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Genetic studies of two sister species in the *Drosophila melanogaster* subgroup, *D. yakuba* and *D. santomea*

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Summary

We performed genetic analysis of hybrid sterility and of one morphological difference (sex-comb tooth number) on *D. yakuba* and *D. santomea*, the former species widespread in Africa and the latter endemic to the oceanic island of São Tomé, on which there is a hybrid zone. The sterility of hybrid males is due to at least three genes on the X chromosome and at least one on the Y, with the cytoplasm and large sections of the autosomes having no effect. F_1 hybrid females carrying two X chromosomes from either species are perfectly fertile despite their genetic similarity to completely sterile F_1 hybrid males. This implies that the appearance of Haldane's rule in this cross is at least partially due to the faster accumulation of genes causing male than female sterility. The larger effects of the X and Y chromosomes than of the autosomes, however, also suggest that the genes causing male sterility are recessive in hybrids. Some female sterility is also seen in interspecific crosses, but this does not occur between all strains. This is seen in pure-species females inseminated by heterospecific males (probably reflecting incompatibility between the sperm of one species and the female reproductive tract of the other) as well as in inseminated F_1 and backcross females, probably reflecting genetically based incompatibilities in hybrids that affect the reproductive system. The latter 'innate' sterility appears to involve deleterious interactions between *D. santomea* chromosomes and *D. yakuba* cytoplasm. The difference in male sex-comb tooth number appears to involve fairly large effects of the X chromosome. We discuss the striking evolutionary parallels in the genetic basis of sterility, in the nature of sexual isolation, and in morphological differences between the *D. santomea*/*D. yakuba* divergence and two other speciation events in the *D. melanogaster* subgroup involving island colonization.

1. Introduction

Much of our knowledge about the genetics of speciation comes from *Drosophila*, especially the cosmopolitan human commensal *D. melanogaster* and its sister clade comprising *D. simulans* (also a commensal), *D. sechellia* (endemic to the Seychelles archipelago) and *D. mauritiana* (endemic to the island of Mauritius). As shown in Fig. 1, these four species are themselves a monophyletic group within the *D. melanogaster* subgroup (itself containing nine species). Well over a hundred papers have been devoted to genetic analysis of these four species, analysing the number, locations and identities of genes causing reproductive isolation and species differences (e.g. Ashburner, 1989; Zeng *et al.*, 2000; Jones, 2001; Sawamura & Tomaru, 2002; Presgraves, 2003) and this work has in turn shed light on the causes of evolutionary patterns such as Haldane's rule: the greater sterility and inviability of heterogametic than of homogametic hybrids (Haldane, 1922; see Coyne & Orr, 2004). Until recently, however, genetic analysis within the entire subgroup was limited to these four species.

The recent discovery of *D. santomea*, a species endemic to the island of São Tomé (Lachaise *et al.*, 2000), has greatly expanded the opportunities for genetic analysis. Molecular phylogenetic analysis shows that *D. yakuba* and *D. santomea* are sister species within the *D. melanogaster* subgroup (Lachaise

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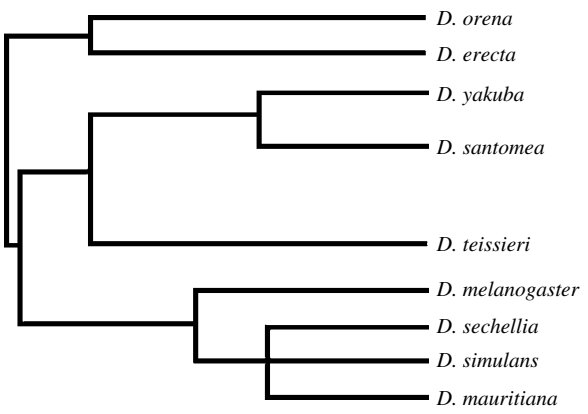

Fig. 1. Phylogeny of the nine species in the *D. melanogaster* subgroup based on allozyme and DNA data (Kliman *et al*., 2000; Lachaise *et al*., 2000; Parsch, 2003). While the branchpoints are based on much concordant data, the relative branch *lengths*, taken from Lachaise *et al*. (2000), may be somewhat inaccurate (for example, Kliman *et al*. (2000) date the divergence of *D. melanogaster* from the *D. simulans* subgroup at about 3 myr, while Llopart *et al*. (2002) date the divergence of *D. yakuba* and *D. santomea* at about 0·4 myr). The phylogeny of the *D. simulans* clade (*D. simulans*, *D. sechellia*, and *D. mauritiana*) is an unresolved trichotomy (Kliman *et al*., 2000), although the last two species probably derived from independent colonizations of Mauritius and the Seychelles by an ancestor of *D. simulans*.

et al., 2000; Cariou *et al*., 2001). As shown in Fig. 1, this pair represents a speciation event independent of the well-studied speciation event separating the ancestor of *D. melanogaster* from that of the *D. simulans* triad, as well as of the two speciation events in which a *D. simulans*-like ancestor produced the two island endemics *D. sechellia* and *D. mauritiana* (Lachaise *et al*., 1988). Moreover, the formation of *D. santomea*, like that of *D. sechellia* and *D. mauritiana*, undoubtedly followed colonization of an oceanic island by a mainland ancestor.

The *D. santomea*/*D. yakuba* pair thus offers us an opportunity to see whether the patterns of speciation found in *D. melanogaster*, *D. simulans*, *D. mauritiana* and *D. sechellia* are repeated in an independent island-colonization event. Here we present genetic and phenotypic data on sterility, sex ratio and developmental anomalies in *D. santomea*/*D. yakuba* and their hybrids. These data offer a comparison with previous work, a basis for future genetic analysis involving both quantitative trait locus (QTL) mapping and more refined molecular analysis, and a set of predictions about which regions of the genome are most likely to introgress between the species in their area of overlap.

D. yakuba is widespread across sub-Saharan Africa and the islands near the continent. *D. santomea*, however, was discovered in 1998, and is endemic to São Tomé, a 860 km² volcanic island lying 255 km from the nearest mainland: the coast of Gabon (Lachaise *et al*., 2000). *D. yakuba* also inhabits São Tomé. On the mountain of Pico do São Tomé, *D. yakuba* occurs at elevations below 1450 m, while *D. santomea* occupies the mist forests at elevations between 1153 and 1800 m (*D. yakuba* is also widespread throughout lowland São Tomé). Between about 1100 and 1450 m in elevation, the ranges of the two species overlap, with the ratio of *D. yakuba*/*D. santomea* shifting from 2 : 1 to 1 : 20 as one moves upward through this zone.

The species show substantial sexual isolation when tested in the laboratory (Lachaise *et al*., 2000) and, using morphological criteria, one finds a low frequency (*c*. 1 %) of hybrids in the zone of overlap. (*D. yakuba* has the black abdominal pigmentation typical of all other species in the *D. melanogaster* group, while *D. santomea* lacks any pigmentation. These species also differ in male genital morphology [Lachaise *et al*., 2000]). The pair fails to show any 'reinforcement', i.e. any increase in sexual isolation between species in the area of sympatry (Coyne *et al*., 2002). In interspecific crosses, F_1 male hybrids are sterile but female hybrids are fertile; the latter can be backcrossed to either parental species (Lachaise *et al*., 2000; Cariou *et al*., 2001). Besides behavioural isolation and intrinsic hybrid sterility, the species also show conspecific sperm precedence: when *D. yakuba* females are multiply inseminated by both conspecific and *D. santomea* males, they produce very few hybrid progeny, regardless of the order of mating (Chang, 2004).

Molecular evidence puts the divergence between *D. yakuba* and *D. santomea* at about 400 000 years ago (95 % confidence interval 250 000–560 000 years; see Llopart *et al*., 2002), a divergence time similar to that estimated between *D. simulans* and each of its two island-dwelling sister species (*c*. 260 000–410 000 years; Kliman *et al*., 2000). The *D. melanogaster* subgroup thus includes three episodes of speciation following island colonization, all occurring at roughly the same time.

2. Materials and methods

(i) *Fly stocks*

(a) *D. yakuba*

The isofemale line BOSU 1153.1 was collected in February 2001 at an altitude of 1153 m, within the area of overlap with *D. santomea*. Lines SJ1, SJ2, SJ3 and SJ4 were collected in March 2000 in São Joao dos Angolares, in the lowlands of southern São Tomé island at an altitude of 320 m. This is outside the range of *D. santomea*. The isofemale line Cam 115 was collected in April 1967 in the Kounden Plateau in western Cameroon. The isofemale lines Taï 6, 18, 30 and 47 were collected in 1981 in the Taï rainforest on

the border between Guinea and the northwest Ivory Coast. The isofemale lines Ab 2, 34, 45, 60, 96 and 160 were collected in March 1999 outside Abidjan, in the southeastern Ivory Coast. The isofemale line Anton-2 was collected in 2001 on Santo Antonio on the northeastern coast of Principé Island, 175 km northeast of São Tomé. Isofemale line 0261.0 was collected from the Ivory Coast, Africa, and was obtained from the National Drosophila Species Resource Center at Tucson, Arizona.

All wild-type strains not explicitly mentioned in the Results section were used for measurements of sex-comb tooth number.

The *D. yakuba* multiple-mutant stock w^{or}; *no*; *se*, containing one recessive mutation on each of the three major chromosomes, was constructed using mutants found in isofemale lines from the Ivory Coast and Gabon (Llopart *et al.*, 2002). Mapping studies (see below) show that *white-orange* (w^{or}), a recessive, X-linked mutation producing light orange eyes, is located at 1–6·2; the gene is identical to the *white* locus of *D. melanogaster* (Llopart *et al.*, 2002). The recessive mutation *notch* (*no*), which produces nicked wings, is roughly 10 cM from the base of the right arm of the second chromosome, at about 2–150. The recessive mutation *sepia* (*se*) in *D. yakuba*, identical to the third-chromosome mutation *sepia* of *D. melanogaster*, is about 9 cm from the tip of the left arm of chromosome 3. This multiple-marker stock is described in more detail by Llopart *et al.* (2002).

To map sterility effects on the X chromosome, we used a multiply-marked X stock of *D. yakuba*: y, w^{or}, *g*, *sn*, constructed from mutants that arose in isofemale lines from Taï, Abidjan, and Principé. The origin and identification of *y* and w^{or} (identical to the *D. melanogaster* loci of the same name) were described by Llopart *et al.* (2002). The mutants *garnet* (*g*) and *snipped* (*sn*), a purple eye mutant and a serrated wing mutant respectively, arose spontaneously in a stock from the island of Principé (Coyne *et al.*, 2002; we do not know whether *garnet* maps to the *garnet* locus of *D. melanogaster*).

A recombinational analysis using a wild-type stock of *D. yakuba* show that the map positions of y, w^{or}, *g*, and *sn* on the X are 0, 6·2, 17·5 and 51·1 respectively ($N = 1114$). Takano-Shimizu (2001) reported that the *D. yakuba* X chromosome is 100·6 cM long, approximately twice the recombinational length of the *D. melanogaster* X, so that in this stock roughly half of the chromosome is unmarked. Nevertheless, we should be able to detect genes of large effect over roughly three-quarters of the chromosome. As the X chromosomes of both species are homosequential, and no inversions have been seen by ourselves and others (Lemeunier & Ashburner, 1976; Lachaise *et al.*, 2000), it is safe to conclude that X-linked markers recombine freely in hybrid F_1 females.

To produce 'unbalanced' F_1 hybrid females and to search for effects of the Y chromosome on hybrid male sterility (see Coyne, 1985*a*) we constructed an attached-X stock of *D. yakuba*. This stock, C(1)RM *y*, w^{or} females, + males, was made by irradiating 1-day-old virgin females of the *y*, w^{or} stock with 4000 rads. Females were then crossed to wild-type Taï 18 males. Out of roughly 24 000 irradiated females, we obtained 75 *y*, w^{or} individuals; 23 behaved like attached-X females in further crosses. This chromosome, like the one we constructed in *D. santomea* (see below), was not verified cytologically; nevertheless, its matrilineal inheritance leaves little doubt that it is in fact an attached-X. We chose the most vigorous of these 23 lines for our crosses.

(b) *D. santomea*

The isofemale lines STO.4, STO.5, STO.7 and STO.10 were collected on March 1998 in the Obo Natural Reserve on São Tomé at 1300 m altitude (Lachaise *et al.*, 2000). *D. santomea* STO.18 was collected at 1153 m, and STO.15 at 1450 m. These lines all came from the zone of overlap with *D. yakuba*. Lines CAR 1566.3 and CAR 1600.3 were collected in February 2001 on Pico Calvario, São Tomé, at elevations of 1566 m and 1600 m respectively, above the zone of overlap with *D. yakuba*. The '2001 synthetic' stock of *D. santomea* ('2001') was made by combining four isofemale lines collected in 2001 in the area of overlap with *D. yakuba*; none of these was identical to the isofemale lines described above.

The *D. santomea garnet* stock was derived from a mutant individual in the STO.18 line; it proved identical to the *garnet* mutation described in *D. yakuba* (see above).

The attached-X stock of *D. santomea*, C(1)RM *g* females, + males, was produced by irradiating virgin *g* females using the protocol for constructing the *D. yakuba* attached-X stock described above. Only one attached-X female was obtained. To increase the viability of this stock, we outcrossed these attached-X *garnet* females for two generations to wild-type males from the STO.4 line.

All strains of *D. yakuba* and *D. santomea* used in analysis of male or female fertility or sex ratios were tested for *Wolbachia* infection using PCR on the *wsp* Wolbachia primer set (Jeyaprakash & Hoy, 2000) and the TRAP100 gene (found in all *Drosophila*) as a positive control for the DNA preparation. No strain used in these studies was infected.

(c) *D. simulans*

Florida City (FC): This stock was derived from a single female collected in Florida City, Florida in June 1985.

Beadex: This stock is homozygous for the dominant, X-linked wing mutation *Beadex* (1–62·2), which maps to the same locus as *Beadex* in *D. melanogaster*.

(d) *D. teissieri*

This species was used as an outgroup to determine whether the larger number of sex-comb teeth seen in *D. santomea* (compared with *D. yakuba*) was a derived condition. We used the following six isofemale strains: 128·2, collected by H. E. Paterson in 1970 on Mount Silinda, Eastern Zimbabwe (formerly Rhodesia; Lemeunier & Ashburner, 1976); BRZ11, collected in Brazzaville, Congo by P. Capy in 1990; UZ11, UZ12 and UZ47, collected in the Uzungwa Mountains, Southern end of the Eastern Arc Mountains, Tanzania by D. Lachaise in 1995 (Cobb *et al.*, 2000); and line TR 103, Lopé Forest Reserve, Middle Ogooué, Central Gabon, collected by D. Lachaise in 2000.

(ii) *Rearing and crosses*

All flies, both stocks and hybrids, were reared on cornmeal/agar/corn syrup medium at 24 °C on a 12 h light/dark cycle. Flies were raised under uncrowded conditions, usually in bottles founded by either 18 *D. yakuba* individuals or 25 *D. santomea* individuals of each sex.

Crosses used to produce interspecific hybrids are described in the Results section. All crosses were made using 10–12 individuals of each species in 8-dram vials and reared under the conditions described above.

(iii) *Measurements of fertility*

Males were tested for fertility as 4-day old virgins by lightly crushing their testes in Ringer's solution and examining these under a compound microscope. Two protocols were used. The first, similar to that we used previously (e.g. Coyne & Charlesworth, 1989) is dichotomous: determining whether a male has motile sperm. Using this criterion, 'fertile' males are those having at least one motile sperm. 'Sterile males' are those entirely lacking motile sperm; these can include males without spermatids, males with spermatids but no sperm, or males with completely immotile sperm. To refine this protocol, in some analyses we used a four-category measure of sperm development: 'no spermatids' (males with no sign of any sperm precursors), 'only spermatids' (males with spermatids but no individuated sperm), 'non-motile sperm' (males with individuated sperm, none of which were motile), and 'motile sperm' (males with individuated sperm, at least one of which was motile).

Females were also tested for fertility. One-day old virgins were confined in 8-dram vials with virgin males for 3 days (depending on the test, a group of 12 females was confined with either 24 males of a single species or 12 males of *D. yakuba* and 12 of *D. santomea*). The females were then placed individually in vials with two males (depending on the test, either both males were from the same species or one was *D. santomea* and the other *D. yakuba*). These vials were inspected for the presence of larvae after 4 days. If larvae were present, females were scored as fertile. If no larvae were present, the female was dissected in Ringer's solution and her seminal receptacle and spermathecae were examined for the presence of sperm. If no sperm were present, the female was discarded and not included in the analysis. If sperm were present, the vial from which the female came was retained and inspected for larvae after another 2 days. Inseminated females producing no larvae over this 6-day period were considered sterile.

(iv) *Sex-comb tooth number*

Sex combs are clumps of stiff bristles found on the forelegs of males in many species of *Drosophila*. In the *D. melanogaster* subgroup they occur on the first tarsal segment. Their function is unknown, but they may help males grasp and mount females before copulation (Coyne, 1985*a*). As in previous studies, we measured tooth number by dissecting both legs of a male and counting the number of teeth on one randomly selected leg using a compound microscope (Coyne, 1985*a*). We counted 50 males from each of eight isofemale lines of *D. santomea* and 19 isofemale lines of *D. yakuba* (all reared at low density) collected from São Tomé, the Taï rainforest, Abidjan, Cameroon, and the island of Príncipe. We also scored 20 males from each of six lines of an outgroup species, *D. teissieri*.

(v) *Sex ratio and other hybrid anomalies*

Details for measuring sex ratio and detecting morphological anomalies in wide crosses are described in the Results section.

3. Results

(i) *Hybrid male sterility*

Table 1 gives measurements of fertility in pure-species, F_1 and backcross hybrid males using the dichotomous criterion of motile sperm ('fertile') versus non-motile sperm ('sterile'). As expected, pure-species males of all strains show almost complete fertility, while F_1 hybrid males with a *D. yakuba* mother (genotype 4) are completely sterile, as reported by

Table 1. *Fertility of males of pure-species, F_1 hybrids and backcross progeny*

Genotype	Fertile	Sterile	Total
1. *D. yakuba* Taï 18	115	3	118
2. *D. yakuba* w^{or}; *no*; *se*	99	1	100
3. *D. santomea* STO.4	94	6	100
4. F_1 (yak w^{or}; *no*; *se* × san STO.4)	0	100	100
Backcrosses with wild-type flies			
5. (yak Taï 18 × san STO.4) × san STO.4	7	255	262
6. (yak Taï 18 × san STO.4) × yak Taï 18	56	260	316
7. (san STO.4 × yak Taï 18) × san STO.4	7	163	170
8. (san STO.4 × yak Taï 18) × yak Taï 18	30	144	174
9. (yak Ab 96 × san STO.18) × san STO.18	17	88	105
10. (yak Ab 96 × san STO.18) × yak Ab 96	32	72	104
11. (san STO.18 × yak Ab 96) × san STO.18	13	100	113
12. (san STO.18 × yak Ab 96) × yak Ab 96	26	81	107
Backcross (yak w^{or}; *no*; *se* × san STO.4) × yak w^{or}; *no*; *se*			
13. w^{or}; *no*; *se*	74	126	200
14. +; *no*; *se*	7	193	200
15. w^{or}; +; *se*	93	107	200
16. w^{or}; *no*; +	51	149	200
17. w^{or}; +; +	87	113	200
18. +; *no*; +	6	194	200
19. +; +; *se*	15	185	200
20. +; +; +	20	180	200

See text for details. In each cross, the female parent is given first. In this and all following tables, 'yak' is *D. yakuba* and 'san' is *D. santomea*.

Lachaise *et al.* (2000). Table 3 also shows that F_1 males from the reciprocal cross are likewise sterile.

Two sets of wild-type strains from each species were used to perform eight backcrosses: these fell into two groups, each of which contained all four possible backcrosses (Table 1, genotypes 5–12). Although the two sets of backcrosses differed somewhat in absolute fertility, the results were similar in several respects. First, F_1 females backcrossed to *D. santomea* males invariably produced male offspring that were more sterile than when they were backcrossed to *D. yakuba* males. This effect of the male parent was significant in all four pairwise comparisons (genotypes 5 vs 6, 7 vs 8, 9 vs 10, and 11 vs 12; all $P<0{\cdot}015$ according to Fisher's exact test). These results, together with the observation that crosses between *D. yakuba* females and *D. santomea* males occur much more readily than the reciprocal hybridization, have implications for which genomic regions are most likely to introgress through the hybrid zone (see Discussion).

In neither set of crosses did we see any cytoplasmic effects on sterility: male backcross progeny whose fathers are from a given same strain have similar fertility, regardless of which interspecific cross produced the mother. None of the four comparisons testing this possibility showed a significant cytoplasmic effect (Table 1: genotypes 5 vs 7, 6 vs 8, 9 vs 11, and 10 vs 12; all $P>0{\cdot}35$ according to Fisher's exact test). Hybrid sterility thus cannot involve species-specific interactions between nuclear genes and either mitochondrial genes or other cytoplasmic factors.

To estimate the effects of segments of the three major chromosomes on male sterility, we produced F_1 hybrid females by crossing *D. yakuba* w^{or}; *no*; *se* females to *D. santomea* STO.4 males. These females were backcrossed to *D. yakuba* w^{or}; *no*; *se* males, producing eight classes of backcross males having all three combinations of the species' chromosomes (Table 1, genotypes 13–20). X-linked segments are hemizygous for either *D. santomea* or *D. yakuba* genome, while autosomal segments are either homozygous for the segment of *D. yakuba* genome linked to the marker, or heterozygous for one segment from each species. We scored sperm motility in 200 4-day old males from each of these eight genotypes (Table 1, genotypes 13–20).

It is immediately apparent that the X-chromosomal segment linked to the w^{or} marker has by far the largest effect on fertility: backcross males with this mutation (and thus having the associated *D. yakuba* genome) show substantial fertility (305/800 males with motile sperm), while males with the wild-type *D. santomea* marker are largely sterile (48/800 males).

Frequency data from these backcrosses were analysed using the log-likelihood CATMOD procedure in SAS (SAS/STAT User's Guide; SAS Institute, 1988). This procedure measures the effect of a marker substitution by comparing all genotypes that differ in

Table 2. *Analysis of male sterility among backcross progeny using the* D. yakuba *chromosomal marker stock* w^{or}*;* no*;* se

Source	χ^2	Probability
Intercept	340·65	<0·001
w^{or} (X chromosome)	168·35	<0·001
no (chromosome 2)	20·16	<0·001
se (chromosome 3)	0·48	0·4899
$w^{or} \times no$	1·45	0·2288
$w^{or} \times se$	1·25	0·2644
$no \times se$	1·49	0·2244
$w^{or} \times no \times se$	0·01	0·9393

Analysis was performed using the CATMOD procedure of SAS (see text). All tests have one degree of freedom.

this substitution. The X-chromosomal segment has by far the largest effect, with the second-chromosomal segment *no* having a smaller but significant effect, while there is no significant effect of the third-chromosomal segment (*se*) or of any interactions between chromosomal segments (Table 2). This large X-effect on male sterility is well known, and is a common feature of genetic analyses of *Drosophila* (Coyne & Orr, 1989*a*). As we note in the Discussion, this large X effect may reflect either a higher density of 'male sterility genes' on this chromosome, the recessivity of such genes, or both factors.

We also show below that this X-effect is due to a minimum of three genes. While the effect of the *notch*-linked segment is highly significant, it is in fact in the direction *opposite* to that predicted: males carrying *notch* are significantly less fertile than those with the wild-type segment from *D. santomea* (*notch*: 138/800 fertile, +: 215/800 fertile). We have no definitive explanation for this result, but suggest that homozygosity for the *notch* mutation causes semisterility of males on a hybrid as opposed to a pure-species genetic background (as shown in Table 1, the pure-species w^{or}; *no*; *se* stock is perfectly fertile). This study thus identifies at least one X-linked gene causing hybrid male sterility; since this *white*-linked gene must interact with a gene or genes in the *D. yakuba* genetic background, there are obviously at least two genes responsible for hybrid sterility – the minimum required under the Dobzhansky–Muller epistatic theory of sterility (Orr, 1996).

Using the formulae of Naveira & Barbadilla (1992) and estimates of map distances in the *D. yakuba* genome (Takano-Shimizu, 2001), we estimate that the length of the chromosomes linked to these genes is roughly 40 cM for w^{or}, 36–50 cM for *notch* (depending on whether this gene is contained within the chromosome arrangement *2Rn*; Llopart *et al*., 2002), and 44 cM for *sepia*. As the recombinational lengths of chromosomes X, 2 and 3 are roughly 100, 160 and 130 cM, respectively (Takano-Shimizu, 2001), we are able to detect sterility effects of genes on roughly 30–40% of each chromosome. Thus, while we are undoubtedly missing genes causing sterility – indeed, further analysis described below shows at least three X-linked loci and one Y-linked locus causing hybrid sterility – it is clear that either major segments of the autosomes do not harbour sterility genes of large effect, that the effects of these genes are recessive and cannot be seen as heterozygotes in a genetic background derived largely from *D. yakuba*, or that both of these factors operate.

We conducted more refined analyses of hybrid male sterility using the four-category ranking of sperm formation described in Materials and Methods; these analyses included pure species and F_1 hybrids, a genetic dissection of sterility effects of the X chromosome, and a study of the effect of the Y chromosome on male fertility. We discuss these results in order.

As shown in Table 3 (genotypes 1–4), all three strains of *D. yakuba* examined (one wild-type and two mutant strains) and the single strain of *D. santomea* showed a high proportion of males having motile sperm (94–100%). Reciprocal crosses between the Taï 18 strain of *D. yakuba* and the STO.4 strain of *D. santomea* revealed that, while F_1 males were always sterile (having no motile sperm), the degree to which spermatogenesis proceeded depended strongly on the direction of the cross. A high proportion of F_1 males with *D. yakuba* mothers had spermatids and non-motile sperm, while virtually all (99%) F_1 males with *D. santomea* mothers showed *no* spermatogenesis, having neither spermatids nor sperm. This difference is highly significant (Table 3, genotypes 5 vs 6, $\chi^2 = 141$, 3 d.f., $P < 0{\cdot}0001$). The more advanced spermatogenesis in males with a *D. yakuba* mother is also seen in genotypes 8 and 9: F_1 hybrids having *D. yakuba* parents from a different strain.

The absence of spermatogenesis in genotype 6 (Table 3) could in principle be due to deleterious interactions between *D. santomea* cytoplasm and *D. yakuba* autosomes and/or Y chromosome, between the *D. santomea* X chromosome and the *D. yakuba* autosomes and/or Y chromosome, or a combination of these interactions. We can rule out cytoplasmic involvement by comparing genotypes 7 and 8; these both involve crosses between *D. yakuba* females carrying the *y* and w^{or} mutations that come from the same strain, but in one case (genotype 7) the mother carries attached-X chromosomes and a Y chromosome. Thus, male genotypes 7 and 8 both carry *D. yakuba* cytoplasm, but genotype 7 has the X chromosome from *D. santomea* and the Y from *D. yakuba*, while in genotype 8 these origins are reversed. The difference between the fertility of these genotypes is highly significant: $\chi^2 = 177$, 3 d.f., $P < 0{\cdot}0001$. This is

Table 3. *Male fertility of pure-species, F_1 hybrids and backcross males* (*tests of the X and Y chromosomes*)

Male genotype	No spermatids	Only spermatids	Non-motile sperm	Motile sperm	Sample size
1. *D. yakuba* Taï 18	0	0	0	67	67
2. *D. yakuba y*, w^{or}, *g*, *sn*	2	2	1	95	100
3. *D. yakuba y*, w^{or}	0	0	2	98	100
4. *D. santomea* STO.4	1	2	3	94	100
5. F_1 (yak Taï 18 × san STO.4)	16	68	16	0	100
6. F_1 (san STO.4 × yak Taï 18)	99	1	0	0	100
7. F_1 (yak C(1)*RM y*, w^{or} × san STO.4)	99	1	0	0	100
8. F_1 (yak *y*, w^{or} × san STO.4)	5	65	30	0	100
9. F_1 (yak *y*, w^{or}, *g*, *sn* × san STO.4)	10	80	10	0	100
Backcross (*y*, w^{or}, *g*, *sn* × san STO.4) × yak Taï 18					
10. *y*, w^{or}, *g*, *sn*	16	96	1	114	227
11. +, w^{or}, *g*, *sn*	2	57	0	89	148
12. +, +, *g*, *sn*	34	104	3	89	230
13. +, +, +, *sn*	71	141	0	15	227
14. *y*, +, +, +	104	15	0	3	122
15. +, +, +, +	202	23	0	2	227
Backcross testing effect of *D. yakuba* Y chromosome (see Fig. 2)					
16. *y*, w^{or}, *g*, *sn*, *D. santomea* Y	16	138	32	14	200
17. *y*, w^{or}, *g*, *sn*, *D. yakuba* Y	11	65	0	124	200
Backcross testing effect of *D. santomea* Y chromosome (see Fig. 2)					
18. Wild-type, *D. yakuba* Y	69	122	20	25	236
19. Wild-type, *D. santomea* Y	44	126	42	51	263

In all crosses, the female parent is given first. See text for details.

the same difference in fertility seen in F_1 males from reciprocal crosses: the $X^{san}Y^{yak}$ males show no spermatogenesis, while the $X^{yak}Y^{san}$ males produce both spermatids and non-motile sperm in proportions close to those seen in other hybrids of this genotype. Thus, $X^{san}Y^{yak}$ males show no spermatogenesis regardless of whether their cytoplasm comes from *D. santomea* or *D. yakuba*. The lack of a cytoplasmic effect on male sterility is supported by data from Table 1 described above. The difference in spermatogenesis thus involves interactions between chromosomes. We show below that, at least in backcrosses, both the X and Y chromosomes have large effects on fertility.

To dissect the X chromosome more finely, we crossed *D. yakuba* y, w^{or}, *g*, *sn* females to *D. santomea* STO.4 males, and backcrossed the heterozygous F_1 hybrid females to *D. yakuba* Taï 18 males. Males from this backcross have a Y chromosome from *D. yakuba*, three-quarters of their autosomes from *D. yakuba*, and an X chromosome that is a mixture of *D. santomea* and *D. yakuba*; the species composition of the X can be discerned from the markers present. With four markers, one obtains 16 distinguishable genotypes of the X chromosome. We examined six of these (genotypes 10–15 in Table 3), chosen so that each chromosomal segment could be assessed in at least one comparison between pairs of genotypes.

The effect of the small segment between *yellow* and *white*, about 6·2 cM long, can be judged by comparing the fertility of two genotypes that differ only in the presence of the yellow mutant: genotype 10 vs 11 and 14 vs 15 in Table 3. Although the first comparison shows a significant effect of the segment on sterility ($\chi^2 = 8{\cdot}6$, 3 d.f., $P = 0{\cdot}034$), it is in the wrong direction, with y, w^{or}, *g*, *sn* males showing slightly less-advanced spermatogenesis than +, w^{or}, *g*, *sn* males. The comparison of genotypes 14 with 15 (*y*, +, +, + vs +, +, +, +) shows no significant effect on spermatogenesis ($\chi^2 = 1{\cdot}8$, 3 d.f., $P = 0{\cdot}40$). We conclude that this X-linked segment carries no genes affecting hybrid male fertility. However, the other three segments tested have highly significant effects in the expected direction: the 11·3 cM-long segment between w^{or} and *g* (genotypes 11 vs 12; $\chi^2 = 28{\cdot}7$, 3 d.f., $P < 0{\cdot}0001$), the 33·6 cM-long segment between *g* and *sn* (genotypes 12 vs 13; $\chi^2 = 74{\cdot}2$, 3 d.f., $P < 0{\cdot}0001$) and the 50 cM-long segment extending from the *sn* marker to the tip of the X chromosome (genotypes 13 vs 15; $\chi^2 = 157{\cdot}7$, 3 d.f., $P < 0{\cdot}0001$). The X chromosome thus harbours at least three genes whose divergence between these species causes hybrid male sterility.

The effect of the Y chromosome on male fertility can be judged by constructing two genotypes that are genetically similar but which differ in the source of the Y chromosome: in this case, backcross males whose genome is largely from *D. yakuba* but have either a *D. yakuba* or *D. santomea* Y chromosome. This scheme, which uses attached-X chromosomes, is identical to

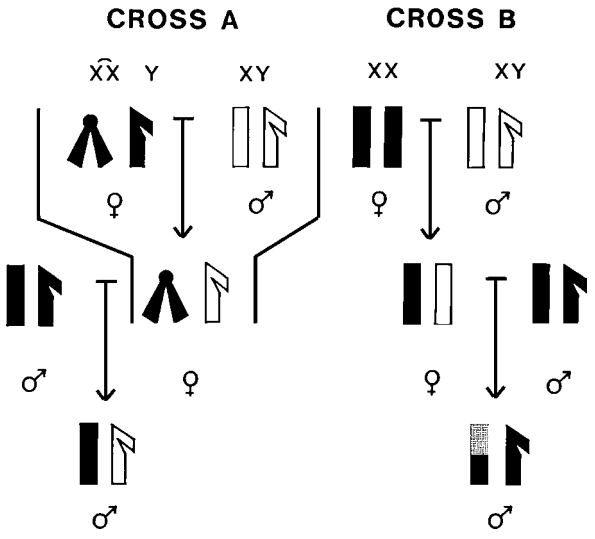

Fig. 2. Interspecific crosses used for two purposes: (1) to examine the effect of unbalanced X chromosome/autosome complement on the fertility of F_1 hybrid females, and (2) to examine the effect of foreign Y chromosomes on the fertility of backcross males. This diagram shows the crosses involving *D. yakuba* attached-X chromosomes and the effect of the *D. santomea* Y. Only sex chromosomes are shown, with those of *D. yakuba* in black and those of *D. santomea* in white. Hatched segments are those that are possibly recombinant regions containing genome of both species. In both crosses A and B, hybrid females were produced by crossing *D. yakuba* females to *D. santomea* males, and the F_1 hybrid females backcrossed to *D. yakuba* males. In cross A, however, attached-X females of *D. yakuba* were used. (See text for further description of crosses.) A similar pair of crosses were used to study the effect of *D. santomea* attached-X chromosomes on hybrid female fertility and of *D. yakuba* Y chromosomes on hybrid male fertility (see text).

that used by Coyne (1985*b*) to show a strong Y-effect on male sterility in *D. simulans*/*D. mauritiana* hybrids.

Fig. 2 displays the two crosses used in this comparison. In cross A, C(1)*RM y*, w^{or} *D. yakuba* females (with sex chromosomes shown in black) were crossed to *D. santomea* STO.4 males (sex chromosomes in white); and the F_1 females (who carry two *D. yakuba* X chromosomes and a *D. santomea* Y) were backcrossed to *D. yakuba y*, w^{or}, *g*, *sn* males. This backcross produces male offspring having roughly three-quarters of their autosomes, their entire X chromosome and their cytoplasm from *D. yakuba*, but one-quarter of their autosomal material and a Y chromosome from *D. santomea* (shown at the bottom of Fig. 2). In cross B, *D. yakuba* y, w^{or}, *g*, *sn* females (sex chromosomes shown in black) were crossed to *D. santomea* STO.4 males (sex chromosomes in white), and the F_1 females backcrossed to *D. yakuba* y, w^{or}, *g*, *sn* males. From this cross, we collected *y*, w^{or}, *g*, *sn* males for testing. These males differ genetically from those in cross A only by having a Y chromosome from *D. yakuba* and additional X-linked genes from *D. santomea*. The foreign X-linked genes should lead to increased sterility. Thus, if the source of the Y chromosome had no effect on fertility, we would expect males from cross B to have less advanced spermatogenesis than those from cross A.

In fact, we saw the opposite result. Comparing genotypes 16 (from cross A) and 17 (from cross B) in Table 3, one sees that males from cross B are much *more* fertile than those from cross A, despite their having additional foreign genes on the X chromosome. (Of 200 flies scored for each genotype, 124 males from cross B had motile sperm, but only 14 from cross A.) This difference is highly significant ($\chi^2 = 146{\cdot}9$, 3 d.f., $P < 0{\cdot}0001$). (As expected, there is no difference in fertility between the *y*, w^{or}, *g*, *sn* males produced in backcross B and males of identical genotype produced in the independent backcross dissecting the X chromosome (comparison of genotypes 10 vs 17 in Table 3: $\chi^2 = 6{\cdot}63$, 3 d.f., $P = 0{\cdot}08$). Given that we expect *y*, w^{or}, *g*, *sn* males to be more fertile in cross A than in cross B if there is no Y effect, the enormous difference in fertility in the opposite direction indicates that, in a genetic background largely from *D. yakuba*, a *D. santomea* Y chromosome substantially reduces male fertility.

A similar set of crosses was made to test the effect of the *D. yakuba* Y chromosome in a genetic background largely from *D. santomea*. Fig. 2 would also represent this cross if the sex chromosomes of *D. santomea* were depicted in black and those of *D. yakuba* in white. In cross A, *D. santomea* C(1)*RM g* females were crossed to *D. yakuba y*, w^{or}, *g*, *sn* males, and the F_1 females backcrossed to *D. santomea* STO.4 males. This backcross produces male offspring having roughly three-quarters of their autosomes, their entire X chromosome and their cytoplasm from *D. santomea*, and one-quarter of their autosomal material and their Y chromosome from *D. yakuba*. In cross B, *D. santomea* STO.4 females were crossed to *D. yakuba y*, w^{or}, *g*, *sn* males, and the F_1 females backcrossed to *D. santomea* STO.4 males. From this cross we collected wild-type males for testing. As in the test of the *D. santomea* Y effect described above, we would expect that, if there were no effect of the *D. yakuba* Y on male fertility, males of genotype A would be more fertile than those of genotype B (males of the latter have on average a large portion of their X chromosome from *D. yakuba*).

Again, the results are the opposite of this expectation, implying that the *D. yakuba* Y substantially reduces fertility in a genetic background largely from *D. santomea*. Males from cross A (Table 3, genotype 18) were much less fertile than those from cross B (Table 3, genotype 19); this lower fertility is seen across all four classes of sperm development. The difference between these genotypes is highly

Table 4. *Female fertility*

Genotype	Fertile	Sterile	Total
Pure species (tested with conspecific male unless indicated otherwise)			
1. *D. santomea* STO.4	66	0	66
2. *D. santomea* 2001	127	1	128
3. *D. yakuba* Taï 18	74	2	76
4. *D. yakuba* Cam 115	130	2	132
5. *D. santomea* STO.4 (yak Taï 18 male)	27	13	40
6. *D. yakuba* Taï 18 (san STO.4 male)	86	10	96
7. *D. santomea* 2001 (yak Cam 115 male)	41	1	42
8. *D. yakuba* Cam 115 (san 2001 male)	137	0	137
F_1 females (tested with males of both species unless indicated otherwise)			
9. yak Taï 18 × san STO.4	114	29	143
10. san STO.4 × yak Taï 18	98	2	100
11. yak 96 × san STO.18	62	6	68
12. san STO.18 × yak Ab 96	69	1	70
13. yak Cam 115 × san 2001	129	31	160
F_1 female test of 'imbalance hypothesis' (Fig. 1)			
14. yak C(1)*RM y*, w^{or} × san STO.4	191	0	191
15. yak *y*, w^{or} × san STO.4	203	0	203
16. san C(1) *RM g* × yak Taï 18	23	0	23
17. san *g* × yak Taï 18	50	0	50
Backcross females (tested with males of both species unless indicated otherwise)			
18. (yak Taï 18 × san STO.4) × san STO.4 (STO.4 males)	68	34	102
19. (yak Taï 18 × san STO.4) × san STO.4 (Taï 18 males)	57	26	83
20. (yak Taï 18 × san STO.4) × yak Taï 18 (Taï 18 males)	104	0	104
21. (san STO.4 × yak Taï 18) × yak Taï 18	119	3	122
22. (san STO.4 × yak Taï 18) × san STO.4	139	1	140

When females result from a cross, the species and genotype of the female parent is given first.

significant ($\chi^2 = 20{\cdot}9$, 3 d.f., $P < 0{\cdot}001$). The sterilizing effect of the *D. yakuba* Y in a *D. santomea* background, however, is less severe than the converse effect.

(ii) *Hybrid female sterility*

During speciation in *Drosophila*, male hybrids almost invariably become sterile before females (Coyne & Orr, 1989*b*, 1997). *Partial* female sterility, however, can sometimes be missed because it is difficult to score. We assayed the fertility of pure-species females when crossed to males of the other species, and of F_1 and backcross hybrid females when crossed to pure-species males. Because we determined whether or not inseminated females produced offspring (see Materials and Methods), 'partial' fertility means that some proportion of females of a genotype fail to produce any offspring. We may thus have missed female sterility resulting in a *reduced number* of offspring per female.

Table 4 gives the data on female fertility. In crosses involving pure species, F_1 and backcross hybrid females, sterility was seen in some strains but not others. There is significant heterogeneity in the proportion of fertility among pure-species females crossed to heterospecific males (Table 4, genotypes 5–8; $\chi^2 = 16{\cdot}3$, 3 d.f., $P < 0{\cdot}001$); this is due to the substantial sterility of *D. santomea* STO.4 females inseminated by *D. yakuba* Taï 18 males (genotype 5; 32% sterile) and the more moderate sterility in the reciprocal cross (genotype 6; 10% sterile). The sterility of females in these crosses is obviously not an intrinsic property of pure-species females, which are fully fertile when tested with conspecific males (genotypes 1 and 3). The absence of offspring from these cross-inseminated females probably involves some form of postmating, prezygotic isolation, which has been seen in other closely related species in the subgroup (Price *et al*., 2001).

Likewise, some F_1 females were also sterile. Three strains of each species were used to make five crosses (Table 4, genotypes 9–13); all F_1 females were given the opportunity to mate with males of both species. There was significant heterogeneity in fertility among these crosses (Table 4, genotypes 9–13; $\chi^2 = 33{\cdot}0$, 4 d.f., $P < 0{\cdot}001$), which largely disappeared when both genotype 9 (*D. yakuba* Taï 18 females × *D. santomea* STO.4 females; 20% sterile) and genotype 13 (*D. yakuba* Cam 115 females × *D. santomea* 2001 females; 19% sterile) were removed. (The remaining genotypes, 10–12, showed only 1–8% sterility; $\chi^2 = 6{\cdot}9$, 2 d.f., $P = 0{\cdot}04$.) The sterility of F_1 females in

cross 9 was not seen in females from the reciprocal cross (genotype 10). Since these females were genetically identical, this implies the existence of a cytoplasmic effect on female fertility – a suggestion supported in analyses of backcross females described below.

We tested the fertility of backcross females in five crosses involving the strains *D. yakuba* Taï 18 and *D. santomea* STO.4, which showed female sterility in both pure-species and F_1 crosses. Backcross females (genotypes 18–22) were again heterogeneous in fertility ($\chi^2 = 118{\cdot}1$, 4 d.f., $P < 0{\cdot}0001$), a heterogeneity due entirely to the high sterility of genotypes 18 and 19. These two groups of females are genetically identical, differing only in that their fertility was tested with either pure *D. yakuba* or pure *D. santomea* males. Because their high sterility (31–33 %) does not depend on the source of sperm (Fisher's exact test, $P = 0{\cdot}87$), it is probably innate (i.e. a developmental defect in females) rather than caused by interactions between the female reproductive tract and foreign sperm. A cytoplasmic effect is further supported by the observation that backcross females of genotype 22 – genetically identical to females of the largely sterile genotypes 18 and 19 but differing in their source of cytoplasm – are nearly completely fertile. As with F_1 females, sterility is seen only when the cytoplasm of hybrids comes from *D. yakuba*. These observations imply that female sterility in hybrids involves a deleterious interaction between *D. yakuba* cytoplasm and *D. santomea* chromosomes. This supposition of course needs confirmation using crosses between other strains. It is possible that this female sterility is due to 'hybrid dysgenesis': the mobilization of transposable elements in species crosses (Kidwell, 1985). Although this phenomenon has been described *within* species, studies show no evidence that it causes interspecific hybrid sterility (e.g. Coyne, 1986; Hey, 1989).

We conclude that, although male sterility is substantial in interspecific hybrids between *D. yakuba* and *D. santomea*, there has also been some genetic divergence causing female sterility. This sterility appears only in certain combinations of strains. It is not due to *Wolbachia* infection, for none of these strains carried this parasite. This sterility is also clearly an interspecific phenomenon, for intraspecific, interstrain crosses produced completely fertile females (data not shown).

Finally, we used the attached-X strains of both *D. yakuba* and *D. santomea* to test the 'balance' hypothesis of Haldane's rule: the theory that males are sterile in species crosses because they have an X chromosome from only one species but a complete set of autosomes from both species, while hybrid females are fertile because they have a 'balanced' genotype, containing a complete haploid genome from each species. (This is, in effect, a test of whether sterility is completely caused by recessive alleles on the X chromosome of one species that are masked in heterozygous hybrids.)

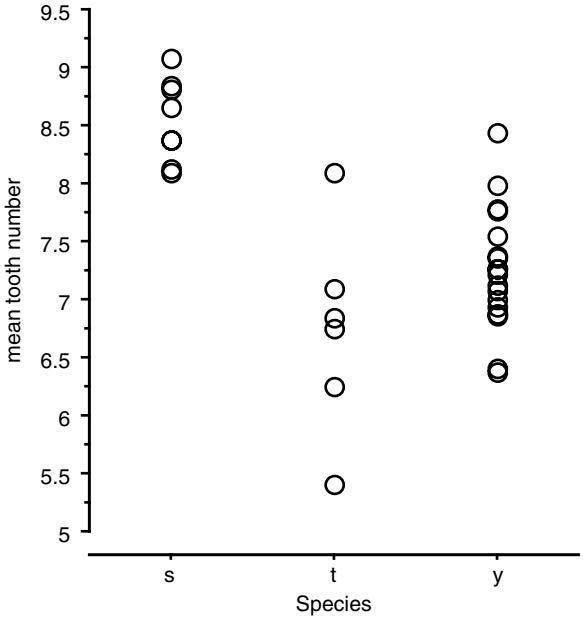

Fig. 3. Mean sex-comb tooth number among eight isofemale lines of *D. santomea* ('s'), 19 isofemale lines of *D. yakuba* ('y') and six isofemale lines of the outgroup species *D. teissieri* ('t').

Our test is straightforward, and identical to that described by Coyne (1985*b*): we simply compare the fertility of 'normal' F_1 hybrid females with that of hybrid females carrying either two X chromosomes from *D. yakuba* or two X chromosomes from *D. santomea*. If the 'imbalance' theory is correct, the last two female genotypes (identical in chromosome 'balance', autosomal complement and cytoplasm to sterile F_1 males) should express recessive alleles and thus show more sterility than 'balanced' F_1 hybrid females. The females used in this comparison were generated in the tests of a Y-effect on male sterility described above (Fig. 2, cross A). These two genotypes, however, are, like 'normal' F_1 hybrid females, completely fertile when mated to a mixture of *D. yakuba* and *D. santomea* males (Table 4; genotypes 14 vs 15 and 16 vs 17). As we note below, this result implies a faster accumulation of genes affecting the sterility of male than of female hybrids.

(iii) *Sex-comb tooth number*

Fig. 3 shows the variation in sex-comb tooth number among strains within species and between the two species. Within each species there is significant variation in sex-comb tooth number. For *D. santomea*, the range of mean tooth number per strain was 8·12–9·02, with a grand mean of 8·54 (standard errors

ranged from 0·11 to 0·19); this inter-strain variation was significant ($F_{7,392}=6{\cdot}52$, $P<0{\cdot}0001$). Among strains of *D. yakuba*, mean tooth number varied from 6·38 to 8·44, with a grand mean of 7·24; this inter-strain variation was also significant ($F_{18,931}=16{\cdot}01$, $P<0{\cdot}0001$). Nevertheless, *D. santomea* males have on average a significantly higher number of sex-comb teeth than do *D. yakuba* males: the ANOVA shows a significant effect of species origin on sex-comb tooth number ($F_{1,98}=33{\cdot}16$, $P<0{\cdot}0001$).

The mean difference between the species (1·3 bristles) is too small to allow a complete genetic analysis of chromosome effects, but we can estimate the effect of the X chromosome by comparing the tooth number of males from reciprocal interspecific crosses. In this analysis, we used two isofemale lines with bristle numbers close to the overall means of their species: *D. santomea* STO.4, with a mean of $8{\cdot}38\pm0{\cdot}13$ bristles (SE) and *D. yakuba* Taï 18 with a mean of $7{\cdot}26\pm0{\cdot}14$ ($N=50$ from each strain).

The mean tooth numbers of males from the reciprocal F_1 crosses between these strains were $X^sY^y=8{\cdot}27\pm0{\cdot}08$ and $X^yY^s=7{\cdot}88\pm0{\cdot}08$ (superscripts refer to species source of sex chromosome; standard errors are given, and $n=100$ males in each cross). The difference between these genotypes, $0{\cdot}39\pm0{\cdot}11$ bristles (SE of difference) is significant ($t_{198}=3{\cdot}43$, $P=0{\cdot}0007$). The X chromosome thus carries genes affecting this species difference. The effect of the X is roughly 35% of the mean strain difference of 1·12 bristles, or somewhat larger than the recombinational length of this chromosome relative to others (based on *D. melanogaster* we assume that X comprises roughly 20% of the genome; Lindsley & Zimm, 1992).

By looking at an outgroup species, we can infer whether the higher tooth number in *D. santomea* (compared with *D. yakuba*), represents a derived trait (i.e. whether an increase has evolved in the *D. santomea* lineage). Fig. 3 shows variation in this trait among the six lines of the outgroup species *D. teissieri* (see Materials and Methods). Twenty males, raised at an uncrowded density at 24 °C, were scored from each line. The mean tooth number among these lines ranged from 5·40 to 8·10, with an overall mean of 6·74 (standard errors ranged from 0·10 to 0·23). This mean is slightly lower than that of *D. yakuba* (7·26), and well below that of *D. santomea* (8·54). From this we can tentatively infer that the higher tooth number in *D. santomea* is a derived condition that has evolved since its common ancestor with *D. yakuba* colonized São Tomé.

(iv) *Sex ratio*

Although there have been no reports of distorted sex ratios in this interspecific cross (Lachaise *et al*., 2000; Cariou *et al*., 2001), we measured sex ratios in pure-species cultures and interspecific crosses to determine whether there may be subtle distortions of sex ratios indicating a deficit of males (Haldane's rule for viability). Crosses were made in multiple vials at low density, and offspring scored daily until all had eclosed. Table 5 gives the results of these tests. Clearly, pure-species cultures produce males and females in a ratio not differing from 1 : 1, while both interspecific crosses show a slight but statistically significant excess of females. In no set of crosses was there a significant correlation between offspring number and sex ratio among vials, nor was there a significant difference between the number of offspring produced by females of a given genotype when mated to either conspecific or heterospecific males. Thus the differences in sex ratio are not attributable to different degrees of larval crowding between crosses that affect the viability of the sexes. Rather, these sex ratios probably reflect a slightly reduced viability of hybrid males, in line with Haldane's rule.

Table 5. *Sex ratios of pure species and F_1 hybrids*

Genotype	Males	Total	Freq. male	χ^2
D. yakuba Taï 18	1028	2082	0·494	0·32
D. santomea STO.4	1052	2064	0·510	0·51
F_1 (STO.4 × Taï 18)	980	2055	0·477	4·39*
F_1 (Taï 18 × STO.4)	929	2098	0·442	27·45**

* $P<0{\cdot}05$; ** $P<0{\cdot}001$.

(v) *Wide crosses of* D. santomea *to other species*

As Cariou *et al*. (2001) report, when *D. santomea* females are crossed to males from the other eight species in the subgroup, they produce offspring only with *D. yakuba* males (fertile females and sterile males) or with *D. mauritiana* males (sterile males and females). *D. santomea* males, on the other hand, produce offspring only with *D. yakuba* females (fertile female and sterile males) and *D. simulans* females (only sterile females).

In contrast to the results of Cariou *et al*. (2001), we were (1) unable to produce offspring in a variety of crosses between *D. santomea* males and *D. simulans* females, but (2) obtained offspring from the cross of *D. santomea* females to some strains of *D. simulans* males. In this latter cross, offspring were all female (74 total). In some interspecific crosses in *Drosophila* and *Caenorhabditis*, hybrids that are genetically male can be phenotypically transformed into either females or intersexes (Sturtevant, 1946; Baird, 2002). To determine whether some offspring from the *D. santomea* × *D. simulans* cross might be sex-reversed males, we crossed *D. santomea* STO.18 females to

D. simulans males hemizygous for the dominant X-linked mutation *Beadex*. These crosses yielded 11 female offspring, all with the Beadex phenotype. The absence of wild-type females implies that none of these hybrids are actually sex-reversed males, so that Haldane's rule in this cross is truly obeyed. All offspring from this cross, however, are morphologically anomalous whether raised at 24 °C or 21 °C, with most having irregularly spaced abdominal microchaetae, rough eyes, outspread wings, abnormally chitinized abdomens, and missing dorsocentral and scutellar bristles. Virtually no such anomalies were seen in F_1 hybrids between *D. santomea* and *D. yakuba*. Coyne (1985*a*) reported similar anomalies in hybrids between *D. melanogaster* and *D. simulans*, which diverged around 3 million years ago (Hey & Kliman, 1993), but not in hybrids between *D. simulans* and *D. mauritiana*, which diverged around 260 000 years ago (Kliman *et al*., 2000).

4. Discussion

We begin with two caveats. First, except for the study of sex combs, our work involves a limited number of strains – sometimes only one pair – from each species. Thus some of our conclusions must be seen as tentative, particularly those involving sex ratio distortion and female sterility. Hybrid female sterility, for example, appears only when some strains are used, and further work is needed to determine the ubiquity of this phenomenon. Second, some of our results may be compromised if there is extensive introgression between the species. For example, a genomic region that has no effect on hybrid male sterility may actually contain *identical* genome due to recent hybridization. We are resolving this question with quantitative trait locus (QTL) mapping of sterility and other aspects of reproductive isolation using a high density of molecular markers.

Our analysis of genetic and morphological divergence between the *D. yakuba* and *D. santomea* shows several features in common with the evolutionary divergence between the mainland species *D. simulans* and its two sister species *D. sechellia* and *D. mauritiana*. First, all three divergences are of similar ages: 393 000, 413 000 and 263 000 years respectively (Kliman *et al*., 2000; Llopart *et. al*., 2002). Although the branching order of the three species in the clade of the *D. simulans*, *D. mauritiana* and *D. sechellia* triad has not been resolved, biogeography suggests that the two island species are derived from independent island colonizations by the mainland ancestor of *D. simulans*. Thus we are probably dealing with three independent speciation events occurring on different islands at about the same time.

The genetic and phenotypic sequelae of these colonizations show several parallels. In all three sets of crosses between mainland and island species, male hybrids are sterile and females are fertile, and the male sterility appears to be primarily associated with both the X chromosome (a ubiquitous observation among closely related species of *Drosophila*; Coyne & Orr, 1989*a*, 2004) and the Y chromosome (Johnson *et al*., 1993). As Coyne & Orr (2004) note, whether or not the Y carries genes causing hybrid sterility varies among other species of *Drosophila* in the *melanogaster* group as well as in more distantly related groups. In all three pairs of species, 'unbalanced' F_1 females having both X chromosomes from a single species are perfectly fertile despite having a genotype similar to that of sterile F_1 males (Coyne, 1985*b*). In addition, all three pairs show 'cryptic' sterility of either hybrid females or pure-species females mated to heterospecific males. This can involve either intrinsic sterility of females (Hollocher & Wu, 1996; this study), or postmating prezygotic isolation caused by poor storage of sperm, by inefficient use of sperm or by 'conspecific sperm precedence', the inability of heterospecific sperm to compete with conspecific sperm when a female is doubly fertilized (Price, 1997; Price *et al*., 2001; Chang, 2004).

Moreover, all three cases show fairly strong sexual isolation between the island species and its mainland relative – isolation that is asymmetrical, being stronger between males of the mainland species and females of the island species (Coyne, 1989, 1992; Coyne *et al*., 2002). These observations violate the theory of Kaneshiro (1980) that isolation will be stronger between mainland females and island males than vice versa. Finally, all the island species have more sex-comb teeth than do their mainland relatives (Coyne, 1985*a*; Macdonald & Goldstein, 1999), an increase that appears in each case to be a derived condition.

These parallels may mean that both the phenotypic and genetic trajectories of speciation is repeatable in the *D. melanogaster* subgroup, although male-limited sterility caused largely by the X chromosome is ubiquitous not only in *Drosophila* but in a wide variety of animals (Laurie, 1997).

(i) *Hybrid male sterility*

Although there are several possible explanations for male-limited sterility in species crosses, two are most plausible: the 'dominance theory', which posits that genes causing hybrid sterility and inviability are recessive in hybrids, and the 'faster-male' theory, which posits that genes ultimately causing hybrid male sterility diverge faster than those causing hybrid female sterility, perhaps through sexual selection. Both of these theories are supported by substantial evidence (reviewed in Coyne & Orr, 2004).

Either of these theories can explain both the large X and Y effects seen in our analysis compared with the

effects of the two autosomal segments analysed, which had no discernible effects on fertility. Alternatively, the X effects (but not male-limited sterility) could reflect a higher density of 'sterility genes' on the X chromosomes than on the autosomes, as seen in the *D. simulans*/*D. mauritiana* hybridization (Tao *et al*., 2003). However, the complete fertility of F_1 hybrid females having two X chromosomes from *D. yakuba* or *D. santomea* (the genetic equivalent of sterile F_1 males) implies that recessivity of sterility genes cannot be the entire story. The fertility of these 'unbalanced' females, which should show the effects of any sex-linked recessive alleles that cause hybrid sterility, implies that sterility genes have evolved faster in males than in females.

Including the present study, the unbalanced-female test has now been performed in six evolutionarily independent hybridizations in *Drosophila*, and in all cases these females remain fertile (Coyne, 1985*b*; Orr, 1987, 1989; Orr & Coyne, 1989). This consistency provides strong evidence for the 'faster-male' theory, although the dominance theory, for which there is copious independent evidence (Coyne & Orr, 2004), is almost certainly responsible for the large effects of X and Y chromosomes on the sterility of backcross males. An alternative theory, the 'faster-X' hypothesis, posits that genes causing hybrid sterility evolve faster on the X chromosome than on the autosomes (Charlesworth *et al*., 1987). Although the unbalanced X experiment, which does not test the effects of autosomes, cannot rule out the faster-X theory, this latter hypothesis alone cannot explain the faster evolution of sterility of the heterogametic sex, as reflected in Haldane's rule (Orr, 1997). Nevertheless, the faster-X theory, the dominance theory and a higher concentration of sterility genes may singly or in combination explain the large X-chromosome effect seen in our analysis.

The genetical analysis of sterility in males (the sex contributing more to postzygotic isolation between these species) makes several predictions about what segments of the genome are most likely to introgress between the species in the hybrid zone, predictions that we are testing through DNA-marker analysis of flies from São Tomé. The absence of a cytoplasmic effect on male fertility implies that the mitochondria of a female carry no genes affecting the fertility of her hybrid offspring, and thus mitochondrial (mt) DNA should move fairly readily between the species. If the asymmetry of sexual isolation measured in the laboratory also holds in nature, wild F_1 hybrid females (the vehicle for introgression) are far more likely to be produced by crosses between *D. yakuba* females and *D. santomea* males than vice versa. Given that F_1 hybrid females cross as readily to *D. yakuba* as to *D. santomea* males (Coyne *et al*., 2002), we thus predict that *D. yakuba* mtDNA will introgress more readily into *D. santomea* genome than vice versa. (This asymmetry should hold despite the deleterious interactions between *D. yakuba* cytoplasm and *D. santomea* genome causing female sterility, which is weaker and strain-specific.) For similar reasons, we would expect that autosomal regions closely linked to the *notch* and *sepia* loci would introgress readily from *D. santomea* into *D. yakuba*. We cannot predict the pattern of introgression in the opposite direction since we did not analyse sterility in backcrosses to *D. santomea*. However, given that F_1 hybrid females produce more-sterile backcross males when mated to *D. santomea* than to *D. yakuba* males (Table 1), we expect that, all else being equal, introgression of genes will occur more readily from *D. santomea* into *D. yakuba* than vice versa. The exception will be the mitochondria (for reasons noted above) and the Y chromosome, for the *D. santomea* Y shows more incompatibility with the *D. yakuba* genome than does the *D. yakuba* Y with the *D. santomea* genome. (Of course, the reciprocal sterility makes introgression of the Y chromosome unlikely in either direction.)

Finally, we would predict less introgression of X-linked segments than of autosomal segments between the species because – except for the small 6 cM region linked to *yellow* at the base of the X chromosome – X-linked segments tested have substantial hemizygous effects on the sterility of males backcrossed to *D. yakuba* (we expect that this will also be true in backcrosses to *D. santomea*), while there is no discernible effect of heterozygosity for the two large autosomal segments tested. Several studies of hybrid zones in mice, butterflies and birds have shown that X chromosomes introgress less readily than do autosomes across these zones (Coyne & Orr, 2004).

(ii) *Hybrid female sterility*

Although it is clear that in *Drosophila* the evolution of male sterility precedes that of female sterility (Coyne & Orr, 1989*a*), the degree of female sterility may be underestimated. It is relatively easy to detect male sterility: typically F_1 hybrids are intercrossed, their failure to produce offspring indicates that one or both sexes are completely sterile, and further work shows that sterility is often confined to males. Female fertility, however, is more difficult to measure. As might often occur under Haldane's rule, males could be completely sterile but females only *partially* fertile (either producing relatively few offspring or with only a fraction being completely sterile). In such a case females might be simply scored as 'fertile'. Determining the presence of partial female sterility thus requires the isolation of individuals, determining whether they produce offspring and, if so, whether they produce as many offspring as pure species.

We tested only the ability of females to produce pure-species offspring when crossed to males of either species, and found that some hybrid females from both F_1s and backcrosses are sterile. Because this sterility is independent of the species of male that contributes sperm, it almost certainly involves an inherent inability to produce offspring rather than an incompatibility between sperm and a female's reproductive tract. The latter phenomenon, however, may be responsible for the lack of offspring in some *D. yakuba* females mated to *D. santomea* males.

Finally, the incipient female sterility that we observed was strain-specific: a substantial proportion of fertilized females failed to produce offspring in some but not all interspecific crosses. This was true for pure species (females inseminated by heterospecific males), F_1 females and backcross females. Variation among strains in the degree of hybrid sterility and inviability in interspecific crosses is not a novel phenomenon: it has been seen in other species of *Drosophila*, for example, by Crow (1942) and Patterson & Stone (1952, chapter 10). Although we did not perform a genetic analysis of female sterility, backcross data with a single pair of strains suggest that this sterility is caused at least partly by deleterious interactions between *D. yakuba* cytoplasm and *D. santomea* chromosomes.

(iii) *Sex-comb tooth number*

As with *D. sechellia* and *D. mauritiana*, *D. santomea* has a higher number of sex-comb teeth than its closest mainland relative. *D. santomea* has an average of 8·5 teeth per male, compared with a mean of 7·2 in *D. yakuba*. In the *D. simulans* subgroup, *D. simulans* has an average of around 10·2 teeth, *D. mauritiana* 13·5 and *D. sechellia* 11·7 (Coyne, 1985*a*; Macdonald & Goldstein, 1999). The higher tooth numbers in *D. sechellia* and *D. mauritiana* probably results from two evolutionary increases in the island lineages rather than a decrease in *D. simulans*, since the outgroup species *D. melanogaster* has a mean tooth number of about 10·1 (Coyne, 1985*a*). Likewise, the higher number in *D. santomea* as compared with *D. yakuba* also appears to be a derived condition. But we have no idea of the adaptive significance, if any, of increases in tooth number, or why colonization of an island might promote them.

There appears to be little consistency in the genetic basis of differences in sex-comb tooth number among species. In *D. santomea*/*yakuba*, the effect of the X chromosome is roughly 35% of the total species difference, with the autosomes showing the remainder of the effects. In contrast, in *D. mauritiana*/*simulans* the X chromosome carries genes causing only 5% of the species difference, with genes on the third chromosome having the largest effect (Coyne, 1985*a*; True *et al*., 1997). In *D. sechellia*/*simulans*, the X chromosome carries genes that affect tooth number in the direction *opposite* to that expected (i.e. the *D. sechellia* X reduces tooth number), while the second chromosome has the largest effect and the third no effect (Macdonald & Goldstein, 1999). There is thus no indication that genes involved in the evolution of this male-limited trait are disproportionately concentrated on the X chromosome. This is true of other secondary sexual traits in *Drosophila* (Coyne & Orr, 1989; Zeng *et al*., 2000), in contrast to male sterility genes, which in the single well-documented case are more concentrated on the X (Tao *et al*., 2003).

(iv) *Developmental anomalies in wide crosses*

Finally, wide crosses between *D. santomea* and its more distant relatives show the appearance of complete male inviability as well as morphological anomalies. The latter, which involve abnormalities in traits that are identical among these species (bristle number, appearance of eyes and abdominal tergites, and so on) imply that either the genetic basis of morphological similarities has changed since these species diverged, or that some other genetic interaction in hybrids 'poisons' pathways leading to the formation of normal traits. In contrast to the divergence of reproductive systems that causes male sterility, which may evolve within a few hundred thousand years (perhaps due to rapidly acting sexual selection), the divergence of developmental pathways that produce morphological anomalies and hybrid inviability appears to require several million years in *Drosophila*.

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