The Genetics of Reproductive Isolation in the *Drosophila simulans* Clade: *X vs.* Autosomal Effects and Male *vs.* Female Effects

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Manuscript received October 14, 1995
Accepted for publication April 1, 1996

ABSTRACT

A strong effect of homozygous autosomal regions on reproductive isolation was found for crosses between the species in the *Drosophila simulans* clade. Second chromosome regions were introgressed from *D. mauritiana* and *D. sechellia* into *D. simulans* and tested for their homozygous effects on hybrid male and hybrid female sterility and inviability. Most introgressions are fertile as heterozygotes, yet produce sterile male offspring when made homozygous. The density of homozygous autosomal factors contributing to hybrid male sterility is comparable to the density of *X* chromosome factors for this level of resolution. Female sterility was also revealed, yet the disparity between male and female levels of sterility was great, with male sterility being up to 23 times greater than female sterility. Complete hybrid inviability was also associated with some regions of the second chromosome, yet there were no strong sex differences. In conclusion, we find no evidence to support a strong *X* chromosome bias in the evolution of hybrid sterility or inviability but do find a very strong sex bias in the evolution of hybrid sterility. In light of these findings, we reevaluate the current models proposed to explain the genetic pattern of reproductive isolation.

EVALUATING models proposed to explain HALDANE's rule (HALDANE 1922) (the observation that the heterogametic sex is preferentially affected in species hybridizations) requires experimental evidence that is, at best, difficult to obtain. This is primarily true because unraveling HALDANE's rule requires dissecting the genetic patterns of species incompatibilities that, by their very nature, are traits that do not yield easily to genetic analyses. Most genetic studies have implicated the *X* chromosome as being principally responsible for causing hybrid sterility, and to a lesser extent, hybrid inviability in crosses between different species (DOBZHANSKY 1936; BENTLEY and HOY 1972; HENNIG 1977; COYNE and ORR 1989; RICHLER *et al.* 1989). Both this large *X* effect and HALDANE's rule have been explained by a model that predicts that the *X* chromosome will evolve more rapidly than the autosomes because of the increased opportunity for natural selection to act on sex-linked traits in hemizygous individuals, thus leading to a greater number of incompatibility genes in the heterogametic sex than in the homogametic sex in species crosses (CHARLESWORTH *et al.* 1987; COYNE and ORR 1989).

This model of the rapid evolution of the *X* chromosome is by no means the only explanation ever offered for HALDANE's rule. MULLER (1940) originally proposed that hybrid sterility and inviability in the heterogametic sex could be explained by an *X*/autosome imbalance. MULLER intuited that heterogametic F_1 hybrids would show a greater degree of incompatibility than homogametic F_1 hybrids, because they receive their autosomes from both species, yet receive their *X* chromosome from only one species, thus causing an "imbalance" in the relative contribution of *X* chromosome and autosomal genes from the two different species. MULLER envisioned this imbalance could result from a variety of genetic mechanisms such as direct incompatibility between interacting *X* and autosomal genes from the two species or differences in expression levels of *X* and autosomal genes from the two species in heterogametic hybrids. However, it has been implied that MULLER's hypothesis cannot explain hybrid sterility (COYNE 1985; COYNE and ORR 1989), yet may remain a plausible explanation for inviability (ORR 1993a; WU and DAVIS 1993). When hybrid females are made "unbalanced" with respect to their *X* chromosomes, *i.e.*, when females are made homozygous for the *X* chromosome of one species while containing autosome complements from both species, they do not become sterile even when F_1 males are completely sterile, yet they do become inviable when their F_1 male counterparts are inviable. Because of this difference in expression pattern between hybrid inviability and sterility, WU and DAVIS (1993) and ORR (1993a) have proposed that these two traits most likely represent different evolutionary processes that require separate explanations; thus, HALDANE's rule, in actuality, represents a composite phenomenon.

Given that MULLER's original hypothesis (1940) may

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explain the pattern of hybrid inviability, WU and DAVIS (1993) caution against the automatic acceptance of the model of the rapid evolution of the *X* chromosome as the most likely explanation for hybrid sterility, because the original data that indicate the *X* chromosome as having a greater effect on reproductive isolation are not conclusive. All previous studies supporting the large *X* effect on reproductive isolation have employed analyses that replace only one copy of each autosome and, thus, underestimate the autosomal effects if they are recessive (WU and DAVIS 1993; WU and PALOPOLI 1994). Whether the *X* chromosome would have a greater effect on reproductive isolation than the autosomes if the latter were analyzed as homozygotes has never been tested adequately before. Because of this lack of evidence, WU and DAVIS (1993) hypothesize other factors that may contribute to the rapid accumulation of alleles causing hybrid sterility, especially for species in which males are the heterogametic sex, factors such as sexual selection on traits associated with male fertility or increased sensitivity of genes involved in spermatogenesis itself (WU and DAVIS 1993).

More recently, ORR (1993b) and TURELLI and ORR (1995) have proposed the dominance theory, a formalized version of MULLER's original explanation for HALDANE's rule focusing on the dominance relationship of different incompatibility alleles and the effect that relationship has on the expression of *X* and autosomal genes in heterogametic *vs.* homogametic F_1 hybrids. The dominance theory indicates that HALDANE's rule will result if alleles affecting hybrid fitness are, for the most part, partially recessive (ORR 1993b; ORR and TURELLI 1995). Based on this premise, differences in fitness seen between heterogametic and homogametic F_1 hybrid offspring are predicted to reflect a bias in gene expression, rather than resulting from the number of hybrid incompatibility genes having accumulated differentially in the two sexes (ORR 1993b; TURELLI and ORR 1995).

Our study focuses on autosomal patterns of reproductive isolation as a means of deciding which model or set of models more closely approximates the evolutionary process that results in HALDANE's rule. Each of the models presented above makes a different set of predictions about the relative effects of heterozygous *vs.* homozygous autosomes, the relative effects of the *X* chromosome *vs.* the autosomes, the relative abundance of genes causing incompatibility in the heterogametic *vs.* homogametic sexes, and the pattern of sterility *vs.* inviability. All of these issues can be addressed simultaneously by systematically evaluating the heterozygous and homozygous effects of autosomal introgressions. By comparing *X* chromosome effects with *homozygous* autosomal effects, we eliminate the bias of earlier studies that compared *X* chromosome effects with *heterozygous* autosome effects and can test whether autosomal effects have been underestimated in the past. In addition, because crosses between autosomal introgressions produce equivalent genotypes in both males and females, biased gene expression associated with *X* chromosome traits are eliminated. In our autosomal studies, males and females are both effectively "homozygous" for their *X* chromosomes, and therefore any sex differences that we detect in sterility or inviability can be directly attributed to differences in the number or sex-specificity of autosomal genes rather than to differences in expression of hemizygous *vs.* heterozygous genes, which have complicated the interpretation of earlier genetic studies. In this autosomal study, we find that the apparent large *X* effect in reproductive isolation observed in earlier backcross analyses actually reflects an observational bias and not the intrinsic evolutionary dynamics of the *X* chromosome. Additionally, autosomal genes show a strong bias in heterogametic sterility even when equivalent male and female genotypes are compared, yet do not show a strong sex-bias for inviability. These results are discussed in light of the various hypotheses that have been proposed to explain HALDANE's rule.

MATERIALS AND METHODS

Drosophila stocks and culture conditions: The *Drosophila simulans* stock used to construct the autosomal introgressions was homozygous for five recessive markers on the second chromosome: a wing vein mutation, *net* (*n*, 2–0.0); a body-color mutation, *black* (*b*, 2–43.0); an eye facet mutation, *pearly* (*py*, 2–74.0); a wing-position mutation, *spread* (*sd*, 2–80.0); and an eye-color mutation, *plum* (*pm*, 2–103.0). The recombinational map distances for these markers in *D. simulans* are given in parentheses (STURTEVANT 1929), except for *net*, which corresponds to the value given for *D. melanogaster* (LINDSLEY and ZIMM 1992). A second *D. simulans* stock carrying three recessive markers on the *X* chromosome (*yellow: y*, 1–0.0; *vermilion: v*, 1–33.0; and *forked: f*, 1–56.7) and a single stock each of wild-type *D. mauritiana* and *D. sechellia* carrying no visible markers were also used and are discussed more fully in PEREZ *et al.* (1993) and CABOT *et al.* (1994). Fly cultures were reared on cornmeal at 22–23°. All stocks in this study were kindly provided to us by J. COYNE.

Introgression scheme: The crosses used to introgress segments of the second chromosome of *D. mauritiana* and *D. sechellia* into *D. simulans* are outlined in Figure 1. *D. simulans* females homozygous for the second chromosome markers (*net, b, py, sd* and *pm*) were crossed to either *D. mauritiana* or *D. sechellia* males. G_1 hybrid females were then backcrossed *en masse* to *D. simulans* males of the marker strain. Individual G_2 males carrying [+ + +] *sd pm*, *n* [+ + +] *pm*, or *n b*[+ + +] were backcrossed to *D. simulans* females of the marker strain to establish lines. In addition, individual G_2 females carrying these same introgressions were backcrossed to males of the *D. simulans* marker strain to establish lines in cases where none of the G_2 males for a particular introgression type were fertile (*e.g.*, for crosses involving *D. mauritiana*) and also to establish a second set of lines. Starting at these G_2 and G_3 female generations, only males were used subsequently to perform the backcrosses to the marker strain. Because there is no recombination in males, this scheme ensures that the introgressions were not eroded by recombination. Only the [+ + +] *sd pm* introgression of *D. mauritiana* went through an additional G_4 single female backcross, since none of the males from the G_2 and G_3 generations were fertile enough to establish lines. In total, 18 heterozygous autosomal

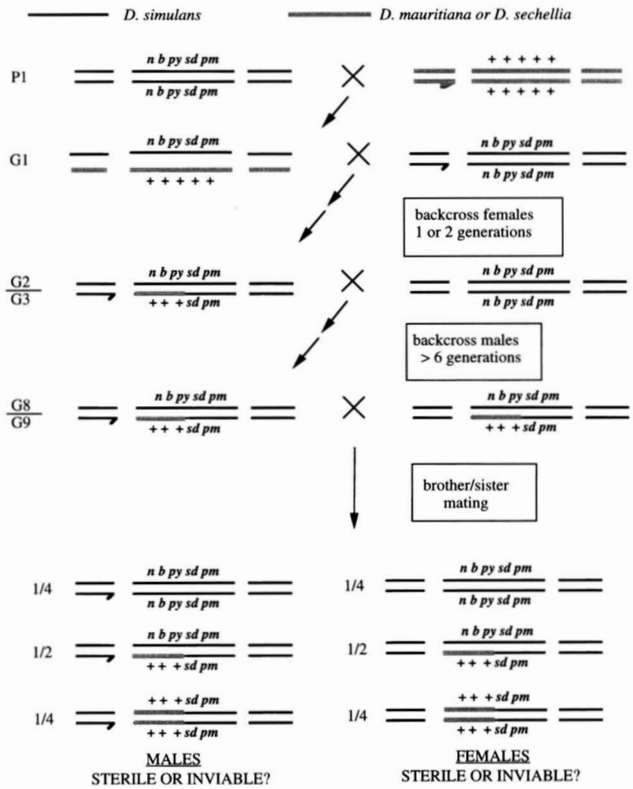

FIGURE 1.—Crossing scheme for constructing autosomal introgressions. The second chromosome visible genetic markers *net* (*n*), *black* (*b*), *pearly* (*py*), *spread* (*sd*), and *plum* (*pm*) were used to keep track of the introgressed segment. See MATERIALS AND METHODS for a more detailed description of the stocks and crosses. The resulting 18 lines were maintained by backcrossing heterozygous males to females of the marker strain. These heterozygous introgressed lines are tested for fertility in Table 1 and later tested for homozygous fertility and inviability effects through brother/sister mating as compiled in Tables 3–6.

lines were established as follows: *D. mauritiana,* two lines for the *n* [+ + +] *pm* introgression, three lines for the *n b* [+ + +] introgression, and a single line for the [+ + +] *sd pm* introgression; *D. sechellia,* four lines for the *n*[+ + +] *pm* introgression, three lines for the *n b* [+ + +] introgression, and five lines for the [+ + +] *sd pm* introgression.

Testing heterozygous introgressions: All males were tested for sterility or fertility by testcrossing them to *D. simulans* virgin females of the marker strain. Because these males are heterozygous for the introgression and there is no recombination in males, this cross produces two types of male and female progeny: those heterozygous for the introgression again and those homozygous for the marker strain alone. In the G_2 generation, 15 males for each introgression type were individually testcrossed. In the G_3 generation, 10 males derived from each of five different G_2 females backcrossed to males of the marker strain were scored for their fertility for a total of 50 males for each introgression from each species. The fertility of males in the G_8/G_9 generations was tested by crossing 12 males each from the heterozygous introgression lines to virgin females of the *D. simulans* marker strain.

Testing homozygous introgressions: Brother/sister matings in the G_8/G_9 backcross generations were performed to create individuals homozygous for the different autosomal introgressions established above. In these types of crosses (*e.g.*, [+ + +] *sd pm*/*n b py sd pm* × [+ + +] *sd pm*/*n b py sd pm*), several different genotypic classes of progeny result, including the recovery of individuals completely heterozygous for the introgression. Note that [+]/*m* and [+]/[+] individuals are not distinguishable until their progeny can be scored, where *m* denotes any of the five markers. Therefore, from these brother/sister matings, we selected 20 virgin females and 20 males that were phenotypically wild type for the three recessive markers used to define each introgression and then testcrossed them to the *D. simulans* marker strain to assess their fertility and determine their genotypes. For fertile individuals, at least 50 progeny from the testcross were scored for the phenotypic markers to determine whether the individual used in the cross was heterozygous or homozygous for parts of the introgressed region.

Single-strand conformation polymorphism (SSCP) analysis: Sterile individuals resulting from brother/sister matings were determined to be homozygous or heterozygous for the autosomal introgression by using SSCP analysis as described in CABOT *et al.* (1994). The three molecular markers used in this analysis were *phosphoglucose isomerase* (*pgi,* 2–58.6), *glyceraldehyde dehydrogenase 1* (*gapdh1, 2–57*) and *daughterless* (*da,* 2–41.5). The recombinational map distances for these markers in *D. melanogaster* are given in parentheses (LINDSLEY and ZIMM 1992). Use of the single *pgi* marker (which lies between phenotypic markers *b* and *py*) was usually sufficient for a minimal estimate of the effects of homozygosity in producing sterile individuals for all three introgressions. Primers for *pgi* that correspond to bases 1138–1157 and 1448–1466 of the *D. simulans* sequence in GenBank (accession number L27550) were used to amplify a 500-bp fragment by annealing at 45° for the first round of amplification and then at 50° for all subsequent rounds. The resulting PCR product shows no size differences between *D. simulans, D. mauritiana,* and *D. sechellia* on an agarose gel, yet shows species-specific migration patterns on SSCP gels. *Gapdh1,* which maps to the same region of the second chromosome as *pgi,* was substituted in a few samples when amplification with the *pgi* primers proved to be difficult. Primers for the 3′ region of *gapdh-1* correspond to bases 1327–1357 and 1871–1890 from the published *D. melanogaster* sequence (TSO *et al.* 1985). The 550-bp fragment of *gapdh1* was amplified by annealing at 60° for the first round of amplification and at 63° for all subsequent rounds. Although the *gapdh1* PCR product is slightly smaller for *D. simulans* than for *D. mauritiana* and *D. sechellia* products when run on agarose gels, heterozygotes were indistinguishable and had to be run on SSCP gels to detect species-specific patterns. The *da* marker (which lies between phenotypic markers *net* and *b*) was used to gain additional information for the [+ + +] *sd pm* introgression in *D. mauritiana.* A 1.8-kb fragment that includes the 1.6-kb intron from *da* was amplified by annealing at 59° for all rounds of amplification using primers corresponding to bases 53–79 and 207–223 of the published *D. melanogaster* sequence (CAUDY *et al.* 1988). The *da* product was digested with *Hha*I at 37° before SSCP analysis, yielding two fragments: one ~550 bp and the other ~1250 bp. No size differences between the three species were detected on agarose gels. SSCP analysis revealed the smaller-sized fragment of the *Hha*I digest to be the most informative in distinguishing the three species. All PCR reactions were 10 μl in volume and contained 5 μCi of [^{32}P-α]dATP. SSCP fragments were analyzed on 0.4 mm gels made of 6% Protogel (National Diagnostics, catalog no. EC-890) with 10% glycerol in 0.6% TBE buffer and run at room temperature at a constant 3.5 W for 14–16.5 hr.

Criteria of fertility/sterility: In all crosses, sterility was defined as the inability to produce progeny in test crosses. Females were scored as fertile if they produced any progeny. Males were only scored as fertile if they were able to produce

progeny of the introgression genotype as well as the marker strain to guard against a nonvirgin female giving a spurious result.

Recombination analysis of the visible markers: To verify that the map distances for the visible markers as determined by STURTEVANT (1929) within *D. simulans* actually reflected the recombinational distances one would get from mapping the markers between two different species, we empirically determined the "between species" map distances for crosses between *D. simulans* and *D. mauritiana* and between *D. simulans* and *D. sechellia*. Mapping of the markers was performed simultaneously with the scoring of heterozygous females from the G_8/G_9 brother/sister matings of the introgression lines. When test crossed to males of the *D. simulans* marker strain, these heterozygous females provide typical three-point cross data, except that in this case, recombination occurs between segments coming from two different species. The distance in cM between a pair of markers can be calculated as cM = $[0.25 \ln\{(1 + 2r)/(1 - 2r)\}]100$, where r is the observed frequency of recombination between the two markers (Crow 1983). To calculate percentage recombination from cM, the reverse equation was used, $r = 0.5 \tanh(2 \text{ cM}/100)$, where tanh represents the hyperbolic tangent (Crow 1983).

Calculating the expected frequencies of the different genotypic classes for the introgression lines: Figure 1 simplifies the expected frequency distribution of progeny resulting from the brother/sister matings by assuming no recombination. Because recombination does occur, the between species recombination data was used to calculate the expected frequencies of the different genotypic classes for each introgression line, assuming no sterility or inviability. For example, the raw recombination data for females heterozygous for the [+ + +] *sd pm* introgression gave the following frequency distribution: nonrecombinants, 0.44; single recombinants between the *b-py* markers, 0.20; single recombinants between the *n-b* markers, 0.27; and double recombinants, 0.09. These raw data were used to calculate the expected frequencies of the different gametes produced by the heterozygous females in the brother/sister matings: [+ + +] *sd pm*, 0.22; *n b py sd pm*, 0.22; [+ +] *py sd pm*, 0.10; *n b* [+] *sd pm*, 0.10; [+] *b py sd pm*, 0.135; *n* [+ +] *sd pm*, 0.135; *n* [+] *py sd pm*, 0.045; and [+] *b* [+] *sd pm*, 0.045. Recombination does not occur in male Drosophila; therefore, the expected frequency of the male gametes is 0.5 for [+ + +] *sd pm* and 0.5 for *n b py sd pm*. Multiplying these frequencies together in a regular Punnett square gives the expected frequencies of all possible progeny produced in the brother/sister matings (16 classes). However, we were only concerned with testing the males and females that were phenotypically wild type for *n-b-py* markers used to define this introgression (nine of the 16 classes). The expected frequencies for this subset of the progeny were obtained simply by dividing each genotypic frequency by the sum of the frequencies for all the classes we were considering. This standardized frequency distribution was then multiplied by the total number of progeny scored to get the expected numbers for each genotypic category. Deviations from these expectations were measured using a chi-square analysis to test for inviability effects of homozygous autosomes. These deviations reflect viability differences rather than meiotic transmission biases in males, because the female gametes are the primary determinants of the genotypes produced (for the most part, the genotypes being considered in the brother/sister matings are only those that result when the male contributes the [+ + +] *sd pm* gamete). Expectations and deviations for the other introgression types were calculated similarly using the appropriate raw data for each introgression type.

Testing *X* chromosome introgression inviability: To compare homozygous autosomal inviability to *X* chromosome inviability, *X* chromosome introgressions of a size comparable to the size of the autosomal introgressions were also analyzed. F_1 females from crosses between *D. mauritiana* or *D. sechellia* and *D. simulans* carrying the *yellow, vermilion* and *forked* markers were backcrossed to males of the marker strain. G_2 males and females were then scored for the presence of the three different markers and these observed numbers were compared to the expected numbers for the different genotypic classes also using a chi square analysis.

RESULTS

Fertility of heterozygous autosomal introgressions: Table 1 shows the number of fertile and sterile males obtained for the introgressions at different backcross generations. The G_2 males in this table are equivalent to males analyzed in a typical backcross analysis. The male sterility observed in these early backcross generations is quite extensive and heterogeneous within and among the three different introgression types. As the introgressions are further purified by backcrossing to the marker strain, however, sterility is absent by the G_8/G_9 backcross generations, indicating that the sterility observed in the G_2 and G_3 generations is due to the background heterogeneity and not necessarily to the individual introgressed segment. As heterozygotes, the second chromosome introgressions have no detectable effect on hybrid sterility and inviability in the purified *D. simulans* background.

The one exception is the heterozygous introgression from *D. mauritiana* covering [+ + +] *sd pm*. Although 6/50 males tested at the G_3 generation showed some fertility, these males were only very weakly fertile. Only one fertile line could be established in the G_4 generation and was determined to be a double recombinant in the region between the *b* and *py* markers based on SSCP analysis of the *pgi* molecular marker. Therefore, introgressions from *D. mauritiana* into *D. simulans* covering this region are not fertile as heterozygotes.

Recombination analysis of the visible markers: Table 2 shows the results of the between species mapping analysis. The recombination frequencies measured for each pair of markers are not significantly different for the two species (*n-b*, $\chi^2 = 2.263$, 1 d.f., $P > 0.10$; *b-py*, $\chi^2 = 0.007$, 1 d.f., $P > 0.90$; *py-sd*, $\chi^2 = 0.198$, 1 d.f., $P > 0.10$; *sd-pm*, $\chi^2 = 0.173$, 1 d.f., $P > 0.10$); therefore, values for *D. mauritiana* and *D. sechellia* were averaged before calculating the cM for the five markers. Although the map distances are very similar to those determined by STURTEVANT (1929) for within *D. simulans* analyses, the values calculated in this analysis for three out of the four sets of markers differ significantly from those reported earlier (*n-b*, $\chi^2 = 2.823$, 1 d.f., $P > 0.05$; *b-py*, $\chi^2 = 9.195$, 1 d.f., $P < 0.01$; *py-sd*, $\chi^2 = 61.103$, 1 d.f., $P < 0.001$; *sd-pm*, $\chi^2 = 5.079$, 1 d.f., $P < 0.05$). Because within species *vs.* between species recombination frequencies differed, the between species frequencies calculated in this study were used for all subsequent analyses.

TABLE 1

Male fertility of heterozygous introgressions in different backcross generations

Backcross generation	$\frac{n\ b\ +\ +\ +}{n\ b\ py\ sd\ pm}$ Fertile	Sterile	Fertility (%)	$\frac{n\ +\ +\ +\ pm}{n\ b\ py\ sd\ pm}$ Fertile	Sterile	Fertility (%)	$\frac{+\ +\ +\ sd\ pm}{n\ b\ py\ sd\ pm}$ Fertile	Sterile	Fertility (%)
D. mauritiana									
G_2	0	15	0	0	15	0	0	15	0
G_3	20	30	40	19	31	38	6	44	12
G_8/G_9	36	0	100	24	0	100	12	0	100
D. sechellia									
G_2	4	11	27	7	8	47	1	14	7
G_3	27	23	54	31	19	62	29	21	58
G_8/G_9	36	0	100	48	0	100	57	3	95

Male fertility of heterozygous introgressions in different backcross generations. All males were tested for sterility or fertility by testcrossing them to *D. simulans* virgin females of the marker strain and scoring progeny production. (See MATERIALS AND METHODS for details.)

Analysis of *D. sechellia* homozygous autosomal introgressions: A subset of the heterozygous lines established above was tested for homozygous effects on sterility and inviability by mating individual males and females resulting from brother/sister matings. For *D. sechellia*, three lines each for the *n* [+ + +] *pm* and *n b* [+ + +] introgressions and five lines for the [+ + +] *sd pm* introgression were tested. In total, ~22,000 progeny were scored to genotype 433 different individuals produced from brother/sister matings of the autosomal introgression lines. Table 3 summarizes the results obtained for *D. sechellia* females. The first important result in this table is that female sterility does not occur to any appreciable extent for any of the introgressions. The second important result is that inviability is very pronounced for all three introgression types, as exhibited by a significant excess of heterozygotes being produced and a deficiency of females homozygous for the entire introgression. All three introgressions show significant deviations from expectations (*n b*: $\chi^2 = 30.101$, 4 d.f., $P < 0.001$; *n pm*: $\chi^2 = 16.295$, 4 d.f., $P < 0.01$; *sd pm*: $\chi^2 = 58.361$, 6 d.f., $P < 0.001$). In each case, the most severe reduction in the observed numbers comes from the genotypic class homozygous for the entire introgression, which contains zero individuals for the *n b* [+ + +] and *n* [+ + +] *pm* introgression types. It can also be seen that the genotype having a larger region of the introgression homozygous is reduced relative to the other recombinant class it is paired with that contains a smaller homozygous region.

Table 4 summarizes the analysis of the *D. sechellia* homozygous autosomal introgressions in males. The greatest difference between the results of Table 3 and Table 4 is the amount of sterility observed for the males as compared to the females. Whereas only two out of a total of 215 females were sterile (0.9%), 47 out of 218 males from equivalent genotypic classes were completely sterile (21.6%). Molecular analysis of the sterile males revealed that for the *n* [+ + +] *pm* introgression, 78% (14/18) of the sterile males were homozygous for the *b-py* region of the introgression. For the [+ + +] *sd pm* introgression, 67% (12/18) of the sterile males were homozygous for the molecular marker. There was no discrepancy between males homozygous or heterozygous for *pgi* for the *n b* [+ + +] introgression. When the observed numbers of heterozygous and homozy-

TABLE 2

Recombination distance in species hybrids

Markers	*D. sechellia*/ *D. simulans* recombinants[a]	*D. mauritiana*/ *D. simulans* recombinants	Hybrids combined Average (%)	cM	Map	*D. simulans*[b] cM	Map
n-b	1565/4312 (36.3)	127/391 (32.5)	35.97	45.3	45.3	43	43
b-py	1982/6827 (29.0)	552/1908 (28.9)	29.01	33.1	78.5	31	74
py-sd	227/5601 (4.1)	119/3086 (3.9)	3.98	4.0	82.4	6	80
sd-pm	711/3086 (23.0)	353/1569 (22.5)	22.86	24.7	107.1	23	103

[a] Recombination between pairs of markers was measured using the standard three-point cross analysis. Results from different three-point crosses containing the same pair of markers were combined for the total percentage of recombination between each pair of markers, shown in parentheses.

[b] The cM distances for the pure *D. simulans* species come from STURTEVANT (1929).

TABLE 3

***D. sechellia* observed and expected female genotypes in crosses using second chromosome introgressions**

n b females			*n pm* females			*sd pm* females		
Genotype	Observed	Expected	Genotype	Observed	Expected	Genotype	Observed	Expected
				Fertile				
$\frac{n\ b\ py\ sd\ pm}{n\ b\ +\ +\ +}$	50	31.79	$\frac{n\ b\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	41	29.56	$\frac{n\ b\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	65	34.98
$\frac{n\ b\ +\ +\ +}{n\ b\ +\ +\ +}$	0	15.89	$\frac{n\ +\ +\ +\ pm}{n\ +\ +\ +\ pm}$	4	14.78	$\frac{+\ +\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	0	17.49
$\frac{n\ b\ py\ sd\ +}{n\ b\ +\ +\ +}$	3	4.88	$\frac{n\ b\ +\ +\ pm}{n\ +\ +\ +\ pm}$	2	6.36	$\frac{+\ +\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	2	7.95
$\frac{n\ b\ +\ +\ pm}{n\ b\ +\ +\ +}$	2	4.88	$\frac{n\ +\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	8	6.36	$\frac{n\ b\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	2	7.95
Others	3	1.55	Others	3	1.93	$\frac{+\ b\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	16	10.73
						$\frac{n\ +\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	3	10.73
						Others	9	7.16
				Sterile				
$\frac{n\ b\ g^{sech}}{n\ b\ g^{sech}}$	1	—	$\frac{n\ g^{sech}\ pm}{n\ g^{sech}\ pm}$	0	—	$\frac{g^{sech}\ sd\ pm}{g^{sech}\ sd\ pm}$	0	—
$\frac{n\ b\ g^{sech}}{n\ b\ g^{sim}}$	0	—	$\frac{n\ g^{sech}\ pm}{n\ g^{sim}\ pm}$	1	—	$\frac{g^{sech}\ sd\ pm}{g^{sim}\ sd\ pm}$	0	—
Total	59	59		59	59		97	97

Females homozygous for portions of the second chromosome introgressions were produced through brother/sister matings as depicted in Figure 1. The observed *vs.* expected numbers of females for each genotypic category are shown for each introgression. Fertile individuals were genotyped by scoring progeny resulting from a testcross and are listed first in the table followed by sterile individuals that were scored using SSCP analysis. (See MATERIALS AND METHODS for details.)

gous males are compared to the expected number, there is a marked inviability effect for the homozygous class for the *n b* [+ + +] and [+ + +] *sd pm* introgressions (*n b:* $\chi^2 = 36.744$, 1 d.f., $P < 0.001$; *sd pm:* $\chi^2 = 16.175$, 1 d.f., $P < 0.001$), but not for *n* [+ + +] *pm* ($\chi^2 = 0.260$, 1 d.f., $P > 0.50$).

Analysis of *D. mauritiana* homozygous autosomal introgressions: For *D. mauritiana,* two lines each for the *n* [+ + +] *pm* and *n b* [+ + +] introgressions and a single line for the [+ + +] *sd pm* introgression were tested. In total, ~10,000 progeny were scored to genotype 200 different individuals produced from brother/sister matings of the autosomal introgression lines. Table 5 summarizes the results obtained for *D. mauritiana* females. As was the case with *D. sechellia,* the introgressions generally show little female sterility, yet substantial inviability when made homozygous (*n b:* $\chi^2 = 18.756$, 4 d.f., $P < 0.001$; *n pm:* $\chi^2 = 10.086$, 4 d.f., $P < 0.05$). However, in contrast to *D. sechellia,* the [+ + +] *sd pm*/ introgression revealed no inviability when made homozygous ($\chi^2 = 4.77$, 4 d.f., $P > 0.30$).

Table 6 summarizes the analysis of the *D. mauritiana* homozygous autosomal introgressions in males. As was seen in the case of *D. sechellia,* male sterility is much greater than that of the females. In fact, the homozygous male sterility effect is even more striking for *D. mauritiana* than it was for *D. sechellia.* For the *n* [+ + +] *pm* introgression, 96% of the total number of fertile males (25/26) generated in the brother/sister matings were still heterozygous for the entire length of the introgression, whereas analysis of the sterile males revealed 86% (12/14) of these to be homozygous for the molecular marker between the *b* and *py.* Similarly, for the [+ + +] *sd pm* introgression, the majority of fertile males were heterozygous for the length of the [+ + +] *sd pm* introgression (6/8), whereas the majority of sterile males (8/12) were homozygous for the molecular marker between *n* and *b.* Again, there was no discrepancy between males homozygous or heterozygous for *pgi* for the *n b* [+ + +] introgression. When the observed numbers of heterozygous and homozygous males are compared to the expected, the same pattern

TABLE 4

***D. sechellia* observed and expected male genotypes in crosses using second chromosome introgressions**

n b males			*n pm* males			*sd pm* males		
Genotype	Observed	Expected	Genotype	Observed	Expected	Genotype	Observed	Expected
				Fertile				
$\frac{n\ b\ py\ sd\ pm}{n\ b\ +\ +\ +}$	50	31.79	$\frac{n\ b\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	27	29.06	$\frac{n\ b\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	48	34.98
$\frac{n\ b\ +\ +\ +}{n\ b\ +\ +\ +}$	0	15.89	$\frac{n\ +\ +\ +\ pm}{n\ +\ +\ +\ pm}$	1	14.53	$\frac{+\ +\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	0	17.49
$\frac{n\ b\ py\ sd\ +}{n\ b\ +\ +\ +}$	1	4.88	$\frac{n\ b\ +\ +\ pm}{n\ +\ +\ +\ pm}$	5	6.25	$\frac{+\ +\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	4	7.95
$\frac{n\ b\ +\ +\ pm}{n\ b\ +\ +\ +}$	0	4.88	$\frac{n\ +\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	6	6.25	$\frac{n\ b\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	6	7.95
Others	1	1.55	Others	1	1.90	$\frac{+\ b\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	13	10.73
						$\frac{n\ +\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	4	10.73
						Others	4	7.16
				Sterile				
$\frac{n\ b\ g^{sech}}{n\ b\ g^{sech}}$	2	—	$\frac{n\ g^{sech}\ pm}{n\ g^{sech}\ pm}$	14	—	$\frac{g^{sech}\ sd\ pm}{g^{sech}\ sd\ pm}$	12	—
$\frac{n\ b\ g^{sech}}{n\ b\ g^{sim}}$	5	—	$\frac{n\ g^{sech}\ pm}{n\ g^{sim}\ pm}$	4	—	$\frac{g^{sech}\ sd\ pm}{g^{sim}\ sd\ pm}$	6	—
ND	(1)	—	ND	(0)	—	ND	(3[a])	—
Total	60	59		58	58		100	97

Males homozygous for portions of the second chromosome introgressions were produced through brother/sister matings as depicted in Figure 1. The observed *vs.* expected numbers of males for each genotypic category are shown for each introgression. Fertile individuals were genotyped by scoring progeny resulting from a testcross and are listed first in the table followed by sterile individuals scored using SSCP analysis. (See MATERIALS AND METHODS for details.) ND, sterile individuals whose genotypes could not be determined molecularly.

[a] One male individual in this analysis was revealed to be a rare recombinant homozygous for g^{sim} and therefore was excluded from the analysis.

of inviability was detected as was seen in the female homozygous introgressions for this species (*n b:* $\chi^2 = 13.189$, 1 d.f., $P < 0.001$; *n pm,* $\chi^2 = 4.844$, 1 d.f., $P < 0.05$; *sd pm:* $\chi^2 = 1.688$, 1 d.f., $P > 0.10$).

Segregation of deleterious alleles within the *D. sechellia* and *D. mauritiana* stocks: There exists the formal possibility that the autosomal inviability and sterility effects could be a result of recessive deleterious alleles segregating within the *D. sechellia* and *D. mauritiana* stocks rather than representing actual hybrid incompatibilities. We do not believe this to be the case for our study for two major reasons. First, the *D. mauritiana* and *D. sechellia* stocks used are inbred isofemale lines that have been through several bottlenecks over the course of routine stock propagation. Second, the inviability and sterility effects were very consistent not only among different lines, but even across different species, which is not expected if different alleles are segregating in the stocks at low frequencies. For example, the chances of isolating three independent chromosomes from *D. sechellia* and two independent chromosomes from *D. mauritiana* all of which cause almost complete lethality when made homozygous in an otherwise *D. simulans* background (as was the case for the *n b* [+ + +] introgression) are very low unless the effect actually represents a genetic incompatibility between these two species and *D. simulans.* To think otherwise, the inbred stocks used to construct the lines would have to be hypothesized as carrying an impossibly high frequency of deleterious alleles on the second chromosome. Although some of the inviability effects may be accounted for by segregating alleles, it is very difficult to envision how segregating alleles could be responsible for the great disparity between male and female sterility effects detected in this study.

TABLE 5

***D. mauritiana* observed and expected female genotypes in crosses using second chromosome introgressions**

n b females			*n pm* females			*sd pm* females		
Genotype	Observed	Expected	Genotype	Observed	Expected	Genotype	Observed	Expected
			Fertile					
$\frac{n\ b\ py\ sd\ pm}{n\ b\ +\ +\ +}$	31	21.55	$\frac{n\ b\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	23	20.04	$\frac{n\ b\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	6	7.21
$\frac{n\ b\ +\ +\ +}{n\ b\ +\ +\ +}$	0	10.78	$\frac{n\ +\ +\ +\ pm}{n\ +\ +\ +\ pm}$	4	10.02	$\frac{+\ +\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	2	3.61
$\frac{n\ b\ py\ sd\ +}{n\ b\ +\ +\ +}$	2	3.31	$\frac{n\ b\ +\ +\ pm}{n\ +\ +\ +\ pm}$	3	4.31	$\frac{+\ +\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	4	1.64
$\frac{n\ b\ +\ +\ pm}{n\ b\ +\ +\ +}$	0	3.31	$\frac{n\ +\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	5	4.31	$\frac{n\ b\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	1	1.64
Others	1	1.06	Others	4	1.31	Others	7	5.90
			Sterile					
$\frac{n\ b\ g^{mau}}{n\ b\ g^{mau}}$	4	—	$\frac{n\ g^{mau}\ pm}{n\ g^{mau}\ pm}$	1	—	$\frac{da^{mau}\ sd\ pm}{da^{mau}\ sd\ pm}$	0	—
$\frac{n\ b\ g^{mau}}{n\ b\ g^{sim}}$	2	—	$\frac{n\ g^{mau}\ pm}{n\ g^{sim}\ pm}$	0	—	$\frac{da^{mau}\ sd\ pm}{da^{sim}\ sd\ pm}$	0	—
Total	40	40	Total	40	40	Total	20	20

Females homozygous for portions of the second chromosome introgressions were produced through brother/sister matings depicted in Figure 1. The observed *vs.* expected numbers of females for each genotypic category are shown for each introgression. Fertile individuals were genotyped by scoring progeny resulting from a testcross and are listed first in the table followed by sterile individuals scored using SSCP analysis. (See MATERIALS AND METHODS for details.)

Inviability of *X* chromosome introgressions: Table 7 shows the results of the *X* chromosome inviability analysis. The G_2 females are heterozygous and G_2 males are hemizygous for the introgressed segments in this analysis. By comparing the frequencies of the different genotypic classes between females and males, we are effectively looking at heterozygous *vs.* homozygous effects of the *X* chromosome on inviability. The *yellow-forked* region of the *X* chromosome covers about 57 cM, approximately the average size of the autosomal introgressions constructed in this study. Ideally, one would want to compare heterozygous female effects with homozygous female effects, as well as making the comparison between males and females, but because males carrying large introgressed segments of the *X* chromosome are invariably sterile, the crosses necessary to produce females that are homozygous for *X* chromosome introgressions can never be performed as they were for the autosomal studies; thus, confounding male and female effects with homozygous and hemizygous effects is unavoidable when dealing with cross-species, *X* chromosome traits. The female recombination frequencies measured for each pair of markers were significantly different for the two species (*y-v*, $\chi^2 = 24.396$, 1 d.f., $P < 0.001$; *v-f*, $\chi^2 = 5.874$, 1 d.f., $P < 0.05$); therefore, all analyses were performed separately for *D. mauritiana* and *D. sechellia*. The map distances calculated in this analysis for the two sets of markers also differ significantly from those reported by STURTEVANT (1929) for the *D. simulans* within species analyses of *v-f* region (*D. sechellia*, *v-f*, $\chi^2 = 32.894$, 1 d.f., $P < 0.001$; *D. mauritiana*, *v-f*, $\chi^2 = 4.675$, 1 d.f., $P < 0.05$) and those reported for *D. melanogaster* (LINDSLEY and ZIMM 1992) for the *y-v* region (*D. sechellia*, *y-v*, $\chi^2 = 3.889$, 1 d.f., $P < 0.05$; *D. mauritiana*, *y-v*, $\chi^2 = 24.126$, 1 d.f., $P < 0.001$). Because within species *vs.* between species recombination frequencies differed, the between species female frequencies calculated in this study were used to evaluate the male recombination frequencies. There was a significant deviation between the expected frequencies of the different recombinant classes based on the female data and the observed numbers for the males (Table 7: *D. sechellia*, $\chi^2 = 19.516$, 7 d.f., $P < 0.01$; *D. mauritiana*, $\chi^2 = 41.234$, 7 d.f., $P < 0.001$), although none of the recombinant classes was missing as was the case for the homozygous autosomal introgressions. The most severe deviation from expectations for both species came from a reduction in the number of males carrying the entire *y-f* introgression from either species (*i.e.*, the + + + genotypes in Table 7 that were reduced 17.7% for *D. sechellia* and 13% for *D. mauritiana*).

DISCUSSION

In deciding between the various models proposed to explain HALDANE's rule, it is helpful to examine a series

TABLE 6

***D. mauritiana* observed and expected male genotypes in crosses using second chromosome introgressions**

n b males			*n pm* males			*sd pm* males		
Genotype	Observed	Expected	Genotype	Observed	Expected	Genotype	Observed	Expected
			Fertile					
$\frac{n\ b\ py\ sd\ pm}{n\ b\ +\ +\ +}$	31	21.55	$\frac{n\ b\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	25	20.04	$\frac{n\ b\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	6	7.21
$\frac{n\ b\ +\ +\ +}{n\ b\ +\ +\ +}$	0	10.78	$\frac{n\ +\ +\ +\ pm}{n\ +\ +\ +\ pm}$	0	10.02	$\frac{+\ +\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	0	3.61
$\frac{n\ b\ py\ sd\ +}{n\ b\ +\ +\ +}$	4	3.31	$\frac{n\ b\ +\ +\ pm}{n\ +\ +\ +\ pm}$	1	4.31	$\frac{+\ +\ py\ sd\ pm}{+\ +\ +\ sd\ pm}$	0	1.64
$\frac{n\ b\ +\ +\ pm}{n\ b\ +\ +\ +}$	0	3.31	$\frac{n\ +\ py\ sd\ pm}{n\ +\ +\ +\ pm}$	0	4.31	$\frac{n\ b\ +\ sd\ pm}{+\ +\ +\ sd\ pm}$	2	1.64
Others	1	1.06	Others	0	1.31	Others	0	5.90
			Sterile					
$\frac{n\ b\ g^{mau}}{n\ b\ g^{mau}}$	2	—	$\frac{n\ g^{mau}\ pm}{n\ g^{mau}\ pm}$	12	—	$\frac{da^{mau}\ sd\ pm}{da^{mau}\ sd\ pm}$	8	—
$\frac{n\ b\ g^{mau}}{n\ b\ g^{sim}}$	2	—	$\frac{n\ g^{mau}\ pm}{n\ g^{sim}\ pm}$	2	—	$\frac{da^{mau}\ sd\ pm}{da^{sim}\ sd\ pm}$	4	—
Total	40	40	Total	40	40	Total	20	20

Males homozygous for portions of the second chromosome introgressions were produced through brother/sister matings as depicted in Figure 1. The observed *vs.* expected numbers of males for each genotypic category are shown for each introgression. Fertile individuals were genotyped by scoring progeny resulting from a testcross and are listed first in the table followed by sterile individuals that were scored using SSCP analysis. (See MATERIALS AND METHODS for details.)

of dichotomies for which the major hypotheses all make a different set of predictions. First, what is the relative contribution of the *X* chromosome versus the autosomes on reproductive isolation? Second, what role does dominance *vs.* recessiveness of sterility and inviability alleles play in reproductive isolation? Third, what are the relative levels of sterility and inviability in males *vs.* females? And, finally, how similar are the patterns of evolution of sterility and inviability? By systematically testing autosomal introgressions for their ability to cause hybrid male and hybrid female sterility and inviability, we can begin to answer the four questions stated above.

In backcross analyses typically employed to evaluate

TABLE 7

Inviability test for *X* chromosome introgression

	D. mauritiana			*D. sechellia*		
	Observed		Expected	Observed		Expected
Genotype	Females	Males	males	Females	Males	males
yvf	448	509	444.3	388	402	323.3
+++	523	386	444.3	428	266	323.3
yv+	206	152	162.5	184	139	136.7
++*f*	149	152	162.5	161	119	136.7
y++	187	169	162.0	197	161	162.4
+*vf*	167	167	162.0	213	181	162.4
y+*f*	29	24	25.2	55	28	45.2
+*v*+	26	29	25.2	59	39	45.2
Total	1735	1588	1588.0	1685	1335	1335.0

G_2 females and males were scored for three visible markers on the *X* chromosome: *yellow* (*y*), *vermilion* (*v*), and *forked* (*f*). The observed numbers of males and females, as well as the expected number of males based on the recombination frequencies measured in the females, are listed for the two different species.

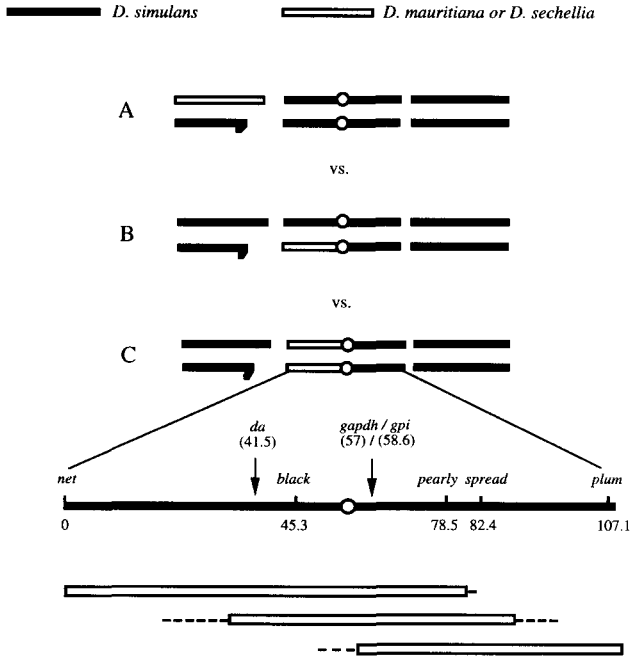

FIGURE 2.—Test of the large *X* effect on reproductive isolation. Black chromosomal regions represent *D. simulans*. White chromosomal regions are either *D. mauritiana* or *D. sechellia*. Sex chromosomes are drawn on the left (*X* chromosome on top, *Y* chromosome on the bottom). The second chromosome is drawn with the centromere indicated. A larger scaled version of the second chromosome is drawn below genotype C to show the visible markers used in this analysis: *net* (*n*), *black* (*b*), *pearly* (*py*), *spread* (*sd*), and *plum* (*pm*). Also shown are the molecular markers used in the SSCP analysis of the sterile individuals: *phosphoglucose isomerase* (*pgi*), *glyceraldehyde dehydrogenase 1* (*gapdh1*) and *daughterless* (*da*). The recombination map distances (in cM) are the between species recombination distances based on a standard three point cross analysis (Table 2).

the genetic basis of reproductive isolation (DOBZHANSKY 1936; HENNIG 1977; ZOUROS 1981; COYNE 1984), the comparison is always made between effects of genotype A and genotype B of Figure 2, yet the magnitude of the autosomal effect will be underestimated because only one of the two copies of autosomes is replaced. Hence, an apparent large effect of the *X* chromosome on reproductive isolation was previously detected (DOBZHANSKY 1936; BENTLEY and HOY 1972; HENNIG 1977; COYNE and ORR 1989; RICHLER *et al.* 1989). However, if male fertility requires positive interactions between the conspecific *X* and the autosomes, then genotype A is expected to be sterile and genotype B fertile. A more appropriate comparison to make is between genotype A and genotype C (Figure 2). If genotype C is also capable of causing reproductive isolation to the same degree as genotype A, then the large *X* effect may not actually reflect the relative rates of accumulation of mutations on the *X* chromosome *vs.* the autosomes.

In addition to underestimating homozygous effects of the autosomes, other difficulties exist in interpreting the results from a backcross analysis (WU and DAVIS 1993; WU and PALOPOLI 1994). Backcrosses typically employ F_1 females to produce the backcross generation, resulting in heterogeneity in genotypes being compared for incompatibility because of recombination. Each male "genotype" is a collection of indistinguishable recombinant classes. Even if F_1 males are fertile and can be backcrossed (eliminating the complication of recombination), the genotypes produced are still genetically complex. Because of background impurity, many different genetic interactions are involved in producing sterility or inviability phenotypes for any given male, making the effects of separate chromosomal regions difficult to assign.

To overcome these limitations, in this study we employed the introgression approach (WU and BECKENBACH 1983; PEREZ *et al.* 1993; WU *et al.* 1993; CABOT *et al.* 1994; PALOPOLI and WU 1994) to evaluate the heterozygous and homozygous effects of the second chromosome on reproductive isolation in the *D. simulans* clade. As summarized in Figure 2, we introgressed three overlapping segments of the second chromosome from *D. mauritiana* and *D. sechellia* into *D. simulans* and tested these for their heterozygous and homozygous effects on reproductive isolation. In this manner we were able to evaluate the homozygous effects of the second chromosome on reproductive isolation to detect hidden homozygosity effects and evaluate the relative contribution of the *X* chromosome *vs.* the autosomes on hybrid sterility and inviability.

Autosomal studies also provide a framework for answering questions pertaining to sex-specificity of reproductive isolation that cannot be easily answered by studying *X* chromosome effects alone. One question that cannot be answered is whether individual *X* chromosome introgressions that are male sterile or inviable cause sterility or inviability in females when made homozygous. Because these males are either sterile or inviable, females homozygous for the same *X* chromosome introgressions cannot be produced and assayed; the crosses simply cannot be performed. Therefore, it is difficult to dissect male/female effects from homozygous/hemizygous effects in *X* chromosome studies. These difficulties are overcome in autosomal studies, because most heterozygous introgressions are completely fertile, allowing males and females of equivalent genotypic classes to be produced simultaneously for a natural comparison between homozygous and heterozygous effects as well as between male and female effects of sterility and inviability.

Lines heterozygous for autosomal introgressions were easily constructed for most regions of the second chromosome in both species. However, fertility of males carrying heterozygous introgressions was not strongly established until several backcrosses had been made to purify the genetic background of the introgression lines (Table 1). Interactions with other genetic elements in the background causes the introgressed segments to exhibit sterility in earlier generations, even though

TABLE 8

Qualitative summary of the homozygous effects of second chromosome introgressions

Introgression type	*D. sechellia*				*D. mauritiana*			
	Inviability		Sterility		Inviability		Sterility	
	Males	Females	Males	Females	Males	Females	Males	Females
n b + + +	Strong	Strong	Moderate	Weak	Strong	Strong	Moderate	Moderate
n + + + *pm*	None	Moderate	Strong	Weak	Weak	Weak	Strong	Weak
+ + + *sd pm*	Strong	Strong	Strong	None	None	None	Strong	None

Males and females homozygous for portions of the second chromosome introgressions from either *D. sechellia* or *D. mauritiana* in an otherwise pure *D. simulans* background were produced through brother/sister matings as depicted in Figure 1. The qualitative effects of these homozygous introgressions on hybrid inviability and sterility in males and females are summarized for both species.

these segments do not cause sterility when analyzed in isolation. This result confirms that heterogeneity and complexity of the genotypes produced in the G_2 generation in a typical backcross analysis cannot provide a reliable estimate of the relative contribution of different chromosomal regions to reproductive isolation. The one introgression that was not fertile, even as a heterozygote, was the [+ + +] *sd pm* region in *D. mauritiana*. The only fertile line that was established was a double recombinant in the *b-py* region. Therefore, the [+ + +] *sd pm* introgression in its pure form represents the only real autosomal sterility effect that could be picked up in a typical backcross analysis. Although individuals heterozygous for second chromosome introgressions generally do not suffer sterility or inviability, individuals homozygous for these same regions show dramatic increases in both, with the pattern being strikingly different between males and females as well as between sterility and inviability (summarized in Table 8).

Overall, sterility was very pronounced in homozygous males of both species, yet occurred only sporadically in females. For *D. sechellia*, sterile males occurred 23.2 times more frequently than sterile females. For *D. mauritiana*, sterile males were 4.3 times more frequent than their female counterparts. The inviability pattern, on the other hand, was virtually identical between the two sexes with the only exception being the *n* [+ + +] *pm* introgression in *D. sechellia* that showed female inviability when none was detected for the males. This result suggests sex-specific inviability may exist for this introgression; however, greater mapping resolution is needed before we can say definitely that the males for this introgression do not exhibit inviability.

Although the general pattern of sterility for the two sexes is similar for the two species, it is apparent that *D. mauritiana* has accumulated more sterility genes than *D. sechellia* (*D. sechellia:* male sterility 22%, female sterility 0.9%; *D. mauritiana:* male sterility 30%, female sterility 7%). In contrast, the two species show remarkable similarity in the pattern of inviability except for the [+ + +] *sd pm* introgression that shows inviability for *D. sechellia* males and females but none in *D. mauritiana*. However, this difference is more likely a reflection of the fact that the *D. mauritiana* introgression is a double recombinant line, rather than evidence that inviability has evolved to a greater degree in *D. sechellia*.

For the most part, inviability and sterility effects are clearly separable in the two sexes and from each other, with sterility being strongly sex-specific and inviability not. However, these effects were confounded in the *n b* [+ + +] introgression, which may represent a situation in which genes causing inviability can cause sterility pleiotropically. For this introgression, almost all individuals recovered from brother/sister mating were heterozygous for this introgression in both species, indicating that inviability is rather complete even when only partially homozygous and is more severe than for the other introgressions. Both species gave the exact same percentage of sterile males, which does not fit the general pattern for sterility seen for the other two introgressions nor for other studies of these two species (COYNE and CHARLESWORTH 1986, 1989; JOHNSON *et al.* 1992, 1993; NAVEIRA 1992; PEREZ *et al.* 1993; WU *et al.* 1993; CABOT *et al.* 1994; PALOPOLI and WU 1995). Molecular analysis of sterile individuals that were recovered showed them to be equally likely to be homozygous or heterozygous. In addition, for *D. mauritiana*, males and females gave identical patterns of inviability and sterility, and, in fact, this female sterility represents the bulk of the total female sterility detected in this study. We interpret these results as evidence that on occasion hybrid sterility results in a nonspecific manner from genes affecting the general viability of the flies. It may be that sterility stemming from a pleiotropic effect of inviability is not common, yet it does contribute to the overall pattern of reproductive isolation and could only be detected in an autosomal design such as this one.

A direct comparison between homozygous regions of the second chromosome and the *X* chromosome reveals them to be comparable in their ability to cause sterility, yet distinct in terms of inviability. For *D. sechellia*, 14 out of the 27 males homozygous for different regions of the *n* [+ + +] *pm* introgression are completely sterile, depending on which regions are involved (Table 4).

This is comparable to the level of sterility seen for the *X* chromosome for *D. sechellia,* where several regions of the *D. sechellia X* chromosome exist that do not cause sterility when introgressed into *D. simulans* (PEREZ *et al.* 1993; WU *et al.* 1993). For the *D. mauritiana n* [+ + +] *pm* introgression, 92% (12/13) of the males known to carry homozygous material from *D. mauritiana* are sterile (Table 6). Again, this is comparable to the level of sterility seen for *X* chromosome introgressions of a similar size marked by *yellow-vermilion* (WU *et al.* 1993; CABOT *et al.* 1994) in which very few regions can be introgressed from *D. mauritiana* that do not cause sterility in *D. simulans.* The results for the [+ + +] *sd pm* introgressions from both species also confirm this general pattern. As long as the introgressed second chromosome is made homozygous, sterility is quite pronounced in males (Tables 4 and 6). Inviability, on the other hand, tends to be more complete for homozygous autosomal introgressions than for the *X* chromosome. Although decreases in the relative abundance of males hemizygous for the entire *X* chromosome introgressed segment did occur, these decreases were in the range of only 13–18% below expectations, considerably less than the 40–100% reduction seen for the homozygous introgressions on the second chromosome.

It seems clear that for this level of genetic resolution the second chromosome hybrid sterility effect approaches that of the *X* chromosome when comparing genotype A *vs.* genotype C in Figure 2 and is not orders of magnitude less, which is the conclusion one draws from comparing genotypes A and B. In fact, the autosomes show hybrid inviability effects that are more severe than those of the *X* chromosome. The apparent large *X* chromosome effect in reproductive isolation observed in traditional backcross analyses is largely due to the insensitivity of the approach for detecting autosomal sterility effects. Overall, for both species introgressions, *homozygous* segments of the second chromosome cause a significant amount of sterility as well as inviability in *D. simulans.* In other words, genotypes like type C of Figure 2 are largely sterile or inviable, whereas those like type B are not. What is also clear from this study is that males have accumulated a significantly greater number of sterility factors than females in this species clade, yet the two sexes do not differ greatly in the levels of inviability that they exhibit. These results have recently been corroborated by the work of TRUE *et al.* (1996), who have compared *X* chromosome and homozygous autosomal introgressions from *D. mauritiana* tagged by *P*-element markers. Although they used an entirely different approach, they come to the same general conclusion that male sterility far outstrips female sterility when autosomal introgressions are made homozygous, whereas there is only a 50% increase in the number of genes causing hybrid male sterility on the *X* chromosome *vs.* the autosomes.

The hypothesis that rapid evolution of the *X* chromosome can account for HALDANE's rule predicts that the *X* chromosome should have a disproportionate effect on reproductive isolation for both sterility and inviability (CHARLESWORTH *et al.* 1987; COYNE and ORR 1989). We find that this effect is not as strong as was predicted and in fact the autosomes exhibited far greater inviability than the *X* chromosome. The model also predicts that male sterility genes should outnumber female sterility genes, yet there should be no difference in the relative number of male and female inviability genes. Although, male sterility genes are predicted to outnumber female sterility genes, this effect is always predicted to be less than the relative effect of the *X* versus the autosomes on male sterility (COYNE and ORR 1989). We do, indeed, see that male sterility genes outnumber females sterility genes as the model predicts, yet both in our study as well as in TRUE *et al.* (1996), the hybrid male sterility effect is much stronger than the *X* chromosome effect that is predicted to be the dominant contributing factor to reproductive isolation. Therefore, the model of the faster accumulation of mutations on the *X* chromosome with respect to hybrid sterility (or inviability) as an explanation for HALDANE's rule is not supported by our observations.

Unlike the model of rapid evolution of the *X* chromosome, the dominance theory allows the *X* chromosome effect on either sterility or inviability to be comparable to homozygous autosomes (ORR 1993b; TURELLI and ORR 1995) as was seen in this study. However, the dominance model states that the apparently more rapid evolution of heterogametic incompatibility actually reflects a bias in the expression of partially recessive incompatibility genes in the F_1, and not that heterogametic and homogametic sexes have accumulated different numbers of loci with sex-limited effects. Therefore, when testing homozygous autosomes, the accumulation of sterility and inviability genes are predicted to be more equal in males and females of equivalent genotypes. This latter prediction of the dominance model is true when referring to inviability, yet is not borne out by the autosomal data with respect to sterility. It is clear that differences in male and female sterility reflect actual differences in the number of genes that have accumulated in the two sexes and not a bias in expression of these alleles. This discrepancy between male and female sterility is much too great to be explained by the dominance theory.

It seems evident from this study that no single explanation offered for HALDANE's rule so far is sufficient to account for all the autosomal data nor is it likely that one ever will or should necessarily be developed. The autosomal results add support to the hypothesis that HALDANE's rule actually represents a composite phenomenon, a belief held by WU and DAVIS (1993) and previously by ORR (1993a). MULLER's original hypothesis (1940) and TURELLI and ORR's reformulation (1995) may account for the evolution of inviability, but neither

alone can account for the extensive amount of sterility found in hybrid males. Because homozygous autosome effects approach those of the *X* chromosome, the model of the more rapid evolution of the *X* chromosome is not sufficient to explain hybrid sterility either. Other possible contributing factors such as sexual selection should be carefully examined for their role in the evolution of hybrid male sterility, especially for species in which males are the heterogametic sex. Rejection of any single model as being sufficient to explain HALDANE's rule should not be construed to mean that none of these mechanisms plays a role in the evolution of reproductive incompatibility. It is very likely that all the mechanisms proposed so far contribute to the evolution of reproductive isolation to varying degrees: *X*/autosome imbalance, recessive nature of alleles acting in foreign backgrounds, different selection pressures for *X* versus autosomal genes, sexual selection, differences in the physiology of fertility and viability, and differences in the very nature of male and female gametogenesis. Just as the complexities inherent in HALDANE's rule are best unraveled by analyzing hybrid sterility and inviability patterns separately as well as *X* chromosome and autosomal effects separately, the various factors that have been proposed to contribute to the evolution of reproductive isolation should also be studied individually to evaluate their relative importance in shaping different types of incompatibilities for different groups of species.

We thank J. H. MCDONALD and A. W. DAVIS for supplying us with the primers used in this study and J. A. COYNE for providing the Drosophila stocks. We greatly appreciate comments provided by A. G. CLARK, C. C. LAURIE and two anonymous reviewers, which helped improve the manuscript. We also thank J. R. TRUE, B. S. WEIR and C. C. LAURIE for providing a copy of their manuscript before publication. This work was supported by a National Science Foundation and National Institutes of Health grant awarded to C.-I.W.

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Communicating editor: A. G. CLARK