Genetic Complexity Underlying Hybrid Male Sterility in Drosophila

Kyoichi Sawamura,*[,1] John Roote,[†] Chung-I Wu[‡] and Masa-Toshi Yamamoto*

**Drosophila Genetic Resource Center, Kyoto Institute of Technology, Kyoto 616-8354, Japan, [†]Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom and [‡]Department of Ecology and Evolution, University of Chicago, Chicago, Illinois 60637*

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ABSTRACT

Recent genetic analyses of closely related species of Drosophila have indicated that hybrid male sterility is the consequence of highly complex synergistic effects among multiple genes, both conspecific and heterospecific. On the contrary, much evidence suggests the presence of major genes causing hybrid female sterility and inviability in the less-related species, *D. melanogaster* and *D. simulans*. Does this contrast reflect the genetic distance between species? Or, generally, is the genetic basis of hybrid male sterility more complex than that of hybrid female sterility and inviability? To clarify this point, the *D. simulans* introgression of the cytological region 34D–36A to the *D. melanogaster* genome, which causes recessive male sterility, was dissected by recombination, deficiency, and complementation mapping. The 450-kb region between two genes, *Suppressor of Hairless* and *snail*, exhibited a strong effect on the sterility. Males are (semi-)sterile if this region of the introgression is made homozygous or hemizygous. But no genes in the region singly cause the sterility; this region has at least two genes, which in combination result in male sterility. Further, the males are less fertile when heterozygous with a larger introgression, which suggests that dominant modifiers enhance the effects of recessive genes of male sterility. Such an epistatic view, even in the less-related species, suggests that the genetic complexity is special to hybrid male sterility.

GENETIC bases of hybrid inviability and sterility have been analyzed in Drosophila species since the pioneering works of Sturtevant (1920) and Dobzhansky (1936). Conceptually, single-gene-based isolating mechanisms are difficult to achieve because populations have to cross adaptation valleys; A_1A_1 genotype can evolve to A_2A_2 genotype only through transitional individuals, A_1A_2, who are supposedly inviable or sterile. Thus, Dobzhansky (1937) and Muller (1940) advocated a two-locus model of genetic incompatibility (the two-locus Dobzhansky-Muller model). In this classical model, $A_1A_1B_1B_1$ and $A_2A_2B_2B_2$ genotypes (species 1 and species 2, respectively) can be derived independently from $A_1A_1B_2B_2$, bypassing individuals equivalent to F_1 or backcross hybrid; A_2 is incompatible with B_1 here (note that inviable or sterile genotypes depend on the dominance of the alleles). Empirical data have been explained on the basis of this simple model of complementary genes, although more than two loci may be involved in some cases. This is still widely accepted as the standard model of reproductive isolation (Gavrilets 1997; Hutter 1997; Orr 1997; Nei and Zhang 1998; Turelli 1998; Sawamura 1999; Johnson 2000; Orr and Presgraves 2000; Hayashi and Kawata 2001).

While the two-locus Dobzhansky-Muller model can be extrapolated to arbitrarily complex interactions involving both heterospecific and conspecific interactions as theoretical works have rigorously modeled (Orr 1995; Orr and Turelli 1996; Gavrilets 1997; Turelli and Orr 2000), its popularity has much to do with its perceived simplicity (see Wu and Palopoli 1994). According to this model, the genetics of hybrid incompatibility can be due to a very small number of loci, plausibly to one locus per species, whereas conspecific genic interactions are entirely missing. It is this most common interpretation of the Dobzhansky-Muller model that we wish to address in this study.

The two-locus Dobzhansky-Muller hypothesis of reproductive isolation implicitly predicts that interspecific introgressions of one of the complementary genes, *e.g.*, homozygous B_1 introgression to species 2, would result in inviability or sterility. Thus, evolutionary biologists have tried to identify such major genes of reproductive isolation. But recent analyses of introgressions between *Drosophila simulans* and its sibling species, *D. mauritiana* or *D. sechellia*, have actually indicated that a large number of genes affect male fertility and that the sterility is a consequence of synergistic effects of multiple genes (Wu and Palopoli 1994; Wu *et al.* 1996; Naveira and Maside 1998). For example, the *D. mauritiana* gene *Odysseus* (*Ods*) alone does not reveal its male sterility effect in the *D. simulans* genetic background, but the co-introgression with a second-site gene or genes linked to it does (Perez and Wu 1995; Ting *et al.* 1998). Thus, the genetic nature of hybrid male sterility is not as simple as what the two-locus Dobzhansky-Muller model assumes.

The three species used in the previous analyses (the

[1]*Corresponding author:* Institute of Biological Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8572, Japan.
E-mail: sawamura@biol.tsukuba.ac.jp

D. simulans clade) are relatively young and produce fertile female hybrids, allowing potential gene flow in nature. Molecular phylogenetic analyses have indeed detected such an ancient event (Ballard 2000; Kliman *et al.* 2000). Having diverged beyond the point of fusion, the three species thus are "good species," but could still share a portion of their genomes, *i.e.*, those in stage III of Wu (2001). This might be why no major genes of reproductive isolation have evolved. Then, what is the situation in highly diverged species that are completely isolated, *i.e.*, in stage IV of Wu (2001)? Are there genes whose sole introgression, not co-introgression of multiple genes, causes inviability or sterility between *D. melanogaster* and *D. simulans*? [*D. melanogaster* is the outgroup of the *D. simulans* clade (Lachaise *et al.* 1988)]. In fact, the presence of such major genes causing inviability and female sterility has been suggested (Presgraves 2003; Sawamura *et al.* 2004), and one of the hybrid inviability genes was molecularly identified in this particular species pair (Presgraves *et al.* 2003). However, it is not clear whether the contrast is closely related species *vs.* less-related species or hybrid male sterility *vs.* hybrid inviability (and female sterility). Investigation of genes causing hybrid male sterility between *D. melanogaster* and *D. simulans* will clarify this point.

The cross of *D. melanogaster* and *D. simulans* produces only sterile unisexual hybrids (Sturtevant 1920). Because backcross hybrids, which are necessary for conventional genetic analyses, are not obtainable, genetic tricks were introduced to map genes of hybrid sterility and inviability (Muller and Pontecorvo 1940; Sawamura 2000; Presgraves 2003). The discovery of "rescue mutations" that prevent hybrid inviability has facilitated the analyses of mechanisms of hybrid inviability (Watanabe 1979; Hutter and Ashburner 1987; Sawamura *et al.* 1993a,c). The fact that single mutations have such dramatic effects on hybrid viability seems consistent with the two-locus Dobzhansky-Muller model of reproductive isolation (Hutter *et al.* 1990; Sawamura *et al.* 1993b). But it cannot be guaranteed that the rescue mutations are equivalent to the ancestral alleles. In fact, two of the rescue mutations have been shown to be loss of function (Sawamura and Yamamoto 1993; Barbash *et al.* 2000, 2003) and difficult to believe as ancestral. Interspecific introgressions would directly tell whether the two-locus Dobzhansky-Muller model is valid in both hybrid inviability and sterility.

In this analysis, a recently obtained *D. simulans* introgression to *D. melanogaster*, *Int(2L)S* (Sawamura *et al.* 2000), is utilized. This introgression (cytologically 30F3-31E1–36A2-36A7; see materials and methods for the details) causes male sterility when homozygous. Previously the recessive genes responsible for this sterility have been mapped by examining fertility of males heterozygous for the introgression and a series of deficiencies. If the responsible genes of *D. simulans* are made hemizygous, males must be sterile. Thus, five genes of male sterility were mapped in this introgression (Sawamura *et al.* 2000; see Figure 1). In the present analysis, the genes were further mapped by molecular-marker-assisted recombination and genetically mapped by complementation with an additional series of deficiencies and male sterility mutations of *D. melanogaster*. The introgression tested here includes the *Alcohol dehydrogenase* (*Adh*) gene region, one of the best-characterized regions in *D. melanogaster* (Ashburner *et al.* 1999). This analysis of hybrid male sterility genes is therefore at a higher resolution than that of previous studies.

MATERIALS AND METHODS

Description of the original introgression: As described previously (Sawamura *et al.* 2000), the introgression chromosome, *Int(2L)D+S*, carries two discrete regions of the left arm of chromosome 2 (*2L*) of *D. simulans* in an otherwise *D. melanogaster* genome. One of the introgressed segments, *Int(2L)D*, carries the *2L* tip region and *Int(2L)S* the mid-*2L* region. The proximal boundary of *Int(2L)D* is between *anterior open* (*aop*, 22C3–D1) and *Phosphoglycerate kinase* (*Pgk*, 23A6–B1), and the distal boundary of *Int(2L)S* has been mapped between *big brain* (*bib*, 30F3–5) and *daughterless* (*da*, 31D11–E1; Sawamura *et al.* 2000; cytology according to FlyBase 2003; http://flybase.org). The proximal boundary of *Int(2L)S* has been precisely determined in this study between *dachshund* (*dac*, 36A2) and *grapes* (*grp*, 36A6–7). Flies homozygous for *Int(2L)D+S* or *Int(2L)S* are sterile in both females and males, although *Int(2L)D* alone does not cause sterility (Sawamura 2000). The introgression is kept balanced with the *CyO* chromosome. For detailed information on marker genes, deficiencies, and other chromosomal aberrations used in the present analysis, see FlyBase (2003).

Making recombinants: Females heterozygous for *Int(2L)S* and a multiply marked chromosome, *wg b pr pk cn tuf ltd*, were crossed to *wg b pr pk cn tuf ltd/CyO* males. From the progeny, strains carrying a recombinant or nonrecombinant second chromosome were established by using *CyO* as a balancer chromosome. Each strain was checked for the marker mutations, *wingless* (*wg*, 27F1–3), *black* (*b*, 34D1–3), and *purple* (*pr*, 38B4–6). These mutations are located distally, within and proximal to the region of the introgression, respectively (Figure 1). Therefore, recombinant introgressions are recovered effectively.

To obtain smaller introgressions, a second recombination cross was set up with females heterozygous for chromosome 37 marked with *wg* and *b* (see results) and a chromosome marked with *reduced ocelli* (*rdo*, 36E1-3; Figure 2). Two markers, *b* and *rdo*, are useful to recover recombinant introgressions, because the locations are close to the distal and proximal boundaries of the introgression, respectively. Recombinant lines were again established from single flies using *CyO* as a balancer chromosome.

Molecular determination of recombinant introgressions: Genomic DNA was extracted from five flies of recombinant/*CyO* heterozygotes with phenol/chloroform, precipitated by ethanol, dissolved in 30 μl of Tris-EDTA buffer, and diluted 100 times in water. The *wg b pr pk cn tuf ltd/CyO* strain of *D. melanogaster* and the C167.4 strain of *D. simulans* [the latter was the progenitor of the introgression (Davis *et al.* 1996)] were used as controls. Polymerase chain reaction (PCR) mixture was prepared according to the manufacturer's instructions. PCR conditions were 30 cycles of denaturation (described in Table 1). Digested DNA was separated in 1% or 2% agarose gel. The loci examined (Figure 2) were *Angiotensin converting enzyme* (*Ance*, 34D6–8), *spellchecker 1* (*spel1*, 35A1),

TABLE 1

Molecular markers (RFLP) used in the present analysis

Gene	GenBank accession no.	PCR primers[a] Forward	PCR primers[a] Reverse	Annealing temperature	Restriction enzyme
Ance	U25344	144	1621	55°	*Hin*fI
spel1	U17893	939	2297	55°	*Hin*dIII
Adh–Adhr	X78384	2255	4132	55°	*Pst*I
Su(H)	M94383	204	2665	60°	*Xho*I
gft	AA391453	161	464	55°	*Pst*I
sna	Y00288	207	1055	70°	*Bgl*I
CycE	X75026	915	2365	65°	*Pst*I
twe	M94158	139	1185	55°	*Pvu*II
dac	U19269	3872	4906	65°	*Hae*III

[a] The starting nucleotide positions of 20-mers are indicated.

Adh (35B3), *Suppressor of Hairless* [*Su(H)*, 35B10–C1], *guftagu* (*gft*, 35C4), *snail* (*sna*, 35D2–3), *Cyclin E* (*CycE*, 35D4–6), *twine* (*twe*, 35F6), and *dac* (36A2).

Fertility test: The male fertility of flies (1) heterozygous for each recombinant and the original intact introgression, *Int(2L)D+S*; (2) homozygous for each recombinant; and (3) *trans*-heterozygous for recombinant introgressions of different sizes was tested. Introgression-carrying flies were crossed to flies carrying a series of deficiencies or male-sterile mutations, and the fertility of the *trans*-heterozygotes was tested. For initial fertility measurement, the five males to be examined were crossed with five females from the Oregon-R strain of *D. melanogaster* (at least four replicates were made) as described previously (Sawamura *et al.* 2000). Male fertility was also measured by pair matings of 3-day-old virgin flies transferred every 3–5 days until day 21 (when the flies were discarded).

RESULTS

Making recombinant introgressions: The *Int(2L)S* chromosome, which carries the mid-*2L* region introgression from *D. simulans*, causes recessive male sterility. First, to make smaller introgressions, 120 lines carrying chromosomes descended from females heterozygous for *Int(2L)S* and *wg b pr* were established. When the chromosomes were made heterozygous with the original *Int(2L)D+S* chromosome, nonintrogression controls (*wg b pr*) produced fertile males (chromosome 3) whereas intact introgression controls (+++) produced sterile males. Thirty single crossovers between *wg* and *pr* were isolated (Figure 1). Among them, 2 ++ *pr* and 4 *wg* ++ chromosomes apparently inherited male sterility genes detected in the previous analysis (Sawamura *et al.* 2000). On the other hand, 7 + *b pr* chromosomes produced fertile males (but there is a possibility that none of these seven carried a *D. simulans* introgression). The results of *wg b* + chromosomes are of interest. Among the 17 chromosomes tested, 3 (chromosomes 5, 37, and 101) resulted in male sterility and 1 (chromo-

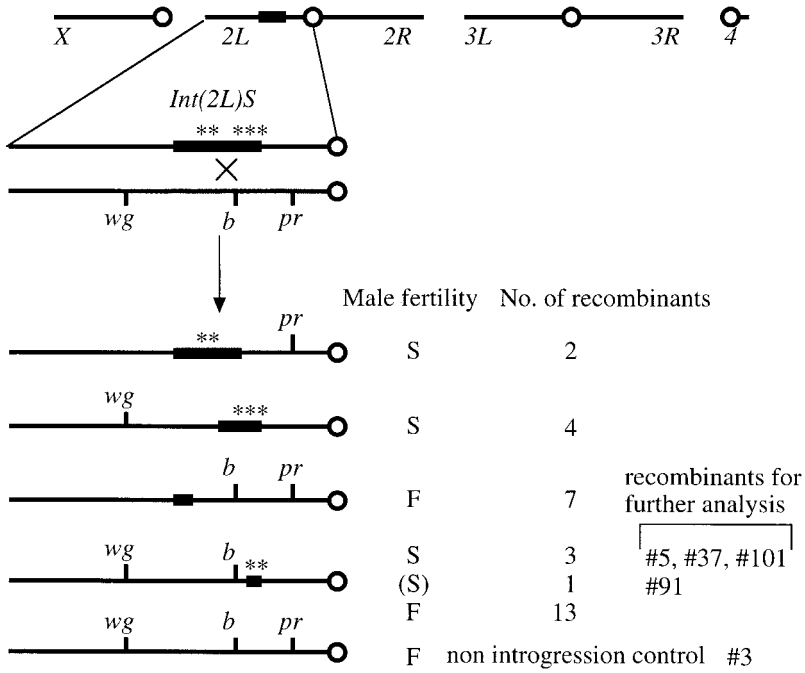

Figure 1.—Mapping male sterility genes on *Int(2L)S* by recombination between the introgression-carrying chromosome and a multiply marked chromosome, *wg b pr*. The *Int(2L)S* carries *D. simulans* genes on the mid-*2L* region (indicated by a thick bar) in otherwise *D. melanogaster* genes (thin bars). Asterisks (*) represent putative male sterility genes detected in the previous analysis of deficiency mapping (Sawamura *et al.* 2000). Male fertility was tested in flies heterozygous for each recombinant and the original introgression, *Int(2L)D+S*. F, fertile; S, sterile; (S), semisterile.

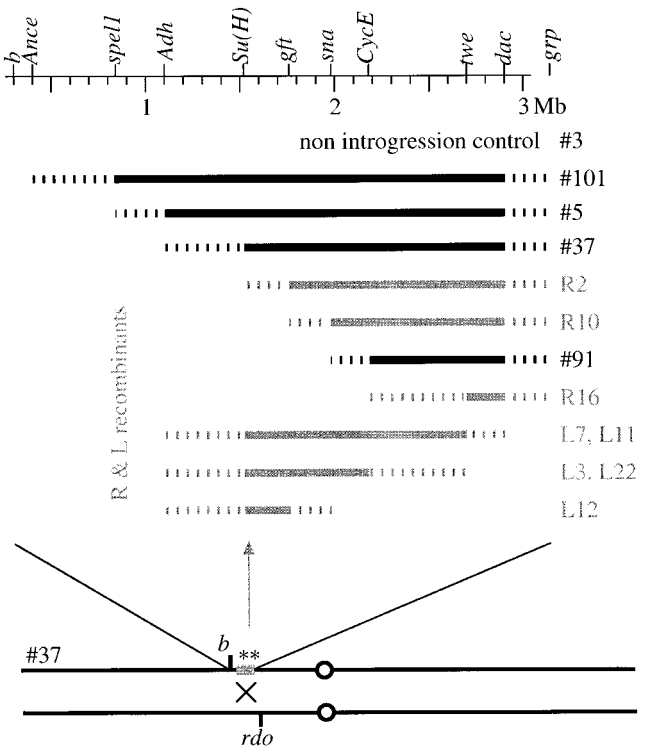

FIGURE 2.—Molecular demarcation of recombinant introgressions. The megabase scale at the top [the 2.9-Mb *Adh* region (ASHBURNER *et al.* 1999)] indicates the physical distance between the known molecular markers used in this study (the start point, 0, is in the *B4* gene). Thick bars denote *D. simulans* introgressions on each recombinant (the species boundaries should be located in the shaded regions). The origin of the R and L series of recombinants is also shown at the bottom.

some 91) resulted in male semisterility (0.6 progeny from a pair). These 4 chromosomes were used to map the male sterility genes more precisely.

Chromosomes 91 and 101 have recessive lethals, which presumably were floating in the original multiply marked strain or introgression strain and were crossed into our experimental lines. Because the two lethals did not complement each other, the chromosomes must carry the same mutation. The lethal gene was eliminated from chromosome 91 by recombination with Oregon-R, and the newly established chromosome utilized when necessary.

Among the three male-sterile introgressions, chromosome 37 is the smallest in size (see below). Smaller introgressions were made by further recombination between this chromosome (marked with *b*) and *rdo* ("R" and "L" recombinants; see Figure 2). Among 23 *b rdo* and 24 + + recombinant chromosomes established, molecular mapping (see below) indicated that 5 *b rdo* chromosomes (L3, L7, L11, L12, and L22) and 3+ + chromosomes (R2, R10, and R16) carried smaller introgressions than did chromosome 37 (R and L recombinants carry the right- and left-hand tip of introgression 37, respectively).

TABLE 2

Fertility of males with recombinant introgressions

Recombinant	No. of offspring per male: Recombinant/*Int(2L)D+S* heterozygotes[a]	No. of offspring per male: Recombinant homozygotes[b]
3	82.0 ± 13.6 (24/25)	—
101	0	X
5	0	0
37	0 (0/10)	0.7
R2	1.3 ± 0.38 (8/11)	4.4
R10	68.2 ± 9.40 (29/30)	213.6
91[c]	24.0 ± 9.97 (13/18)	34.6
R16	194 ± 24.7 (30/31)	3.4
L7	0.4 ± 0.24 (3/9)	0
L11	0.4 ± 0.15 (5/12)	0.1
L3	0.7 ± 0.26 (5/12)	0
L22	1.7 ± 0.90 (7/13)	0
L12	33.5 ± 4.46 (24/27)	96.6

—, not determined; X, inviable.

[a] Pair matings to Oregon-R females: average ± standard error (number of pairs producing offspring/total). Recombinants 101 and 5 are mass matings.

[b] Mass matings to Oregon-R females.

[c] The chromosome on which the linked lethal gene was eliminated.

Molecular-marker-assisted mapping of male sterility genes: Molecular demarcation of the recombinant introgressions is depicted in Figure 2 and fertility of males heterozygous for *Int(2L)D+S* and each recombinant introgression is shown in Table 2. The data are explicable if we predict a gene or genes having strong effects on male sterility in the region between *gft* and *sna* [or maybe between *Su(H)* and *sna*]. An exception is chromosome 91, which resulted in low fertility (or semisterility in the preliminary test mentioned above). This suggests that there might be more genes responsible for low male fertility between *sna* and *twe*, although the effect is detectable in a certain genetic background (chromosome 91 has a different genetic background than chromosomes R2, R10, and R16). The proximal boundary of introgression L12 is between *gft* and *sna* and this introgression may not include the whole region having strong effects on male sterility. This might be why the introgression L12 exhibited low male fertility.

Fertility was also examined in males homozygous for the recombinant introgressions (Table 2). (We have no data for introgression 101 because homozygotes are lethal.) A similar conclusion above is met; a gene or genes having strong effects on male sterility exist in the region between *gft* and *sna*. In this case the region between *Su(H)* and *gft* is excluded because L12 homozygotes were fertile. Exceptionally the fertility of chromosome 91 homozygotes was lower than that of R10 homozygotes, which is parallel to the data of heterozygotes with *Int(2L)D+S*. Another exception is R16 homozygotes, which were semisterile. But this might be caused

TABLE 3

Fertility of males *trans*-heterozygous for introgressions of different sizes

Genotype	No. of offspring per male[a]
101/5	0
101/37	0.1
5/37	0.3
101/91[b]	61.0
5/91	54.0
37/91	86.0

[a] Mass matings to Oregon-R females.
[b] The chromosome on which the linked lethal gene was eliminated.

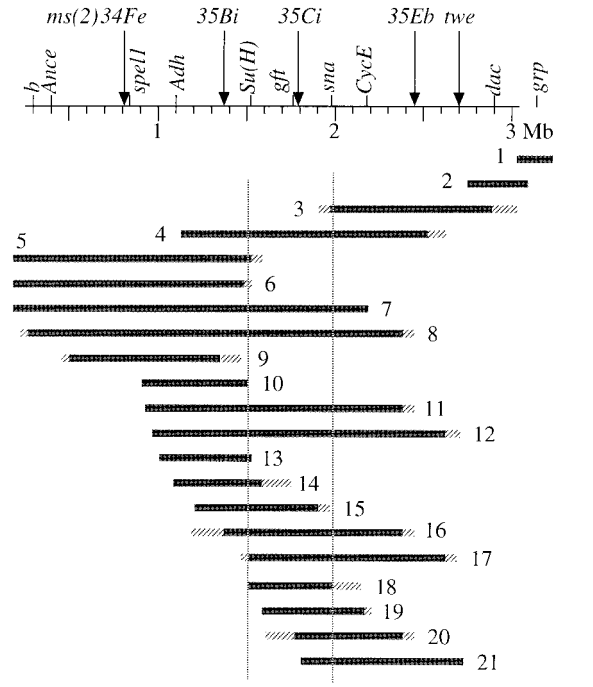

FIGURE 3.—Deficiencies and male-sterile mutations used in these analyses. To make this figure easily comparable with Figure 2, the same scale is presented at the top. Male sterility loci of *D. melanogaster* are indicated by arrows, and the deleted region of each deficiency chromosome is shown by hatched bars (uncertainty boundaries indicated by shaded bars). The deficiency breakpoints are from FLYBASE (2003) and M. ASHBURNER and J. ROOTE (unpublished results). Deficiencies 1–5 are the deficiencies used in the previous analysis (SAWAMURA *et al.* 2000). See Table 4 for the name of each deficiency.

by a recessive male-sterile mutation linked to the chromosome not by the introgression itself because *Int(2L)D+S/*R16 heterozygous males exhibited normal fertility. The fact that L12 homozygotes were fertile but *Int(2L)D+S/*L12 heterozygotes exhibited low fertility suggests that heterozygous *D. simulans* genes on *Int(2L)D+S* may be involved in this low male fertility. In other words, dominant modifiers of recessive male sterility exist on the introgression (but not necessarily in the region now studied).

The fertility of males *trans*-heterozygous for introgressions of different sizes was examined (Table 3). Males *trans*-heterozygous for any combination of the introgressions 101, 5, and 37 are almost completely sterile. Interestingly, males *trans*-heterozygous for those and 91 were fertile. This contrasts with *Int(2L)D+S/*91 heterozygous males, which were semisterile. Male sterility genes apparently exist in introgression 91, but the effect can be detected only when heterozygous with a very large introgression. This again suggests the presence of dominant modifier(s) of recessive male sterility genes on *Int (2L)D+S.*

Deficiency and complementation mapping of male sterility genes: The male sterility genes in the introgression generally behave as recessives; if *D. simulans* alleles of such genes are made homozygous or hemizygous by deficiencies, then males are sterile. In fact, five overlapping deficiencies uncovered male (semi-)sterility genes in this region of the introgression (proximal to the *b* gene) when tested against the original introgression, *Int(2L)D+S* (SAWAMURA *et al.* 2000; Figure 3, deficiencies 1–5; Table 4). Three male sterility genes were tentatively located in this region by assuming that such genes are within the overlap of deficiencies uncovering male sterility. But the actual number of male sterility genes can be much larger. When 16 other deficiencies covering a similar region (Figure 3, deficiencies 6–21) were examined against *Int(2L)D+S,* most of the deficiencies uncovered male sterility genes: sterile, semisterile, or weakly fertile in *trans*-heterozygous males (Table 4). It is difficult to map major genes of male sterility from these data; some blocks not including male sterility genes were necessary to localize the genes.

Then what is the situation when the same series of deficiencies are made heterozygous with smaller introgressions like 101, 5, or 37? Because dominant modifiers of recessive male sterility genes exist on the original introgression (mentioned above), male fertility must be higher when the smaller introgressions are utilized. This was the case in most of the deficiencies. The extreme cases are *Df(2L)H20* (deficiency 1) and *Df(2L)TE35B-9* (deficiency 9), which resulted in constantly high male fertility. Such regions may not have male sterility genes with a strong effect, although the effect can be detected when heterozygous with the larger introgression. Contrastingly, some of the deficiencies exhibited semisterility when heterozygous with an introgression, such as 101, 5, or 37. Those are *Df(2L)osp29* (deficiency 4), *Df(2L)b88c75* (deficiency 7), *Df(2L)Sco7* (deficiency 8), *Df(2L)TE35BC-8* (deficiency 11), *Tp(2;2)A446* (deficiency 12), *Df(2L)TE35D-5* (deficiency 16), *Df(2L)TE-35BC-24* (deficiency 17), and *Df(2L)TE35BC-3* (18). The deletions generally make the *Su(H)-sna* region hemizygous. Thus, male sterility genes with a strong effect exist in this region of introgression, which is consistent with the conclusion from the recombination mapping above.

TABLE 4

Fertility of males heterozygous for an introgression and a deficiency chromosome

Deficiency[a]	Introgression:	No. of offspring per male[b]				
		D + S	101	5	37	91
1 *Df(2L)H20*		0	97.9	92.1	98.5	149.5
2 *Df(2L)cact-255rv64*		0	51.0	44.4	59.5	156.1
3 *Df(2L)r10*		0	9.7	22.1	39.4	173.4
4 *Df(2L)osp29*		0	0.7	0.2	2.3	154.8
5 *Df(2L)b87e25*		0.9	37.8	14.5	46.2	101.1
6 *Df(2L)b80k*		0.9	55.0	98.2	93.4	164.4
7 *Df(2L)b88c75*		0	2.1	11.6	2.9	115.2
8 *Df(2L)Sco7*		0	0.6	6.8	1.2	59.7
9 *Df(2L)TE35B-9*		17.2	160.7	213.1	161.4	225.1
10 $T(Y;2)A80^{D}R15^{P}$		<0.1	11.4	17.6	14.9	79.7
11 *Df(2L)TE35BC-8*		<0.1	0.5	0	0.4	128.4
12 *Tp(2;2)A446*		0	0.9	<0.1	4.2	127.6
13 *Df(2L)A267*		2.4	97.9	35.3	73.3	162.5
14 *Df(2L)A72*		12.9	43.9	86.2	51.1	219.5
15 $In(2L)C158^{L}Sco^{rv11R}$		36.9	100.5	48.0	30.3	112.2
16 *Df(2L)TE35D-5*		0	0.3	<0.1	0.1	104.3
17 *Df(2L)TE35BC-24*		0	3.3	2.4	0	158.1
18 *Df(2L)TE35BC-3*		0.6	17.8	12.8	11.2	170.0
19 *Df(2L)TE35D-4*		21.7	61.7	105.7	92.9	185.9
20 *Df(2L)TE35D-6*		<0.1	12.9	10.8	32.4	161.1
21 *Df(2L)TE35D-13*		0.3	25.2	37.5	76.4	198.0

[a] Numbers represent deficiencies depicted in Figure 3.
[b] Mass matings to Oregon-R females.

But it should be stressed here that the semisterility is not caused by a single gene. This region can be subdivided to two overlapping deficiencies exhibiting high male fertility: *e.g.*, $In(2L)C158^{L}Sco^{rv11R}$ (deficiency 15) and *Df(2L)TE35D-4* (deficiency 19). Thus, the *Su(H)-sna* region has at least two genes that, in combination, are sufficient to cause male semisterility.

The same series of deficiencies were made heterozygous with the smaller introgression 91. Almost all resulted in male fertility comparative to the controls. [The deleted regions of *Df(2L)b87e25* (deficiency 5), *Df(2L)b80k* (deficiency 6), *Df(2L)TE35B-9* (deficiency 9), $T(Y;2)A80^{D}R15^{P}$ (deficiency 10), *Df(2L)A267* (deficiency 13), and *Df(2L)A72* (14) do not overlap with the introgression region of 91 and thus can be controls.] This is presumably because this small introgression does not have enough dominant modifiers of recessive male sterility to be detected.

The 2.9-Mb chromosome region from *kuzbanian* (*kuz*, 34C1–4) to *dac*, including *Adh*, has been extensively characterized in *D. melanogaster* at the molecular and genetic level (Ashburner *et al.* 1999). The *D. simulans* introgression studied in the present analysis is almost all included in that region. Five loci whose mutation or deficiency causes recessive male sterility are known in the region between *b* and *dac* as shown in Figure 3. Complementation tests were performed with mutations of the loci against the original introgression, *Int(2L)D+S*. Mutations tested are $ms(2)34Fe^{1}$, $ms(2)35Bi^{z0090}$, $ms(2)35Ci^{02316}$, $ms(2)35Eb^{z3860}$, and twe^{1} [($ms(2)35Bi^{z0090}$ and $ms(2)35Eb^{z3860}$ are newly discovered mutant alleles of genes previously predicted from the sterility of overlapping deletions (Ashburner *et al.* 1999; D. Lindsley and J. Roote, unpublished results)]. None resulted in male sterility in heterozygotes (results not shown).

DISCUSSION

The *D. simulans* introgression of the mid-*2L* region, *Int(2L)S*, causes male and female sterility when homozygous in an otherwise *D. melanogaster* genetic background (Sawamura *et al.* 2000). To identify recessive genes involved in male sterility, this introgression was genetically dissected, especially for the region around the *Adh* locus. First, molecular-marker-assisted recombination mapping indicated that the *gft-sna* region [or maybe the *Su(H)-sna* region, ~450 kb] has at least one gene with strong effects, although other regions (*e.g.*, the introgression region of 91) may also have male sterility genes whose effect is detectable in a certain genetic background. Interestingly, dominant enhancers of the effects of the recessive genes have been detected on the introgression. Small recombinant introgressions (*e.g.*, 91 and L12) resulted in low male fertility only when

heterozygous with a larger introgression, *Int(2L)D+S*. This fits the type II incompatibility of Johnson (2000) or the H_1 incompatibility of Turelli and Orr (2000) (between a homozygote at one locus and a heterozygote at the other).

Second, deficiency mapping was performed. Most of the deficiencies resulted in male semisterility when tested against *Int(2L)D+S* and the responsible genes could not be localized. Because dominant enhancers exist on the introgression, a more sensitive test is possible if such elements are decreased by utilizing smaller introgressions. In fact, *trans*-heterozygous males were more fertile when introgression 101, 5, 37, or 91 was used. The results from 101, 5, and 37 were consistent with the conclusion from recombination mapping: *trans*-heterozygous males were semisterile when the *Su(H)-sna* region was deleted. (Introgression 91 exhibited normal fertility when heterozygous with any deficiencies, presumably because this introgression does not have enough dominant enhancers to be detected in this analysis.)

It is important to note that male sterility is not caused by a single gene. Deficiencies including the whole *Su(H)-sna* region result in male semisterility when heterozygous with introgressions 101, 5, or 37, but those partially including the region (which can overlap each other) do not. Thus, this region has at least two genes that, in combination, cause male semisterility. The complementation tests against male sterility mutations in this region also support that any single genes are not responsible for male sterility. In summary, no single gene substitutions between species in this region of the genome result in male sterility, which contrasts with what the two-locus Dobzhansky-Muller model of reproductive isolation assumes. The epistatic aspect of postzygotic reproductive isolation is not specific to the early stage of speciation (*cf.* Wu and Palopoli 1994; Wu *et al.* 1996; Naveira and Maside 1998), but is general for hybrid male sterility. This contrasts with the genetic system of hybrid inviability and female sterility, where major genes that individually cause inviability and sterility have been suggested or identified (Presgraves 2003; Presgraves *et al.* 2003; Sawamura *et al.* 2004). It is possible that functional divergence of genes involved in male reproduction is accelerated by sexual selection (Coulthart and Singh 1988; Tsaur and Wu 1997; Nurminsky *et al.* 1998; Singh and Kulathinal 2000; Swanson *et al.* 2001).

A concern remains that the male sterility genes detected in this analysis might be simple null mutations. In fact, a recessive lethal detected on the introgression was shown to be caused by a new transposable element (TE) insertion; TEs might have been mobilized as a result of the initial species cross (Sawamura *et al.* 2000). However, in this analysis we detected dominant enhancers of male sterility genes and showed that not a single gene but instead a combination of at least two genes causes male sterility. We believe that not all the genes detected in this analysis are new mutations but rather true hybrid male sterility genes.

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