Complex Epistasis and the Genetic Basis of Hybrid Sterility in the *Drosophila pseudoobscura* Bogota-USA Hybridization

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ABSTRACT

We analyzed the genetic basis of postzygotic isolation between the Bogota and USA subspecies of *Drosophila pseudoobscura*. These subspecies diverged very recently (perhaps as recently as 155,000 to 230,000 years ago) and are partially reproductively isolated: Bogota and USA show very little prezygotic isolation but form sterile F_1 males in one direction of the hybridization. We dissected the basis of this hybrid sterility and reached four main conclusions. First, postzygotic isolation appears to involve a modest number of genes: we found large chromosome regions that have no effect on hybrid fertility. Second, although apparently few in number, the factors causing hybrid sterility show a remarkably complex pattern of epistatic interaction. Hybrids suffer no hybrid sterility until they carry the "right" allele (Bogota *vs.* USA) at at least *four* loci. We describe the complete pattern of interactions between all chromosome regions known to affect hybrid fertility. Third, hybrid sterility is caused mainly by *X*-autosomal incompatibilities. Fourth, hybrid sterility does not involve a maternal effect, despite earlier claims to the contrary. In general, our results suggest that fewer genes are required for the appearance of hybrid sterility than implied by previous studies of older pairs of Drosophila species. Indeed, a maximum likelihood analysis suggests that roughly 15 hybrid male steriles separate the Bogota and USA subspecies. Only a subset of these would act in F_1 hybrids.

OUR understanding of speciation has grown dramatically over the last 15 years. Attention has focused on a number of problems, including reinforcement (Liou and Price 1994; Servedio and Kirkpatrick 1997; Noor 1999), sympatric speciation (Schliewen *et al.* 1994; Kondrashov and Kondrashov 1999), and the ecological context of speciation (Schemske and Bradshaw 1999; Rundle *et al.* 2000). But the greatest progress has been made in the study of intrinsic postzygotic isolation (hybrid sterility and inviability) and, in particular, in the genetics of postzygotic isolation. We now understand a good deal about the chromosomal locations and densities of hybrid lethals, hybrid male steriles, and hybrid female steriles (Hollocher and Wu 1996; True *et al.* 1996). We have also learned a great deal about the dominance and sex specificity of the genes causing postzygotic isolation (Turelli and Orr 1995; True *et al.* 1996; Orr 2000). Moreover, a remarkably strong consensus has emerged on the causes of Haldane's rule, the preferential sterility and inviability of hybrids of the heterogametic sex (Zeng and Singh 1993; Wu *et al.* 1996; Laurie 1997; Orr 1997; Turelli 1998). Finally, at least two candidate genes causing postzygotic isolation, *Tu* and *OdsH*, have been cloned and partially characterized (Wittbrodt *et al.* 1989; Ting *et al.* 1998; reviewed in Orr and Presgraves 2000).

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Despite this progress, several key problems remain unresolved. Perhaps the most important concerns the number of genes required for the evolution of hybrid sterility or inviability. The traditional neo-Darwinian view, which holds that reproductive isolation is a byproduct of gradual genetic change within populations, posits that speciation involves a large number of genes, each having a small effect on reproductive isolation (Dobzhansky 1936, 1937). Mayr (1963, p. 543) summarized this view in his well-known assertion that "most species differences . . . seem to be controlled by a large number of genetic factors with small individual effects. The genetic basis of the isolating mechanisms, in particular, seems to consist largely of such genes."

Many genetic studies of speciation in Drosophila appear to support this view. In particular, many backcross analyses have found that every marker used in genetic analysis of postzygotic isolation is linked to one or more factors causing hybrid sterility or inviability (*e.g.*, Dobzhansky 1936; Muller and Pontecorvo 1942; Naveira and Fontdevila 1986; Orr 1987, 1989a,b; Coyne and Charlesworth 1989; see also Naveira and Maside 1998). Similarly, recent introgression experiments—in which chromosome regions from one species are introduced into the genetic background of another by repeated backcrossing—have shown that a large number of chromosome regions can cause postzygotic isolation (usually, hybrid male sterility) when made homozygous on a foreign genetic background (Hollocher and Wu 1996; True *et al.* 1996). Extrapolating from such

studies as well as from fine-scale analyses of chromosome regions known to cause hybrid sterility, Davis and Wu (1996) and Wu *et al.* (1996) estimate that the species *Drosophila simulans* and *D. mauritiana* are separated by as many as 120 hybrid male steriles. Postzygotic isolation would seem complex.

While the finding of a large number of genes causing hybrid sterility in Drosophila is interesting and important, its proper evolutionary interpretation is less clear. The problem is that the species pairs that have been genetically analyzed thus far are fairly old; *i.e.*, they diverged from a common ancestor long ago (see Coyne and Orr 1989, 1997). Almost certainly, therefore, these taxa evolved complete hybrid male sterility or inviability in the distant past. Subsequently, these species surely continued to diverge at additional loci that affect the fitness of backcross or introgression hybrids studied in genetic analyses. Inclusion of these later genes might, then, be misleading as a smaller number of genes initially killed or sterilized hybrids. The evolutionarily interesting issue, after all, is not how many genes can cause reproductive isolation (presumably a large number), but how many are required to do so.

This overcounting concern is not hypothetical. We know that some studies supporting the polygenic view include factors that diverged after the attainment of complete hybrid sterility or inviability. Introgression analyses, in particular, are designed to detect genes that cause complete or nearly complete sterility or inviability when moved *alone* onto a foreign genetic background. But as each of these small chromosome regions singly causes complete fitness loss, all are obviously not required for the expression of sterility or inviability. And given that some of these factors must have diverged before others, enumeration of all of them may be misleading.

Recent theoretical work suggests this overestimation problem may be more serious than it first appears. Hybrid sterility and inviability in animals appear to evolve as described by the "Dobzhansky-Muller" model (Dobzhansky 1937; Muller 1942): although all evolutionary substitutions must be compatible with their normal within-species genetic background (as natural selection will not tolerate the fixation of strongly deleterious mutations), we have no guarantee that alleles that have never been "tested" together will function properly when brought together in hybrids. Instead, one locus from one species might well be incompatible with another locus from a second species, giving rise to sterility or inviability, either partial or complete. Mathematical analysis of the Dobzhansky-Muller model shows that the number of hybrid incompatibilities grows at least as fast as the square of time, the so-called snowball effect (Orr 1995a; Orr and Turelli 2001), reflecting the fact that postzygotic isolation involves interactions between pairs or triplets, etc., of loci. Doubling the time since divergence will, therefore, at least *quadruple* the number of genes seen in genetic analyses. Study of old species pairs might, then, lead to considerable overestimates of the number of genes required to cause hybrid problems.

There are also direct empirical grounds for believing earlier experiments may have overestimated the number of genes causing postzygotic isolation. Drosophila geneticists have discovered a number of "hybrid rescue genes," single mutations that restore the viability or fertility of normally inviable or sterile species hybrids. In the case of hybrid viability, five mutations have been discovered to date, all involving hybrids produced when *D. melanogaster* is crossed to species belonging to the *simulans* subgroup (*D. simulans*, *D. mauritiana*, and *D. sechellia*; reviewed in Hutter *et al.* 1990; Sawamura *et al.* 1993). The fact that single mutations can restore the viability of hybrids suggests that inviability has a simple developmental basis. If lethality involved a large number of independent developmental problems it seems unlikely that a single mutation could reverse them all. But a simple developmental basis in turn suggests a simple genetic basis. If many genes were involved, it seems unlikely that all would act in the same developmental pathway. Interestingly, recent work suggests that rescue mutations, which map to a small number of loci, may be mutant alleles of the actual loci that kill hybrids (Barbash *et al.* 2000; Orr and Irving 2000).

But the most direct test of the idea that analysis of old species pairs leads to overestimation of the number of genes required for postzygotic isolation is obvious. We must genetically analyze young pairs of taxa. Here we present such an analysis. We report the results of a large genetic analysis of male sterility between two subspecies of *D. pseudoobscura*, the Bogota and USA subspecies. The Bogota subspecies is found only at high elevations near Bogota, Colombia, and is geographically isolated by more than 2000 km from the USA populations of North and Central America (Prakash 1972). The Bogota-USA pair represents a young hybridization that is often viewed as paradigmatic of the early stages of speciation (*e.g.*, Lewontin 1974). Indeed, DNA sequence analysis shows that Bogota and USA may have separated as recently as 155,000 to 230,000 years ago (Schaeffer and Miller 1991; Wang *et al.* 1997). These subspecies are separated by Nei's genetic distance of only $D = 0.194$, smaller than the distances separating several other pairs of Drosophila taxa that have been subject to intensive genetic analysis (*e.g.*, *D. melanogaster-D. simulans* show $D = 0.55$, *D. pseudoobscura-D. persimilis* show $D = 0.41$, and *D. simulans-D. mauritiana* show $D = 0.30$; see Coyne and Orr 1989 for a review of such data). Not surprisingly, Bogota and USA are incompletely reproductively isolated. They show very weak prezygotic isolation (Prakash 1972; Noor 1995) and produce completely fertile female hybrids. Male hybrids are also fertile in one direction of the hybridization (USA mothers) but are completely sterile in the reciprocal direction (Bogota mothers).

This hybrid sterility has been the subject of several previous genetic studies (Prakash 1972; Dobzhansky 1974; Orr 1989a,b). The present analysis extends the results of these studies and, in several places, corrects previous errors (including our own). Building on our earlier analyses, we have now constructed a more complete picture of the basis of Bogota-USA hybrid sterility. Our conclusions rest on the use of 17 mapped markers that provide good genomic coverage (especially as we take advantage of several balancer chromosomes to suppress recombination over large chromosome regions).

Our experimental approach differs from that of quantitative trait locus (QTL) analysis in which a large number of markers segregate simultaneously in a single backcross or F_2 population. Instead, we perform a series of separate backcross analyses. In general, we proceed in three steps. First, backcrosses are used to detect the presence of hybrid steriles in large chromosome regions. Second, additional crosses are used to refine the position of hybrid steriles within these regions using a larger number of flanking markers (*e.g.*, see Perez and Wu 1995). Third, further crosses are used to disentangle the pattern of interactions between the Bogota and USA factors mapped in these earlier experiments. This strategy—unlike typical QTL analysis—allows repeated (at least three) tests of the effects of particular chromosome regions. Indeed, in many cases, more than three independent tests are performed, effectively ruling out false positives. Also, unlike typical QTL analysis, our strategy allows for very large sample sizes, sometimes in the thousands of flies per contrast. These large sample sizes allow us to determine with considerable confidence if a region has no effect on hybrid fertility, a matter of special interest in a young species pair.

As we will see, our results show that the genetic basis of hybrid sterility is simple in one respect (number of genes involved) but complex in another (pattern of epistatic interactions between these genes).

MATERIALS AND METHODS

Our methods generally follow those of Orr (1989a,b). Briefly, male fertility was measured by assessing sperm motility. Testes were dissected from 4-day-old virgin males and examined under a compound microscope with dark-field optics. This method allows rapid scoring of many males and ensures that measures of male fertility are not confounded with male mating ability (which can occur if fertility is assessed by offspring production). Initially, an attempt was made to classify males into three sperm motility classes: Many, wherein a male possesses a large number of motile sperm that essentially fill the field of vision; Few, wherein small pockets of motile sperm were seen; and None, wherein no motile sperm were seen. But because classification of males into the Many *vs.* Few classes is unavoidably somewhat subjective, we ultimately pooled data according to Coyne's (1984) binary method: males possessing *any* motile sperm are deemed fertile while those possessing none are deemed sterile. The fertility of a genotype is thus reported as the percentage of males possessing any motile sperm, an approach that is standard in the study of hybrid sterility (*e.g.*, Coyne 1985; Vigneault and Zouros 1986; Orr 1987; Orr and Coyne 1989; Davis and Wu 1996). While the presence of motile sperm is not equivalent to fertility, the two are strongly correlated (Orr 1987). Although we present results in Coyne's binary form, all of our statistical conclusions remain unchanged whether males are classified into two or three motility classes.

Statistical analysis of the effect of chromosome regions on hybrid fertility is complicated by frequent qualitative interactions among regions. A region may have no effect on almost all genetic backgrounds, but a very large effect on one particular background. These interactions are expected on theoretical grounds (Orr 1995a) and are often seen in empirical studies of hybrid sterility (Wu and Palopoli 1994). In general, we tested the effects of chromosomes or chromosome regions on hybrid fertility by simple χ^2 statistics, as described below. In the case of especially complicated analyses, we also performed multivariate analyses, *i.e.*, PROC CATMOD (SAS Institute). These tests (not shown) almost always supported the results of our simpler contrasts. We discuss the single case in which PROC CATMOD contradicted the results of our simpler analyses.

All crosses were performed, and all males aged, at 22°. The markers used and their map positions are provided in the results as each cross is described. All map positions are from Anderson and Norman (1977), except on the *X* chromosome, which are from Orr's (1995b) revised map.

RESULTS

***X* chromosome mapping:** Pure Bogota and USA males are fertile, as expected (Table 1). Table 1 also shows that visible markers do not affect male fertility in pure species (although one exception is discussed below). Also as expected, hybrid males who have Bogota mothers are almost always sterile, where we show a sample of results using different combinations of stocks (Table 1). As Prakash (1972) and Orr (1989a,b) noted, F_1 males with Bogota mothers who are considered "fertile" (Table 1) possess very few and very short motile sperm. These males do not produce offspring (Prakash 1972; Orr 1989a,b), a finding that is not surprising as small sperm classes in *D. pseudoobscura* are incapable of fertilization (Snook *et al.* 1994). Table 1 also shows that the cross of *D. pseudoobscura* Bogota females to the outgroup *D. persimilis*, a hybridization that apparently has not been previously reported, also produces sterile F_1 males; this hybridization is not pursued further here.

To map genes on the Bogota *X* causing Bogota-USA F_1 hybrid sterility, we performed a series of backcross analyses. Figure 1 shows a linkage map of the *D. pseudoobscura X* including all of the markers used in these analyses; the reader will find it useful to refer to Figure 1 throughout this section. We first crossed Bogota-ER females to multiply marked USA *ct y se sh* males (map positions in Figure 1) and backcrossed the resulting F_1 females to USA *ct y se sh* stock males. Backcross males having recombinant *X* chromosomes were genotyped and scored for fertility. As previous work (Orr 1989a,b) showed that the ct^+-y^+ interval harbors a hybrid male sterile, this interval was manipulated as a unit; *i.e.*, all

TABLE 1

Fertility of pure species and hybrid F_1 males

Genotype	No. fertile	No. sterile	% fertile
Bogota-ER	252	0	100.0
USA *ct sd y se*	267	3	98.9
USA *Ba/Dl*	89	0	100.0
USA *ct y*	115	0	100.0
USA *Pt y*	126	1	99.2
F_1 (Bog-ER × *ct y*)	5	204	2.4
F_1 (Bog-ER × *ct y se sh*)	13	175	6.9
F_1 (Bog-ER × *ct sd y se*)	9	252	3.4
F_1 (Bog-ER × *D. per* Kalana)[a]	0	37	0.0

In crosses, females are shown first.
[a] D. per, *D. persimilis*.

TABLE 2

Backcross analysis of *X* chromosome

Genotype	No. fertile	No. sterile	% fertile
ct y se sh	92	2	97.9
ct y se sh$^+$	99	2	98.0
ct y se$^+$ *sh*	81	12	87.1
ct y se$^+$ *sh*$^+$	80	9	89.9
ct$^+$ *y*$^+$ *se*$^+$ *sh*$^+$	9	111	7.5
ct$^+$ *y*$^+$ *se*$^+$ *sh*	8	82	8.8
ct$^+$ *y*$^+$ *se sh*$^+$	86	9	90.5
ct$^+$ *y*$^+$ *se sh*	87	6	93.5

Mutant alleles are from USA and wild-type alleles are from Bogota.

males scored were either *ct-y* (having mostly or all USA material in the interval) or *ct*$^+$-*y*$^+$ (having mostly or all Bogota material in the interval). As expected, the *ct*$^+$-*y*$^+$ region has a large and significant effect on hybrid fertility (Table 2; $\chi^2 = 191.7$, 1 d.f., $P < 0.0001$, summing over all contrasts). Also as expected, the *sh*$^+$ region at the tip of *XR* has no effect on hybrid fertility ($\chi^2 = 2.09$, 1 d.f., $P = 0.15$). Surprisingly, however, the previously untested Bogota *se*$^+$ region has the largest effect on fertility ($\chi^2 = 227.0$, 1 d.f., $P < 0.0001$). Indeed, this region appears essential for hybrid sterility. Males whose markers all derive from Bogota but who are *se* are almost always fertile (90.5% fertility), while males whose markers all derive from Bogota but who are *se*$^+$ are almost always sterile (7.5% fertility).

The *se*$^+$ region's effect was missed in all previous studies of Bogota-USA hybrid sterility (Prakash 1972; Dobzhansky 1974; Orr 1989a,b). Looking across these studies, it is clear that a small portion of the *X* (including *se*) remained unlinked to any markers used in these analyses. As luck would have it, this region harbors a gene or genes of major effect.

The *se*$^+$ region: We want to know if the *XR* factor(s) near *se*$^+$ maps to the left or right (or both) of this marker. To test the region to the right of *se*$^+$, we crossed Bogota-ER females to USA *ct se ll sp tt* males and backcrossed the resulting F_1 females to USA *ct se ll sp tt* males. As the *ll* marker cannot be reliably scored and as *tt* resides in a region known to have no effect, we scored the remaining three markers. We thus determined if recombination between *se* and *sp* affects hybrid fertility: it does not (Table 3). Controlling for genotype at *se*, *sp* genotype has no effect on fertility: *ct*$^+$ *se*$^+$ *sp*$^+$ males, for example, are no more sterile than *ct*$^+$ *se*$^+$ *sp* ones ($\chi^2 = 0.225$, 1 d.f., $P = 0.64$); similarly, *ct*$^+$ *se sp*$^+$ males are no more sterile than *ct*$^+$ *se sp* ones ($\chi^2 = 3.23$, 1 d.f., $P = 0.07$). Similar results were obtained in an independent test in which USA *ct sd y se sp* males were crossed to Bogota-ER females (not shown).

To test the region to the left of *se*, we crossed Bogota-ER females to USA *y co se* males and backcrossed the resulting F_1 females to USA *y co se* males. While addition of *co*$^+$ has no effect on a *se* background (Table 4; $\chi^2 = 0.01$, 1 d.f., $P = 0.95$), it *does* have an effect on a *se*$^+$ background (Table 4; $\chi^2 = 4.00$, 1 d.f., $P = 0.04$). A factor causing hybrid male sterility thus resides to the left of *se*$^+$. This factor (or at least one factor in the region), however, must be tightly linked to *se*$^+$. Otherwise, one cannot explain why *ct*$^+$ *y*$^+$ *se sh*$^+$ males are nearly always fertile while *ct*$^+$ *y*$^+$ *se*$^+$ *sh*$^+$ males are nearly always sterile (Table 2).

Note that this tight linkage between hybrid sterility and *sepia* cannot be explained by suppression of recombination in the region (*e.g.*, by an inversion). Tables 3 and 4 instead show that recombination rates both to the right and left of *sepia* in hybrids are normal or even slightly higher than expected. Similarly, repeated cytological examination of Bogota-USA hybrid salivary gland chromosomes confirmed normal pairing along the entire *X* (not shown).

We now confirm the existence of an essential hybrid sterile(s) near *sepia* in a much larger experiment involving 2500 hybrid males distributed over 16 *X* chromosome genotypes.

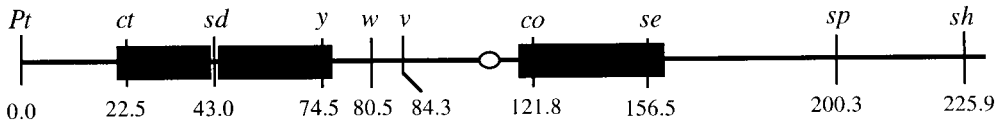

Figure 1.—Linkage map of the *D. pseudoobscura X* chromosome. Markers used in this and our previous analyses of Bogota-USA hybrid sterility are shown. The small circle gives the approximate position of the centromere (material to the left resides on *XL* and material to the right on *XR*). The solid bars represent chromosome regions known to play a role in hybrid male sterility. The remaining regions appear to have no effect on hybrid fertility.

TABLE 3

Recombination to the right of *sepia* has no effect on hybrid fertility

Genotype	No. fertile	No. sterile	% fertile
ct se sp	110	6	94.8
ct se sp$^+$	98	3	88.2
ct se$^+$ *sp*	64	35	65.6
ct se$^+$ *sp*$^+$	81	71	53.2
ct$^+$ *se sp*	83	4	95.4
ct$^+$ *se sp*$^+$	39	6	86.6
ct$^+$ *se*$^+$ *sp*	38	48	44.1
ct$^+$ *se*$^+$ *sp*$^+$	37	54	40.6

Conspecific epistasis on the *X*: We constructed a multiply marked USA stock that carries the *ct sd y se* markers (Figure 1). We crossed males from this stock to Bogota-ER females and backcrossed the resulting F_1 females to the *ct sd y se* stock. We scored the fertility of all hybrid backcross males. Our results reveal several important points (Figure 2). First, the *XR se*$^+$ region is required for hybrid sterility. As Figure 2 shows, *se* genotype is a near perfect predictor of fertility; *e.g.*, the top half of the plot corresponds to *se* males, who are nearly always fertile. Second, the *se*$^+$ region from Bogota is necessary but not sufficient for sterility—several of the *se*$^+$ genotypes in the bottom half of the plot are highly fertile. Put differently, hybrid male sterility involves strong conspecific epistasis. To be sterile, a hybrid must carry Bogota material on both *XR* and *XL*. Remarkably, neither region alone has *any* effect on hybrids. Genotype 8, for instance, carries Bogota material at all three *XL* markers, but is completely fertile (98%). Genotype 9 carries Bogota material at the *XR* marker but is completely fertile (95%). But genotype 16 carries Bogota material at *both* the *XL* and *XR* markers and is essentially completely sterile (3%). Thus conspecific epistasis between *XL* and *XR* is complete. Bogota-USA hybrid male sterility must, therefore, require the right genotype at at least three loci, because the Bogota *XL* and *XR* regions must interact with at least one locus from USA.

The data in Figure 2 also allow us to dissect the known effect of *XL* on hybrid sterility. Previous work showed that no steriles of substantial effect reside between *Pt* (*1*-0.0) and *ct* (*1*-22.5; Orr 1989b) or between *y* (*1*-74.5) and *v* (*1*-84.3; Orr 1989a). The effect of *XL* is therefore due to material between *ct*$^+$ and *y*$^+$. Because the *sd* marker resides between these genes, we now ask if the *ct*$^+$-*y*$^+$ region's effect is due to material to the left or right of *sd* or both. The answer is that both the *ct*$^+$-*sd*$^+$ and the *sd*$^+$-*y*$^+$ regions appear to affect male fertility. This is most easily seen by comparing particularly informative pairs of genotypes. Comparison of genotypes 9 and 10 (Figure 2), for instance, shows that recombination to the left of *sd* affects fertility ($\chi^2 = 10.9$, 1 d.f., $P = 0.0009$), while comparison of 9 and 12 shows that recombination to the right of *sd* affects fertility ($\chi^2 = 184.1$, 1 d.f., $P < 0.0001$). The *ct* marker effect represents the one case in which our results were not fully confirmed by the multivariate PROC CATMOD analysis (see materials and methods): although PROC CATMOD confirms highly significant effects of *se*, *y*, and *sd*, it yields borderline-significant to nonsignificant effects of *ct*, depending on model details. Nonetheless, we feel that the weight of the evidence, including data that emerge later (*e.g.*, Table 7), suggests that a hybrid sterile(s) resides between *ct* and *sd*, as indicated in Figure 1. This issue is discussed later when the relevant new data appear.

We have thus identified a total of three regions of the Bogota *X* causing hybrid male sterility (Figure 1). The simultaneous presence of all three on a largely USA background is sufficient to cause complete hybrid male sterility (genotype 16). We now roughly map the USA factors that interact with these Bogota *X* regions to cause hybrid sterility.

Role of the USA autosomes: As sterile F_1 males do not carry a USA *X* or cytoplasm, USA hybrid steriles causing F_1 sterility must be restricted to the *Y* and/or autosomes. Orr (1989a) showed that the *Y* plays little or no role in Bogota-USA hybrid sterility; instead both he and Dobzhansky (1974) suggested that sterility reflects an interaction between the Bogota *X* and USA autosomes. We now test the role of each autosome.

Only those autosomal factors that are partially dominant can affect F_1 hybrids. To locate such factors we must compare the fertility of *Bog/Bog* homozygotes with *Bog/USA* heterozygotes, which requires backcrossing to Bogota and the use of dominant USA markers. We do not attempt to fine map dominant USA hybrid steriles here. Instead we ask: (1) if entire autosomes affect hybrid fertility; (2) how these autosomes interact with each other; and (3) if there are large regions of these autosomes having no effect on fertility.

To test the role of the second and third chromosomes, we crossed USA *Ba* (*2*-62.1, associated with an inversion); *L* (*3*, associated with medial Santa Cruz inversion) females to Bogota-ER males; we then chose the phenotypically Ba; L F_1 males and backcrossed them to Bogota-ER females. Because we backcross through F_1 males who show no recombination, single mutations mark the origin of entire chromosomes.

TABLE 4

Recombination to the left of *sepia* affects hybrid fertility

Genotype	No. fertile	No. sterile	% fertile
y$^+$ *co se*	122	27	81.9
y$^+$ *co*$^+$ *se*	139	30	82.2
y$^+$ *co se*$^+$	18	71	20.2
y$^+$ *co*$^+$ *se*$^+$	27	206	11.6

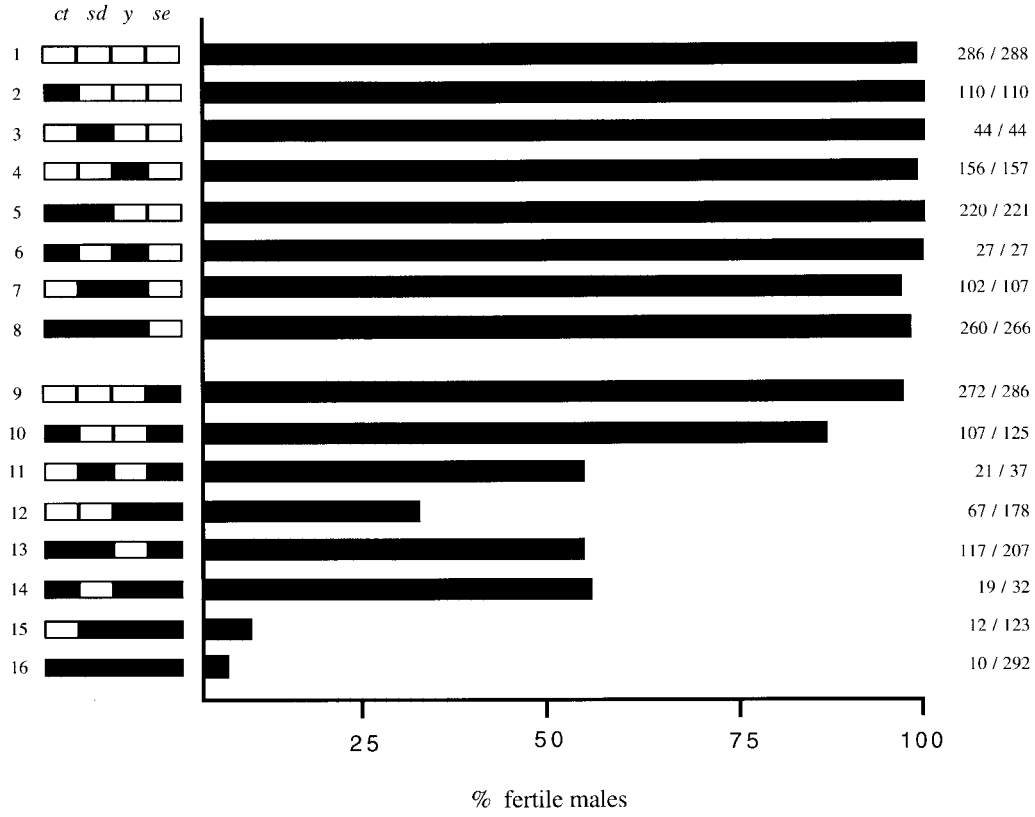

FIGURE 2.—Genetic dissection of the effect of the *X* on hybrid male sterility using the *ct*, *sd*, *y*, and *se* markers. Chromosome regions from Bogota are solid and those from USA are open. The number of males of a genotype that are fertile over the total number of males scored is shown at the far right. Hybrid sterility involves strong epistatic interaction between *XL* (marked by *ct*, *sd*, and *y*) and *XR* (marked by *se*).

Both the USA second and third affect hybrid fertility (Figure 3). [Neither effect can be due to marker effects as preliminary tests confirmed that marked USA flies are fully fertile (not shown).] Remarkably, we again find evidence of conspecific epistasis. Although the USA third chromosome has no fertility effect when moved alone into a Bogota background (Figure 3; compare genotypes 1 and 2), it has a large effect when present with the USA second chromosome (Figure 3; compare genotypes 3 and 4).

To test the role of the fourth chromosome (a major chromosome in *D. pseudoobscura*), we crossed USA *Cy* (*4*-67.2, associated with inversion)/+ females to Bogota-ER males and backcrossed phenotypically Cy F_1 males to Bogota-ER females. Backcross males inherit a complete Bogota *X* as well as unrecombined USA or Bogota fourth chromosomes. Our results suggest that the fourth has a modest (13.8%) but significant effect on hybrid fertility (Table 5; $\chi^2 = 9.5$, 1 d.f., $P = 0.002$). Unfortunately, within-subspecies controls show that this effect is due to the *Cy* marker *per se* or something linked to it: pure USA *Cy*/+ males are fertile 78.2% of the time ($N = 129$), while their +/+ brothers are fertile 90.7% of the time ($N = 161$), a significant effect of 12.4% ($\chi^2 = 8.7$, 1 d.f., $P = 0.003$). The *Cy* chromosome thus has almost exactly the same effect on fertility within as between subspecies—the only instance of such a marker effect in our analysis—and we thus have no evidence for a role of the fourth in hybrid sterility. This conclusion agrees with that of DOBZHANSKY (1974).

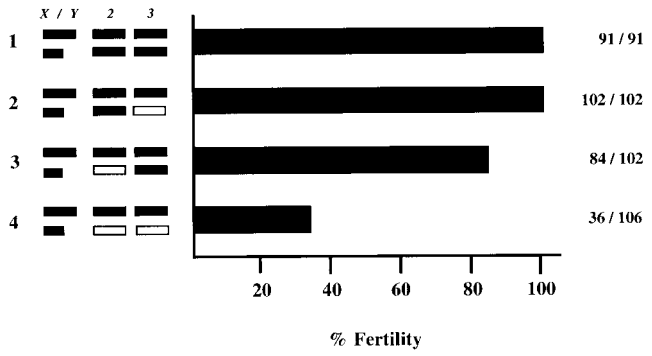

FIGURE 3.—Test of the effect of the USA second and third chromosomes on hybrid male sterility. All chromosomes are unrecombined. Those from Bogota are solid and those from USA are open. Chromosome *3* has no effect on hybrid fertility unless *2* is also present.

No dominant markers are available on the dot fifth chromosome. Although it seems unlikely that such a small chromosome would play a major role in hybrid sterility (but see ORR 1992), we tested for the presence of any partially recessive USA factors affecting fertility via use of the *spa* (*V*) recessive marker. Because the dot chromosome does not recombine, *spa* marks the entire chromosome. Our results show that the fifth has no effect on hybrid fertility. *spa/spa* and *spa/Bog* backcross males show almost exactly the same fertility: 75.9% ($N = 141$) and 73.7% ($N = 179$), respectively ($\chi^2 = 0.19$, 1 d.f., $P = 0.66$).

TABLE 5

Test of chromosome *4*'s role in hybrid sterility

Genotype	No. fertile	No. sterile	% fertile
Hybrid individuals			
Cy/Bog	118	153	43.5
Bog/Bog	132	98	57.4
Within-species individuals			
Cy/USA	101	28	78.2
USA/USA	146	15	90.7

Within-species data derive from pure USA stock males.

TABLE 6

The proximal region of the USA second chromosome has no effect

Genotype	No. fertile	No. sterile	% fertile
y^+ se^+ *; Ba/Bog*	131	104	55.7
y^+ se^+ *; Bog/Bog*	135	70	65.9
y^+ se^+ *; Dl/Bog*	101	28	78.3
y^+ se^+ *; Bog/Bog*	153	45	77.2

Our only attempt to localize hybrid steriles within autosomes involved the USA second. To map factors to the proximal *vs.* medial regions of the chromosome, we used the widely separated markers *Dl* (*2*-8.4; proximal) and *Ba* (*2*-62.1; medial and associated with an inversion). We crossed Bogota-ER females to *y se; Ba/Dl* males and separately backcrossed phenotypically Ba and phenotypically Dl females to Bogota-ER males. Scoring y^+ se^+ backcross males (who carry the *X* chromosome material required for sterility), we find that hybrid steriles are limited to the medial region of the chromosome. Table 6 shows that *Bog/Bog* males are significantly more fertile than *Ba/Bog* ones ($\chi^2 = 4.68$, 1 d.f., $P = 0.03$). *Bog/Bog* males are not, however, more fertile than *Dl/Bog* ones ($\chi^2 = 0.047$, 1 d.f., $P = 0.83$).

Interactions among hybrid steriles: We have found five regions causing hybrid male sterility: three from Bogota (two on *XL* and one on *XR*) and two from USA (one medial on *2* and one on *3*). In this and previous work we have also uncovered chromosome regions having no discernible effect on hybrid fertility: three regions of the Bogota *X* (Figure 1), the *Y*, the proximal end of *2, 4*, and *5*. Because we have good marker coverage—the entire Bogota *X* has been searched for hybrid steriles using 10 markers and the USA autosomes have been tested without recombination—it seems likely that we have identified most chromosome regions having a substantial effect on F_1 hybrid fertility. (We have not, of course, fine mapped these regions, but that is a separate issue.) In particular, Figure 3 shows that the combination of an (unrecombined) Bogota *X* and (unrecombined) USA second and third causes a 70% drop in fertility. The above five regions thus explain the majority, though not all, of Bogota-USA hybrid sterility.

We now want to know how these five chromosome regions interact to cause hybrid sterility. While we already have some information on these interactions, the genetics of sterility appears simple enough that we can disentangle the entire network of interactions among the above five regions.

To do so, we performed a large backcross analysis in which all five regions were simultaneously marked. In particular, we crossed Bogota-ER females to *ct sd y se*; *Ba/+; L/+* males and collected phenotypically Ba and L F_1 females and backcrossed them to Bogota-ER males. The resulting backcross males carry all possible combinations of the five known hybrid sterile regions. Because, unlike in the above autosomal crosses, we use recombining F_1 females, single markers do not mark the subspecies origin of whole autosomes (despite inversions) and we thus have no guarantee that extreme genotypes will show a "complete" (*i.e.*, 70%) drop in fertility. Because this backcross produces a very large number (64) of genotypes, we simplified our analysis in one way. Because we know that the se^+ region from Bogota is required for sterility, *se* males are uninformative and we thus scored only se^+ flies. To again ensure that se^+ is required for sterility, we made one exception to this rule—scoring the fertility of ct^+ sd^+ y^+ *se*; *Ba/Bog; L/Bog* males for reasons explained below. In total, then, we scored 33 backcross genotypes.

Our results from 2500 genotyped and phenotyped males are shown in Table 7. For ease of presentation, Table 7 is broken into sets of *X* chromosome genotypes. For each *X* genotype, we present results for males who carry each of the four possible autosomal genotypes. First note that our exceptional ct^+ sd^+ y^+ *se*; *Ba/Bog; L/Bog* males are fully fertile (genotype 33). Because these males carry all the Bogota and USA regions required for sterility *except* the se^+ region from Bogota, this result confirms our previous finding that the se^+ region is required for sterility. The new and important point that emerges from Table 7 is simple: only 3 of 33 genotypes show any sterility. Indeed, no hybrid sterility appears until males carry at least four of the right regions from Bogota and USA. In particular, genotype 28 carries the sd^+-y^+ and se^+ regions from Bogota as well as the USA second and third. Similarly, genotype 30 carries the ct^+-sd^+, sd^+-y^+, and se^+ regions from Bogota as well as the USA second. Either of these *X*-autosomal combinations causes 8–14% sterility. But not until males carry all five of the appropriate regions do we see substantial hybrid sterility: genotype 32 shows 30% sterility. Thus the pattern of epistasis underlying Bogota *X*-USA autosome hybrid sterility is remarkably complex. Hybrids must carry the proper genotype at at least four regions to show any sterility at all. [Note that Table 7 also confirms the existence of a hybrid sterile(s) in the ct^+-sd^+ region;

TABLE 7

Epistasis between chromosome regions causing hybrid sterility

Genotype	No. fertile	No. sterile	% fertile
1 *ct sd y +; +; +*	123	0	100
2 *ct sd y +; Ba; +*	103	0	100
3 *ct sd y +; +; L*	93	0	100
4 *ct sd y +; Ba; L*	90	1	99
5 *ct + y +; +; +*	29	0	100
6 *ct + y +; Ba; +*	20	0	100
7 *ct + y +; +; L*	18	0	100
8 *ct + y +; Ba; L*	19	0	100
9 *+ sd y +; +; +*	37	0	100
10 *+ sd y +; Ba; +*	52	0	100
11 *+ sd y +; +; L*	20	0	100
12 *+ sd y +; Ba; L*	35	0	100
13 *ct sd + +; +; +*	100	0	100
14 *ct sd + +; Ba; +*	93	1	99
15 *ct sd + +; +; L*	56	0	100
16 *ct sd + +; Ba; L*	96	0	100
17 *+ + y +; +; +*	104	1	99
18 *+ + y +; Ba; +*	110	1	99
19 *+ + y +; +; L*	90	0	100
20 *+ + y +; Ba; L*	107	0	100
21 *+ sd + +; +; +*	20	1	95
22 *+ sd + +; Ba; +*	22	0	100
23 *+ sd + +; +; L*	11	0	100
24 *+ sd + +; Ba; L*	11	0	100
25 *ct + + +; +; +*	71	0	100
26 *ct + + +; Ba; +*	70	0	100
27 *ct + + +; +; L*	52	0	100
28 *ct + + +; Ba; L*	42	7	86
29 *+ + + +; +; +*	223	2	99
30 *+ + + +; Ba; +*	139	12	92
31 *+ + + +; +; L*	147	1	99
32 *+ + + +; Ba; L*	114	50	70
33 *+ + + se; Ba; L*	84	0	100

For ease of presentation, Bogota alleles are shown as + symbols.

e.g., while the extreme genotype 32 (ct^+ sd^+ y^+ se^+*; Ba; L*) is often sterile, removal of the ct^+ allele significantly (genotype 28) improves fertility ($\chi^2 = 5.25$, 1 d.f., $P = 0.026$); see also genotypes 30 *vs.* 26 ($\chi^2 = 5.88$, 1 d.f., $P = 0.015$).]

The number of hybrid steriles: Although hybrid sterility involves complex epistasis, Table 7 includes data confirming that the total number of factors causing postzygotic isolation between these taxa is fairly modest. Genotype 1, for instance, is hemizygous for the entire *XL* from Bogota and is homozygous for much of the second and third autosomes from USA. Despite this extreme hemizygous-homozygous genotype, it remains perfectly fertile.

To test the generality of this finding, we produced two other extreme homozygous-homozygous hybrid genotypes. In particular, we crossed *y; Ba/Dl; or/or* females to Bogota-ER males and then crossed *y/Bog; Ba/Bog; or/Bog* females to their *y; Dl/Bog; or/Bog* brothers, forming F_2 hybrids. We scored the fertility of three F_2 genotypes, with the following results. First, *y; Bog/Bog; Bog/Bog* (or *or/Bog*) hybrid males are highly fertile (90.9%, $N = 398$). This shows that the USA *X* region near *yellow* is compatible with much of the Bogota second chromosome, despite the fact that both regions are effectively homozygous. Second, there is no significant difference between the fertility of *y; Ba/Bog; or/or* and *y; Bog/Bog; or/or* males (83.5%, $N = 139$ and 82.0%, $N = 167$, respectively; $\chi^2 = 0.11$, 1 d.f., $P = 0.74$), despite the fact that the latter genotype is homozygous for much of the second from Bogota and homozygous for a region of the third from USA. The fact that such extreme homozygous-homozygous genotypes remain fertile is particularly surprising and strongly suggests that the Bogota and USA subspecies have diverged at a fairly modest number of loci causing hybrid sterility.

Tests of maternal effect: Backcross males who carry the appropriate regions of the Bogota *X* on a largely USA background are essentially completely sterile (Table 1; Figure 2). This finding differs from those obtained in previous studies. Neither Prakash (1972), Dobzhansky (1974), nor Orr (1989a) were able to recover backcross males that were as sterile as F_1 males, a finding that suggested hybrid sterility might involve a maternal effect (Dobzhansky 1974; Orr 1989a). This conclusion now appears unnecessary. But the fact that a maternal effect is not necessary does not, of course, mean that it is not present. Maternally acting genes might still exist and affect hybrid fertility.

To test this possibility, we screened the entire Bogota genome (except the dot fifth chromosome) for maternal effect genes. In particular, we screened for regions that cause greater male sterility when homozygous (*Bog/Bog*) than heterozygous (*Bog/USA*) in mothers, where the zygotic genotype of the son is held constant across the contrast. This difference in maternal genotype corresponds to the one that would be required to contribute to the greater fertility of backcross than F_1 males.

To test *XL*, we first crossed Bogota-ER females to USA *Pt y* males and backcrossed the F_1 females to Bogota-ER males. This produced two classes of backcross females, *Pt/Bog* and *Bog/Bog*. Each was separately crossed to USA *Pt y* males and the resulting Pt^+ y^+ sons were scored for fertility. Maternal genotype had no effect on male fertility: *Pt/Bog* mothers produced sons showing 39.6% fertility ($N = 111$), while *Bog/Bog* mothers produced sons showing 39.8% fertility ($N = 236$; $\chi^2 = 0.001$, 1 d.f., $P = 0.97$). Although we have no dominant markers on *XR* we tested its role in the following way: we produced hybrid females who were *Bog/USA* heterozygotes for the entire *X* by backcrossing F_1 males from *Pt y* females ×

TABLE 8

Test of autosomal maternal effect

Maternal genotype	No. fertile	No. sterile	% fertile
Ba/Bog; L/Bog	112	7	94.1
Ba/Bog; Bog/Bog	194	14	93.3
Bog/Bog; L/Bog	180	13	93.3
Bog/Bog; Bog/Bog	306	22	93.3

Genotype given is that of hybrid mother. Data reflect fertility of Ba^+ L^+ sons.

TABLE 9

Wolbachia plays no role in Bogota-USA hybrid male sterility

Genotype	No. fertile	No. sterile	% fertile
F_1 (Bog-ER × *ct sd y se*)	7	112	5.9
F_1 (Bog-ER [TET] × *ct sd y se* [TET])	14	194	6.7

TET refers to stocks of Bogota and USA that were reared for several generations on medium containing tetracycline, following Hoffmann and Turelli's (1988) protocol.

Bogota-ER males to Bogota-ER females. We then produced hybrid females who had a 50:50 mixture of *Bog/USA* or *Bog/Bog* material at *XR* by performing the same cross but by backcrossing through F_1 females. Females from each cross were crossed to USA wild-type (SC) males and Pt^+ y^+ sons scored for fertility. Once again, maternal genotype has no effect: sons of *Bog/USA* females at *XR* enjoy 64.8% fertility ($N = 182$), while sons of the mixed *Bog/USA* and *Bog/Bog* mothers enjoy 62.0% fertility ($N = 205$; $\chi^2 = 0.35$, 1 d.f., $P = 0.56$).

To test the autosomes for maternal effect genes, we crossed USA *Ba/+; L/+* females to Bogota-ER males and crossed phenotypically Ba L F_1 males back to Bogota-ER females. This produced four classes of backcross females: *Ba/Bog L/Bog*; *Ba/Bog Bog/Bog*; *Bog/Bog L/Bog*; and *Bog/Bog Bog/Bog*. Because backcrossing proceeds through F_1 males, markers mark the origin of whole chromosomes. Each class of female was crossed to wild-type USA SC males and the fertility of their Ba^+ L^+ sons was scored. Maternal genotype again had no effect on male fertility. Table 8 shows that all four female genotypes produced sons of identical fertility (heterogeneity $\chi^2 = 0.001$, 3 d.f., $P = 0.99$).

To test the fourth chromosome, analogous crosses were performed but with the *Cy* marker, where *Cy* again marked the subspecies origin of the entire chromosome. Once again, maternal genotype had no effect on male fertility: *Cy/Bog* mothers produced sons showing 90.0% fertility ($N = 150$), while *Bog/Bog* mothers produced sons showing 94.2% fertility ($N = 189$), where only Cy^+ sons were scored in each case ($\chi^2 = 2.06$, 1 d.f., $P = 0.15$).

In sum, neither the *X* nor the major autosomes harbor maternal factors having a discernible effect on hybrid male fertility. Last, we tested whether Wolbachia (or any other tetracycline-susceptible endosymbiont) might play a role in Bogota-USA male sterility. It does not. As Table 9 shows, the cross of Bogota females × USA males invariably produces sterile F_1 males, whether or not the stocks used were reared on tetracycline for several generations, a result that confirms that of Noor and Coyne (1995). *D. pseudoobscura* Bogota-USA hybrid male sterility is caused by zygotically acting nuclear genes, not by maternal effect genes or endosymbionts.

DISCUSSION

We have reached four main conclusions. First, *D. pseudoobscura* Bogota-USA hybrid sterility appears to involve a fairly modest number of genes. Although the basis of hybrid sterility is more complex than suggested by earlier work, it appears that the number of factors of substantial effect on hybrid fertility is not very large. In particular, use of a larger number of genetic markers—17, including 10 on the *X*, where we sum over this and our previous analyses (Orr 1989a,b)—allows us to better define the boundaries of chromosome regions that do and do not include hybrid steriles. This work reveals several regions of large effect on hybrid fertility and, more important, several large regions of no discernible effect. As Figure 1 shows, for instance, three regions of the Bogota *X* chromosome play a role in hybrid sterility, but several large regions do not, including the tip of *XR* [which has been repeatedly tested (Orr 1989a and above)], the region near *vermilion*, and the tip of *XL*. Similarly, our results show that the proximal end of the second, the entire fourth, and the *Y* chromosomes have no apparent effect on hybrid fertility. Last and most surprising, additional tests show that at least three combinations of homozygous Bogota *vs.* homozygous USA genotypes—extreme genotypes that would suffer the full brunt of all hybrid steriles, including recessive ones—are fertile.

These findings appear inconsistent with a highly polygenic basis for sterility. Moreover, these results differ dramatically from those seen in studies of other species pairs, *e.g.*, *D. pseudoobscura-D. persimilis* (Orr 1987) and *D. simulans-D. mauritiana* (reviewed in Wu *et al.* 1996). (Our finding of fertile homozygous-homozygous extreme genotypes is particularly unimaginable in these other species pairs.) As emphasized in the Introduction, the likely reason for this difference seems clear. Bogota-USA is a young hybridization (Schaeffer and Miller 1991; Wang *et al.* 1997). The fact, therefore, that Bogota-USA hybrid sterility is characterized by large chromosome regions of no effect, while *D. pseudoobscura-D. per-*

similis and *D. simulans-D. mauritiana* are not, suggests that analysis of older species pairs may lead to overestimates of the number of genes required for postzygotic isolation. Note that this difference in results holds even when restricting attention to the hemizygous *X*, where we need not be concerned with the effects of dominance on detection of hybrid steriles.

We can go farther and estimate the number of genes causing hybrid male sterility. This is best done via the higher resolution *X* chromosome data. In particular, we can perform a maximum likelihood analysis asking what number of hybrid steriles most often yields the observed data when randomly sprinkled on the map shown in Figure 1. The point is that the sizes of regions of no effect can be used to infer the true number of steriles: the probability of observing so many such regions obviously declines as hybrid steriles grow too common. A simple Monte Carlo simulation (involving one million simulations at each of $i = 3, 4, \ldots$ hybrid steriles) shows that the most likely number of hybrid steriles on the Bogota *X* is, in fact, 3. Using the 2-unit support limit rule, *i.e.*, rejecting likelihood values that are $< e^{-2}$ as likely, the number of hybrid steriles could be as high as 6. As the *X* represents ~40% of the *D. pseudoobscura* genome, our best guess is that ~15 hybrid steriles separate Bogota and USA ($= 3/0.4 \times 2$ subspecies), although we cannot reject a total of 30. Many of these factors, however, probably would not contribute to F_1 fitness problems as our estimate derives from the hemizygous *X*, and partially recessive factors will, if autosomal, make little contribution to F_1 hybrids. The *X* chromosome may not, of course, be representative of the rest of the genome. But, if anything, the density of *X*-linked hybrid steriles is likely to be higher than that on the autosomes (Charlesworth *et al.* 1987; True *et al.* 1996), making our value an overestimate. More dangerously, we have assumed that the map positions of hybrid lethals are independent, *i.e.*, that they show no tendency to cluster. This may or may not be true (see below). In any case, our estimate should not be taken too literally. The important point is that it is considerably smaller than the similarly rough estimate (~120) obtained from the older *D. simulans-D. mauritiana* species pair, a result that provides some support for the rapid "snowballing" of the number of hybrid steriles and lethals with time (Orr 1995a; Orr and Turelli 2001). Only future fine-scale analysis can provide a more accurate estimate of gene number.

Our second main conclusion is that, despite their fairly modest number, the genes causing hybrid sterility show a complex pattern of epistasis. Indeed, hybrid steriles on the Bogota *XL* have no effect on sterility without those on *XR* and vice versa (Figure 2). Similarly, the USA third chromosome has no effect without the USA second (Figure 3). All told, hybrids must carry the right alleles (Bogota *vs.* USA) at at least four loci before any hybrid sterility appears (Table 7). Strong hybrid sterility appears only when hybrids carry the right alleles at at least five loci, where sterility reflects an incompatibility between the Bogota *X* and the USA autosomes. It is important to emphasize, therefore, that our analysis has uncovered a single hybrid incompatibility. It cannot, then, be vulnerable to overcounting factors that accumulated after the evolution of complete hybrid male sterility—all mapped factors are required for complete sterility. It is also worth noting that this pattern of complex epistasis is seen whether fertility is measured in all or none, as above, or in three classes (Many, Few, None; see materials and methods). This suggests, although does not prove, that the pattern seen is not an artifact (at least completely) of the unit of measurement, *i.e.*, is not a scale effect.

Epistasis for fitness is, of course, expected for intrinsic postzygotic isolation. Under the Dobzhansky-Muller model (Dobzhansky 1937; Muller 1942), alleles that cause hybrid sterility or inviability cannot have such effects on their normal within-species genetic background as natural selection will not allow the substitution of plainly deleterious alleles. Nonetheless, alleles that have not seen each other during their evolutionary histories may well cause sterility or inviability (partial or complete) when brought together in hybrids. Under this view, epistasis is required among the genes causing intrinsic postzygotic isolation, and its repeated observation in genetic analyses of speciation is rightly taken as support for the Dobzhansky-Muller model. But while epistasis must characterize postzygotic isolation, this argument does not require that it take the form of the very complex conspecific epistasis seen here. Interestingly, such complex epistasis appears common, at least in Drosophila (see Muller 1942, who early emphasized the role of complex epistasis in hybrid sterility; for other examples, see Orr and Coyne 1989; Cabot *et al.* 1994; Davis *et al.* 1994; Davis and Wu 1996). The present example, however, represents one of the most complex examples of hybrid epistasis described to date.

There has been a good deal of speculation about the causes of complex hybrid epistasis. Cabot *et al.* (1994) and Orr (1995a) discussed the problem at length and emphasized one possible explanation. All else being equal, a greater fraction of imaginable paths to the evolution of postzygotic isolation between taxa is allowed by natural selection when incompatibilities are complex. That is, the mathematics of the Dobzhansky-Muller mechanism show that there are more ways of "getting" to two isolated species without passing through a sterile or inviable intermediate when incompatibilities involve more, rather than fewer, factors. More recently, Davis and Wu (1996) argued that complex conspecific epistasis involves tightly linked factors that have little or no individual effect but that cause strong postzygotic isolation when moved as a block onto a foreign background. Indeed, Palopoli and Wu (1994) and Wu and Palopoli (1994) suggested that tight physical linkage

may play a causal role in the evolution of hybrid incompatibilities: within species, such factors may have a favorable effect only when all of the relevant alleles are present simultaneously. If so, physical linkage helps maintain the integrity of these coadapted complexes, easing the conditions for their invasion. The present data call this linkage hypothesis into question. The complete conspecific epistasis seen between Bogota *XL* and *XR*, for instance, involves factors that are essentially freely recombining. Similarly, the conspecific epistasis seen between the USA second and third chromosomes involves factors that reside on separate chromosomes. Similar results were obtained or discussed by Muller (1942; in the *obscura* group), Orr and Coyne (1989; in the *virilis* group), and Davis *et al.* (1994; in the *melanogaster* group). Thus, while there are clear cases in which conspecific epistasis involves tightly linked factors, there are also many cases in which it involves unlinked ones. Physical linkage does not, therefore, play a necessary role in the evolution of complex incompatibilities. This finding casts doubt on the notion that selection of alleles in linkage disequilibrium within species plays a causal role in the invasion of mutations that ultimately cause reproductive isolation.

Incidentally, it is worth noting that the existence of complex hybrid incompatibilities may explain the easy recovery of hybrid rescue mutations (Sawamura *et al.* 1993; Davis *et al.* 1996). In the case of complex hybrid interactions, mutation at *any one* of the relevant loci may suffice to undo hybrid lethality or sterility. Thus complex hybrid incompatibilities may not only be easier to evolve but easier to undo.

Our third main conclusion is that the sterility of *D. pseudoobscura* Bogota-USA hybrids is due largely to *X*-autosomal incompatibilities, in particular to interactions between the Bogota *X* and USA autosomes. While *X*-autosomal interactions have been assumed to play an important role in postzygotic isolation, especially in Haldane's rule (Muller 1942; Orr 1997; Singh 2000), few studies have mapped the location of both *X*-linked and autosomal partners in such incompatibilities.

Last, we have found that one of our previous conclusions was mistaken. Bogota-USA hybrid sterility does not involve a maternal effect. Previous work by Prakash (1972), Dobzhansky (1974), and Orr (1989a) had shown that backcross male genotypes are never as sterile as F_1 males. Prakash attributed this to undetected recombination that separated markers from hybrid steriles while Dobzhansky (1974) and Orr (1989a) favored a maternal effect: F_1 males have pure Bogota mothers while backcross males do not. The present data show that Prakash was correct. This is shown by two findings. First, we can recover backcross male genotypes that are as sterile as F_1 males (Figure 2). Previous workers failed to recover such genotypes because they failed to mark the *sepia* region of the Bogota *X*, a region that we now show is essential for hybrid male sterility. [This also explains why Orr (1989a) saw ~50% fertility in his backcross analysis: marked backcross males segregate for the independently assorting *sepia* region.] Second, we performed a genome-wide screen for maternal effect factors on hybrid sterility and found none. We also confirm (following Noor and Coyne 1995) that neither Wolbachia nor any other tetracycline-sensitive microbe plays a role in Bogota-USA hybrid sterility. [Similarly, see Zeng and Singh (1993) who show that the cytoplasm plays no role in the sterility of *simulans* clade hybrid males.]

It is worth noting that the previously undetected hybrid sterility effect near *sepia* provides promising material for future fine-scale mapping. The region is required for sterility and the factors involved are very tightly linked to the *sepia* locus. Indeed, Tables 2 and 7 show that the *sepia* genotype is a near perfect predictor of hybrid fertility (on the appropriate genetic background). It will be interesting to see if the large effect of this region is due to a single gene or to several linked ones.

In sum, the sterility of Bogota-USA hybrid males appears to involve a fairly modest number of zygotically acting factors. But while few in number, these factors show a complex pattern of epistasis. *D. pseudoobscura* Bogota-USA hybrid sterility is thus simple in one respect (number of factors) but complex in another (pattern of epistasis).

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