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The minimal interspecific introgression resulting in male sterility in *Drosophila*

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Summary

Introgression of *Drosophila simulans* genes into the *Drosophila melanogaster* genome provides an ideal system for analysing genetic incompatibility between species. Females and males homozygous for the introgression *Int(2L)S* (cytologically, 30F3-31C5 to 36A2-7) are sterile. Genetic dissection of the proximal part of the introgression (34D1-3 to 36A2-7) has indicated that introgressions of 0·7–1·6 Mb size result in male sterility when homozygous. In the present analysis we examine the distal part of the introgression (30F3-31C to 34D1-3) and reveal that introgressions with similar DNA content (1·8–2·1 Mb) result in male sterility. Compared with introgressions between the more closely related species *Drosophila mauritiana* and *D. simulans*, the minimal introgression resulting in male sterility is smaller by several-fold.

1. Introduction

In order to understand genetic mechanisms of post-zygotic reproductive isolation, interspecific introgressions have been made in *Drosophila*, mice, barley, wheat and rice (e.g. Wu & Palopoli, 1994; Shi & Endo, 1999; Matsubara *et al*., 2003; Oka *et al*., 2004). Among them, introgressions of *Drosophila simulans* genes to the *Drosophila melanogaster* genome, which became available recently (Sawamura *et al*., 2000), offer an especially suitable system for analysis.

It has generally been impossible to make interspecific introgressions between *D. melanogaster* and *D. simulans* because the reciprocal crosses produce sterile unisexual hybrids (Sturtevant, 1920). The discovery of strains which restore hybrid fertility (Davis *et al*., 1996; Barbash & Ashburner, 2003) brought the hope that introgressions could be introduced by repeated backcrosses. In fact, Sawamura *et al*. (2000) succeeded in introgressing two segments of the left arm of chromosome 2 (2L) from *D. simulans* into *D. melanogaster*; one at the tip of 2L (*Int(2L)D*; cytologically, 21A1 to 22C1-23B1) and the other in the middle of 2L (*Int(2L)S*; 30F3-31C5 to 36A2-7).

One of the introgression segments, *Int(2L)S*, causes female and male sterility when homozygous. The recessive genes responsible for the female sterility have been genetically mapped (Sawamura *et al*., 2004*a*). Recombinants between the *Int(2L)S* and *D. melanogaster* chromosomes were made and the boundary of each recombinant introgression was determined by species-specific RFLPs (restriction fragment length polymorphisms). The fertility of females heterozygous for *Int(2L)D+S* and each introgression was examined. Further, the *Int(2L)D+S* chromosome was made heterozygous with a series of deficiency chromosomes of *D. melanogaster*. Because the sterility genes are recessive, females should be sterile if the *D. simulans* genes are made hemizygous by the deficiencies. The female sterility was attributed to a single gene or a cluster of genes in a 170 kb region of the introgression.

The introgression, *Int(2L)S*, also causes inviability in a specific genetic background. Male hybrids from the cross of *D. melanogaster* females and *D. simulans* males are lethal (Sturtevant, 1920), and are rescued by the *Lethal hybrid rescue* mutation of *D. simulans* (Watanabe, 1979), but the male hybrids are not rescued if they retain the introgression (Sawamura,

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2000). The inviable hybrids are homozygous for genes from *D. simulans* in the region of the introgression. Thus, *Int(2L)S* has recessive lethal genes whose effect is detectable in the male hybrid genotype. The genes have been mapped by recombination and complementation tests with deficiencies (Sawamura *et al.*, 2004*a*). The gene(s) of female sterility and those of inviability were not separable with the mapping resolution achieved. They must be tightly linked, or the same gene(s) might be responsible (i.e. pleiotropy).

The proximal part of the introgression to the *black* gene (*b*, 34D1-3) has been dissected for male fertility by recombination and deficiency mapping (see Fig. 1). The region includes the *Alcohol dehydrogenase* (*Adh*) gene and to date is the most suitable region for molecular cytogenetic analysis (Ashburner *et al.*, 1999). The male sterility caused by the introgression was shown to be the consequence of synergistic effects of multiple genes (Sawamura *et al.*, 2004*b*). The fact that more genes are involved in male sterility than female sterility (and inviability) in *D. melanogaster*/*D. simulans* hybrids is consistent with the previous analysis of *Drosophila mauritiana*/*D. simulans* hybrids (Wu *et al.*, 1996). Genes of male reproduction may evolve rapidly or spermatogenesis may be easily perturbed by genetic manipulation (Wu & Davis, 1993; Wu *et al.*, 1996).

A genome-wide analysis of *D. melanogaster* has shown clustering of testis-specific genes on chromosomes (Boutanaev *et al.*, 2002). Because the proximal part of the introgression might be such a specific region, other introgression regions should be analysed to evaluate the generality of the above observations. Here we dissect the introgression distal to the *b* gene, where the gene(s) of hybrid female sterility and inviability were located (Sawamura *et al.*, 2004*a*). In order to identify the minimal introgression causing male sterility, we tested the fertility of males carrying the same series of recombinant introgressions as those used to map the gene(s) of female sterility and inviability. Such systematic analyses have been done in the *D. mauritiana*/*D. simulans* species pair (Hollocher & Wu, 1996; True *et al.*, 1996). We then compared the present results with the previous analyses of the proximal part of *Int(2L)S* (Sawamura *et al.*, 2004*b*) and the autosomal introgression between *D. mauritiana* and *D. simulans* (Tao *et al.*, 2003*a*, *b*).

2. Materials and methods

(i) *Chromosomes*

We used the same series of recombinant introgressions as did Sawamura *et al.* (2004*a*). Briefly, the introgression *Int(2L)S* (cytologically, 30F3-31C5 to 36A2-7) was recombined with the $P\{w^{+mC}=lacW\}l(2)k04603^{k04603}$ *b* chromosome (see Fig. 1). In the latter chromosome, a *P* element vector carrying the mini-*white*$^+$ (w^+) gene, which complements the *w* mutant phenotype, is inserted at the *string-of-pearls* locus (*sop*, 30E1-4). The chromosome also bears another visible marker, *black* (*b*, 34D1-3). The chromosome is abbreviated as $P\{w^+\}sop\ b$ hereafter. Recombinants were screened for among the offspring of $+Int(2L)S+/P\{w^+\}sop+b$ heterozygous mothers, and the recombinant chromosomes maintained by using *CyO* as a balancer chromosome. In the present analysis, we used only sop^+ *b* recombinants, which presumably retain the distal part of the introgression. This is because the reciprocal $P\{w^+\}sop\ b^+$ recombinants must always carry male sterility genes on the introgression proximal to *b* (Sawamura *et al.*, 2004*b*).

The following deficiency chromosomes were also utilized (the deleted region is indicated cytologically in parentheses; see Fig. 3): *Df(2L)Mdh* (30D-F; 31F), *Df(2L)J2* (31B1; 32A1-2), *Df(2L)J3* (31D; 31F2-5), *Df(2L)J39* (31C; 32D1-E5), *Df(2L)FCK-20* (32D1; 32F1-3), and *Df(2L)Prl* (32F1-3; 33F1-2). For detailed descriptions of genes and chromosomes see FlyBase (2003) (http://flybase.org).

(ii) *Fertility test*

To test the effect of recombinant introgressions on male fertility, males heterozygous for each recombinant and the *Int(2L)D+S* chromosome were examined. (Instead of *Int(2L)S*, *Int(2L)D+S* was utilized for the test because sterility could be enhanced with the extra introgression *Int(2L)D*.) The fertility of viable males homozygous for each recombinant introgression was evaluated. Males heterozygous for *Int(2L)D+S* and each deficiency chromosome were also made. Five males to be examined and five Oregon-R females of *D. melanogaster* were mated in a single vial with four replicates. Flies were transferred every 3–5 days until all females died, and the offspring were counted. The numbers of offspring per male were truncated at 100, because differences in fertility beyond 100 may not be reliable. Males with >100 offspring were categorized as fertile.

(iii) *Molecular analysis*

Genomic DNA was extracted, amplified by polymerase chain reaction (PCR), and digested by restriction enzymes to detect RFLPs following the method described previously (Sawamura *et al.*, 2004*b*). The loci examined (see Fig. 1; for detailed descriptions see Sawamura *et al.*, 2004*a*) were: *basket* (*bsk*, 31C1-5), *daughterless* (*da*, 31D11-E1), *CG4705* (32D2-3), *CENP-meta* (*cmet*, 32E3-4) and *paired* (*prd*, 33B13-14). The *big brain* locus (*bib*, 30F3-5) is outside the introgression.

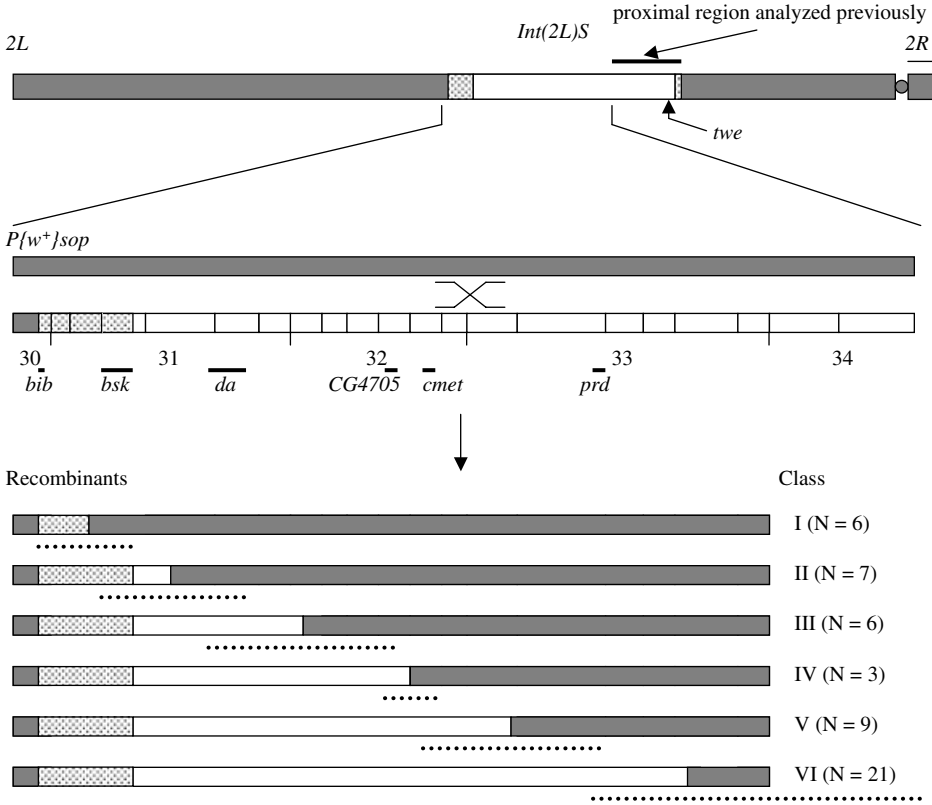

Fig. 1. Recombinant chromosomes used in the present analysis. Recombinants were isolated from offspring of females heterozygous for *Int(2L)S* and *P{w+}sop b*. Fifty-two recombinants were classified into six classes according to the molecular markers indicated below the polytene chromosome map. The chromosome regions from *D. melanogaster* and *D. simulans* are indicated by filled and open bars, respectively. The origin of genes has not been specified in the grey region of the distal end of the introgression. The recombination sites indicated are not known exactly; they are located somewhere in the dotted line regions. The proximal part of the introgression analysed previously (Sawamura *et al.*, 2004*b*) is indicated as a reference.

To check the possibility of double recombination, the *twine* locus (*twe*, 35F6) was analysed (for details see Sawamura *et al.*, 2004*b*). If *D. simulans*-specific DNA sequence was detected, the chromosome was attributed to a double recombinant carrying the proximal part of the introgression.

3. Results

(i) *Recombination mapping*

We classified 52 recombinant chromosomes into classes I to VI by molecular markers (Fig. 1). The proximal boundaries of each class of introgression are: between *bib* and *bsk* (class I), *bsk* and *da* (class II), *da* and *CG4705* (class III), *CG4705* and *cmet* (class IV), *cmet* and *prd* (class V) and *prd* and *b* (class VI).

The fertility of males heterozygous for *Int(2L)D+S* and each recombinant introgression is shown in Fig. 2. It should be added here that the order of recombinants within each class is arbitrary with respect to the recombination site. The data are plotted accordingly to the fertility (higher fertility on the left). Males were fertile when introgressions of classes I to

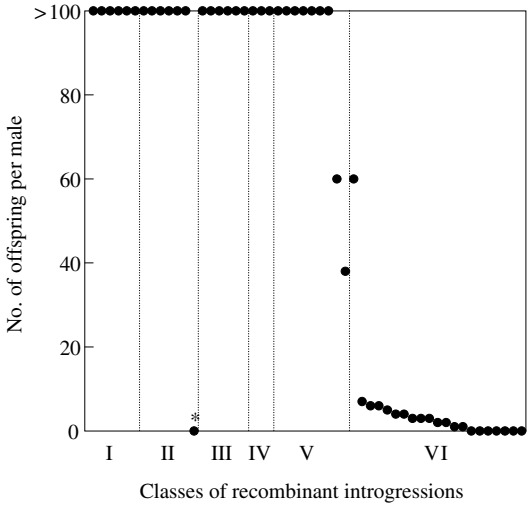

Fig. 2. Fertility of males heterozygous for *Int(2L)D+S* and each recombinant introgression. The mean number of offspring per male is shown. Note here that the order of recombinants within each class is arbitrary with respect to the recombination site. The data are plotted according to fertility (higher fertility on the left). An asterisk (*) indicates a double recombinant chromosome; this chromosome presumably retains male sterility genes in the introgression proximal to the *b* gene.

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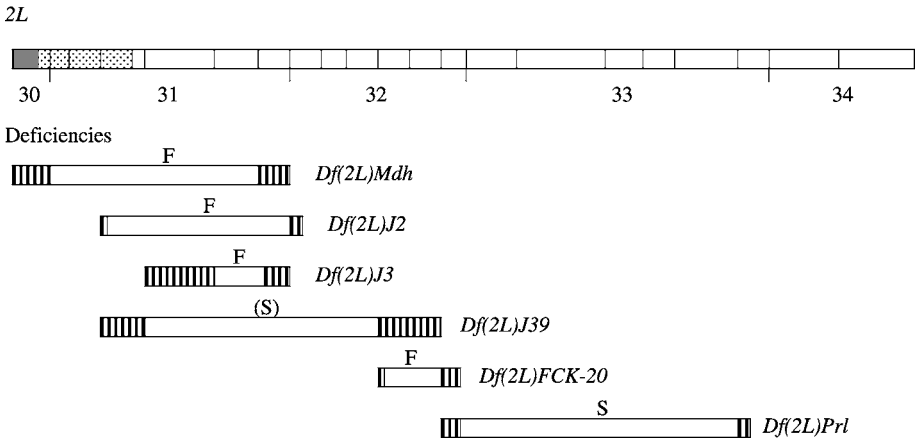

Fig. 3. Deficiency chromosomes used in the present analysis. The deleted region of each deficiency chromosome is indicated by open boxes (uncertainty of boundaries is indicated with hatching). The fertility of males heterozygous for *Int(2L)D+S* and each *Df(2L)* is indicated above the deficiency (F, fertile; S, sterile). See text for *Df(2L)J39* data.

Table 1. *Male fertility of heterozygous introgressions over* Int(2L)D+S *and of homozygous introgressions. Mean number of offspring per male is shown*

Heterozygotes over *Int(2L)D+S*[a]	Homozygotes
(i) 1 recombinant of class V[b]	
37·9 (IF)	134·6
(ii) 8 recombinants of class VI[b]	
59·9 (IF)	187·6
5·5 (SS)	10·4
2·6 (SS)	0
2·5 (SS)	0·2
1·7 (SS)	0
1·5 (SS)	18·4
0·8 (SS)	5·4
0·4 (SS)	0·2

[a] Data plotted in Fig. 2. IF, intermediate fertility; SS, semi-sterility.
[b] Classes as depicted in Fig. 1.

IV were utilized (more than 100 offspring per male produced), except for one case (no offspring, indicated by asterisk). But the molecular analysis of the *twe* locus indicated that this exceptional chromosome also contain another introgression. We assume that this chromosome retained male sterility genes on the proximal introgression by double recombination (Fig. 1).

Among nine recombinants of class V, seven were fertile (>100) but two showed reduced fertility (59·9 and 37·9 offspring). Among 21 recombinants of class VI, one showed reduced fertility (59·9 offspring) but the others were almost completely sterile (2·3±2·26 (SD)). We assume that the two exceptional recombinants in class V carry larger introgressions than the others and that the one exceptional recombinant in class VI carries a smaller introgression than the others, although we do not have molecular evidence for these assumptions. There seems a transition zone around the *prd* locus. Generally, large introgressions covering far beyond *prd* lead to male sterility. The transition zone suggests the presence of at least two genes which synergistically cause male sterility. This is consistent with the view that hybrid male sterility is multi-genic and epistatic (Wu & Palopoli, 1994; Wu *et al*., 1996; Naveira & Maside, 1998). The proximal part of the introgression was also found to be such a case (Sawamura *et al*., 2004*b*).

The fertility of males homozygous for the introgressions of classes V and VI was tested. Only one and eight recombinants among those of classes V and VI, respectively, were examined (Table 1). If males with an introgression over *Int(2L)D+S* exhibited semi-sterility (seven introgressions of class VI), homozygous introgressions also resulted in male semi-sterility. On the other hand, if males with an introgression over *Int(2L)D+S* exhibited intermediate fertility (one each of classes V and VI), homozygous males were fertile (>100). This confirms that dominant enhancers of male sterility exist somewhere in the introgression, which has been suggested previously (Sawamura *et al*., 2004*b*).

(ii) *Deficiency mapping*

Males heterozygous for *Int(2L)D+S* and a series of deficiency chromosomes (Fig. 3) were examined for their fertility. Males were fertile when heterozygous with *Df(2L)Mdh*, *Df(2L)J2*, *Df(2L)J3* or *Df(2L)FCK-20*, but were sterile over *Df(2L)J39* or *Df(2L)Prl* (data for *Df(2L)Mdh*, *Df(2L)J39* and *Df(2L)Prl* are from Sawamura *et al*., 2000). (Only seven males were tested in *Df(2L)J39* because the deficiency causes semi-lethality. As this region might contain loci of haplo-insuficiency (even *D. melanogaster* flies with this deficiency are semi-lethal), the

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male sterility might not be caused by the introgression but by a side-effect of the deficiency.) We can, at least, conclude that the introgression regions 30F3-31C5 to 32A1-2 and 32D1 to 32F1-3 do not contain recessive genes solely responsible for male sterility.

4. Discussion

The smallest introgression resulting in male sterility when homozygous is in class VI. According to FlyBase (2003) the physical size of the introgression (*bsk/bib–prd*) is 1·8–2·1 Mb and covers 86–93 polytene chromosome bands. Previously, Sawamura *et al.* (2004*b*) indicated that homozygous introgressions covering the region of *Adh/Su(H)–CycE/twe* (0·7–1·6 Mb; 12–27 bands) cause male sterility. The minimal introgression resulting in male sterility is similar at the physical level, although very different at the cytological level (the density of bands varies depending on the chromosome region).

Here we compare the *D. melanogaster/D. simulans* data with the previous analyses of introgression from *D. mauritiana* to *D. simulans*. Precise mapping of hybrid male sterility genes at a comparable level has been done for chromosomes X and 3 (Cabot *et al.*, 1994; Perez & Wu, 1995; Davis & Wu, 1996; Tao *et al.*, 2003*a*, *b*), but the resolution of the previous mapping on the chromosome 2 does not reach the same level (Hollocher & Wu, 1996; True *et al.*, 1996). Therefore, we use data from different chromosomes to compare the physical length of introgressions which result in male sterility. Because there still remains the controversy of whether the density of hybrid male sterility genes is similar between the X chromosome and the autosomes (Hollocher & Wu, 1996; True *et al.*, 1996; Tao *et al.*, 2003*a*), comparison among and within autosomes seems most reasonable. Data for chromosome 3 (Tao *et al.*, 2003*a*) are used for the comparison.

In the *D. simulans/D. mauritiana* pair the minimal introgression resulting in male sterility includes 138 polytene chromosome bands (Tao *et al.*, 2003*a*). This is 5·1 times larger than that in the *D. melanogaster/D. simulans* pair, where at most 27 polytene chromosome bands are enough to cause male sterility (the present calculation). Because only two regions of *D. melanogaster/D. simulans* introgression have been tested, the present calculation might underestimate the value. We therefore introduce another method of calculation. According to Tao *et al.* (2003*a*), the genome has 15 times the minimum number of incompatibility factors necessary to cause complete hybrid male sterility (HMS "equivalents") in the *D. simulans/D. mauritiana* pair. Because the euchromatin size of the species seems similar to *D. melanogaster* (i.e. 120 Mb; Adams *et al.*, 2000), every 8 Mb must have 1 HMS "equivalent" on average. This is 4·7 times larger than that in the *D. melanogaster/D. simulans* pair, where a 1·7 Mb size of introgression corresponds to 1 HMS "equivalent" (the present calculation). There is much molecular evidence suggesting that *D. melanogaster* and *D. simulans* diverged 2–3 MYA (million years ago), and that *D. simulans* and *D. mauritiana* diverged 0·3–0·4 MYA (Lachaise & Silvain, 2004). The minimal introgression resulting in male sterility is several times smaller in the species pair that has been isolated from each other for several-fold longer time period.

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