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A three-locus system of interspecific incompatibility underlies male inviability in hybrids between *Drosophila buzzatii* and *D. koepferae*

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Abstract

In hybrids between the sibling species *D. buzzatii* and *D. koepferae*, both sexes are more or less equally viable in the F_1. However, backcross males to *D. buzzatii* are frequently inviable, apparently because of interspecific genetic incompatibilities that are cryptic in the F_1. We have performed a genetic dissection of the effects of the X chromosome from *D. koepferae*. We found only two cytological regions, termed *hmi-1* and *hmi-2*, altogether representing 9% of the whole chromosome, which when introgressed into *D. buzzatii* cause inviability of hybrid males. Observation of the pattern of asynapsis of polytene chromosomes (incomplete pairing, marking introgressed material) in females and segregation analyses were the technique used to infer the X chromosome regions responsible for this hybrid male inviability. The comparison of these results with those previously obtained with the same technique for hybrid male sterility in this same species pair indicate that in the X chromosome of *D. koepferae* there are at least seven times more regions that produce hybrid male sterility than hybrid male inviability. We have also found that the inviability brought about by the introgression of *hmi-1* is suppressed by the cointrogression of two autosomal sections from *D. koepferae*. Apparently, these three regions conform to a system of species-specific complementary factors involved in an X-autosome interaction that, when disrupted in backcross hybrids by recombination with the genome of its sibling *D. buzzatii*, brings about hybrid male inviability.

Introduction

Interspecific animal hybrids are frequently sterile or inviable, these disharmonies constituting the postzygotic barriers to gene exchange between species. Models for their evolution (Wu & Beckenbach, 1983; Wu & Palopoli, 1994; Zeng & Singh, 1993; Zouros, 1989) are generally an extension of the ideas first put forward by Dobzhansky (1937) and Muller (1942), and involve the gradual build-up of species-specific systems of complementary genes. These genes may be mutually incompatible if present in the same genome, thus causing hybrid-specific disharmonies while leaving the parental species unaffected. A relatively simple two-locus model for the evolution of hybrid inviability (Coyne, 1994) involving an X-linked and an autosomal locus is presented in Figure 1. A base population (0) of an ancestral species would be fixed for two interacting, complementary alleles, X^0 in the X chromosome and A^0 in the autosome. A derived population (1) may diverge first in the X-linked component, which would finally become fixed for allele $X^1{}_1$. Another population (2) may happen to diverge first in the autosomal component instead, and become fixed for allele $A^2{}_1$. These independent substitutions may create the conditions for new alleles to become fixed in the complementary locus, which, in turn, would affect the probability of new substitutions in the former. Thus, the two loci are expected to evolve in concert, with virtually no fitness loss through the different steps. The two derived populations may finally become two different species, one of them fixed for allele $X^1{}_m$ and $A^1{}_m$, the other

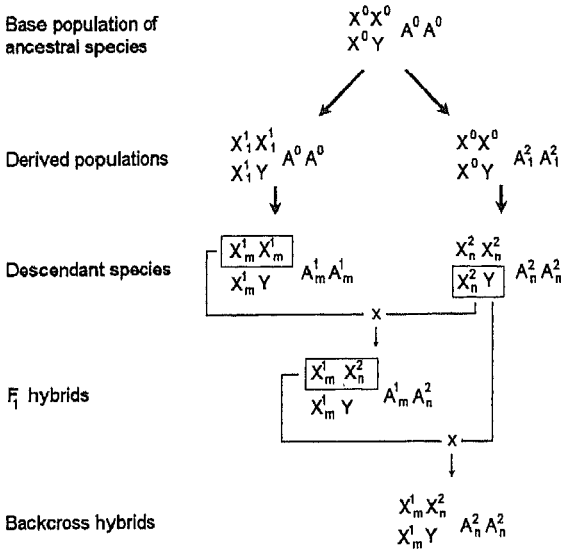

Figure 1. Two-locus model for the evolution of hybrid male inviability, involving an X-linked and an autosomal locus. X and Y designate the sex-chromosomes, with X^0, X^1, and X^2 meaning the alleles present in the base population of the ancestral species, and in the two derived populations (later, descendant species), respectively. A^0, A^1, and A^2 have the same meaning for the autosomal locus. Subindexes are intended to suggest an indeterminant number of successive allelic substitutions in the corresponding loci. XX and XY designate females and males, respectively (both with the same autosomal complement).

for $X^2{}_n$ and $A^2{}_n$, where m and n are intended to illustrate the successive episodes of allele replacement in each species. In the hybrid offspring, F_1 males, for example, may be inviable because of an incompatibility between the male dose of $X^1{}_m$ and the single copy of $A^2{}_n$. However, in other kinds of interspecific hybrids, F_1 hybrid males may happen to be viable, inviability being deferred to certain kinds of backcrosses, due now to an incompatibility between the male dose of $X^1{}_m$ and two copies of $A^2{}_n$. Notwithstanding the simplicity of the model, no experimental study has yet succeeded in the identification of both interacting components $X^1{}_m$ and $A^2{}_n$ (for a review see, Wu & Palopoli, 1994). The gene system that leads to the development of malignant melanoma in *Xiphophorus* interspecific hybrids (Wittbrodt et al., 1989) would have been a perfect illustration of this model, were it not for the fact that melanomas apparently can be produced also by interpopulation crosses (Kallman, 1975, Table 5 and p. 105), so that this system may be acting deleteriously in the source species too, although not as much as in the interspecific hybrids. In *Drosophila*, the study that has been nearest the goal of indentifying both interacting components is a report by Pantazidis and Zouros (1988) on a Y-autosome interaction underlying hybrid male sterility in backcross hybrids between *D. mojavensis* and *D. arizonae*. However, the precision attained in mapping the autosomal component is insufficient to rule out polygenic factors in favor of a single major element, and the Y chromosome contribution was not dissected at all.

Hybrid males produced by most interspecific crosses in *Drosophila* are sterile; conversely, hybrid females are frequently fertile (Bock, 1984). This kind of discrepancy between the heterogametic and the homogametic sex is frequently observed in animal hybrids, and constitutes a part of what is usually known as 'Haldane's rule' (Haldane, 1922). The other possible manifestation of this rule, consisting of crosses that give rise only to hybrid females with males dying before the adult stage, is about 10 times less frequently observed in *Drosophila*, according to Wu and Davis (1993). Besides, it may be argued whether Haldane's rule actually applies to hybrid inviability in *Drosophila*, since there are 14 reported cases that conform to the rule, but also 9 where the reverse is true, i.e., where only female hybrids die (Wu & Davis, 1993, p. 191). These exceptions apparently result from maternal effects, which at least in some cases are temperature-dependent (Orr, 1993a). In a strict sense, Haldane's rule was enunciated for F_1 hybrids, but it may be valid for backcross hybrids, too. F_1 hybrids receive a full chromosome complement from each species (Figure 1), introducing a bias in the kind of interactions observed between the genomes of the two hybridized species. The analysis of recombinants in backcrosses, on the other hand, opens up the possibility of investigating the interactions between introgressed factors from the donor species and chromosome regions made homozygous for the alleles of the recipient species (Figure 1, $X^1{}_m$ vs. $A^2{}_n$). When this second kind of analysis is performed, hybrid sterility and/or inviability determinants that were cryptic in the F_1 are frequently unveiled (see, for inviability, Hennig, 1977; Schäfer, 1979; Zouros, 1981). Even in the case of the hybrids with *D. melanogaster*, it is well known that besides factors such as *Hmr* and *Lhr*, or *mhr* and *Zhr*, which rescue the viability of F_1 hybrids with *D. simulans* (Hutter & Ashburner, 1987; Sawamura et al., 1993a, 1993b; Watanabe, 1979), there are at least nine factors that form complementary groups of recessive lethals and their recessive suppressors (Pontecorvo, 1943). These factors may be more important for the understanding of speciation genetics than those with effect

already in the F_1 hybrids (Provine, 1991). However, little is known about them. On the one hand, with respect to viability, in addition to pseudobackcrosses between *D. melanogaster* and *D. simulans* (Pontecorvo, 1943), only backcross hybrids from *D. arizonensis* x *D. mojavensis* (Zouros, 1981), *D. hydei* x *D. neohydei* (Henning, 1977; Schäfer, 1979), and *D. virilis* x *D. lummei* (Lumme & Heikkinen, 1990), have been studied with certain detail. On the other hand, the genetic analysis of the involved factors rarely has gone beyond their mere adscription to a chromosome, thus permitting neither an evaluation of their number nor their relative effects on fitness. In both of these aspects, information on hybrid sterility is considerably better (Wu & Palopoli, 1994). Therefore, before attempting to compare the relative rates of evolution of hybrid inviability and hybrid sterility or the genetic architecture underlying them, more detailed information on hybrid inviability factors is needed.

In this paper we investigate the genetic basis of backcross male inviability in hybrids between *D. koepferae* and *D. buzzatii*. F_1 hybrids between these two species are more or less equally viable in both sexes, but in backcrosses there is a significant excess of hybrid females over males. This excess points to deleterious interactions of recombinant chromosomes on male viability. Actually, previous results (Naveira & Fontdevila, 1986, p. 853) indicated that the substitution of a relatively large, distal segment of the X chromosome of *D. buzzatii* for its homolog in *D. koepferae* was lethal in males. Now, as an extension of those former results, we have arrived at a relatively precise localization of two *D. koepferae* factors on the X chromosome (*hmi-1* and *hmi-2*) that independently determine the inviability of hybrid males. The rest of the X chromosome seems to have no other factors of this kind. Hybrid males introgressed with X chromosome segments containing *hmi-1* from *koepferae* on an otherwise *buzzatii* genetic background die as pupae, either in early or late stages. In addition, we have localized two autosomal factors from *koepferae* that rescue the viability of *hmi-1* hybrid males when simultaneously cointrogressed into *D. buzzatii*, thus building up a three-locus system of complementary genes. This system seems to fit the general model depicted in Figure 1, except for the involvement of two autosomal elements instead of a single one.

Some of the implications of these findings for the current theories on the genetics of speciation in *Drosophila* are discussed.

Materials and methods

Drosophila *species*

D. buzzatii and *D. koepferae* (formerly *D. serido* from Argentina) are two sibling species of the *repleta* group that coexist in many of the arid and semiarid zones of Andean Bolivia and Northwest Argentina (Ruiz, Fontdevila & Wasserman, 1982; Fontdevila et al., 1988). Hybrids between them are not found in the wild, but they can be obtained in the laboratory, although only by crossing *D. koepferae* females with *D. buzzattii* males. The hybrid F_1 consists of sterile males and usually fertile females that can be backcrossed with males of either parental species but most easily with *D. buzzatii*. Both species have the standard *D. repleta* group polytene karyotype consisting of five rod-like chromosomes and a tiny dot-like chromosome. Number 1 corresponds to the X, numbers 2-5 to the long acrocentric autosomes, and number 6 to the dot. Each chromosome is subdivided into cytological intervals, identified by capital letters and numbers (see Figure 3), while the polytene bands in each interval are identified by lower-case letters; alphabetical and numerical order follows from telomere to centromere.

Stocks

The origin of the fly strains is essentially as described in Naveira and Fontdevila (1991a). Eighteen strains of *D. koepferae* and 18 of *D. buzzatii* were derived from independent samples of the populations of these species in the Argentinian locality of 'San Luis'. These strains were crossed in pairs, one from each species, to study the variation in sex-ratio of the hybrid offspring; then, the F_1 hybrid females were backcrossed to the *D. buzzatii* parental strain, with the same objective. Only one of these strains of *D. koepferae* (koeSL.8) and one of *D. buzzatii* (buzSL.8) were used for the detailed introgression experiment described in this paper, together with a *white* mutant (buzSL.101) that arose spontaneously in a strain derived from buzSL.10 (see Fontdevila et al., 1982 for a description of collection sites; koeSL.8 and buzSL.8 are not the strains used in Naveira & Fontdevila, 1986). All the stocks were founded in 1981, except for buzSL.10 and buzSL.101, which date from 1983, and koeSL.8 and buzSL.5, which were established in 1990; all of them were derived from single inseminated females and were kept by mass-matings (10–50 flies) thereafter. Chromosomes X, 3, 4, and 6 of *D. buzzatii* and *D. koepferae* are homosequential in the populations of San Luis, whereas chromosomes 2

and 5 are fixed for species-specific inversions which, in the hybrid females, suppress crossing-over on 70% and 50% of the chromosome, respectively (Ruiz, Fontdevila & Wasserman, 1982). All the stocks were kept in small vials with 5 ml Drosophila Instant Medium, formula 4–24 (Carolina Biological Supplies), at 25°C.

Interspecific hybrids and chromosome asynapsis
The sterility of F_1 hybrid males is the only apparent manifestation of Haldane's rule in this pair of species. The genetic basis of sterility has been investigated with considerable detail (Naveira & Fontdevila, 1986, 1991a, 1991b), using a method of cytogenetic mapping based on the asynapsis (incomplete pairing) of homologous chromosomes in hybrids (Naveira, Plà & Fondevila, 1986), a well known property of many interspecific hybrids of *Diptera*. This method only works well with relatively distant species, because the extent of the asynapsis appears to be correlated with genetic distance (Riede & Renz, 1983). Thus, it can not be applied to species of the *melanogaster* subgroup, but it allows a relatively high resolution in the chromosomal localization of any fixed genetic difference between species such as *D. buzzatii* and *D. koepferae*. In this respect, this technique has proved to be not too much inferior to rather more sophisticated, and considerably more expensive, methods of fine genetic mapping (Wu & Palopoli, 1994). The rationale of the method is quite simple: any part of the genome of a fly that comes to be polytenized in the salivary glands of third instar larvae can be diagnosed as introgressed or not, according to the pairing pattern with the homolog (see Figures 5 and 7). Then, the presence or absence of different introgressed chromosome regions may be correlated with the species-specific phenotypical trait under analysis, and accordingly the genetic factors involved can be referred to the polytene chromosome map.

Introgression into D. buzzatii
General design. The mating protocol was essentially as described in Naveira and Fontdevila (1986, 1991a, 1991b), and it is described schematically in Figure 2. Crosses were performed in groups of 20 females of *D. koepferae* with 20 males of *D. buzzatii*. The first backcrosses also consisted of groups of 10–20 F_1 hybrid females mated with 20 *D. buzzatii* males. Beginning with the second backcross, randomly chosen females in the offspring of the former generation were individually mated with 2 males of *D. buzzatii*. Most females from first backcrosses are recombinant flies whose hybrid constitution is cryptic in the adults, but can be inferred by analyzing the polytene chromosome of several (usually 8) third-instar larvae in their offspring. Each individually mated female is accordingly identified as hybrid (heterozygote) or not for the different chromosome regions of the polytene karyotype. The adult offspring from the different individual crosses (introgression lines) were reserved until the information on the hybrid constitution of all the lines was collected. Those exhibiting the desired introgression were selected to establish the next generation by individually backcrossing several randomly-chosen offspring females (usually 10) with 2 *D. buzzatii* males. This selective process was repeated for several generations, up to the complete elimination of undesired chromosome regions from *D. koepferae*.

Genetic analysis of inviability in recombinant hybrid males. Interspecific crosses were performed between koeSL.8 and buzSL.8. After the first backcross, whenever different lines happened to be introgressed with the same X chromosome segment, those exhibiting the least introgressed fraction of their autosomes were selected to establish the next generation (Figure 2). This selective process was repeated for as many generations as necessary, until only separate chromosome segments, altogether representing the entire X polytene chromosome of *D. koepferae*, were left introgressed into *D. buzzatii* (Figure 2). Then, each introgression line should segregate for hybrid (heterozygous females and hemizygous males) and pure *D. buzzatii* flies. A total sex-ratio (males/females) in the adults of these introgression lines was determined and used as a rough indicator of inviability restricted to hybrid males: if the introgression does not affect the viability of hybrid males, a sex-ratio value near 1 is expected; conversely, the sex-ratio should be around 0.5 if the introgression causes the death of all hybrid males before the adult stage. However, it is already known that when hybrid males introgressed with X chromosome segments get to the adult stage, they are always sterile (Naveira & Fontdevila, 1986, 1991b) with a distinctive atrophy of the testes that can be easily observed through the tegument. This extreme phenotypic manifestation of sterility was also observed in hybrids resulting from the introgression of very large autosomal segments, but it was never observed in *D. buzzatii* or *D. koepferae* controls (Naveira et al., 1984; Naveira & Fontdevila, 1991b). Therefore from among the adult males produced by an X chromosome introgression line, those showing atrophy of the testes can be safely assumed

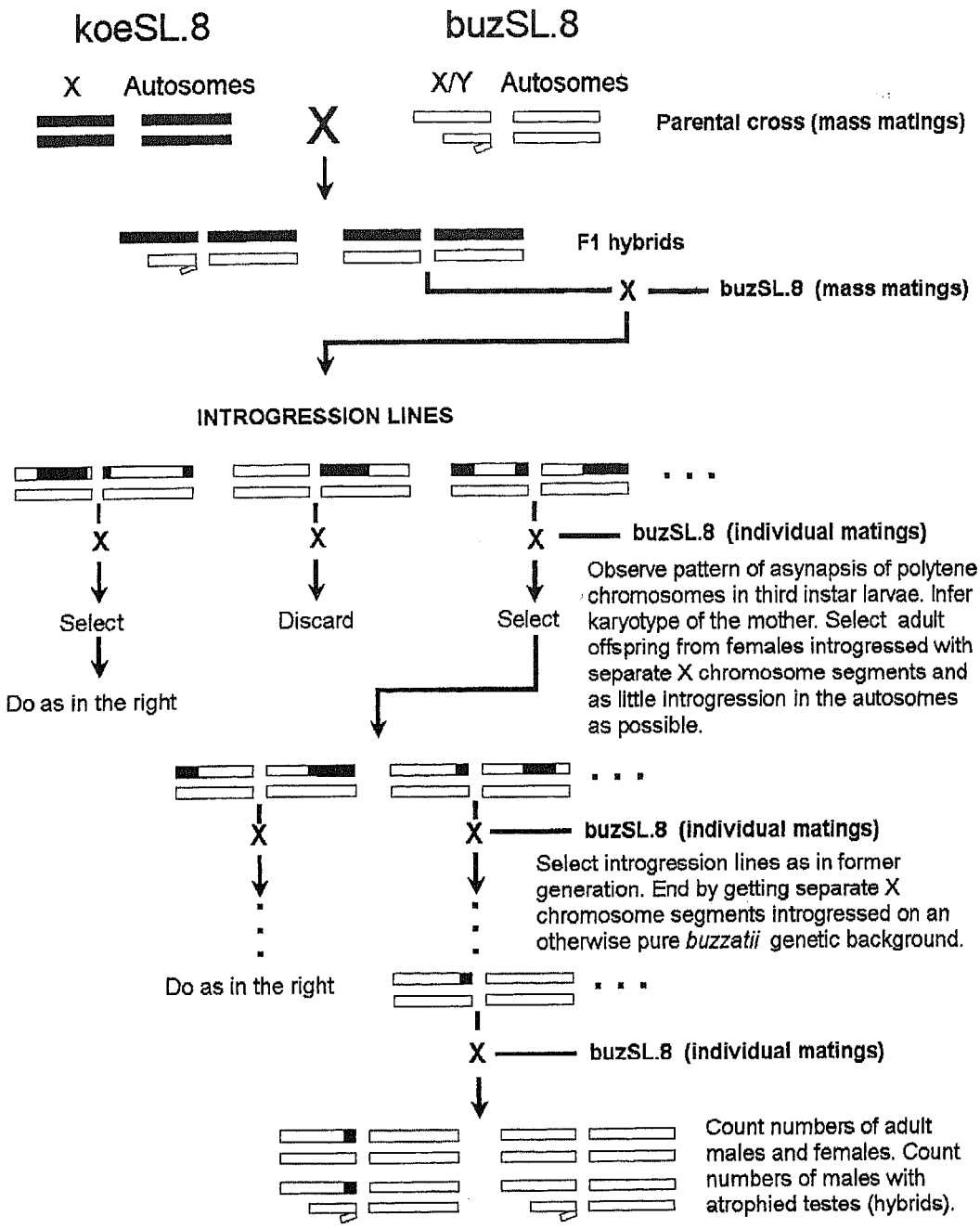

Figure 2. Mating scheme used to investigate the effect of separate X chromosome segments of *D. koepferae* introgressed into *D. buzzatii* on the viability of hybrid males. The chromosomes of *koepferae* are shown in black and those of *buzzatii* in white. Sex chromosomes are shown at the left (X on top; Y, with 'hook', on the bottom). Haploid sets of autosomes are shown at the right.

to be hybrid. A sex-ratio in hybrids can be accordingly estimated simply by dividing the number of males with atrophy by half the total number of females. The extreme values of this sex-ratio would be 0 (when all hybrid males are lethal) and approximately 1 (when all hybrid males are viable). This procedure is expected to produce a positive correlation between the size of introgressed segments and the relative frequency

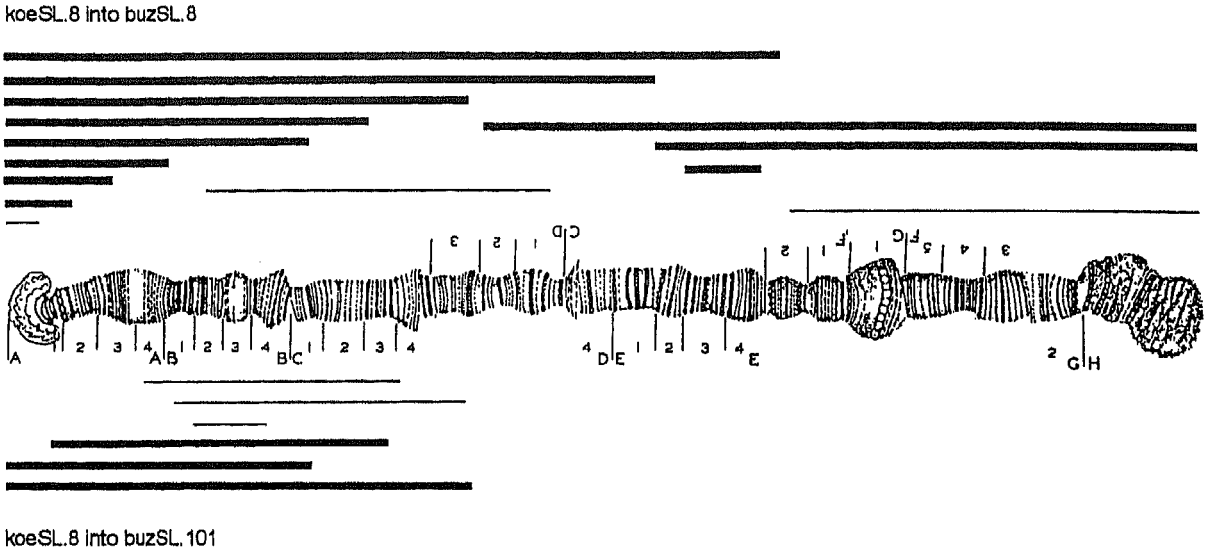

Figure 3. Graphical distribution of X chromosome introgression lines of *D. koepferae* into *D. buzzatii*. Bars represent length and position of *D. buzzatii* segments separately substituted for their corresponding homologs from *D. koepferae*. Segments depicted above the X chromosome correspond to introgression of koeSL.8 into buzSL.8; those below, to introgression of koeSL.8 into buzSL.101. Gross bars correspond to segments that contain factors of hybrid male inviability; thin bars, to segments that do not contain such factors. The polytene chromosome map was constructed on the drawings from Wharton (1942), following Ruiz et al. (1982).

of hybrid males, simply because the longer the segment introgressed in the mother, the more frequent the opportunities for crossing over to give rise to hybrid males (i.e., males with atrophied testes). By overlapping in the polytene chromosome map those regions whose introgression causes a deficit of males and those whose introgression does not, an approximate localization can be given for the genes involved.

In order to improve the resolution of the mapping protocol and investigate the distribution of deaths among the different life stages, we introgressed the telomeric X chromosome segment A-D3b from koeSL.8, which apparently contained a distal factor of hybrid male inviability, *hmi-1*, into buzSL.101 (*white* mutant). The *white* locus has been localized by *in situ* hybridization on polytene band B4a of the X chromosome (M. Labrador, personal communication), not far from the telomere (see Figure 3). The segment we introgressed into *D. buzzatii* included this polytene band, and therefore, as expected, hybrid females were also heterozygotes w/w^+, the wild-type allele marking the X chromosome segment from *D. koepferae*. The introgression line, which we will call from now on Xd[*hmi-1*,w^+], was maintained by crossing individual hybrid females (wild-type, red eyes) with 2 *D. buzzatii* males (white eyes), and selecting those crosses that, according to the analysis of polytene chromosomes in their larval progeny, still contained *hmi-1*. Then, an estimation of the recombination distance between the inviability factor and the white locus could be obtained simply by the ratio of adult w^+ males to w^+/w females from the introgression line (see Figure 4). Furthermore, given that the white phenotype is also manifest in the colour of Malpighian tubules, either white (usually non-hybrid) or wild-type (hybrid) males and females can be easily scored in larval stages, a most useful condition for the analysis of inviability through development.

Determination of inviable life-stages. To study viability from egg to the prepupa stages we collected eggs from the line Xd[*hmi-1*,w^+] in an egg chamber. The eggs were transferred in groups of 50 to small vials with 5 ml Drosophila Instant Medium and allowed to develop at 25°C. All the resulting individuals were collected in the stage of white prepupae and scored as either male or female and as wild-type phenotype (hybrid) or white phenotype (usually non-hybrid) according to the size of their gonads and the colour of the Malpighian tubules. The number of individuals in the four classes was counted and their relative viabilities estimated.

To study the viability from prepupa to adult, we collected third-instar larvae from the line Xd[*hmi-1*,w^+]. The larvae were examined in saline solution (C1 Na

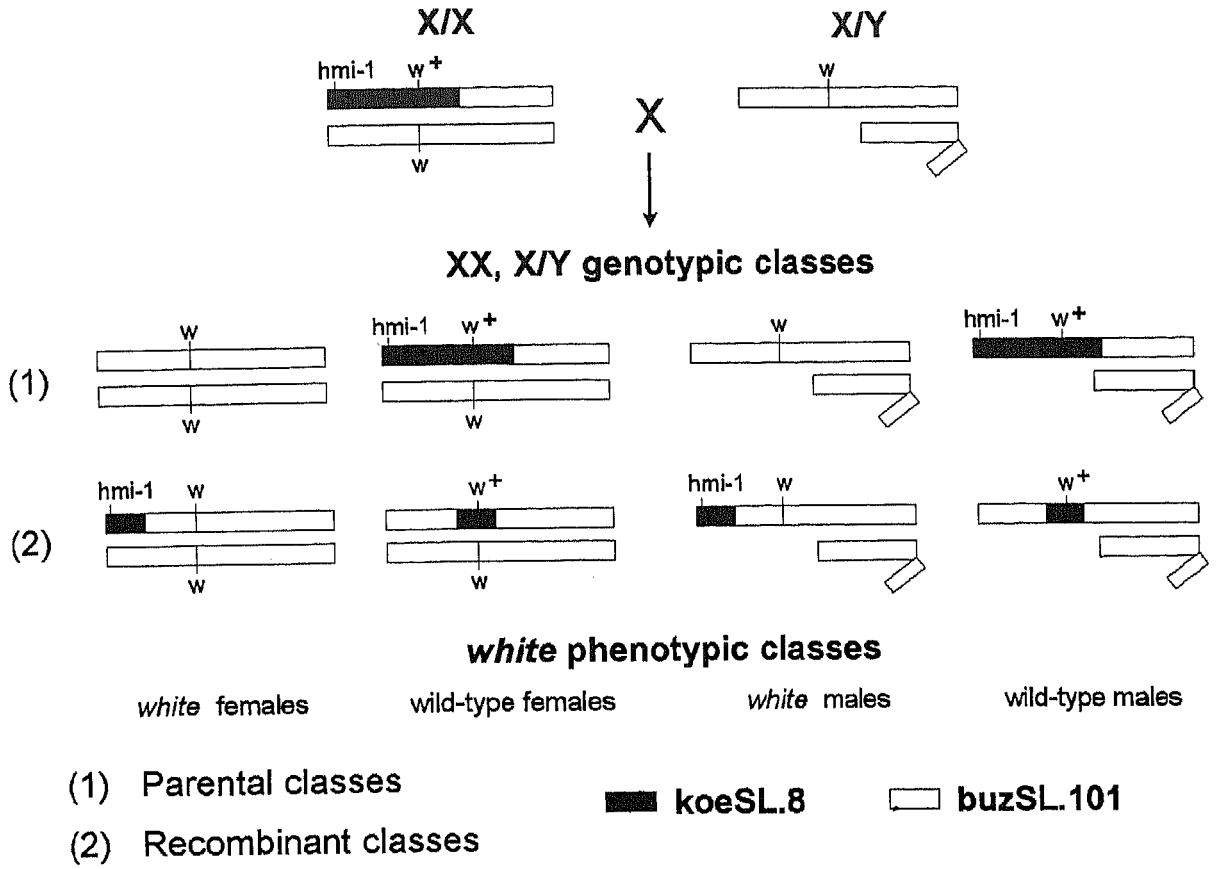

Figure 4. Mating scheme used to estimate the frequency of recombination between the locus *white* and the distal factor of hybrid male inviability, *hmi-1*, in the offspring from hybrid females of the introgression line Xd[*hmi-1*,w^+]. See Materials and methods for more detailed descriptions of stocks and crosses.

0.9%) on a white background, using an Olympus dissecting microscope with lateral illumination close to the stage. Wild-type and white males were identified as before, and two groups of 50 individuals of each kind were placed in different pupation plates consisting of sealed Petri dishes lined with moist filter paper (Bainbridge & Bownes, 1981). The plates were stored at 25°C during metamorphosis. The number of wild-type and white males emerging as adults was compared and an estimation of their relative viabilities obtained. Finally, the number of dead pupae in each of the main stages of pupal development (Bainbridge & Bownes, 1981) was scored.

Localization of complementary factors of hmi-1. In the framework of the simple model depicted in Figure 1, we have tried to identify in *D. koepferae* any autosomal elements ($A^1{}_m$) that complement *hmi-1* ($X^1{}_m$). The crossing protocol is depicted in Figure 6. Wild-type *koepferae* females from the Argentinian locality of San Luis (strain koeSL.8) were crossed in pool with males from a *buzzatii* white mutant (strain buzSL.101). F_1 hybrid females were backcrossed in the same way to *buzzatii* (see Figure 6). Beginning with the second backcross, randomly chosen wild-type females in the offspring of selected crosses from the former generation were individually mated with 2 males of *buzzatii*. Except for the w^+ allele, the hybrid constitution of these females is cryptic in the adults but can be inferred by analyzing the polytene chromosomes of third-instar larvae in their offspring. Each individually mated female is accordingly identified as hybrid (heterozygote) or not for *hmi-1* (cytological interval A1g-A2c of the X chromosome). The adult offspring from the different individual crosses (introgression lines) were reserved and counted. Crosses that, in spite of

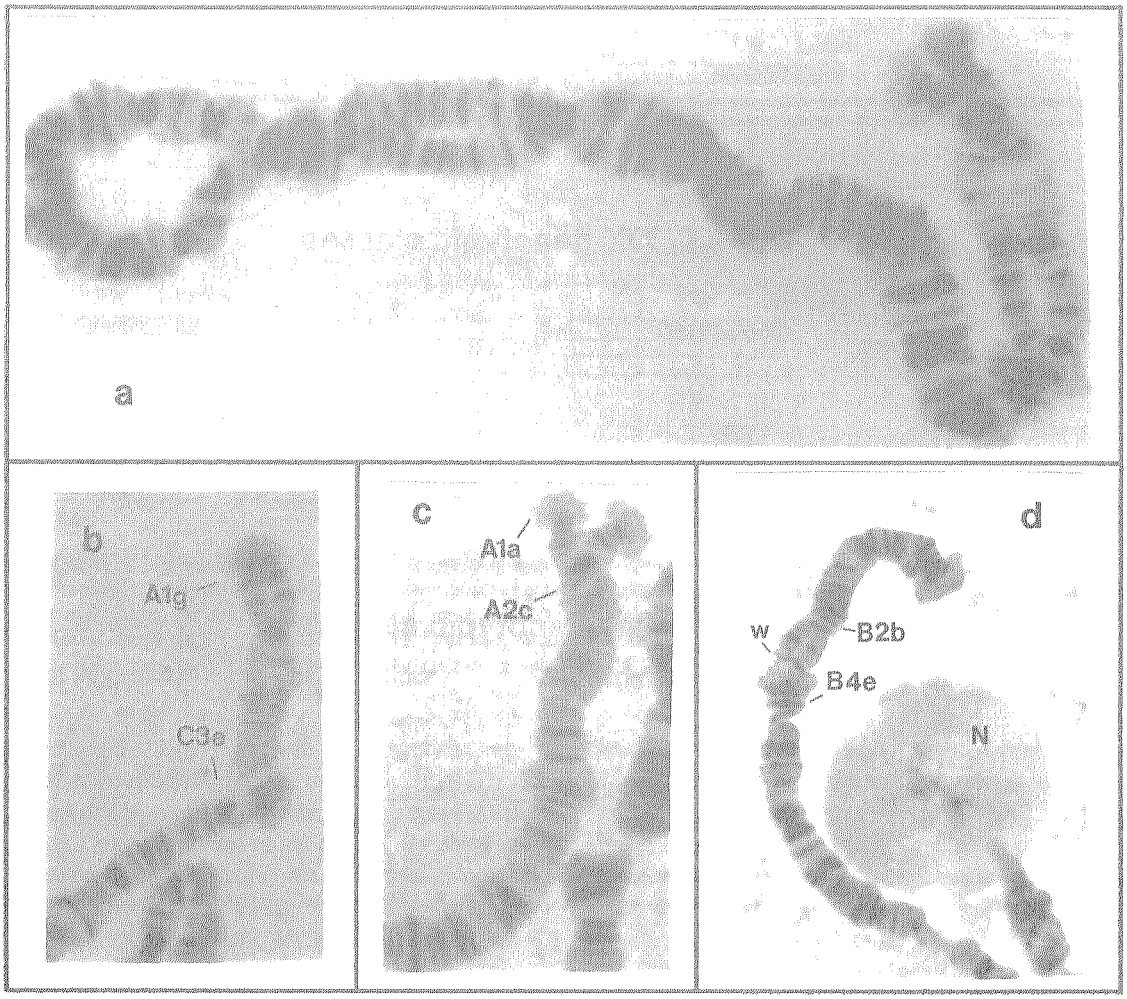

Figure 5. Polytene X chromosomes of salivary glands from hybrid female larvae, showing different introgressions from *D. koepferae* in heterozygosis with *D. buzzatii*. The introgressed segment in each case is easily diagnosed by its characteristic asynapsis (incomplete pairing). **a**, introgression of the whole chromosome. **b**, introgression of A1g-C3e. **c**, introgression of A-A2c. **d**, introgression of B2b-B4e; w-locus *white*; N-nucleolus.

the introgression of *hmi-1*, yielded relatively high frequencies of wild-type (hybrid) males were selected, while the others were discarded. Selection was continued for several generations to get rid of all the *koepferae* material that was irrelevant for hybrid male rescue, ending up with introgression lines that should contain only the complementary factors of *hmi-1* and linked chromosome regions. The chromosomal position of these factors could be determined by the characteristic asynapsis of the chromosome segments that remained introgressed (see also Figure 7).

Results

Backcross hybrid males are often relatively less viable than hybrid females

Interspecific crosses between *D. koepferae* and *D. buzzatii* usually yield roughly equal numbers of males and females in their hybrid offspring. In an attempt to survey the genetic variation for sex-ratio in hybrids within natural populations of these two species, we established crosses involving strains derived from 18 independent samples of each species from their populations in 'San Luis' (Argentina). We obtained a total of 957 males and 1150 females (sex-ratio = 0.832).

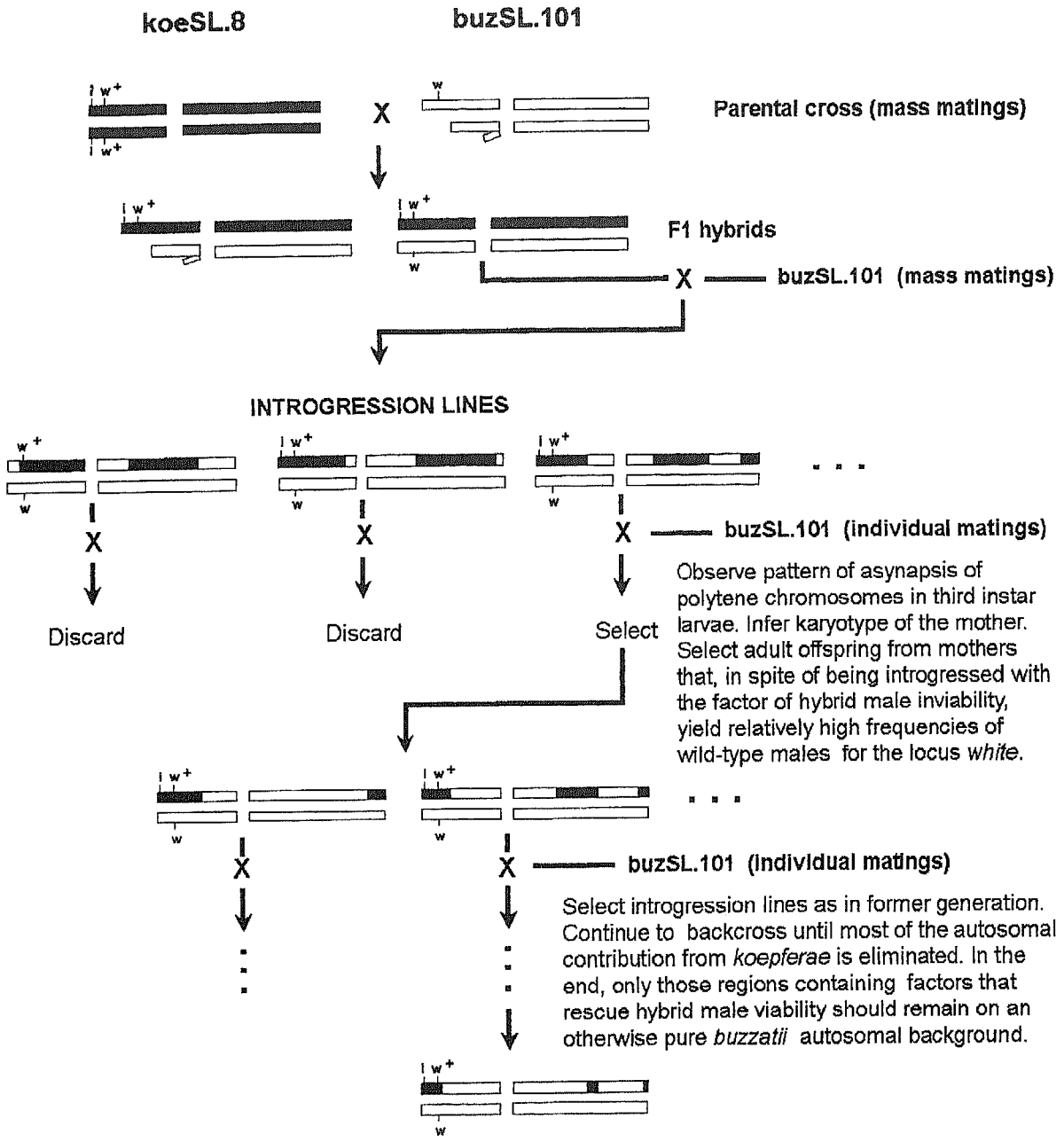

Figure 6. Mating protocol for the isolation of factors from *D. koepferae* that rescue the viability of hybrid males bearing *hmi-1* from this species into the genetic background of *D. buzzatii*. Symbols on the chromosomes: letter 'i' on the left tip of the X chromosome of *koepferae* designates position of the inviability factor *hmi-1*; letter 'w' designates the locus *white*, with + indicating the wild-type allele.

Although the total number of hybrid offspring differed considerably among crosses, the heterogeneity in the relative frequencies of hybrid males and females was not significant (χ^2 = 10.618, 17 d.f., P = 0.8757), with an average sex-ratio of 0.855, which indicates a slight predominance of hybrid females over males. However, a highly significant heterogeneity in the relative frequencies of males and females was observed in the offsprings of the corresponding backcrosses to *D. buzzatii* (χ^2 = 39.056, 17 d.f., P = 0.0018). This hetero-

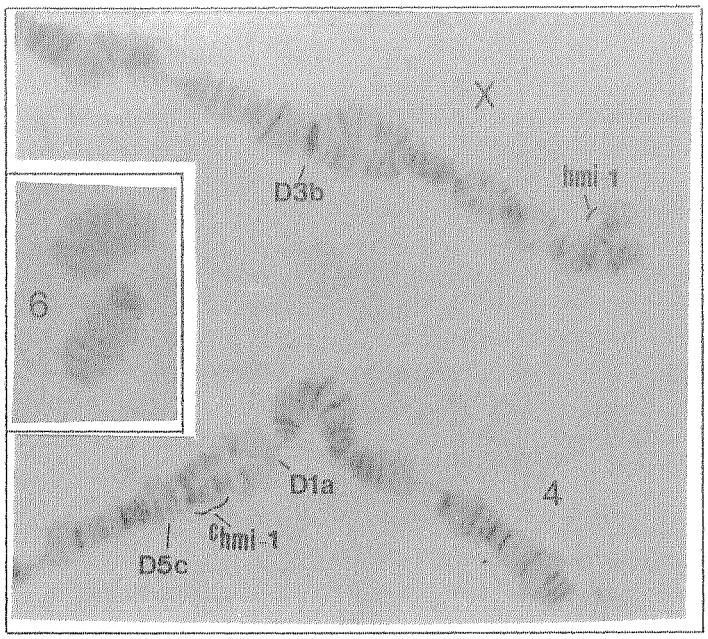

Figure 7. Diagnosis of hybrid male rescue factors in third instar female larvae by the characteristic asynapsis of introgressed segments from *koepferae* into *buzzatii*. The X chromosome is introgressed with segment A-D3b (the localization of *hmi-1* is indicated); chromosome 4 is introgressed with segment D1a-D5c (the localization of ^{c}*hmi-1* is also indicated); chromosome 6 (the 'dot') is entirely hybrid (i.e., each homolog from a different species), and it is shown at a higher magnification.

geneity was mainly due to the difference between two groups of backcrosses, each with considerably homogeneous results. One group had an average sex-ratio of 0.838 ($\chi^2 = 4.606$, 7 d.f., P = 0.7080); the other had an average sex-ratio of 0.542 ($\chi^2 = 6.502$, 9 d.f., P = 0.6888). While the sex-ratio in the first group is similar to that observed in the F_1 and indicates only slightly less viability for hybrid males than for hybrid females, the value of the sex-ratio for the second group seems to indicate a considerable loss of viability for hybrid males.

In the X chromosome of D. koepferae *there are at least two factors of hybrid male inviability*

Previous results (see Introduction) indicated the association of hybrid male inviability to X chromosome introgressions. For a more precise localization of the implicated factors, we have now substituted separate segments of the X chromosome of *D. buzzatii* buzSL.8 for their corresponding homologs in *D. koepferae* koeSL.8 (Figure 2). The upper part of Figure 3 shows the X chromosome constitution of the different introgression lines we obtained, and Table 1 contains the observed numbers of males and females with corresponding sex-ratios and controls for all of them. Where appropriate, two kinds of sex-ratios are given: a total sex-ratio, produced by dividing total numbers of males and females; and a sex-ratio in hybrids, obtained by dividing the number of hybrid males (males with atrophied testes) by half the total number of females, assuming that in this sex, hybrid and pure *D. buzzatii* genotypes are equally viable (see next section). In the first backcross, the atrophy of the testes has no diagnostic value for X chromosome introgressions because several kinds of autosomal introgressions also give rise to this extreme phenotype; therefore, only the total sex-ratio is given.

As shown in Table 1, the sex-ratio observed in both parental stocks, koeSL.8 and buzSL.8, was nearly equal to 1. In F_1 hybrids, its value was reduced to 0.704. It was further reduced in the first backcross generation, reaching a value of 0.497 (total sex-ratio). Regarding the lines introgressed with different X chromosome segments, a simple heterogeneity test on total sex-ratios reveals two significantly different groups of homogeneous data: on the one hand, that formed by the introgression of segments A-A1c, B2c-D1b and F2e-H, with an average total sex-ratio of 0.893 ($\chi^2 = 0.505$, 2 d.f., P = 7770); on the other, the rest of the

Table 1. Observed numbers of adult males and females and corresponding sex-ratio from parental stocks, interspecific crosses, and introgression lines of X chromosome segments of *D. koepferae* (koeSL.8) into *D. buzzatii* (buzSL.8)

Type of flies	Total no. of males	Total no. of females	Total sex-ratio	No. of hybrid males	Hybrid sex-ratio
Parental stocks:					
koeSL.8	201	194	1.036	–	–
buzSL.8	783	801	0.978	–	–
Interspecific hybrids:					
F_1	50	71	0.704	50	0.704
First backcross	85	171	0.497	–	–
Introgression lines:					
A-F2i	100	211	0.474	10	0.095
A-E2a	44	107	0.411	11	0.206
A-D3d	51	106	0.481	8	0.151
A-C3a	29	50	0.580	4	0.160
A-C1e	47	85	0.553	5	0.118
A-B1a	41	89	0.461	0	0.000
A-A3d	144	308	0.468	1	0.006
A-A2c	17	35	0.486	0	0.000
A-A1c	93	106	0.877	44	0.830
D2f-H	80	196	0.408	24	0.245
E2b-H	63	122	0.516	15	0.246
E3a-E4i	34	83	0.410	0	0.000
B2c-D1b	98	102	0.961	52	0.020
F2e-H	110	131	0.840	61	0.931

introgressions, with an average total sex-ratio of 0.477 ($\chi^2 = 4.172$, 10 d.f., P = 0.9393). These two groups can also be easily differentiated by the values of the relative frequencies of hybrid males. In the first group, an average of 51.9% of all the males are hybrids, according to the atrophy of their testes. In the second group, only 12% of the males are hybrids. As expected, a positive correlation between the size of the introgressed segment and the relative frequency of hybrid males is clearly observed in this second group (see Materials and methods). Regarding the sex-ratio among hybrids, the average value is 0.927 for the first group, whereas it is only 0.111 for the second one. All these results indicate that the first group corresponds to chromosome segments from koeSL.8 that usually do not produce male lethality when introgressed into buzSL.8 (Figure 3, thin bars). The second group should correspond to segments that quite often do produce hybrid male lethality (Figure 3, gross bars). By overlapping the two kinds of segments (upper part of Figure 3), it may be concluded that the X chromosome of *D. koepferae* koeSL.8 contains at least two independent factors

Table 2. Introgression of the X chromosome segment A-D3b from *D. koepferae* koeSL.8 into *D. buzzatii* buzSL.101 (*white* mutant). Observed numbers of individuals for each genotype in the offspring introgression line Xd[*hmi-1*,w^+]. P (parental cross): koeSL.8 females x buzSL.101 males, giving rise to F_1 hybrids; 1 (first backcross): F_1 hybrid females x buzSL.101 males; 4–15 (successive backcrosses): X(A-D3b) hybrid females x buzSL.101 males

Type of cross	Adult offspring				Wild-type sex-ratio
	Females		Males		
	w^+/w	w/w	w^+	w	
P	33	–	34	–	0.971
1	12	15	2	9	0.167
4	273	210	11	254	0.040
5	756	673	30	696	0.040
6	597	623	23	577	0.039
7	256	245	13	268	0.051
8	251	227	7	214	0.028
9	441	406	19	434	0.043
10	231	210	12	218	0.052
11	181	160	9	162	0.050
12	79	80	3	65	0.038
13	30	21	1	23	0.033
14	50	40	2	60	0.040
15	189	183	5	190	0.026
$Total_{4-15}$	3334	3078	135	3161	0.040

of hybrid male inviability: a distal one, localized in interval A1d-A2c, and a more proximal one, situated in E3a-E4i. Hereafter, these two factors will be called *hmi-1* and *hmi-2*, respectively.

The factor hmi-1 of hybrid male inviability recombines with a frequency of 4–5% with the locus white in hybrids

Given the proximity between the locus *white* and the distal factor of hybrid male inviability (see Materials and methods), we decided to introgress a telomeric X chromosome segment containing both loci from *D. koepferae* koeSL.8 into *D. buzzatii* buzSL.101 (*white* mutant). We finally obtained the segment XA-D3b, which included both the wild-type allele of the *white* locus and the factor of inviability linked to A1d-A2c. The introgression line thus obtained, called Xd[*hmi-1*,w^+], was therefore heterozygous for both loci, and it could be used to estimate the recombination distance between them (see Figure 4). Table 2 gives the distribution of the *white* phenotype for adult males and females in successive generations of introgression. Excluding the F_1 and the first backcross, the relative frequencies observed are similar for all the crosses (χ^2 = 24.983,

Table 3. Viability from egg to adult for different genotypes produced by introgression line Xd[*hmi-1*,w^+]. Observed numbers of individuals for each genotype in the indicated life-stage (in parenthesis, % of total number), after egg collection, and development in low-density cultures. The wild-type allele is usually linked to the introgression of the *hmi-1* factor

Life-stage	Genotypes			
	Females		Males	
	w^+/w	w/w	w^+	w
White prepupae	126(26.7)	139(29.5)	112(23.8)	94(20.0)
Adults	219(33.0)	224(33.8)	5(0.8)	215(32.4)

33 d.f., P = 0.8405). As expected, wild-type males are always relatively infrequent, since they should be the result of crossing over between the two loci, whereas the other three phenotypes appear in more or less equal frequencies. Assuming that recombinant male and female are equally frequent, recombination distance can be simply estimated by the ratio of wild-type males to wild-type females. This ratio is 0.040 in backcross 4 and stays roughly the same up to the end of the experiment, in backcross 15.

The total number of wild-type males and females from backcross 4 to 15 is included in the results shown in Table 5 for the introgression line Xd[*hmi-1*,w^+]. This line also incorporates the results from other counts obtained later, giving a final sex-ratio of 0.039. Table 5 also shows the counts of male and female hybrids between koeSL.8 and buzSL.101 from the F_1 and first backcross. While in the F_1 the sex-ratio is close to 1, it drops to 0.448 in the offspring from the first backcross. Both parental stocks show sex-ratios slightly above 1.

An independent estimate of the recombination distance between the locus *white* and the factor of inviability could also be obtained by the analysis of the polytene karyotype of hybrid females. Out of 295 wild-type females from the line Xd[*hmi-1*,w^+] backcrossed to *D. buzzatii*, 15 of them (i.e., 5.1%) had lost a telomeric region of the introgressed segment by recombination with the *D. buzzatii* homolog. Correspondingly, the number of wild-type males in their offspring increased significantly, reaching values close to those observed for wild-type females (sex-ratio = 0.869), as shown in Table 5 (introgression line Xd[w^+]). Therefore, these females most probably correspond to wild-type recombinants produced by crossovers in between the loci of *hmi-1* and *white* (i.e., to flies that lost the *hmi-1* allele from *D. koepferae*). Based on both estimates (5.1% from females, 3.9% from males), it may be finally concluded that in hybrid females the locus *white* recombines with the inviability factor with a frequency of roughly 4–5%.

More precise cytological localization of hmi-1

Following the same rationale that we used to map *hmi-1* through the introgression of koeSL.8 into buzSL.8, we have found several distal X chromosome segments whose introgression into buzSL.101 through the line Xd[*hmi-1*,w^+] brings about hybrid male inviability, and several others that do not. We show in the lower part of Figure 3 three segments of the first class (gross bars: A-D3b, A-C1e, and A1g-C3e), and three of the second (thin bars: B2b-B4e, B1e-D3b, and A4b-C4a), including the most interesting ones. A-D3b is the segment that we continually introgressed in the line Xd[*hmi-1*,w^+], whereas the five others were produced by new crossing-over events. The segment B2b-B4e (Figure 5d), B1e-D3b, and A4b-C4a, which do not determine bybrid male lethality, correspond to 3 of the above mentioned 15 females that had lost telomeric region of the introgressed X chromsome, and thus recovered normal frequencies of hybrid male offspring. The segment A1g-C3e (Figure 5b) is particularly interesting. Hybrid males bearing this introgression are inviable, and therefore it must be concluded that *hmi-1* is contained in this cytological interval. But we had shown previously in the introgression into buzSL.8 (Table 1, and upper part of Figure 3) that the segment A-A2c (Figure 5c) also included *hmi-1*. So, simply by overlapping both segments, a final cytological localization within the interval A1g-A2c (five polytene bands) can be given for *hmi-1*.

hmi-1 *hybrid males die during the pupal stage*

Viability from egg to adult of the *hmi-1* hybrid males was investigated in the introgression line Xd[*hmi-1*,w^+] under near optimal conditions. Table 3 shows the observed numbers of hybrid and pure flies in the

Table 4. Viability of w^+ (usually bearing *hmi-1*) and *w* (usually not bearing *hmi-1*) males from the introgression line Xd[*hmi-1*,w^+] through pupal stages of development. Each pupation plate received 50 third-instar larvae of each class. Numbers correspond to records of dead pupae in the corresponding life-stage

	No. of dead males					
	w^+			*w*		
	Plates		Total	Plates		Total
Life stages	1	2		1	2	
P1-P2	15	16	31	2	7	9
P4(ii)	1	3	4	0	0	0
P7-P8	11	10	21	0	0	0
P10-P11	1	0	1	0	0	0
P15(i)	19	14	33	2	0	2
P15(ii)(eclosion)	0	0	0	1	3	4
Total	47	43	90	5	10	15

Table 5. Effect of the introgression of *hmi-1* on hybrid male inviability. Xd[*hmi-1*,w^+]: introgression of a distal X chromosome segment containing both *hmi-1* and w^+ from *koepferae* in a *buzzatii* genetic background. Xd[w^+]: same as before, but with chromosome segments that lost *hmi-1* by crossing-over, as determined by analysis of polytene chromosomes

Type of flies	No. of males	No. of females	Sex-ratio
Parental stocks:			
koeSL.8	201	194	1.036
buzSL.101 (*white*)	1415	1199	1.180
Interspecific hybrids:			
F_1	84	86	0.977
First backcross	30	67	0.448
Xd[*hmi-1*,w^+]	209*	5325*	0.039
Xd[w^+]	1434*	1650*	0.869

*Observed number of wild-type flies for the locus white (X chromosome hybrids).

stage of white prepupae and in adults reared from egg collections using the w^+ allele as marker for the introgression of the distal X chromosome segment from *D. koepferae*. Hybrid males represent 23.8% of the examined prepupae, very close to the expected 25%, but they represent only 0.8% of the examined adults. Therefore, there is no indication of hybrid male lethality before the stage of white prepupa; most hybrid male deaths must take place later, during the pupal metamorphosis.

For a more precise staging of the lethal effect of *hmi-1*, we harvested third instar male larvae, both hybrid and pure *D. buzzatii*, from introgression line Xd[*hmi-1*,w^+] and studied their development in pupation plates. As in the experiment above, the wild-type and white phenotypes should correspond to flies bearing and not bearing *hmi-1*, respectively (except for recombinants). The results are shown in Table 4. Only 10% of w^+ males completed their pupal metamorphosis and emerged as adults. Deaths took place mostly in three periods, namely P1-P2 (before bubble prepupa, 34% deaths), P7-P8 (yellowing pigmentation of the eye, 23% of deaths), and P15(i) (ptilinium expansion, 37% of deaths). Death during this last stage is particularly striking, since the hybrids look like normal animals, showing movement of the legs within the pupal case and expansion in their ptilinium; however, they do not emerge and fail to survive for more than a few hours even when removed mechanically from the pupal exuviae. The control *w* males had an adult emergence rate of 85%. The distribution of deaths among the stage of pupal development was also significantly different from w^+ males. Most of the deaths took place in P1-P2 (60%), but also in P15(i) (13% of deaths), and P15(ii) (during eclosion, 27% of deaths).

hmi-1 *adult hybrid males can be rescued by cointrogressing autosomal factors from* D. koepferae

Following the mating protocol depicted in Figure 6, after 10 generations of backcrossing to buzSL.101, the *hmi-1* female lines that continued to produce adult wild-type males showed only two regions, apart from the distal X chromosome segment, that came from *koepferae* (Figure 7): the cytological interval *D1a-D5c* of chromosome 4, and chromosome 6 (the microchromosome in these species). Apparently, for *hmi-1* hybrid males to be viable, both these autosomal regions must be cointrogressed. To confirm this finding, white hybrid males bearing these two regions in heterozygosis (males *4D1a-D5c;6A-H* in Table 6) were individually crossed with females Xd[*hmi-1*,w^+]. In the offspring, the sex-ratio in hybrids was 0.278, compared to 0.039 in controls, which is indeed in agreement with the expected rescue from two independently segregating interacting loci (further genetic characterization of these rescued males is not possible, for they are completely sterile). Similar results were obtained from crosses of females Xd[*hmi-1*,w^+];*4D1a-D5c;6A-H* with males buzSL.101 (data not shown). For a more precise mapping of the factor on chromosome 4, we set up crosses between females Xd[*hmi-1*,w^+] and hybrid males introgressed both with chromosome 6

Table 6. Results of crosses to investigate the incidence of the hybrid male inviability brought about by the introgression of *hmi-1* from *D. koepferae* into *D. buzzatii* and its complementation with factors from either of these species

Type of cross			No. of	Adult hybrid offspring		
Female	x	Male	crosses	No. of males	No. of females	Sex-ratio
Xd[*hmi-1*,w^+](101)	x	buzSL.101	200	209	5325	0.039
Xd[*hmi-1*,w^+](101)	x	buzSL.101	55	1434	1650	0.869
Xd[w^+](101)	x	4*D1a-D5c;6A-H*(101)	50	414	1492	0.278
Xd[*hmi-1*,w^+](101)	x	4*D1a-D3d;6A-H*(101)	28	30	585	0.051
Xd[*hmi-1*,w^+](101)	x	buzSL.5	10	336	436	0.771
Xd[*hmi-1*,w^+](5)	x	buzSL.5	4	60	59	1.017
Xd[*hmi-1*,w^+](101)	x	buzSL.101/buzSL.5	9	85	449	0.189

The strain buzSL.101 is a *buzzatii white* mutant; buzSL.5 is wild-type. The parenthetical number refers to the genetic background of the introgression lines. Hybrids were identified by their wild-type phenotype for this locus whenever the type of cross made it possible. When not, hybrid males were identified by the atrophy of their testes (cross Xd[*hmi-1*,w^+](5) x buzSL.5) and hybrid females were assumed to be half the total number of this sex in the offspring (crosses with buzSL.5).

and segment *D1a-D3d* of chromosome 4 (males 4*D1a-D3d;6A-H* in Table 6). This time, no rescue of *hmi-1* hybrid males was observed and the sex-ratio was only 0.051, not significantly different from the 0.039 observed in controls (χ^2 = 1.797, 1 df, p = 0.1800). It must be concluded that hybrid males introgressed with *hmi-1* from *koepferae* are viable whenever they are cointrogressed (in heterozygous conditions) with two other factors from the same species: one of them within interval 4*D3e-D5c* (twelve polytene bands), the other one in the 'dot' chromosome. On these grounds, both these factors may be considered interacting complementary genes of *hmi-1* (i.e., ^{c}*hmi-1* genes), anologous to A^1m in Figure 1. The disruption of this system of complementation from *koepferae* by recombination with homologous genes from *buzzatii* is at least one of the causes of the inviability observed in backcross hybrid males.

hmi-1 *adult hybrid males can be rescued by factors segregating in* D. buzzatii *populations*

Not every *buzzatii* strain contains a genetic background incompatible with *hmi-1* from *koepferae*. We give in Table 6 some preliminary data on this. Females Xd[*hmi-1*,w^+] on the genetic background of strain buzSL.101 were crossed with males from buzSL.5, a different *buzzatii* strain that was derived, as was strain buzSL.101, from the Argentinian population of San Luis. These crosses yielded large numbers of adult hybrid males with an estimated sex-ratio in hybrids of 0.771, which indicates a nearly complete rescue of *hmi-1* hybrid males by complementary factors either on the autosomes or the Y chromosome of this *buzzatii* strain. This finding was later confirmed by the introgression of the region Xd[*hmi-1*,w^+] (i.e. the X chromosome segment *A-D3b* from *koepferae*) into the genetic background of buzSL.5. Our results, which correspond to crosses between females Xd[*hmi-1*,w^+](5) and males buzSL.5 in Table 6, indicate that the factor *hmi-1* from *koepferae* is effectively complemented by *buzzatii* factors fixed in this strain (sex-ratio in hybrids = 1.017). To estimate the number of these factors, we crossed females Xd[*hmi-1*,w^+](101) with white males from crosses between both stocks (heterozygous buzSL.101/buzSL.5). The sex-ratio observed in the hybrid offspring was 0.189. Assuming equal segregation of buzSL.101 and buzSL.5 homologs, this result indicates that, as in *koepferae*, two independently segregating interacting factors are involved in the rescue of *hmi-1* hybrid males by buzSL.5 (expected sex ratio would be 0.193, i.e., 0.771 × 0.5^2).

Discussion

Hybrid lethal phenotypes

Hybrid males from the introgression of the X-linked factor *hmi-1* of *D. koepferae* into *D. buzzatii* die as pupae, both in early and late stages, with a substantial proportion of males getting to nearly complete meta-

morphosis but being unable to eclose from the pupal case. The causes of this hybrid death are unknown, but a good guess is that, like hybrid males from the cross of *D. melanogaster* to sibling males, which die as third-instar larvae or pseudopupae (Hutter, Roote and Ashburner, 1990), failure of metamorphosis is due to the absence of the correct hormonal stimuli and not to the inability of the hybrid imaginal disks to develop properly in a suitable host (Sánchez & Dübendorfer, 1983). On this respect, Madhavan (1973) observed that application of juvenile hormone (JH) to pharate pupae of *Drosophila* blocked adult emergence (ecdysis) without preventing earlier processes of moulting, and suggested that JH could influence the synthesis and release of an eclosion hormone, analogous to that found in silkmoths (Truman, 1971). However, the effects of JH, limited to the pupal-adult transformation, cannot explain the substantial lethality observed in early stages of pupal development. In these cases, the most probable cause is an alteration in the levels of the molting hormone (20-OH-ecdysone), which in *D. melanogaster* is known to have a peak of activity at pupariation and two or three more during the mid-pupal period (Handler, 1982).

Hybrid inviability factors in Drosophila

The total number of factors of hybrid inviability localized so far in *Drosophila* is surprisingly small, given the experimental effort devoted to this matter, particularly in recent years. Most advances in this field come from studies in the *melanogaster* subgroup. F_1 hybrid males from the cross of *D. melanogaster* females to males of the sibling species are larval lethal, while hybrid females from the reciprocal cross are embryonic lethal. These kinds of lethality can be rescued by genes that are present only in some populations, either in the autosomes of *D. simulans* (Watanabe, 1979; Sawamura, Taira & Watanabe, 1993a) or in the X of *D. melanogaster* (Hutter & Ashburner, 1987; Sawamura, Yamamoto & Watanabe, 1993b), thus suggesting a simple genetic base for hybrid inviability. However, from pseudobackcrosses of these same interspecific hybrids, Pontecorvo (1943) concluded that at least nine complementary recessive factors were concerned with hybrid viability. Reports from other interspecific hybrids reveal X chromosome factors affecting the viability of either females (Patterson & Griffen, 1944; Schäfer, 1979) or males (Lumme & Heikkinen, 1990) and also report autosomal factors affecting only females (Mitrofanov & Sidorova, 1981) or both sexes (Henning, 1977; Zouros, 1981). With the exception of three *melanogaster* subgroup factors that have been mapped with considerable precision, the localization of the others corresponds to rather large chromosome intervals, most of the times not going beyond a simple chromosome adscription. However, the detailed introgression study described in this paper has allowed the genetic dissection of the effects on hybrid inviability of different regions from a single chromosome, enabling us to trace major effects to cytological intervals of only a few polytene bands. It may be instructive to contrast the results of this analysis with those from a previous similar one on hybrid male sterility (Naveira & Fontdevila, 1986).

The number of hybrid male inviability factors versus sterility factors

After our analysis of most of the X chromosome of *D. koepferae*, we have found only two small regions that cause male inviability when either of them is separately introgressed into *D. buzzatii*; the introgression of the rest of the X chromosome apparently has no effect. In principle, each of these regions may harbor either a single, major inviability factor or a cluster of closely linked factors segregating as a single unit, but this point will be discussed later. What we want to point out now is that a similar search of the hybrid male sterility factors on the X chromosome of *D. koepferae* produced a very different picture: no chromosome region, no matter how small, enabled hybrid male fertility when introgressed into *D. buzzatii* (Naveira & Fontdevila, 1986). It is clear that in this last case we have reached the limit in the resolution power of our method of cytogenetic mapping, based on the observation of asynapsis. Except for introgressions of the chromosome ends (telomere and centromere), which can be easily diagnosed even when exceptionally short, a conservative estimate of the smallest size that an introgressed segment should have to be detected in any of our routine surveys is in the order of two cytological intervals (16 polytene bands). This means that, if the underlying factors are uniformly spaced on the X chromosome, the maximum number of non-overlapping regions that we can find to be associated with the trait is 15 (any deviation from strictly uniform distribution should lead to an increase in this number). Therefore, it may be concluded that, in the pair *buzzatii-koepferae* X chromosome regions with major effects on hybrid male sterility are at least 7 times more frequent than those with effect on hybrid male inviability. A ratio 1 : 10 for hybrid invi-

ability versus hybrid sterility factors has already been pointed out in a review by Wu and Davis (1993), from hybridization data and genetic analyses at the whole chromosome level, but this is the first time that this assertion is supported by a detailed genetic dissection of a single chromosome. As Wu and Davis remark, this ratio contrasts with the 7 : 1 or 10 : 1 of lethal relative to male sterility mutations observed in mutagenesis studies in *Drosophila*, both for the X chromosome and the major autosomes (Lindsley & Tokuyasu, 1980; Cooley, Berg & Spradling, 1988). We are thus confronted with the fact that there are either too few loci of hybrid male inviability, or too many of hybrid male sterility (or both). In the next section we will make clear why we favor the second alternative.

The nature of hybrid male inviability factors versus sterility factors

In the last five years (see Wu & Palopoli, 1994 for a review) it has become increasingly clear that the genetic basis of hybrid male sterility in *Drosophila* is extremely complex. What appeared to be genes of major effect have turned out to correspond to located polygenic effects, whose interacting components have not been identified so far. Hybrid male sterility would thus be produced by the synergy of an undetermined number of minor effect factors. Based on the evidence from recombining introgressed segments from very distant chromosome locations, we favor the hypothesis of an unspecific interaction, i.e., the different factors would be interchangeable, each one being able to act in concert with any others, no matter the distance among them (Naveira & Fontdevlia, 1991a; Naveira, 1992). However, as postulated by other authors (Cabot et al., 1994; Palopoli & Wu, 1994; Pérez & Wu, 1995), it is possible that at least some of the located polygenic effects are brought about by a set of closely linked factors engaged in a specific interaction. In principle, something similar could happen with the hybrid inviability factors mapped so far. Thus, in the long run it may turn out that both *hmi-1* and *hmi-2* actually correspond to a cluster of closely linked, specifically interacting polygenes, but there is no evidence in favor of this hypothesis, except that hybrid sterility factors may work in that way. An indirect proof that the genetic architecture of hybrid male sterility and inviability may indeed be very different is rendered by our analysis of autosomal factors in the pair *buzzatii-koepferae*. No chromosome region has been found to be associated with hybrid male inviability, although introgressions that dissected the full autosomal complement have been analysed; on the contrary, all chromosome regions seem to make an unspecific contribution to hybrid male sterility, as a result of numerous polygenic factors distributed all along the different chromosomes (Naveira & Fontdevila, 1986, 1991a, 1991b). Therefore, as far as we know at this moment, it is perfectly possible that the polygenic basis of hybrid male sterility coexists with a classical (major gene) genetic architecture of hybrid male inviability in the pair *buzzatii-koepferae*.

The composition and evolution of incompatibility systems

F_1 hybrid males from the cross between *D. koepferae* females and *D. buzzatii* males are viable. Nevertheless, backcross hybrids introgressed with either *hmi-1* or *hmi-2* from *D. koepferae*, in an otherwise *D. buzzatii* genetic background, are inviable. Something similar happens with all cases of backcross hybrid inviability discussed above: F_1 hybrids are perfectly viable, but some combinations of the chromosomes of the two species in backcross individuals are lethal, or nearly so. Thus, some kind of gene complementation is expected to be operating in these F_1 hybrids, as well as in the parental species. Apparently, in our case, several systems are involved. First, the two hybrid male inviability factors of *D. koepferae* identified in this study, *hmi-1* and *hmi-2*, do not interact in any way that we could detect, and, therefore, they most probably correspond to at least partially different systems of complementary genes. Secondly, we have already localized two autosomal factors from *D. koepferae* that rescue hybrid males introgressed with *hmi-1*, making up a system of three interacting complementary components that essentially correspond to the model depicted in Figure 1 (except for the involvement of two autosomal factors instead of a single one). Third, we have also some evidence (unpublished results) that this may not be the only complementation system involving *hmi-1* in *D. koepferae*. Finally, we have presented some evidence for complementation of *hmi-1* with gene sets that are polymorphic in *D. buzzatii*. This last kind of complementary action may be the explanation for the heterogeneity we observed in sex-ratios of offsprings from first backcrosses (see first section of Results). Therefore, the total number of factors contributing to backcross hybrid inviability may be still large, but then they would be subdivided into different, perhaps partially overlapping, gene sets, each set consisting of only

a few interacting components (lethals and their conspecific suppressors), which would give rise to hybrid inviability whenever they were substituted for incompatible alleles from another species.

It must be stressed that the small number of factors involved in the system of complementation described in this paper and the large effect associated with the interspecific substitution of any of these factors do not mean that speciation has been instantaneous. Many allele substitutions in these loci may have taken place since the genomes of these two species began to diverge (Figure 1), with the system evolving as a whole, through a divergent path of successive balanced steps with no deleterious effects on fitness for the carriers in the intermediate stages within each species.

A final point that deserves consideration is the nature of the interacting components in this system. The role played by the 'dot' chromosome is particularly intriguing. This chromosome has not ever been associated with hybrid inviability, but it has been reported to be involved in development (Orr, 1990) and regulatory (Bicudo, 1981) anomalies in hybrids, although both males and females are affected.

Haldane's rule

As originally stated (Haldane, 1922), the application of Haldane's rule to *Drosophila* would make reference to male inviability or sterility exclusively in the hybrid F_1 of an interspecific cross. From this strict point of view, cases such as the one studied in this paper – where hybrid disharmonies are not manifest in the F_1 but only in certain backcrosses that combine homospecific and heterospecific chromosome regions – should not be considered an appropriate material to investigate the genetic basis of Haldane's rule. However, most discussions on the factors underlying this rule incorporate the evidence from those types of crosses (see, for example, Coyne & Orr, 1989; Wu & Davis, 1993). This is more prevalent in the case of hybrid inviability, where, except for the hybrids with *D. melanogaster*, the vast majority of results are produced by crosses that yield viable F_1 but inviable backcross males. Therefore, although not explicitly stated, it is common practice to assume that both manifestations of hybrid inviability reflect the same underlying pattern of genetic divergence between closely related species. It is not difficult to envisage a common genetic frame. For example, those cases showing F_1 hybrid male inviability could be produced by dominant autosomal factors, acting as lethal genes in hybrids, whereas those cases where hybrid males are viable in the F_1 but inviable in backcrosses could be produced by recessive autosomal factors of analogous function. In both kinds of crosses, the X chromosome should contribute factors acting as lethal suppressors for the conspecific autosomal lethals. But another possibility is a system involving X-linked lethals and autosomal suppressors, either recessive (F_1 males inviable) or dominant (F_1 viable, but backcross males inviable).

We do not know whether the cause of the male-restricted inviability described in this paper is a genotypic difference between males and females (the X-chromosome imbalance hypothesis, Wu & Davis, 1993), as may be the case with F_1 hybrid male inviability in the *melanogaster* group (Orr, 1993a), or a problem associated with sex-specific genes, as seems to be the general case for hybrid sterility (Coyne, 1985; Coyne & Orr, 1989). The evidence that most *Drosophila* mutants affecting viability do not have sex-limited effects, except for those involved in sex determination or dosage compensation (Baker & Belote, 1983), would seem to argue against this second possibility, but in principle nothing precludes hybrid inviability factors from being concerned with sexual differentiation. Besides, some of the factors associated with backcross hybrid inviability in other *Drosophila* species are autosomal, and still their effects are restricted to only one sex, thus apparently corresponding to sex-specific lethals (see references cited in the discussion of hybrid inviability factors in Drosophila). The easiest way to solve this question would be to produce homozygous *hmi-1* or *hmi-2* hybrid females on a *buzzatii* genetic background, but unfortunately this is not possible, because rescued *hmi-1* hybrid males are invariably sterile.

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