Genetics of Hybrid Male Sterility Between Drosophila Sibling Species: A Complex Web of Epistasis Is Revealed in Interspecific Studies

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Manuscript received March 11, 1994
Accepted for publication June 6, 1994

ABSTRACT

To study the genetic differences responsible for the sterility of their male hybrids, we introgressed small segments of an *X* chromosome from *Drosophila simulans* into a pure *Drosophila mauritiana* genetic background, then assessed the fertility of males carrying heterospecific introgressions of varying size. Although this analysis examined less than 20% of the *X* chromosome (roughly 5% of the euchromatic portion of the *D. simulans* genome), and the segments were introgressed in only one direction, a *minimum* of four factors that contribute to hybrid male sterility were revealed. At least two of the factors exhibited strong epistasis: males carrying either factor alone were consistently fertile, whereas males carrying both factors together were always sterile. Distinct spermatogenic phenotypes were observed for sterile introgressions of different lengths, and it appeared that an interaction between introgressed segments also influenced the stage of spermatogenic defect. Males with one category of introgression often produced large quantities of motile sperm and were observed copulating, but never inseminated females. Evidently these two species have diverged at a large number of loci which have varied effects on hybrid male fertility. By extrapolation, we estimate that there are at least 40 such loci on the *X* chromosome alone. Because these species exhibit little DNA-sequence divergence at arbitrarily chosen loci, it seems unlikely that the extensive functional divergence observed could be due mainly to random genetic drift. Significant epistasis between conspecific genes appears to be a common component of hybrid sterility between recently diverged species of Drosophila. The linkage relationships of interacting factors could shed light on the role played by epistatic selection in the dynamics of the allele substitutions responsible for reproductive barriers between species.

In dim outline evolution is evident enough. But that particular and essential bit of the theory of evolution which is concerned with the origin and nature of species remains utterly mysterious.–BATESON (1922)

THE architects of the modern evolutionary synthesis provided a framework for investigating "the origin and nature of species" when they introduced the biological species concept. In sexually reproducing organisms, a species can be defined as a group of populations possessing inherent differences that prevent genetic exchange with other such groups (DOBZHANSKY 1935, 1937; MULLER 1938; MAYR 1940). The process of speciation, accordingly, is the separation of lineages into permanently isolated fields of genetic recombination; one approach to understanding this evolutionary process is to study the genetics of reproductive barriers between closely related species (DOBZHANSKY 1936).

The neo-Darwinian view is that these reproductive barriers arise as incidental by-products of the numerous genetic differences that accumulate inevitably between geographically isolated populations (*e.g.*, MAYR 1942; COYNE 1992). This verbal scenario has enjoyed widespread acceptance among biologists, probably because of its plausibility–diverging genomes simply become less and less likely to produce fit hybrids. There remains, however, a heated debate concerning almost every aspect of the dynamics of this process (TEMPLETON 1980; NEI *et al.* 1983; CARSON and TEMPLETON 1984; BARTON and CHARLESWORTH 1984; CHARLESWORTH *et al.* 1987; BARTON 1989; WHITLOCK and WADE 1994). Debated questions include: What are the relative roles played by natural selection *vs.* genetic drift in the fixation of those alleles that contribute to reproductive isolation? How quickly do reproductive barriers arise once populations are isolated? How important are factors such as population structure, genetic linkage, and epistasis in the dynamics of the inevitable divergence of separated gene pools?

Such abundant disagreement about fundamental assumptions suggests that we lack a sufficient empirical foundation for constructing genetical theories of speciation. Ideally, we would like to know the number, locations, effects on hybrids, and likely adaptive functions of the hereditary factors that reduce gene flow between incipient species (COYNE 1992). As a first step, numerous researchers have examined F_2 backcross hybrids between closely related species in the genus Drosophila (*e.g.*, DOBZHANSKY 1936; HENNIG 1977; ZOUROS 1981; COYNE 1984, 1985; COYNE and KREITMAN 1986; VIGNEAULT and ZOUROS 1986; ORR 1987, 1989; ZOUROS *et al.* 1988; HEIKKINEN and LUMME 1991; KHADEM and KRIMBAS 1991). Most of these studies have discovered at least one gene that contributes to hybrid sterility associated with every

genetic marker used, resulting in lower bounds that range from 5 to 9 loci (COYNE 1992). The implication, however, is that many more genes are involved, and this agrees with indirect estimates (*e.g.*, 50–200) based on the reduction in hybrid viability and measurements of gene flow across hybrid zones (BARTON and HEWITT 1981, 1985; SZYMURA and BARTON 1986, 1991). The F_2 backcross analyses have also demonstrated that, whereas morphological and behavioral differences between Drosophila species are due to genes distributed more or less equally among all chromosomes, the *X* chromosome has a disproportionate effect on hybrid fertility and viability (CHARLESWORTH *et al.* 1987; COYNE and ORR 1989). Whether this discrepancy between traits is actually due to a higher concentration of genes affecting hybrid fertility and viability on the *X* chromosome than on the autosomes, or is instead due to inherent differences between fertility/viability *vs.* morphology/behavior in the type of gene action that underlies phenotypic variation for these traits in natural populations, is not yet clear (WU and DAVIS 1993; H. HOLLOCHER and C.-I WU, unpublished results).

A related approach to studying the genetics of reproductive barriers is to introgress a defined chromosomal segment from one species into the genetic background of a closely related species (WU and BECKENBACH 1983; COYNE and CHARLESWORTH 1986, 1989; NAVEIRA and FONTDEVILA 1986; JOHNSON *et al.* 1992, 1993; ORR 1992; WU *et al.* 1993; PEREZ *et al.* 1993; CABOT *et al.* 1994). The purified introgression can then be subjected to detailed analyses of its effects upon hybrid fertility and/or viability. By manipulating the length of the introgressed segment, and determining the lengths of introgressions more precisely with molecular markers, these phenotypic effects can be attributed to small chromosomal regions. Although this sort of approach promises to provide many of the genetical details required by theoreticians, analyses of sufficient resolution have only been attempted in a small number of cases. As a result, most points of contention regarding the speciation process remain unresolved (*e.g.*, WU and DAVIS 1993; WU and PALOPOLI 1994). High resolution analyses of the hereditary factors that constitute reproductive barriers between closely related species are still sorely needed.

The three sibling species of the *D. simulans* complex provide excellent material for such work (COYNE 1984, 1985). Because hybrid females are fertile, genes can be moved between species by repeatedly backcrossing hybrid females to males of either pure species. Because they are homosequential to each other and closely related to *D. melanogaster* (LEMEUNIER and ASHBURNER 1976, 1984), it is relatively easy to acquire molecular markers for a chromosomal region of choice (WU *et al.* 1993). Although the species complex is believed to have originated in Africa, *D. simulans* is a cosmopolitan, human commensal; in contrast, both *D. mauritiana* and *D. sechellia* are endemic to small islands in the Indian Ocean (LACHAISE *et al.* 1986). Studies of DNA sequence variation at arbitrarily chosen loci have shown that there is little divergence between these species (many polymorphisms are shared), and the phylogeny remains unresolved (COYNE and KREITMAN 1986; HEY and KLIMAN 1993; KLIMAN and HEY 1993).

With respect to hybrid male sterility between *D. simulans* and *D. mauritiana*, previous studies have determined the following: (1) there is at least one factor that contributes to hybrid male sterility on every major chromosome arm, including the *Y* chromosome (COYNE 1984; JOHNSON *et al.* 1993); (2) there are at least seven such factors on the *X* chromosome of *D. mauritiana* that cause male sterility when introgressed into a pure *D. simulans* background (COYNE and CHARLESWORTH 1989; WU *et al.* 1993; PEREZ *et al.* 1993; CABOT *et al.* 1994); (3) the sterility factors that have been mapped may actually represent linked polygenic effects (NAVEIRA 1992); and (4) at least two of these factors appear to exhibit strong epistasis (CABOT *et al.* 1994).

In this study, we conducted a high resolution analysis of some of the genetic differences between *D. simulans* and *D. mauritiana* responsible for the sterility of their male hybrids. Specifically, we introgressed small segments from the proximal region of a *D. simulans X* chromosome into a pure *D. mauritiana* background. We then used recombination to create heterospecific introgressions of different sizes, determined their lengths more precisely using molecular markers, and conducted detailed analyses of their effects on male fertility. These were the first purified introgressions from the genome of the mainland species into the genetic background of one of the island endemics. The same segment was introgressed in the reciprocal direction (*i.e.*, from the island species into a genetic background from the mainland species) in a previous high resolution analysis of hybrid male sterility in the *D. simulans* clade (PEREZ *et al.* 1993). This allowed us to compare our results with that found for the reciprocal introgression, and in conjunction with this previous study it provides the first relatively complete picture of the genetics of hybrid male sterility between these species for a defined chromosomal segment. The results are discussed in relation to SEWALL WRIGHT's conviction that gene interactions play an important role in adaptive evolution (*e.g.*, WRIGHT 1931, 1965), as well as the general lack of convincing evidence for multilocus interactions as an important factor contributing to phenotypic variation within species (BARKER 1979; CLARK 1987; BARTON and TURELLI 1989).

MATERIALS AND METHODS

Stocks: The parental *D. simulans* stock was homozygous for three visible *X*-linked mutants [see LINDSLEY and ZIMM (1992) for detailed descriptions]: a recessive eye-color mutant,

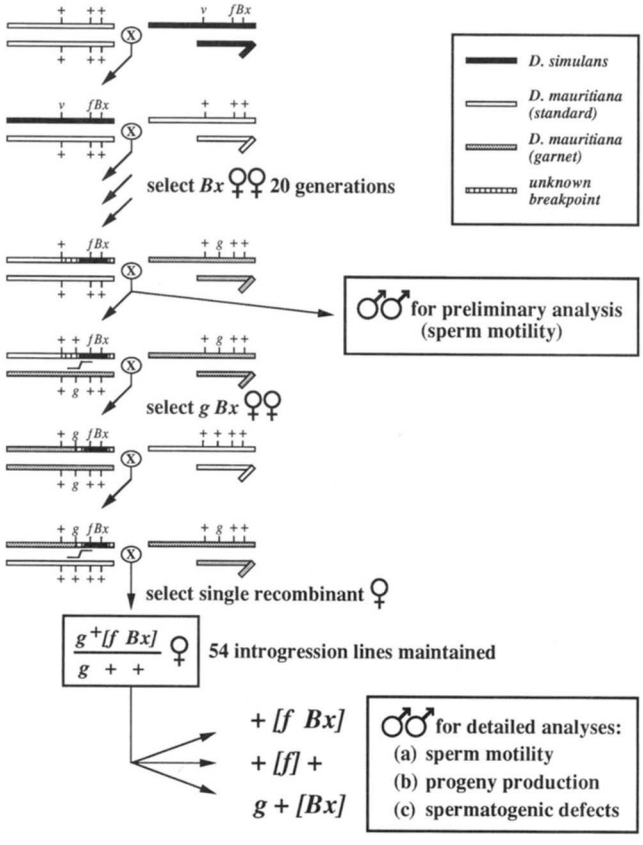

FIGURE 1.—Mating scheme used to create *X*-linked introgressions. The visible genetic markers *forked* (*f*) and *Beadex* (*Bx*)were used to keep track of the introgressed segment initially. Later, the marker *garnet* (*g*) was introduced for recombination analysis. See MATERIALS AND METHODS for more detailed descriptions of the stocks and crosses. The resulting 54 introgressions were maintained in females, and these are the introgressed lines compiled and categorized according to effects on male fertility in Table 1.

vermilion (*v*, 10A, 1–33.0); a recessive bristle-morphology mutant, *forked* (*f*, 15F, 1–56.7); and a dominant wing-morphology mutant, *Beadex* (*Bx*, 17A, 1–59.4). The cytological locations given (*e.g.*, 10A) are the band positions along the salivary gland polytene chromosomes, and recombination locations given (*e.g.*, 1–33.0) are the map positions, for the same genes in *D. melanogaster*. The map positions are known to differ slightly from those in *D. simulans* but retain the same linear order (STURTEVANT 1929; LEMEUNIER and ASHBURNER 1976; LINDSLEY and ZIMM 1992). The parental *D. mauritiana* stock (designated *MAU ST*) did not harbor any visible mutant markers. For recombination analysis, a *D. mauritiana* stock that carried the eye-color mutant *garnet* (*g*, 12B, 1–44.4) was used. The stocks are described in greater detail elsewhere (PEREZ *et al.* 1993). All fly cultures were maintained at 22–23° and reared on standard cornmeal medium.

Crosses: The crossing scheme used to introgress segments of an *X* chromosome from *D. simulans* into a pure *D. mauritiana* background is outlined in Figure 1. We started 60 independent introgressions of the multiply marked *D. simulans* chromosome into the *MAU ST* background. To purify the background of each introgression, we selected virgin females carrying the dominant *Bx* marker every generation and mated them to males from the *MAU ST* stock. We examined the resulting male progeny each generation in order to retain only those lines harboring introgressions that extended from distal of *f* to proximal of *Bx*. After 20 generations of backcrossing, 8 such lines remained. A preliminary analysis of the fertility of males carrying a particular introgression, as well as males harboring only smaller (recombinant) portions of each introgression, was then conducted (fertility assays are described below). In order to analyze the sterility factor(s) between *g* and *f* in greater detail, as well as to maintain the introgressions more conveniently with flanking markers, we used the recessive marker *g* and the dominant marker *Bx* to select 54 recombinant lines from the original 8 parental lines (Figure 1). Finally, we analyzed the resulting 54 lines for the degree of male fertility exhibited by the entire introgression and recombinant portions therefrom.

To verify the epistasis that appeared to be responsible for the male sterility associated with introgression category III, we performed the following experiment. (1) We selected one introgression of this category that rendered males sterile. (2) We selected fertile recombinant males from this parental line to create fertile recombinant lines. Fertile recombinant lines were selected from both the proximal and the distal ends of the original, sterile introgression (5 "distal" and 10 "proximal" fertile recombinant lines resulted). (3) We probed the fertile recombinant lines (the use of DNA markers is described below) and determined that the heterospecific segment in three lines extended from beyond 13F to beyond 16DE, while in two lines it extended from beyond 16DE to beyond 17C. Because both classes of fertile recombinant passed the same internal marker, we inferred that the introgressions overlapped. We chose two fertile recombinants of each type for detailed analyses of male fertility. (4) We made females heterozygous for the two overlapping fertile introgressions, chose from their progeny two recombinant females that had regained the original introgression, and assayed the fertility of the male progeny harboring this reconstituted introgression.

DNA markers: To determine the lengths of introgressions more precisely, as well as to verify the species origin of the internal portion of all introgressions, three DNA clones were used for Southern blot analyses of species-specific restriction fragment patterns. These clones and their cytological locations were as follows: *sd* at 13F, unnamed cosmid at 16DE, and *A57* at 18CD. These molecular markers are described in greater detail elsewhere (PEREZ *et al.* 1993; D. E. PEREZ and C.-I WU, unpublished results; European Drosophila Physical Mapping Consortium). Genomic DNA was prepared and Southern blots were carried out using standard protocols adapted from SAMBROOK *et al.* (1989).

Determination of fertility: We measured the fertility of males with heterospecific segments of different sizes. Three criteria were employed, as follows. (1) Sperm motility: males were separated by genotype and aged 3–5 days without access to females. Testes of individual males were dissected in a drop of Drosophila Ringer's solution, gently squashed under a coverslip and examined under phase-contrast microscopy. Males were classified (following COYNE 1984) as either fertile (at least one motile sperm visible) or sterile (no motile sperm). If the male was sterile, we determined the approximate stage at which spermatogenesis appeared to be arrested (following KEMPHUES *et al.* 1982). (2) Progeny production: one or two virgin males were placed in a vial with three to five virgin females. Both males and females were 3–5 days of age when combined. Vials were checked for presence of progeny at 7 and 10 days after the cross was started. Males were classified as either fertile (progeny of correct genotype present) or sterile (eggs but no larvae/pupae/adults present). Because a particular introgression generally exhibited very little variation in male fertility according to this criterion, no correction for the possibility of multiple fertile males per vial was necessary.

TABLE 1

Introgressions categorized according to effects on male fertility

Category of *[f Bx]* introgression		No. of lines	♂♂ progeny: genotypes and fertilities					
			[f Bx]		*[f] Bx*$^+$		*f*$^+$*[Bx]*	
			Sperm	Progeny	Sperm	Progeny	Sperm	Progeny
I.	*[f Bx]* fertile	9	0.99 (176)	0.96 (121)	1.00 (16)		1.00 (10)	
II.	*[f Bx]* sterile *f*$^+$*[Bx]* recombinants sterile Both with onion stage defects	4	0 (120)	0 (187)	0.45 (31)		0 (46)	
III.	*[f Bx]* sterile *f*$^+$*[Bx]* recombinants fertile *[f Bx]* with motile sperm	18	0.42 (554)	0 (644)	0.68 (71)	0.32 (94)	1.00 (27)	1.00 (34)
IV.	*[f Bx]* sterile *f*$^+$*[Bx]* recombinants fertile *[f Bx]* without motile sperm	23	0 (662)	0 (696)	0.26 (113)	0.06 (75)	0.99 (74)	1.00 (27)

Introgressions were categorized according to the effects of the intact heterospecific segment (*[f Bx]* genotypes) as well as the recombinants therefrom (*[f] Bx*$^+$ and *f*$^+$*[Bx]* genotypes) on male fertility. The "sperm" column for each genotype provides the proportion of males that had motile sperm when dissected. The "progeny" column provides the proportion of males that produced progeny when mated to virgin females. Combined sample sizes for all lines of a given category are in parentheses beneath each proportion. See *Determination of fertility* and *Categorizing [f Bx] introgressions* for additional details.

(3) Copulation and sperm transfer: for one category of introgression, males often managed to produce large quantities of motile sperm but always failed to produce progeny. A sample of 38 of these males were placed individually in vials with three to five virgin females (168 females total) as described in (2), above, and these vials were observed periodically over the next 3 days for the presence of copulating pairs. Then all males and females were sacrificed and dissected. Males were checked for sperm motility and spermatogenic defect as described in (1), above. Female reproductive tracts were examined for presence of stored sperm per LEFEVRE and JONSSON (1962). As a positive control, intraspecific matings (10 males and 32 females in 10 vials) were conducted in parallel with the experiment.

RESULTS

Categorizing *[f Bx]* introgressions: The effects on male fertility of all 54 *[f Bx]* introgressions, as well as that of both the proximal and distal recombinants from each, are presented in Table 1. Four categories of introgression were suggested by the data, as follows. (I) There were nine introgressions that did not sterilize males. (II) There were four introgressions that sterilized males and for which all proximal recombinants also sterilized males. In addition, males harboring either the entire introgressed segment or just the proximal portion exhibited characteristic early defects in spermatogenesis (see *Spermatogenic phenotypes* below). (III) There were 18 introgressions that sterilized males, for which proximal recombinants did not sterilize males, and for which males often managed to produce large quantities of motile sperm. Note that these males, if they harbored the intact introgression, never produced progeny when mated (0 out of 644 males tested) despite producing motile sperm in many cases (233 out of 554 males dissected). Although there was heterogeneity among these 18 lines for the proportion of males producing motile sperm (G_H = 174.7, $P \ll 0.005$; range = 0.17–0.93), suggesting that more than one introgression category might actually be represented, they were combined into one category in order to infer a *minimum* number of factors that contribute to male sterility (see *Inferring sterility factors*, below). (IV) There were 23 introgressions that sterilized males, for which proximal recombinants did not sterilize males, and for which males never produced motile sperm (0 out of 662 males dissected).

Inferring sterility factors: At least four factors had to be invoked to explain the observed relationships between introgression genotype and sterility phenotype. The inferences upon which this minimum estimate rests, as well as the approximate locations for each factor, are depicted in a simplified format in Figure 2. The actual set of comparisons necessary to infer each factor and its approximate location were more complicated (refer to Table 1 throughout).

Sterility factor 1: Because some males carrying *[f Bx]* introgressions were fertile (category I), the introgressed segment between *f* and *Bx* must have been insufficient to cause sterility on its own. For introgressions of category II, in contrast, males harboring either the entire introgression (307 out of 307 males by both fertility criteria) or just the proximal half (46 out of 46 males by dissection) were always sterile, and males of both genotypes exhibited identical, early defects in spermatogenesis (see *Spermatogenic phenotypes*). The implication is that there must be a factor proximal of *Bx* that contributes to male sterility (sterility factor 1, Figure 2). Because introgressions of all other categories exhibited no sterility associated with just the proximal half (categories I, III and IV), and because 1 out of the 4 introgressions of category II did *not* extend beyond 18D in the proximal direction (neither did any of the 50 introgressions of

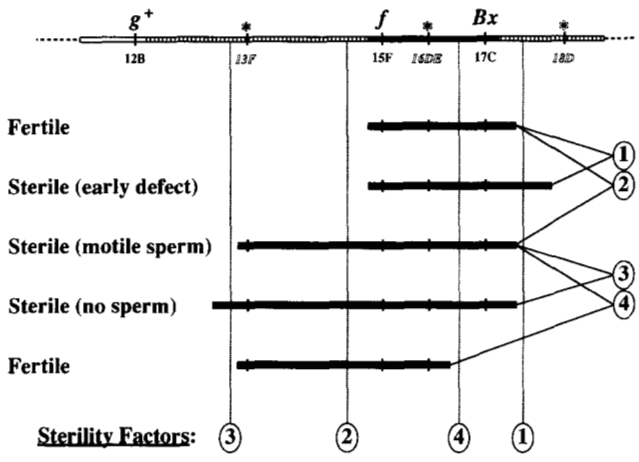

FIGURE 2.—Model depicting the *minimum* number of hybrid sterility factors that must be invoked to account for the categories of introgression and the fertilities of their recombinants. The comparison between introgression categories most relevant to inferring the existence of a particular factor is diagrammed on the right, and the inferred factors were arbitrarily numbered 1–4. The data which suggested this particular categorization of introgressions are detailed in Tables 1 and 2.

other categories), sterility factor 1 must lie between 17C and 18D. This inference was further supported by the fact that, in two of the four introgressions of category II, prolonged maintenance in females resulted in these lines changing to fertile introgressions (the portion of each introgression proximal of *Bx* was always unprotected from further recombination, whereas the portion between *g* and *Bx* was held by flanking markers). In one of these cases, the DNA-marker genotype for this introgression at cytological location 18D changed from *D. simulans* to *D. mauritiana*, suggesting that the proximal factor had been lost due to recombination. Note that 17 out of 31 males carrying only the *distal* half of the introgressions of category II were also sterile; this was attributed to variation among category II introgressions in their distal breakpoints (two out of the four introgressions appeared to carry a sterility factor(s) distal of *f* in addition to sterility factor 1).

Sterility factor 2: The introgressed segment between *f* and *Bx* was insufficient to cause sterility on its own (see *Sterility factor 1*). Males harboring introgressions of category III, however, were always sterile by the criterion of ability to produce progeny (0 out of 644 males tested). Because males carrying only the proximal portions of this category of introgression were always fertile (34 out of 34 by the same criterion), whereas males carrying only the distal portions of the same introgressions were usually sterile (only 30 out of 94 males produced progeny), the implication is that there is something *distal* of *f* that is contributing to male sterility (sterility factor 2, Figure 2). The fertility of a significant fraction of those males carrying just the distal half suggested that sterility factor 2 alone was insufficient to render males sterile, perhaps requiring a second factor located between *f* and *Bx*. This possibility was tested explicitly and confirmed (see *Sterility factor 4*). Note that a significant fraction of males in all 18 lines of category III were able to produce motile sperm, but never produced progeny (see *Categorizing [f Bx] introgressions*). Because DNA markers showed that 16 out of the 18 introgressions of category III did *not* extend distally beyond 13F, sterility factor 2 must lie between 13F and 15F.

Sterility factor 3: There were 23 introgressions that, like category III, appeared to carry at least one factor *distal* of *f* that contributed to male sterility (same reasoning as above, for sterility factor 2); in contrast to category III, however, males carrying category IV introgressions *never* produced motile sperm (0 out of 662 males dissected). This suggested the presence of an additional genetic factor distinguishing introgressions of categories III and IV (*i.e.*, sterility factor 3). This factor appeared to also lie distal of *f*, because males carrying the distal half of these introgressions were of lower fertility than were males carrying the distal half of category III introgressions (*i.e.*, 30 out of 94 for category III, *vs.* 5 out of 75 for category IV, produced progeny). This location for sterility factor 3 was supported by DNA marker results: all introgressions of category IV extended beyond the marker at 13F in the distal direction, whereas only 2 out of 16 introgressions of category III extended this far. Because the mutant marker *g* that was used to select recombinants lines at 12B, it seems likely that sterility factor 3 is situated between 12B and 13F (probably closer to 13F, although the precise recombination distance is not known).

Sterility factor 4: Because males carrying complete introgressions of category III were always sterile, whereas males carrying the proximal half alone were always fertile and males carrying the distal half alone were sometimes fertile, it seemed likely that something between *f* and *Bx* was interacting with sterility factor 2 (see above) to render males sterile. This was tested explicitly by selecting fertile recombinants that overlapped at 16DE, and then reconstituting the original, sterile introgression from these recombinants (see *Testing for epistasis*). Because fertile recombinants of category III that carried the distal half (*i.e.*, genotype *[f] Bx*$^+$) often stretched proximally from *f* to beyond 16DE, but never extended as far as *Bx*, sterility factor 4 was inferred to lie between 16DE and 17C.

Testing for epistasis: Introgressions of category III did not appear to harbor any single factor that was sufficient to cause complete male sterility (*i.e.*, males carrying either the proximal or the distal half of the introgression were often observed to be fertile; see Table 1). Fertile recombinants from both the proximal and distal halves of a category III introgression were probed with a DNA marker at 16DE and shown to overlap (*i.e.*, pass the same internal marker) in some cases. This suggested

TABLE 2

Demonstration that hybrid male sterility is due to strong epistasis between the proximal and distal halves of an introgression

Genotype	Description	Sperm	Progeny	Phenotype
[f 16DE Bx]	Sterile introgression (category III, Table 1)	0.45 (31)	0 (73)	Sterile
[f 16DE] Bx⁺	Distal recombinants (2)	0.98 (43)	0.99 (88)	Fertile
f⁺[16DE Bx]	Proximal recombinants (2)	1.00 (38)	1.00 (75)	Fertile
[f 16DE Bx]	Re-recombinants (2)	0.40 (55)	0 (130)	Sterile

The effects of the original *[f 16DE Bx]* introgression on male fertility are shown in the first row. Overlapping proximal and distal recombinants from this introgression were selected from a larger number of fertile recombinants based on DNA-marker genotypes at *16DE* (see *DNA markers* for details). Re-recombinants that contained the reconstituted (original) introgression were then generated (twice independently). "Sperm" and "progeny" columns are as for Table 1. See *Crosses* and *Testing for epistasis* for additional details.

that epistasis between genes in each half of the original introgression was responsible for the observed sterility (similar to CABOT *et al.* 1994). To verify this result, we measured the fertility of males carrying the overlapping recombinant segments (two proximal and two distal) as well as males carrying a reconstituted version of the original introgression (Table 2). The fertility of both (overlapping) recombinant classes, as well as the recovery of sterility in the reconstituted introgressions, demonstrated clearly that significant epistasis between introgressed segments underlies hybrid male sterility in this case. Males carrying either factor alone were completely fertile, whereas males carrying both factors together were completely sterile (depicted graphically in Figure 3). We concluded that at least two factors interact within this region to cause sterility. The simplest explanation (*i.e.*, that requiring the fewest sterility factors to be invoked) was that sterility factors 2 and 4 in Figure 2 interact to render males sterile.

Spermatogenic phenotypes: Distinct spermatogenic phenotypes were observed for sterile introgressions of different lengths (refer to Table 1 and Figure 2). Males with introgressions of category II never produced motile sperm and always exhibited early defects in spermatogenesis (Figure 4A). In particular, severe asymmetries in both the sizes and numbers of nuclei and mitochondrial derivatives were observed in onion-cell stage cysts (contrast Figure 4, A and B). The tails of the resulting spermatids degenerated into small fragments during elongation. Approximately 42% of the males harboring introgressions of category III managed to produce large quantities of motile sperm (Figure 4C), but these males always failed to produce progeny. The 58% that did not produce motile sperm exhibited the same spermatogenic phenotype as males carrying introgressions of category IV, in which males produced elongated spermatid bundles that appeared normal but the spermatids failed to individualize into mature sperm (Figure 4D). The sterility phenotype produced by introgressions of category III, which requires at least two interacting factors

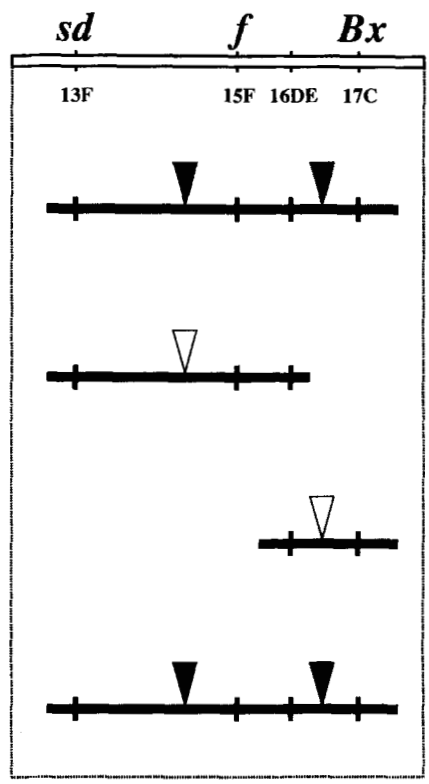

FIGURE 3.—Graphical representation of the experiment demonstrating that strong epistatic interactions between loci are responsible for sterility in male hybrids (data in Table 2). Starting with a sterile introgression (from introgression category III in Table 1), we produced fertile recombinants that overlapped at cytological location 16DE, and then selected for re-recombinants between them. Black inverted triangles represent factors 2 and 4 from Figure 2 interacting to render a male sterile, whereas white inverted triangles represent these same factors as insufficient to induce sterility on their own.

(see *Testing for epistasis*), was analyzed in greater detail (see below). It also appeared that an interaction between conspecific genes influenced the stage of spermatogenic defect: males harboring the entire introgressions of category IV never produced motile sperm, whereas 26% of the males carrying only the proximal

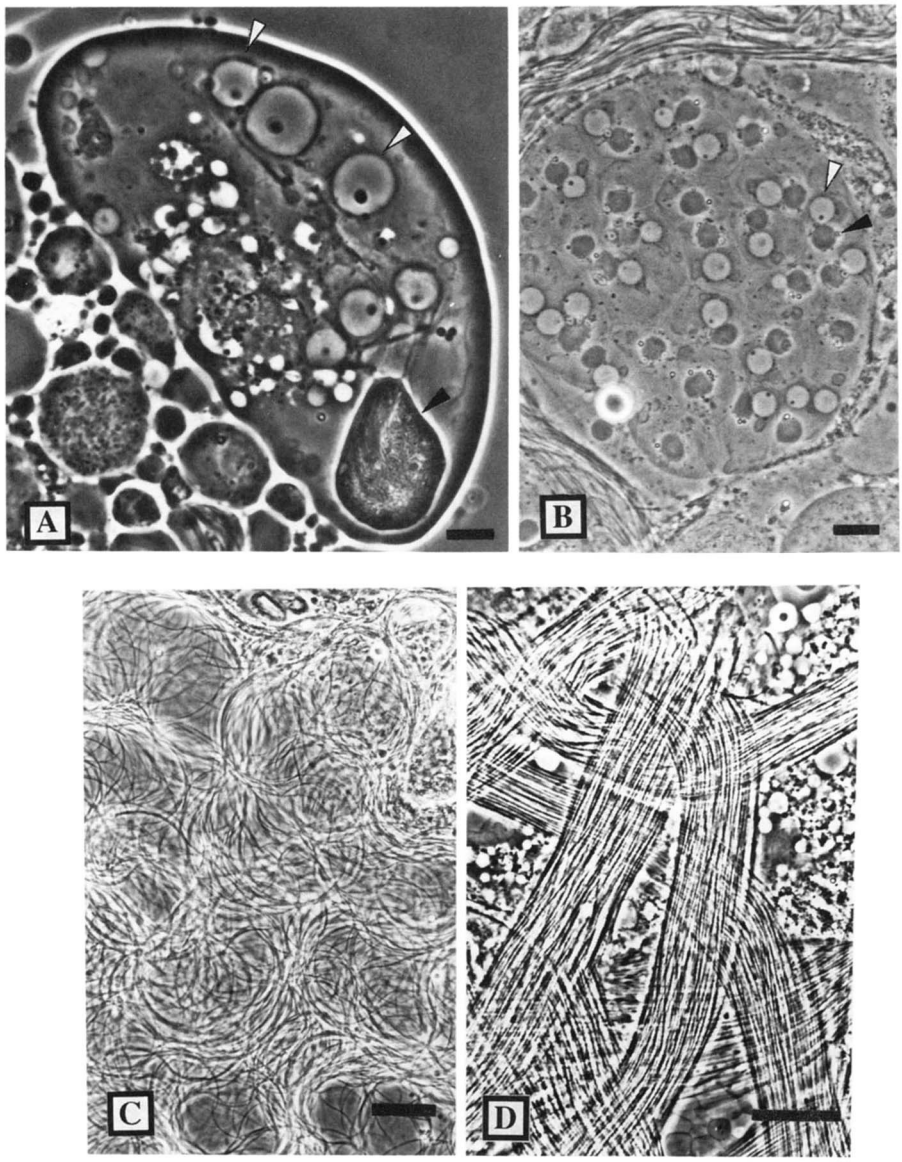

FIGURE 4.—Examples of the spermatogenic phenotypes observed in males harboring heterospecific introgressions (scale bars = 10 μm). (A) Males harboring sterility factor 1 (see Figure 3) exhibited severe asymmetries in both volumes and numbers of nuclei and mitochondrial derivatives of onion-nebenkern stage cysts. The black triangle indicates what is apparently a "giant" mitochondrial derivative that is in the same cyst as several nuclei of varying (abnormal) sizes (two nuclei are indicated by white triangles). The extent of such asymmetries varied among males and even among cysts within a male, but never approached the phenotype of wild-type males. (B) In contrast, males harboring sterility factors 2 + 4 exhibited onion-nebenkern stage cysts that appeared normal. In general, each cell within a cyst contained one nucleus (*e.g.*, white triangle) and one mitochondrial derivative (*e.g.*, black triangle) of approximately the same size. (C) Males harboring sterility factors 2 + 4 often managed to produce large quantities of motile sperm. Shown here are a small fraction of the several hundred motile sperm that were undulating vigorously under the coverslip after dissecting one such male. These males never inseminated females (Table 3). (D) The sperm bundles of males carrying sterility factors 2 + 3 + 4 appeared normal. This is a view of small portions of several sperm bundles (category IV male) that were each fully elongated and exhibited normal morphologies.

half produced motile sperm (Table 1, category III *[f]* Bx^+ recombinants). The most parsimonious explanation is that some combination of factors 2 and/or 3 interact with factor 4 to render males unable to produce motile sperm (analogous to the inferred interaction between factors 2 and 4, discussed above).

Effects of an introgression on a male's ability to copulate and transfer sperm: Males with introgressions of category III (Table 1 and Figure 2) often managed to produce large quantities of motile sperm (Figure 4C) but never produced progeny. In a separate experiment, these males were often observed copulating but never inseminated females (Table 3).

DISCUSSION

This study is one of a relatively small number of high resolution analyses of the genetics of hybrid male sterility, all of which have been conducted between species of Drosophila (ORR 1992; WU *et al.* 1993; PEREZ *et al.* 1993; CABOT *et al.* 1994). By introgressing small segments of an *X* chromosome from *D. simulans* into a pure *D. mauritiana* genetic background and assessing the fertility of males carrying heterospecific introgressions of varying size, we determined that (1) there was an extremely high density of genes that contribute to hybrid male sterility in this region; (2) epistasis between linked conspecific genes in a heterospecific background was responsible for hybrid male sterility in at least one case, and appeared to influence the stage of spermatogenic defect in another; and (3) different introgressions produced distinct sterility phenotypes, including two different spermatogenic defects and one category of males that often produced motile sperm but never inseminated females. Each of these conclusions, as well as their implications for our understanding of the origin and nature of species, is discussed below.

High density of sterility factors: Although this analysis examined only 20% of the *X* chromosome (roughly

TABLE 3

Effects of an introgression on the ability of males to copulate and transfer sperm

Source of males	Sperm	Progeny	Pairs obs. copulating	Sperm transfers
Sterile *[f Bx]* introgression (category III, Table 1)	0.53 (38)	0 (38)	17	0 (155)
D. mauritiana garnet stock	1.00 (10)	1.00 (10)	6	0.91 (32)

Males of introgression category III often produced large quantities of motile sperm but never produced progeny. "Sperm" and "progeny" columns are as for Table 1. "Pairs obs. copulating" is the number of mating pairs observed by simply checking the vials periodically during the three days that the crosses were going on. If each female mated only once, these represent 0.11 (17/155) and 0.19 (6/32) of the total number of copulations that actually occurred in the experimental and control vials, respectively. "Sperm transfers" is the proportion of females that had sperm in their reproductive tracts after three days with males of that type. See *Determination of fertility* for additional details.

5% of the euchromatic portion of the *D. simulans* genome), and the heterospecific segments were introgressed in only one direction, a *minimum* of four factors that contribute to hybrid male sterility were revealed (Figure 2). By extrapolation, and considering introgressions in both directions, we estimate a minimum of 40 such loci on the *X* chromosome alone. This minimum estimate is supported by observations of similar densities of hybrid male sterility factors for introgressions in the reciprocal direction between these species for three separate regions of the *X* chromosome, including the same segment analyzed in this study (PEREZ *et al.* 1993; WU *et al.* 1993; CABOT *et al.* 1994; A. W. DAVIS and C.-I WU, unpublished results). Because the relative densities of hybrid sterility factors on the *X* chromosome *vs.* the autosomes is still debated (COYNE and ORR 1989; WU and DAVIS 1993; H. HOLLOCHER and C.-I WU, unpublished results), at this point it is not clear whether a simple extrapolation to the entire euchromatic genome is valid. Nevertheless, considering that the number of factors invoked is a minimum estimate and that each factor must be interacting with a gene(s) in the *heterospecific* background in order to sterilize hybrid males, our results suggest that the total number of factors contributing to hybrid male sterility between these sibling species is on the order of at least 100 loci. Indirect estimates of the number of loci that reduce hybrid fitness, based on analyses of inviability and obstructed gene flow in the hybrid zones between species of grasshoppers or toads (BARTON and HEWITT 1981, 1985; SYRIMURA and BARTON 1986, 1991), also suggested the presence of many (*e.g.*, 50–200) factors that reduce hybrid fitness. As these authors have argued, the presence of so many factors that reduce hybrid fitness, scattered widely throughout the genome, is expected to serve as an extremely effective barrier to gene flow. The reason is that essentially every chromosomal segment harbors tightly linked factors that reduce fitness in a heterospecific background. As a result, it is unlikely that a segment of DNA would manage to recombine away from all such factors, and it would seem therefore that there is at present little possibility for the transfer of genetic material across the boundary between these two sibling species (despite the fact that hybrid females are essentially completely fertile).

Because these species exhibit little DNA-sequence divergence at arbitrarily-chosen loci (COYNE and KREITMAN 1986; HEY and KLIMAN 1993; KLIMAN and HEY 1993), it seems unlikely that the extensive functional divergence observed could be due mainly to random genetic drift. Even for the relatively small numbers of individuals sampled in DNA-sequencing studies (*e.g.*, 5 from each species in HEY and KLIMAN 1993), many molecular polymorphisms are demonstrated to be shared between these species. This suggests that, if a near-neutral polymorphism was segregating in the ancestor to these species, it is relatively likely to still be segregating in extant populations of both species. Although a fraction of these ancestral polymorphisms will have drifted to fixation for alternative alleles, one must ask what fraction of these neutral fixations would be expected to have significant effects on hybrid male fertility? Put another way, what fraction of alleles having such a drastic effect on fitness in some genetic backgrounds (*i.e.*, in hybrids), are likely to have been associated with a negligible selective value in the genomes of the pure species? Although the answer is not known, it seems unlikely to us that random drift of neutral alleles would result in so many hybrid male sterility factors in such a short time span (relative to the apparent sojourn times of most neutral variants in these species). This observation is particularly striking given that we do not observe many polymorphisms segregating *within* Drosophila species that can interact to render males sterile [*i.e.*, "synthetic sterility," see THOMPSON (1986) for a review]. Overall, there appears to be a discrepancy between the picture emerging from arbitrarily chosen genes, which are supposedly segregating near-neutral polymorphisms, and the picture from functionally divergent genes assayed by examining effects upon male fertility. Although there are many possible explanations for the apparent discrepancy between DNA-sequence and functional analyses, one plausible scenario is that selectively driven alleles have gone to fixation in both lineages independently, and that some fraction of these happen to have pleiotropic effects on hybrid male fertility.

One problem that must be kept in mind when extrapolating from the observed densities of hybrid sterility factors to the numbers of fixed differences between species that affect hybrids is that we have not collected polymorphism data for these factors within species. The experiments were conducted with just two lines from *D. mauritiana* (*MAU ST* and *MAU g*) and a single line from *D. simulans* (just one *X* chromosome) so we have really only measured the genetics of hybrid sterility between these genomes. These data do not, therefore, tell us anything about whether the interacting factors are polymorphic within either species. This is an important issue because the answer has the potential to change rather dramatically our picture of the genetic architecture of hybrid male fertility. For example, if most hybrid sterility factors are highly polymorphic within species, then any apparent discrepancy between the functional and DNA sequence analyses disappears.

The question of the relative frequencies within species of alleles that reduce hybrid fitness is certainly interesting and deserving of further study (*e.g.*, see WADE and JOHNSON 1994). Nevertheless, we feel that it is reasonable to assume, as a first approximation, that the genes being analyzed are representative of the allelic state for the species as a whole if the chromosomes were chosen arbitrarily with respect to the trait under study (the basic approach utilized by developmental geneticists). In fact, virtually all previous studies that have investigated the genetics of hybrid fitness reduction looked at only one line from each species. If, however, a chromosome is selected for study because of a known functional difference (*e.g.*, the macromelanophore phenotype in platyfish; WITTBRODT *et al.* 1989), then polymorphisms must be considered a primary concern. What are the relevant empirical observations? First, as mentioned above, there is the fact that both synthetic lethals and steriles are apparently quite rare within species (THOMPSON 1986). Second, there are at least three recent studies that address the levels of polymorphism of genes that reduce hybrid fitness: (1) The *Hmr* mutation within *D. melanogaster* suppresses the inviability of the hybrids between *D. melanogaster* and *D. simulans/mauritiana/sechellia* (HUTTER and ASHBURNER 1987; HUTTER *et al.* 1990). If one picks a random chromosome within *D. melanogaster*, the chances are greater than 0.99 that one would be studying Hmr^+. (2) JOHNSON *et al.* (1993) showed that *Y*-autosome sterility interactions in the *D. simulans* clade do not vary with either different *Y* chromosomes or different sets of autosomes. (3) D. E. PEREZ, H. HOLLOCHER and C.-I WU (unpublished results) found no functional variation for the *Ods* gene in *D. mauritiana* and only an extremely low level of variation in the interacting elements of *D. simulans*. Although the available data are meager, the relevant work that has been done supports the initial assumption that the levels of polymorphism are generally low for those alleles that alter fitness so dramatically in a hybrid genetic background.

Epistasis is revealed in interspecific studies: At least two of the sterility factors exhibited significant epistasis. In a heterospecific background, either factor alone left males able to produce sperm and progeny consistently; males carrying both factors together, however, usually failed to produce motile sperm and never produced progeny. In addition, gene interactions appeared to influence the stage of spermatogenic defect exhibited by sterile males in a different category of introgression. These results, considered in conjunction with parallel studies (CABOT *et al.* 1994; DAVIS *et al.* 1994; D. E. PEREZ and C.-I WU, unpublished results; A. W. Davis and C.-I Wu, unpublished results), suggests that epistasis between conspecific genes in a heterospecific background is a common component of hybrid fitness reduction in Drosophila. Our estimates of both the density of genes and the number of epistatic interactions are likely to be significant underestimates, as so far it seems that the finer the scale of the genetic and phenotypic analysis that is done, the higher the density of such loci that are uncovered.

The complex web of epistasis underlying hybrid male sterility raises a long-standing question in population genetics. What role does epistasis play in the evolutionary process? FISHER (1930) argued that, unless the loci in question are very tightly linked, selection operates primarily on the average additive effects of single genes; hence, gene combinations are assembled one gene at a time. He dismissed gene interactions as having only minor importance, similar to non-heritable modifications (FISHER 1918), and argued that evolution typically proceeds by the gradual accumulation of favorable alleles, each of which is weighed by natural selection in terms of its average effects across the genetic background of the entire species. Because each allele is tested in many genetic backgrounds, only those genes that are able to *consistently* enhance the fitness of their bearers will be favored by natural selection. As stated by Mayr (1963), "good mixers" are favored in this additive model of a simple cumulative system. Over evolutionary time scales, Fisher envisioned a long succession of "good mixers" marching through the gene pool, each laying but a single brick in what will ultimately become an adaptive edifice. Once gene pools have separated, they will eventually come to exhibit large differences due to the accumulation of many selectively driven allelic substitutions, each of small phenotypic effect, and the force propelling this continued selective response is a constantly deteriorating environment (FISHER 1930).

In contrast, WRIGHT (1931) believed that gene interactions are so universal that going from one favored combination of alleles to another often necessitates passing through genotypes that are of lower fitness. Wright

thus elevated gene interactions to a primary role in evolution and reasoned that geographically isolated gene pools could evolve to alternative, harmonious combinations of alleles most effectively by a combination of random genetic drift and subsequent selection (the shifting-balance process). Each population has the potential to develop its own set of "coadapted gene complexes" rather quickly because of the highly interactive nature of the genome and the importance of peak shifts for the evolutionary process (CARSON and TEMPLETON 1984).

More than a half century after these rival theories were proposed, their relative importance in natural populations remains debatable (*e.g.*, WADE 1992). One approach that has been taken to weigh the relative importance of the two paradigms for adaptive evolution is to measure the standing levels of epistatic variance within species for various phenotypic traits; another approach has been to look for linkage disequilibrium between polymorphic loci in natural populations. Although the results have been mixed, it is fair to say that there is a general lack of convincing evidence for the importance of multilocus interactions in natural populations (BARKER 1979; CLARK 1987; BARTON and TURELLI 1989; but see Zapata and ALVAREZ 1992, 1993).

Before dismissing the Wrightian view of evolution, however, it is important to consider the possibility that the conditions appropriate for the shifting-balance process to occur (*e.g.*, subdivided population structure and standing epistatic genetic variation) may indeed be rare, and therefore difficult to detect in extant populations within a given species, but that *occasional* bouts of evolution triggered by random genetic drift in local populations could nevertheless play a critical role in the evolutionary process. Indeed, it has been demonstrated that the shifting-balance process is possible under laboratory conditions (WADE and GOODNIGHT 1991). Evolution triggered by random genetic drift in local populations might be especially important in the creation of novelties (WRIGHT 1977; TEMPLETON 1986). This brings us to an important advantage gained by observing differences that have accumulated *between* species: even rare events may have left their signature in the genetic architecture of species differences.

It must be stressed that epistasis between *heterospecific* genes in a hybrid background is a well established observation that is consistent with both FISHER's and WRIGHT's views of the evolutionary process. The general neo-Darwinian explanation for the fact that deleterious interactions are observed when divergent gene pools are mixed in hybrids is that the entire series of allelic fixations in species A have proceeded without regard to whether they were "good mixers" with alleles at all of the loci in species B, and vice versa. So although the epistatic "variance" (as measured in hybrids) rises unchecked, this observation does not tell us anything about the forces responsible for the allelic fixations within species.

But what about the apparent epistatisis that we have observed between *conspecific* genes in a hybrid background? One plausible explanation for this observation is that it has nothing to do with the dynamics of the underlying allelic substitutions, and that hybrid fertility is simply a threshold trait (*e.g.*, RENDEL 1968). In other words, it could be that all of the biochemical interactions that are deleterious to hybrids are actually occurring between heterospecific genes, but that the hybrids remain relatively fertile unless the cumulative number of such interactions is greater than some threshold value; at this point, the production of progeny is no longer possible and complete sterility results. This sort of model is also plausible given our simplistic phenotypic measures of male fertility: for testes dissections, the presence of at least one motile sperm resulted in the classification of that male as "fertile"; for mating tests, the presence of at least one progeny resulted in the classification of that male as "fertile." Although males producing precisely one sperm or one progeny were never observed (data not shown), no attempt was made to quantify the relative fertility of individual males. This results in a dichotomous phenotypic classification that might be rather insensitive to the detection of any intermediate effects upon fertility.

Alternatively, it is also possible that some fraction of the observed epistasis between conspecific introgressions for negative pleiotropic effects in hybrids actually represents gene interactions that were involved in the *positive* phenotype(s) of these allelic substitutions and therefore played a critical role in the dynamics of the adaptive process. If so, this would lend credence to the Wrightian paradigm. Although we do not know the answer at this point, the fact that epistasis between conspecific genes is so pervasive in hybrids at least suggests that it is a common enough feature of genetic differentiation to warrant further investigation; this result is particularly interesting given the general lack of convincing evidence for epistatic variance underlying much of the total phenotypic variance in natural populations within species (but see WADE 1992).

Implications for simplistic models of neutral molecular evolution: The complication of epistasis is often dismissed by those studying molecular evolution because of a lack of large-scale linkage disequilibrium (KIMURA 1983; NEI 1987; but see BARKER 1979; ZAPATA and ALVAREZ 1992, 1993). The argument is that recent epistatic selection would be expected to have left a genetic "signature" in the form of non-random associations among loci. This may, however, be an insensitive measure of the importance of epistasis across evolutionary time scales because linkage disequilibrium is expected to decay rapidly due to recombination (LEWONTIN 1974). In other words, because surveys of standing polymorphisms within species are intrinsically biased to detect alleles

with long sojourn times (KIMURA 1983), they are also biased towards the analysis of associations among loci that have had ample time to decay due to recombination In contrast, whenever positive epistatic selection is sufficiently strong to overcome recombination we expect these allele pairs to go quickly to fixation; as a result, intraspecific surveys are unlikely to detect these events. The only cases in which intraspecific studies are not biased against detecting epistatic selection are (1) when recombination rates are extremely low in a region containing genes, and (2) when epistatic selection is acting to maintain a balanced polymorphism. In both of these cases, linkage disequilibrium is often found to be widespread [*e.g.*, Drosophila inversions, see PRAKASH and LEWONTIN (1968); Segregation Distorter system of meiotic drive, see WU and HAMMER (1991)].

In general, current models of molecular evolution assume a complete lack of epistatic selection. The fact that alleles having such strong, epistatic effects in hybrids are so pervasive, even between such closely related species, is at odds with the null hypothesis that most fixed differences are neutral and that every nucleotide can be treated independently. Perhaps something akin to the notion of "covarions" should be revived and incorporated into modern models of molecular evolutionary dynamics (FITCH and MARKOWITZ 1970). KIMURA (1985, 1990), by treating the neutral evolution of compensatory mutations, has at least made a start at incorporating epistasis into models of molecular evolution.

Varied effects on male fertility: The variety of phenotypic effects, in conjunction with the high density of genetic factors detected, agrees with WRIGHT's concept of "universal pleiotropy" and its importance for the evolutionary process (*e.g.*, WRIGHT 1977). Although it is clear from mutagenesis screens within *Drosophila melanogaster* that there are many genes that influence male fertility and their mutants can exhibit a variety of phenotypes (LINDSLEY and ZIMM 1992; FULLER 1994), these screens are only able to detect mutations that are of large effect individually. As a result, it is not clear *a priori* that these results can be extrapolated to the allelic variants that contribute to evolutionary change in natural populations. Our results suggest that there are indeed many genes whose differences, even between closely related sibling species, are sufficient to have significant pleiotropic effects on hybrid male fertility, and that these effects are relatively unpredictable in phenotype.

Linkage relationships of interacting factors: The introgression approach suffers from an intrinsic bias: it is best at detecting tightly linked sterility factors. For this reason, as well as the relatively small number of high resolution analyses that have been completed, we must be cautious in making any generalizations about the linkage relationships among hybrid sterility factors at this point. By combining many different pairs of fertile introgressions, one could in principle distinguish between a generalized polygenic model for this hybrid trait (*i.e.*, male sterility is a threshold trait and there are many cumulative differences that affect this trait approximately additively between species; NAVEIRA 1992) *vs.* a model invoking specific, epistatic interactions between conspecific loci. Unfortunately, many introgression-combinations might have to be tested for their effects on hybrid fertility to distinguish these models. Nevertheless, if epistatic selection was indeed important in the fixation of those alleles that now contribute to reproductive barriers between species, one straightforward prediction is that there will be a tendency for interacting factors to be more tightly linked than are non-interacting factors (WRIGHT 1965; LEWONTIN 1974). This is expected because tighter linkage increases the efficacy of epistatic selection. This prediction could be tested by combining fertile introgressions from different regions in a systematic manner.

We thank NORMAN JOHNSON, ANDREW DAVIS, MIKE WADE and BRIAN CHARLESWORTH for participating in many stimulating discussions. We thank NORMAN JOHNSON, MIKE WADE and MOHAMMED NOOR for providing criticisms of an earlier version of this manuscript. As usual, JERRY COYNE was extremely generous with his collection of Drosophila stocks. This work was supported by an National Institutes of Health (NIH) Genetics Training Grant to M.F.P. and NIH grants (GM39902, RCDA GM00553) to C.-I W.

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Communicating editor: A. G. CLARK