explainable by the polymorphism of the proximal end of the introgression. By the 43rd generation (G_{43}), many chromosomes in the lines had lost this second factor. At G_{43}, three sublines were established, each from a single female that carries an introgression with a different proximal breakpoint. Two of these lines produced families of *[f⁺ Bx⁺]* sons with normal onion cells and one with *[f⁺ Bx⁺]* males showing only "anucleated" onion cells.

DISCUSSION

The question: A central question concerning the genetic basis of reproductive isolation is if hybrid sterility/inviability is caused by the cumulative effect of many minor genes or if there exist discrete loci of complete or very major effect. Many questions such as "How many genes cause reproductive isolation?" or "What do these genes interact with?" (PANTAZIDIS and ZOUROS 1988; JOHNSON *et al.* 1992) have been based on the supposition of the predominance of major genes. Models pertaining to the evolution of reproductive isolation also depend on the assumption of the genetic architecture (*e.g.*, NEI, MARUYAMA and WU 1983; CHARLESWORTH, COYNE and BARTON 1987). Moreover, if major effect genes exist, strategies can be devised to isolate and characterize them by molecular techniques. On the other hand, if the underlying basis for hybrid sterility or inviability is polygenes of relatively minor effects, we can only strive to understand them in broader genetic terms as we do for quantitative trait loci (QTL; PATERSON *et al.* 1988; SHRIMPTON and ROBERTSON 1988).

The quest to identify major genes: There are basically two approaches to analyzing the genetic basis of reproductive isolation. The first approach exploits genes that are polymorphic within well-studied species, such as *D. melanogaster* or *Mus musculus*, in their effect on the viability or fertility of hybrids (WATANABE 1979; HUTTER and ASHBURNER 1987; HUTTER, ROOTE and ASHBURNER 1990; FOREJT *et al.* 1991; SAWAMURA, TAIRA and WATANABE 1993; Sawamura, Yamamoto and WATANABE, 1993). Such mutations are expected to be single genes of major effect because polymorphisms segregating in nature are much less likely to be due to many minor genes in linkage disequilibrium (unless recombination is suppressed). This has indeed been confirmed, within the limit of resolution, by genetic and molecular analyses in these studies. Because these polymorphisms may not necessarily represent true interspecific differences, this approach and a second one described below are complementary.

The second approach is to introgress the genetic materials from one species to another by hybridization. In the majority of studies, there was only one generation of backcross. Such backcross F_2 analysis (DOBZHANSKY 1936; HENNIG 1977; ZOUROS 1981; COYNE 1984) have provided a broad outline of the genetic basis of hybrid sterility. A salient finding of these studies is that many chromosome segments throughout the genome are associated with hybrid sterility, even between closely related species (see COYNE 1992 for a review). It is thus equivocal to interpret the results as due to either major genes linked to the markers or a large number of minor genes scattered through the whole genome (WU *et al.* 1993; WU and DAVIS 1993). A refined procedure is to carry out repeated backcrosses (WU and BECKENBACH 1983; NAVEIRA and FONTDEVILA 1986, 1991; COYNE and CHARLESWORTH 1986, 1989). The result is the introgression of a small segment of chromosome from another species, identified by visible markers, while the rest of the genome has been purified as summarized in Figure 1. The advantage of the introgression approach is the relatively clean genetic makeup of the sterile hybrids. Nevertheless, the introgression may still contain more than one gene affecting male fertility. Most studies showed that the introgressed segments are associated with sterility in some but not all of the males. The interpretation thus can be either (1) the existence of a major gene some distance away from the marker such that only some

introgressions contain this gene (COYNE and CHARLESWORTH 1986); or (2) several minor effect genes exist near the marker and a different degree of sterility is associated with each introgression (NAVEIRA and FONTDEVILA 1986, 1991). The evidence for major genes in these hybridization studies is not conclusive.

The demonstration of a single gene of a major sterility effect within the introgressed segment requires fulfilling three criteria: (1) the sterility is completely penetrant; (2) the sterility factor behaves like a "point mutation" by recombination analysis, and (3) this "point mutation" can be demarcated by physical markers to within a small chromosomal interval. When the interval is made sufficiently small, in the order of 100 kb or smaller, the problem of mapping is equivalent to an attempt at molecular cloning (*e.g.*, POWERS and GANETZKY 1991; VAN DER BLIEK and MEYEROWITZ 1991).

The first criterion of complete penetrance has rarely been applied. In most cases, the interpretation of penetrance is confounded by the heterogeneity in the genotypes analyzed. In the scheme of Figures 1 and 2, each introgression genotype is kept homogeneous by removing flies losing the desired flanking markers. Recombination analysis (criterion 2) was carried out by WU and BECKENBACH (1983), who reported complementarity in one but not in a second region on the *X* chromosome of *D. pseudoobscura*. NAVEIRA and FONTDEVILA (1986) used the synapses of polytene chromosomes as physical markers. Recently, ORR (1992) carried out a deletion complementation study of sterility associated with the introgression of the fourth (dot) chromosome of *D. simulans* into *D. melanogaster*, obtained by MULLER and PONTECORVO (1942). ORR concluded that either one or two major genes exist on the introgressed fourth chromosome. Since the deletion mapping is roughly equivalent to criterion 3 and the absence of crossover on this chromosome precludes recombination analysis, the discussion below on the roles of the three criteria is relevant to his conclusion.

Each of the three criteria plays a distinct role in excluding the alternative interpretations. For example, in comparing the extent of introgression in the 47 (=29 + 18) fertile and the 7 (=3 + 4) sterile $[f^+]$ *Bx* lines of Figure 5, one might conclude that a major gene exists on the original sterile $[f^+ Bx^+]$ line, L2-5D. However, if the 18 lines with an introgression beyond 16C, while fertile, had all been much less so than the other 27 lines, then the polygenic interpretation could not have been ruled out. In this way, criterion 1 complements criterion 3. Moreover, criterion 2 is also needed. It is still possible that sterility would occur in an all-or-none manner if the number of genes within an introgression is above or below a threshold. NAVEIRA and FONTDEVILA (1986) indeed argued that sterility is caused by introgressions above a critical length and NAVEIRA's observations (1992) further supported that interpretation. The requirement for the whole set of linked genes to confer a phenotype such as sterility is not unusual; for example, the Sex-Ratio meiotic drive requires all four genes within the inversion (WU and BECKENBACH 1983). In this view, the region of *Ods* demarcated by the fertile and sterile $[f^+]$ *Bx* introgressions at 16D can be interpreted as the last one in a constellation of minor factors that finally go above the threshold, leading to sterility. The recombination analysis (Table 2) and physical demarcation ($[f^+]$ *Bx* and *f* $[Bx^+]$ fertile lines of Figure 5) employing *two* markers flanking the putative sterility factor rule out the threshold model, which would not have predicted the introgression to exceed the threshold at the same location from both sides. Recombination analysis also confirms that there is only one sterility effect within the introgression. We have shown that, before the backcross generation 32, most of the sterile $[f^+ Bx^+]$ males in fact carry two independent sterility factors (Table 1 and 3). Without applying criterion 2, we might have misinterpreted the sterility phenotype of Figures 7C and 7D to be due to the *Ods* factor itself. Such independent sterility effects were detected by the recombination analysis.

In summary, we were able to show the existence of a major factor that fulfills all three criteria. This gene, *Ods*, is mapped to 16D, an interval of about 500 kb within which 22 introgression breakpoints are now available. It remains to be shown if the factor can be further assigned to an interval between two such breakpoints, 22 kb apart on average. Another important observation is that the introgressions, even relatively short ones, may often be genetically complex, affecting fertility, viability, and crossing-over distance. It appears that many genes of major or minor effects on viability, fertility or other attributes exist even between very closely related species.

The cytological location at 16D is in rough correspondence with COYNE and CHARLESWORTH'S (1986) estimate based on linkage data. Because the main assumption in their estimation procedure that a single major gene exists on only one side of the marker turns out to be valid, the agreement is not unexpected. Apparently, various fitness effects associated with the introgression did not bias the estimation very much. Nevertheless, such an assumption is not always valid as is the case of a second region on the same chromosome (the *v*-region; see Figure 6 of WU *et al.* 1993).

Other genetic effects of introgressions: In our analysis, male sterility is the most obvious phenotype in the hybrids but is hardly the only effect of the introgressions. While it is convenient to assume that the sole effect of the introgressions is on the phenotype of interest and, perhaps, due to a single major

gene, such assumptions need to be tested rigorously because they underlie the entire interpretation. The recombination analysis of long introgressions (Table 1) and short introgressions (Table 2) addresses those assumptions. The comparison shows that the structure of a relatively short introgression of about 15% of the *X* chromosome between two closely related species can be complex. There are at least three factors within the introgression—two lead to male sterility and one affects viability. (We speak of the second sterility factor loosely as we have only partial physical mapping data and have not done recombination analysis on it.) In the analysis of short introgressions, we were able to make the genetic construct relatively simple by recombining off the *Bx*-proximal region. The two reciprocal recombinant types become complementary in Table 2 and the differential recovery of the two types in Table 1 also disappears. The analysis is thus relatively free of confounding effects, such as the secondary male sterility or inviability, and the interpretation is less uncertain.

The observation of partial viability in Table 1 (the low recovery of *f [Bx$^+$]* relative to *[f$^+$] Bx* recombinants) is another element of complexity. There may be an intricate balance within the genome of each species that is disrupted by various combinations of genes from different species. Such a balance is embedded in WRIGHT's (1977) idea of universal epistasis. The phenomenon of F_2 breakdown (DOBZHANSKY 1970) in hybridizations, where F_1 is more fit than many F_2 genotypes, is a clear manifestation. Partial viability of this kind likely involves many genes with epistatic interactions. The observations of NAVEIRA (1992) on partial fertility between *D. simulans* and *D. mauritiana* may be of this category as well. He reported that the combination of two introgressions depress male fertility much more than the sum of the two separate effects and advocated the view that complete hybrid sterility represents the cumulative effect of such partial reduction in fertility. It is possible that complete male sterility in hybrids has many causes, including that of cumulative defects. However, while it remains to be shown that the partial reduction in fertility can, by accumulation, result in complete sterility, there is some evidence for the existence of genes with major effects on sterility. We have also found the partial fertility, associated with a given genotype, to be variable in different *D. simulans* autosomal backgrounds (ranging from 70% to more than 90% male fertile for the same *[f$^+$] Bx* introgression) (PEREZ unpublished results). It is thus preferable to consider partial fertility reduction separately from complete sterility. The former may be polygenic and variable whereas the latter is due to definable genes with major effects.

Evolutionary consideration: The allelic relationship, $Ods^{sim} = Ods^{sec} \neq Ods^{mau}$, suggests that Ods^{mau} may have been derived from Ods^{sim}, if we assume that the three species are nearly equally related (COYNE and KREITMAN 1986; CACCONE *et al.* 1988; SATTA and TAKAHATA 1980). The study of three species with a known phylogeny allows us to infer not only the changes but also the possible direction of such changes (see also JOHNSON 1992).

We are not making the claim that genes like *Ods* are "speciation genes" in the sense that they "caused" reproductive isolation. One of these genes (the first) should have been sufficient for the primary event of reproductive isolation. Besides, neither "speciation" nor its "causes" are easily definable in genetic terms. Regardless of whether the two species in question had ever been sympatric in their entire history (thus, genes like *Ods* would have an opportunity to play a role in reproductive isolation), the structure, function and evolution of such genes is still enormously fascinating. The quest is to understand the genetic bases and evolutionary forces underlying species divergence—how and why two closely related species have evolved such divergent and incompatible genetic pathways for spermatogenesis. Reproductive isolation is the consequence of such divergence in the reproductive biology of the respective species.

There has been considerable debate on the conceptual issues of reproductive isolation and speciation (OTTE and ENDLER 1989). The immediate objective of our research is to carry out the analysis of hybrid sterility in Drosophila strictly as a comparative developmental genetic study of spermatogenesis. While such an approach can be justified in its own right as a legitimate tool to understand an important developmental system, we are confident that the debate on the evolution of reproductive isolation and the concept of speciation will in the long run benefit from a pursuit of this nature.

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