# Haldane's Rule Is Linked to Extraordinary Sex Ratios and Sperm Length in Stalk-Eyed Flies

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**ABSTRACT** We use three allopatric populations of the stalk-eyed fly *Teleopsis dalmanni* from Southeast Asia to test two predictions made by the sex chromosome drive hypothesis for Haldane's rule. The first is that modifiers that suppress or enhance drive should evolve rapidly and independently in isolated populations. The second is that drive loci or modifiers should also cause sterility in hybrid males. We tested these predictions by assaying the fertility of 2066 males derived from backcross experiments involving two pairs of populations and found that the proportion of mated males that fail to produce any offspring ranged from 38 to 60% among crosses with some males producing strongly female-biased or male-biased sex ratios. After genotyping each male at 25–28 genetic markers we found quantitative trait loci (QTL) that jointly influence male sterility, sperm length, and biased progeny sex ratios in each pair of populations, but almost no shared QTL between population crosses. We also discovered that the extant $X^{SR}$ chromosome has no effect on sex ratio or sterility in these backcross males. Whether shared QTL are caused by linkage or pleiotropy requires additional study. Nevertheless, these results indicate the presence of a "cryptic" drive system that is currently masked by suppressing elements that are associated with sterility and sperm length within but not between populations and, therefore, must have evolved since the populations became isolated, *i.e.*, in <100,000 years. We discuss how genes that influence sperm length may contribute to hybrid sterility.

A major challenge to those who study speciation is to determine the causes of early reproductive isolation between incipient species. One avenue for gaining insight into this issue is to investigate the causes of Haldane's rule, *i.e.*, the observation that the heterogametic sex of hybrid offspring are more likely to be sterile or inviable than the homogametic sex (Haldane 1922). This phenomenon has been observed in a wide range of animal taxa and is believed to be a nearly ubiquitous phase of early speciation (Orr 1997). Given that alleles for sterility or inviability are expected to be selected against within populations, models for the evolution of hybrid dysfunction typically assume two or more genes, each of which is neutral or advantageous within a population but have deleterious joint effects when mismatched between populations. Two nonmutually exclusive explanations (Turelli 1998) have been proposed for why these epistatic interactions, known as Dobzhansky–Muller incompatibilities (Dobzhansky 1937; Muller 1940, 1942; Orr 1995), should arise more quickly in the heterogametic sex.

The dominance hypothesis (Muller 1942; Orr 1993; Turelli and Orr 1995) assumes that if genes causing hybrid dysfunction are recessive, then the heterogametic sex will be affected more than the homogametic sex. This result is expected because the degenerate (or missing, in XO taxa) sex chromosome possessed by the heterogametic sex fails to mask recessive alleles present on its homolog. This hypothesis potentially applies to all taxa with a degenerate sex chromosome, whether male or female heterogametic. The faster-male hypothesis (Wu and Davis 1993; Wu *et al.* 1996) states that male-specific sterility factors accumulate faster than female-specific factors, because sexual selection causes more rapid change in loci with male-specific reproductive function. This hypothesis applies to male-heterogametic taxa or taxa where the chromosome that determines maleness is not degenerate (as in *Aedes* mosquitoes, Presgraves and Orr 1998).


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In 1991 two publications independently proposed a third hypothesis to explain incompatibilities in heterogametic hybrids: divergence of sex chromosome meiotic drive systems (Frank 1991; Hurst and Pomiankowski 1991). Sex chromosome meiotic drive refers to non-Mendelian passage of the X (or Y) chromosome into gametes and causes biased progeny sex ratios. Drive is expected to be more common on sex chromosomes than autosomes because the nonrecombining portions of the X and Y each act as a cooperative unit with regard to biased transmission (Dawkins 1982). Consequently, two-locus drive systems that require linkage between target and responder loci to prevent self-destruction can more easily evolve on the sex chromosomes (Hurst and Pomiankowski 1991). Because population sex-ratio bias generates strong selection to restore sex-ratio equality, suppressors elsewhere in the genome are expected to evolve rapidly (Leigh 1971; Hartl 1975). Thus, the drive hypothesis assumes that hybrid incompatibilities arise as a result of coevolution between drive and modifiers of drive that alternately suppress and enhance it within populations over time.

Unlike the faster-male theory, the drive hypothesis can apply to either male- or female-heterogametic taxa (Tao and Hartl 2003) because there are two different mechanisms for producing meiotic drive. In one type, loci attack their counterparts on homologous chromosomes in male gametes and kill the sperm in which they are carried, which could lead to Haldane's rule in male-heterogametic species. In the other type, centromeres compete to reach the ovum during meiosis in females instead of being relegated to a polar body (Novitski 1951; Henikoff and Malik 2002). Covariation in satellite sequence between the centromere and centromeric histones is consistent with an arms race to ensure successful spindle attachment (Malik and Henikoff 2001; Malik *et al.* 2002) and differences in centromeric and telomeric sequences between populations can disrupt meiosis in hybrid males and cause sterility (McKee *et al.* 1998; Henikoff *et al.* 2001). The drive hypothesis could, therefore, potentially apply to diverse taxa.

The drive hypothesis for Haldane's rule was largely dismissed at first (Coyne *et al.* 1991; Johnson and Wu 1992; Coyne and Orr 1993) but several lines of evidence suggest that the evolutionary arms race created by drive can cause hybrid sterility (Johnson 2010; McDermott and Noor 2010). First, multiple studies on *Drosophila* have revealed "cryptic" drive in which hybrids between populations exhibit distorted sex ratios even though distortion is absent in the source population (Mercot *et al.* 1995; Dermitizakis *et al.* 2000; Tao *et al.* 2001; Yang *et al.* 2004). Second, loci involved in drive systems can evolve rapidly (Palopoli and Wu 1996; Capillon and Atlan 1999; Derome *et al.* 2004; Presgraves *et al.* 2009; Bastide *et al.* 2011) and diverge between populations (Carvalho *et al.* 1997). Third, hybrid sterility factors have been linked directly to sex chromosome drive in the mouse and in at least three cases involving *Drosophila*. In *Mus musculus* multicopy gene regions on the X, *Slx/Slxl1*, and the Y, *Sly*, chromosomes have antagonistic effects during sperm differentiation that influence sterility and cause biased sex ratios in hybrids (Cocquet *et al.* 2012). In *Drosophila subobscura*, males that carry an X chromosome with an SR inversion from Tunis but autosomes from another population are sterile while males with the reciprocal arrangement are fertile (Hauschteck-Jungen 1990). Introgression of *D. mauritiana* third chromosome segments into *D. simulans* revealed that an autosomal suppressor of X drive, *Tmy*, also causes sterility in the presence of a modifier, *broadie* (Tao *et al.* 2001). Further, several studies have found genetic associations between sterility and drive in *D. pseudoobscura*. Crosses between *D. pseudoobscura bogatana* and *D. p. pseudoobscura* (Orr and Irving 2001, 2005; Phadnis and Orr 2009) have identified a gene of major effect, *Overdrive*, that affects both drive and sterility with additional X and autosomal genes exhibiting epistatic effects (Phadnis 2011). Likewise, crosses between an SR strain of *D. persimilis* and *D. pseudoobscura* found a QTL for X drive that overlaps with a QTL for sterility (Wu and Beckenbach 1983; McDermott and Noor 2012). Thus, meiotic drive loci can directly affect sterility, but the mechanism by which this occurs remains unclear. To date, examples supporting the drive hypothesis in flies have been reported only in *Drosophila*, so the degree to which this pattern extends to other species is unknown.

The stalk-eyed fly genus *Teleopsis* presents an excellent system in which to investigate sex chromosome meiotic drive and its effect on hybrid dysfunction. Experimental crosses between flies from isolated populations of *Teleopsis dalmanni* and *T. whitei* in southeast Asia have revealed evidence for rapid evolution of male hybrid sterility (Christianson *et al.* 2005) and asymmetric gametic isolation (Rose *et al.* 2014). These populations are also polymorphic for X chromosome meiotic drive in which carrier (or *sex ratio*, SR) males produce predominantly (>90%) female progeny (Presgraves *et al.* 1997) and occur at frequencies of 10–30% (Wilkinson *et al.* 2003; Cotton *et al.* 2014). Recombination is rare or absent between $X^{SR}$ and $X^{ST}$ chromosomes (Johns *et al.* 2005), indicating that the extant drive system is in a paracentric inversion that involves most of the X chromosome (Christianson *et al.* 2011). As a consequence, many X-linked genes have diverged dramatically between $X^{SR}$ and $X^{ST}$ chromosomes with >400 containing fixed differences and 400 showing differential expression (Reinhardt *et al.* 2014). Most genes on the X in *Teleopsis* are on Muller element B, *i.e.*, chromosome 2L in *D. melanogaster* (Baker and Wilkinson 2010), rather than the putative ancestral X–Muller element F (Vicoso and Bachtrog 2013), indicating that the sex chromosomes are independently derived in diopsids. Genes on the X influence sperm length (Johns and Wilkinson 2007), female sperm storage organ size (Wilkinson *et al.* 2005), and eyestalk length (Wolfenbarger and Wilkinson 2001), with $X^{SR}$ and $X^{ST}$ males differing in eyespan in the lab (Wilkinson *et al.* 1998; Johns *et al.* 2005) and field (Cotton *et al.* 2014). Consequently, drive-associated genes could influence hybrid fitness in multiple ways.

Given the presence of extant $X^{SR}$ chromosomes in some *Teleopsis* populations (Wilkinson *et al.* 2003; Christianson *et al.* 2011) and the expectation that an arms race between drivers and suppressors should be ongoing, we postulated that X chromosome drive and sterility could be genetically associated in at least three ways. First, sterile hybrid males might be produced if a female carrying an extant $X^{SR}$ chromosome from one population mated with a male from a different population carrying divergent alleles at loci that interact with the foreign $X^{SR}$ chromosome to cause sterility (*cf*. McDermott and Noor 2012). In this case, sterile males would be expected to carry X-linked drive alleles. Second, cryptic drive loci could become expressed when placed in a foreign genetic background lacking appropriate suppressors and produce either biased sex ratios or sterility or both in hybrids. This scenario would result in X-linked factors that influence sex ratio or sterility in the absence of an extant $X^{SR}$ chromosome. Finally, loci that suppress drive within a population could negatively affect fertility when placed in a foreign genetic background. In this case, variation in sex ratio and sterility should map to the same autosomal or Y-linked location, depending on cross direction and the type of genetic interaction causing sterility. Autosomal and Y-linked suppressors have been detected in many drive systems (Jaenike 2001; Montchamp-Moreau *et al.* 2001) and, as noted above, have been linked to sterility in *D. simulans* (Tao *et al.* 2001) and *D. pseudoobscura* (Phadnis 2011).

To determine which, if any, of these alternatives is occurring, we selected three allopatric monophyletic populations of *T. dalmanni* that exhibit male hybrid sterility but little prezygotic isolation (Christianson *et al.* 2005; Rose *et al.* 2014) and conducted crosses between one of them, Gombak (G), and the other two, Bukit Lawang (B) or Soraya (S). Because G became isolated from B or S ~500,000 years ago, while B diverged from S <100,000 years ago (Swallow *et al.* 2005), similar outcomes in both sets of crosses would indicate shared, and therefore relatively old, genetic factors that must have been present in the lineage prior to separation of B and S, while differences between the crosses provide evidence of recent evolutionary change that must have occurred within the past 100,000 years. To locate genetic factors responsible for drive and sterility we conducted backcrosses using flies from each pair of populations and scored sterility and length of mature sperm bundles from either first- or second-generation male progeny. For males that produced 10 or more offspring we also scored sex ratio. We then genotyped parents and progeny for a set of genetic markers that span all three pairs of chromosomes in the genome and used QTL mapping to detect genomic regions affecting male sterility, brood sex ratio, and sperm length. The results reveal extraordinary variation in sex ratio due to cryptic drive, male sterility caused by both sperm absence and abnormal sperm length, and QTL with joint effects on sterility, sex ratio, and sperm length predominantly within, rather than between, each cross.

## Materials and Methods

### *Populations and parental lines*

The *Teleopsis dalmanni* (synonymized with *Cyrtodiopsis*, Meier and Baker 2002) in this study descend from flies that were collected by hand net along small streams flowing into the Gombak River in peninsular Malaysia (3°12'N, 101° 42'E) or two sites in Sumatra (Bukit Lawang—3°35'N, 98° 6'E and Soraya—2°52'N, 97°54'E) in 1999 or 2000 (Swallow *et al.* 2005) and have subsequently been maintained in large Plexiglass cages at 25°, 70% humidity, and 12 hr L:D with 100 or more individuals. Hybrid females between these populations are fertile while most hybrid males are sterile (Christianson *et al.* 2005) and hybrid inviability is low (13.6 ± 7.0%, Rose *et al.* 2014). However, reciprocal crosses among these three populations exhibit reduced (34.8 ± 8.3%) egg-hatching success, *i.e.*, partial prezygotic incompatibility, much of which appears to be caused by sperm failing to move successfully through the reproductive tracts of females to achieve fertilization (Rose *et al.* 2014). For comparison, egg-hatching success was 67.4 ± 4.0% within these populations. Nevertheless, nearly all (99.0 ± 0.1%) mated females laid some eggs that hatched in crosses within or between these three populations, indicating that complete male sterility is rare.

Larvae were reared in cups containing 25–50 ml of pureed corn and kept in incubators at 25° with a 12 hr L:D cycle. Cups containing pupae were kept for at least 1 week after eclosion ceased to ensure that progeny counts were complete. Eclosed flies were separated into single-sex cages prior to sexual maturity, which occurs at 22 days for females and 25 days for males (Baker *et al.* 2003), and kept for at least 4 weeks before using them in crosses. Adult flies were fed pureed corn in disposable cups twice a week.

To facilitate linkage mapping we created inbred lines from the S and G populations. We created three inbred S lines by pair-mating full-sibs for 10 generations. Similarly, we established two inbred G lines by pair-mating full-sibs for 7 generations but starting with flies taken from a control line that had been maintained with $N_e \leq 50$ (Wilkinson 1993) for 50 prior generations without any known $X^{SR}$ chromosomes (Wilkinson *et al.* 2005, 2006). We then chose one line from each population (S3 and G2) that had high productivity and informative marker loci (see below) for mapping. For the other set of crosses, we used flies from the G2 line and the outbred B population, which had been in culture for 10 years (~40 generations) at the start of the experiment.

### *Mapping crosses*

Because F1 males derived from crosses between G and either S or B flies are sterile (Christianson *et al.* 2005), we conducted two sets of reciprocal backcrosses, *i.e.*, mated hybrid females to either possible parental male, to produce male offspring with all possible combinations of autosomal and X-linked genotypes. For crosses using the B and G

populations both possible hybrid females, *i.e.*, GB females produced by mating a G male to a B female and BG females from a B male mated to a G female, were used. Then to create males segregating for sterility, each type of hybrid female was backcrossed to either G (these crosses are labeled G-BC, Figure 1A) or B (labeled B-BC, Figure 1B) males (see Supporting Information, Table S1 for sample sizes of each replicate family). In these crosses we used reciprocal hybrid females so that any maternal effects, such as mitochondrial–nuclear interactions, could be identified and controlled. All parental males used in the B-G crosses produced unbiased sex ratios, *i.e.*, did not carry an active $X^{SR}$ chromosome.

To determine if an extant $X^{SR}$ chromosome causes male sterility in the presence of autosomal or Y-linked alleles from another population, we initiated crosses between the S and G populations using G males that either contained a $X^{SR}$ or a $X^{ST}$ chromosome. Thus, two types of hybrid females were also created for this cross, GS females derived from crossing a G male with an S female and $G^{SR}S$ females derived by crossing a noninbred Gombak $X^{SR}$ male with an S female. $X^{SR}$ males were identified by production of a highly female-biased sex ratio and presence of a previously identified diagnostic microsatellite haplotype (Wilkinson *et al.* 2006; Christianson *et al.* 2011). The GS and $G^{SR}S$ females were then backcrossed to males from either the G or S inbred lines, creating four sets of backcross progeny. We tested the fertility of 60 male offspring sampled from these four crosses and found only two males that produced pupae after mating three virgin females for 2 weeks. Therefore, to increase the frequency of fertile males for mapping, we conducted a second generation of backcrossing by mating female progeny from each of the four backcrosses to a male from either the G (labeled as G-BC2, Figure 1C) or S (labeled S-BC2, Figure 1D) inbred line. Thus, the G-BC2 crosses included G males mated to $G(G^{SR}S)$ females and G males mated to G(GS) females while the S-BC2 crosses included S males mated to $S(G^{SR}S)$ females and S males mated to S(GS) females. Each of these four crosses was replicated three times to obtain sufficient male progeny for phenotype mapping (see Table S1 for sample sizes of each replicate family). To distinguish male progeny from the two BC2 crosses involving, for example, a G male, below we use the following nomenclature: $G \times G(G^{SR}S)$ or $G \times G(GS)$.

### Phenotype and genotype data

***Sterility and sex ratio:*** We scored sterility and brood sex ratios for males produced in each cross by housing each male with three virgin females from the G population and collecting food cups for at least 2 weeks. We counted number of eclosed male and female flies and used absence of any pupae to indicate sterility. We also calculated the $\log_{10}$(total number of progeny + 1) to obtain a continuous measure of male fertility. We used proportion of males in the brood to quantify sex ratio for males that produced 10 or more offspring. We classified males as having a biased sex ratio if the probability associated with a chi-square goodness-of-fit test, using 50% male for the expected value, was less than a threshold set by applying a Benjamini–Hochberg (Benjamini and Hochberg 1995) false discovery rate (FDR) of 10% to adjust for multiple testing. We chose 10% because it is more stringent, *i.e.*, adjusted alpha ranged from 0.008 to 0.015 for the B-G cross and 0.034 to 0.048 in the S-G cross, but keeps the unbiased sex-ratio category centered on 0.5. Adopting a more stringent test would have the undesired effect of shifting the average sex ratio of the unbiased category away from 0.5 for some of the crosses, as well as for outbred flies from each population (see Figure S1).

***Sperm length:*** After breeding, male abdomens were opened and if testes were present, one was placed in a drop of phosphate-buffered solution (PBS, pH 7.4) on a microscope slide. The testis was then teased apart to separate and spread sperm bundles over the slide. Slides were air dried, fixed with 3:1 methanol/acetic acid for 4 min, rinsed with PBS, stained with Hoechst 33258 for 1 min, and then rinsed again in PBS (Sakaluk and O'Day 1984). After affixing a cover slip, slides were examined under UV illumination with a Nikon E600 microscope at 400× magnification. We used an Insight 4MP camera and the program Spot to take digital images of four mature sperm bundles that were beginning to separate. The length of each bundle was then measured using the segmented line tool in the program ImageJ (Schneider *et al.* 2012) to the nearest micrometer and the average length of the four bundles was recorded.

***Genotypes:*** After testes removal we pulverized each male in a 1.5-ml tube and extracted DNA using Qiagen DNEasy kits (Valencia, CA). Genotype data were generated using primers designed for *T. dalmanni* microsatellites (Wright *et al.* 2004) or genes containing glutamine repeats (Birge *et al.* 2010). Initially, parents were genotyped to identify loci that exhibited variation that could be associated with each parental population and spanned each chromosome. For the B-G crosses, 38 loci were screened and 25 loci were typed while for the S-G crosses, 35 loci were screened and 28 loci were typed. Even though fewer loci were used in the B-G crosses, the loci spanned more of chromosome 2 than in the S-G cross. We amplified loci in 10 μl PCR reactions on a thermal cycler (MJ Research). Each 10 μl reaction contained 1 μl of 10× PCR buffer (200 mM Tris-HCl, 500 mM KCl), 0.5 μl 50 mM $MgCl_2$, 0.25 μl 8 mM mixed dNTPs, 0.05 μl 5 units/μl recombinant Taq DNA polymerase (all of the above Invitrogen, Carlsbad, CA), 0.5 μl each of 10 μM forward (labeled with 6-FAM, NED, or HEX) and reverse primers, and the remainder sterile, deionized water. PCR was started at 94° for 2 min. followed by 32 cycles of 94° for 30 sec, 52° 30 sec, 72° for 30 sec, and terminated with 72° for 7 min. When possible, we amplified three primers in multiplex reactions. Reagent concentrations in multiplex reactions were identical to single-primer reactions with 0.5 μl of each forward and reverse primer, and the quantity of water was adjusted to maintain a reaction volume of 10 μl. We sized

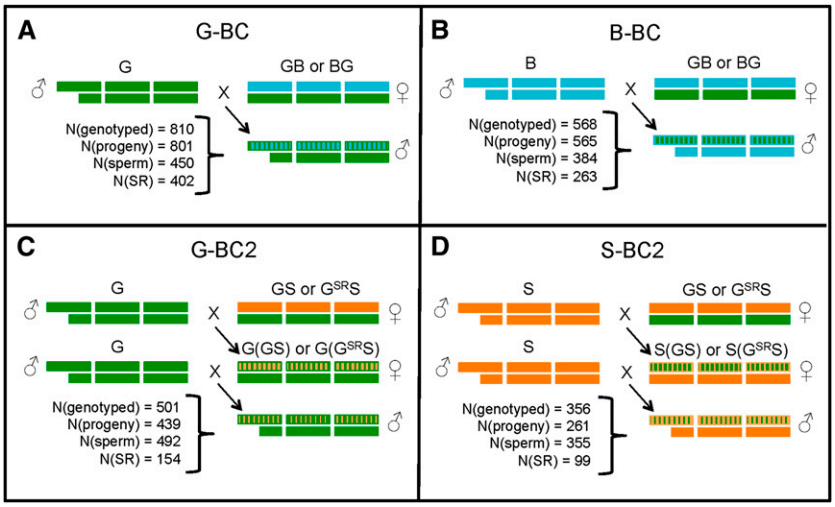


**Figure 1** Crossing scheme to map sterility, sex ratio, and sperm length using two pairs of isolated *T. dalmanni* populations. First pair of bars denote sex chromosomes with short bars indicating a Y chromosome, remaining bars are autosomes, and bar color indicates population origin: Gombak (G), green; Bukit Lawang (B), blue; and Soraya (S), orange, with hatching indicating expected recombinant chromosomes. (A) Backcross to G male (G-BC) using a hybrid female derived from a G male mating a B female (GB) or a B male mating a G female (BG). Number of male progeny scored for genotype, progeny number, sex ratio, and sperm length is listed. (B) Backcross to B male (B-BC) using hybrid females as in A. (C) Second-generation backcross to G male (G-BC2) using either a hybrid female created by mating a G male with a nondrive X chromosome to an S female (GS) or a G male carrying an XSR chromosome (GSRS). (D) Second-generation backcross to S male (S-BC2) using hybrid females as in C. See Table S1 for numbers of flies tested per cross and family.

amplified products using either an ABI 3100 or 3730 genetic analyzer and either Genotyper v. 2.5 or Genescan v. 4.0 (Applied Biosystems, Foster City, CA).

### *Linkage mapping and QTL analysis*

We constructed linkage maps for each chromosome in both sets of crosses. We used parental genotypes for each family to determine the population of origin for each microsatellite allele and then coded male progeny genotypes within each family accordingly. In cases where data from parents were missing we determined allele origin from segregation patterns of linked loci using information from previous mapping studies on *T. dalmanni* (Johns *et al.* 2005; Baker and Wilkinson 2010). Inbreeding was sufficient to create population-specific alleles at many loci; however, it did not eliminate heterozygosity within populations or sharing of alleles between populations. Flies with alleles of ambiguous origin were coded as missing data for that locus in that family. In the relevant S-G crosses the fate of the $X^{SR}$ chromosome was determined, which was possible because most X-linked loci had unique drive-associated alleles (*cf.* Christianson *et al.* 2011).

Linkage maps were first constructed separately for each cross using the multipoint maximum-likelihood algorithm and Haldane mapping function in JoinMap 4.0 (Van Ooijen 2006). Because the loci followed the same order in the reciprocal crosses for each population pair, we used JoinMap 4.0 to estimate a single set of map distances for the two autosomes for each population pair. To determine map distances among the X-linked loci we used segregation expectations for a doubled haploid population and included all X-linked genotypes for each population pair because in every cross X-linked alleles are haploid in males and expected to segregate 1:1.

We then used MapQTL 6.0 (Van Ooijen 2009) to locate chromosomal regions affecting sterility, sex ratio, and sperm length using a mixed-model maximum-likelihood estimation procedure. For each cross, we used a common linkage map (see Figure S3) and performed interval mapping on each of the three chromosomes jointly. We determined significance thresholds using 1000 permutations (Churchill and Doerge 1994) and identified putative QTL at LOD score maxima that exceeded a genome-wide significance threshold corresponding to a probability of 0.05. When interval mapping suggesting a significant association was present on a chromosome, we selected one or more markers with significant LOD scores to set as cofactors and then performed multiple QTL mapping (MQM, Jansen 1993; Jansen and Stam 1994) to identify distinct chromosomal regions that explained the greatest amount of phenotypic variation. Coding sterility as a continuous trait, *i.e.*, $\log_{10}(\text{progeny} + 1)$, produced similar QTL maps, so we report only results for the bivariate trait (*i.e.*, presence/absence of pupae). Experimental replicate was also included as a cofactor in all analyses.

To identify regions containing QTL for each trait, we plot the LOD scores from both reciprocal backcrosses for each population pair. This procedure makes it possible to identify QTL influenced by loci with dominant allelic effects, which would otherwise be detectable only in the backcross direction where the father has a recessive allele, as well as additive effects, which should be detected in either cross direction. To illustrate the magnitude and direction of allelic effects, we plot the mean phenotype for each of the four possible genotypes at the marker nearest the maximum LOD score for the QTL that explained the greatest amount of variation on each chromosome, if one was present, for each trait.

To determine if QTL for the three phenotypic traits occur at independent locations in the genome, we conducted randomization tests in which we counted the number of times significant QTL occurred at the same genomic location for both traits given the number of markers in the map for each of the three possible pairwise combinations of traits and then compared the observed value to a distribution obtained from at least 500 randomly shuffled combinations

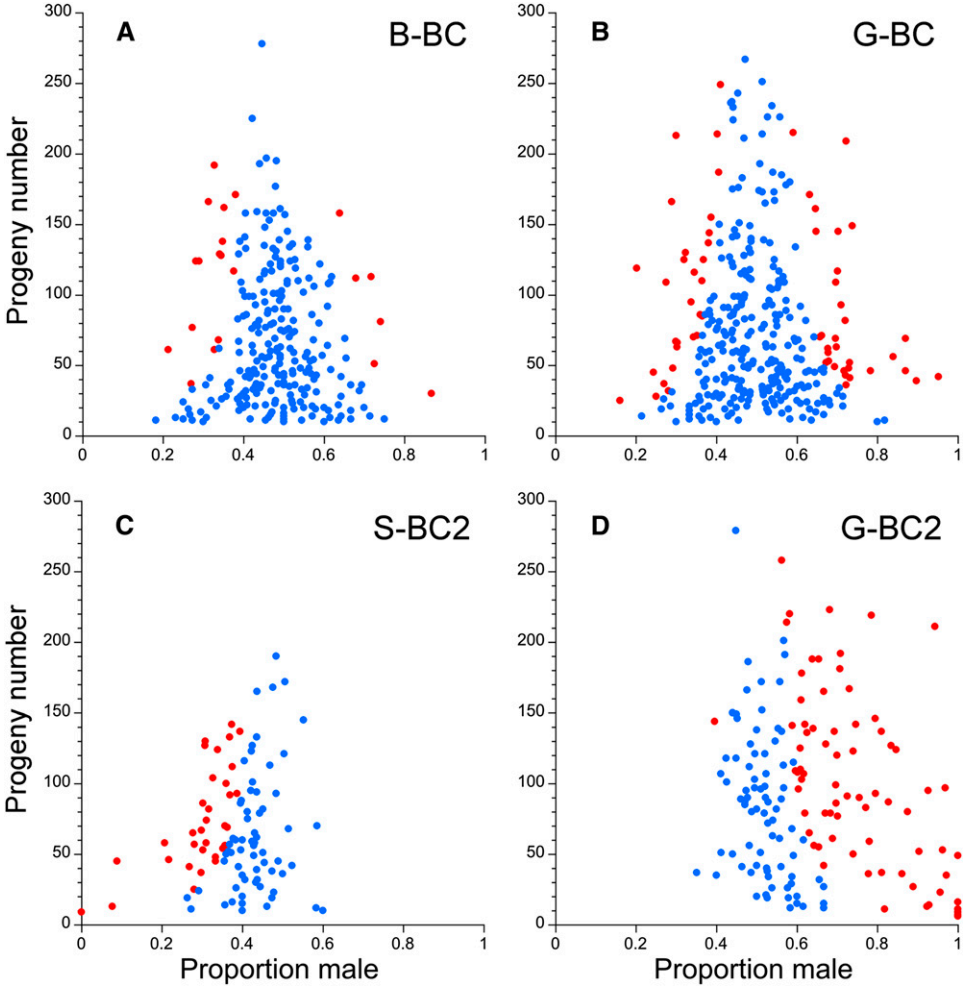


**Figure 2** Scatterplots of progeny number on sex ratio (proportion male brood) for males producing >10 progeny for each type of backcross illustrated in Figure 1, *i.e.*, (A) B-BC, (B) G-BC, (C) S-BC2, and (D) G-BC2. Red indicates males producing brood sex ratios significantly different from 50% male according to chi-square analysis after adjusting for multiple testing as described in the text. See Figure S1 for comparable data from within populations.

of QTL locations, holding the number of significant QTL constant for each trait in each population. We then report the proportion of shuffled combinations that had as many or more significant QTL at the same genome location.

## Results

### *Sterility and X$^{SR}$*

To determine if the extant G X$^{SR}$ chromosome influences sterility when combined with genes from the S population, we scored male progeny produced from the two S-G crosses initiated with a drive male for the presence of diagnostic X$^{SR}$ alleles. In the G × G(G$^{SR}$S) cross, 66 of 241 (27.4%) male progeny carried an X$^{SR}$ allele at all informative X-linked markers; *i.e.*, there was no evidence of recombination between the X$^{SR}$ and X chromosomes and the proportion of X$^{SR}$ males did not differ from 25% ($\chi^2 = 0.73$, d.f. = 1, $P = 0.39$), as expected if transmission of the two types of X chromosomes was equal in hybrid G$^{SR}$S females. In addition, the presence of the X$^{SR}$ haplotype had no effect on fertility of 218 males produced in the G × G(G$^{SR}$S) cross ($\chi^2 = 0.10$, d.f. = 1, $P = 0.75$; 47.0% sterile with X$^{SR}$ *vs.* 49.3% sterile without X$^{SR}$). The X$^{SR}$ haplotype also had no effect on fertility of 112 males from the S × S(G$^{SR}$S) cross because no X$^{SR}$ alleles were present in them or in any of their female parents. Absence of the X$^{SR}$ chromosome in the female parents from this cross could be due to chance given that only four females, each of which had a G$^{SR}$S mother, produced almost all of the males scored in this cross (see Table S1) or to an incompatibility that prevented X$^{SR}$ transmission. Nevertheless, absence of recombination between X$^{SR}$ and X$^{ST}$ chromosomes means that none of the QTL described below are due to factors on the extant X$^{SR}$ chromosome. Consequently, the number of recombinant X chromosomes available for mapping is reduced in the S-G crosses.

### *Sex ratio and X$^{SR}$*

For the B-G crosses, among 664 males that produced more than 10 offspring, the average sex ratio was 0.491 ± 0.004. However, even in the absence of an X$^{SR}$ chromosome, 83 (12.5%) exhibited significantly biased sex ratios, with nearly equal numbers showing female-biased (44, 6.6%) and male-biased (39, 5.9%) sex ratios. All three types of sex-ratio categories, *i.e.*, female-biased, male-biased, or unbiased, were present in both directions of the cross (Figure 2, A and B). The average sex ratio for 251 males screened in the S-G crosses was 0.536 ± 0.011, but in contrast to the B-G crosses only two phenotypic classes were present in

**Table 1 Number of male progeny dissected, tested for fertility, and scored for progeny sex ratios with percentages carrying sperm, producing progeny, and displaying significantly biased brood sex ratios for the four crosses described in Figure 1, A–D**

| Cross | Dissected | % with sperm | Scored for fertility | % fertile | Scored for sex ratio | % female-biased sex ratio | % male-biased sex ratio |
|---|---|---|---|---|---|---|---|
| G-BC | 450 | 78.7 | 801 | 59.9 | 401 | 7.2 | 8.2 |
| B-BC | 384 | 78.4 | 565 | 54.0 | 263 | 5.7 | 2.3 |
| G-BC2 | 492 | 63.0 | 439 | 37.6 | 152 | 0.7 | 46.7 |
| S-BC2 | 355 | 67.6 | 261 | 44.8 | 99 | 33.3 | 0.0 |

each cross direction. In backcrosses to an S male, one-third of 99 male progeny produced significantly female-biased sex ratios (Figure 2C). As noted above, no $X^{SR}$ alleles were present in males from the S × S($G^{SR}$S) cross, so the $X^{SR}$ chromosome had no effect on the presence of female-biased sex ratios in that cross. In backcrosses to a G male, nearly one-half (46.7%) of 152 male progeny produced significantly male-biased sex ratios, with some individuals producing all male broods (Figure 2D). Furthermore, in the G × G($G^{SR}$S) cross the $X^{SR}$ haplotype did not influence biased sex ratios: male-biased sex ratios were produced by 20 of 34 males carrying the $X^{SR}$ haplotype and by 33 of 72 males without the $X^{SR}$ haplotype ($\chi^2 = 1.56$, d.f. = 1, $P = 0.22$). (For comparison, 48 of 50 outbred G males carrying an $X^{SR}$ chromosome produced <5% sons; see Figure S2.)

### *Sperm length and sterility*

Because we scored large numbers of males for both offspring production and sperm length (Table 1), we could distinguish cases of sterility caused by sperm absence from those where sperm were present. In the B-G crosses of 402 sterile males, 153 (38.1%) had no sperm and 249 (61.9%) had sperm. In the S-G crosses 410 males were sterile with 213 (52.0%) lacking sperm and 197 (48.0%) with sperm. Thus, almost half (45.7%) of all cases of male sterility were due to sperm absence. To determine if the remaining cases of sterility were associated with variation in sperm length, we fit a series of general linear models (GLMs) to sperm length for each backcross using paternal population, type of hybrid female, sterility, and their possible interactions (see Table S2). For the B-G crosses, the best GLM contained four parameters, including paternal population ($\chi^2 = 161.6$, $P < 0.0001$), female hybrid type ($\chi^2 = 55.0$, $P < 0.0001$), sterility ($\chi^2 = 24.4$, $P < 0.0001$), and the interaction between paternal population and sterility ($\chi^2 = 59.3$, $P < 0.0001$). When G was the paternal population, sterile males had longer sperm than fertile males but when B was the paternal population, sterile males had shorter sperm than fertile males (Figure 3A). The hybrid female effect was due to males derived from $G^{SR}$S hybrid females having shorter sperm (160.7 ± 0.9 μm) than those of males from GS females (170.8 ± 0.9 μm), consistent with drive causing shorter sperm as previously reported (Johns and Wilkinson 2007). In the S-G crosses, the best GLM again contained four parameters including paternal population ($\chi^2 = 325.4$, $P < 0.0001$), female hybrid type ($\chi^2 = 54.5$, $P < 0.0001$), sterility ($\chi^2 = 5.3$, $P = 0.021$), and the interaction between paternal population and sterility ($\chi^2 = 113.5$, $P < 0.0001$). As in the B-G crosses, sperm length varied with sterility but the pattern was reversed. When G was the paternal population, sterile males had shorter sperm than fertile males, but when S was the paternal population, sterile males had longer sperm than fertile males (Figure 3B). In addition, males from GB hybrid females had shorter sperm (167.2 ± 0.8 μm) than those of males from BG females (178.5 ± 1.3 μm), suggesting a potential maternal effect on sperm length. Nonetheless, sterility was strongly associated with abnormal sperm length in both sets of crosses.

### *Quantitative trait locus analyses*

The preceding results show that extant X drive causes neither sterility nor biased sex ratios in hybrid males. However, dramatic variation in sex ratio is present in hybrid males indicating that cryptic drive must be present. Thus, drive could still influence hybrid sterility, if cryptic drive loci on the X also influence sterility either directly or indirectly through epistatic effects of autosomal suppressors. To assess these alternatives we used QTL mapping to locate chromosomal regions that explained variation in sterility, sex-ratio, and sperm length in each of the two sets of crosses and then tested for the presence of nonrandomly shared QTL.

***Sterility (B-G crosses):*** Male sterility was strongly influenced by at least two regions of the X chromosome but by no autosomal regions in the B-G crosses (Figure 4A). A QTL near *CG31738* explained 21.9% of the phenotypic variation in sterility among the B backcross (henceforth B-BC) males while a QTL near *crc* explained 5.2% of the phenotypic variation in sterility in the G-BC males. The presence of a G allele at *CG31738* in the B-BC males increased sterility to >65% while the presence of a B allele at *crc* in the G-BC males increased sterility to nearly 50% (Figure 4D).

***Sex ratio (B-G crosses):*** Variation in brood sex ratios in the B-G crosses was associated with four genomic regions: three autosomal and one on the X (Figure 4B). A QTL near ms392 on chromosome 1 explained 5.2% of the variation in G-BC male sex ratios while a QTL near *crc* on the X explained 4.4% of the variation in G-BC males and 3.1% of the variation in B-BC males. An additional QTL on chromosome 1 near ms174 explained 3.1% of the variation in both G-BC and B-BC male sex ratios while a QTL near ms39 on

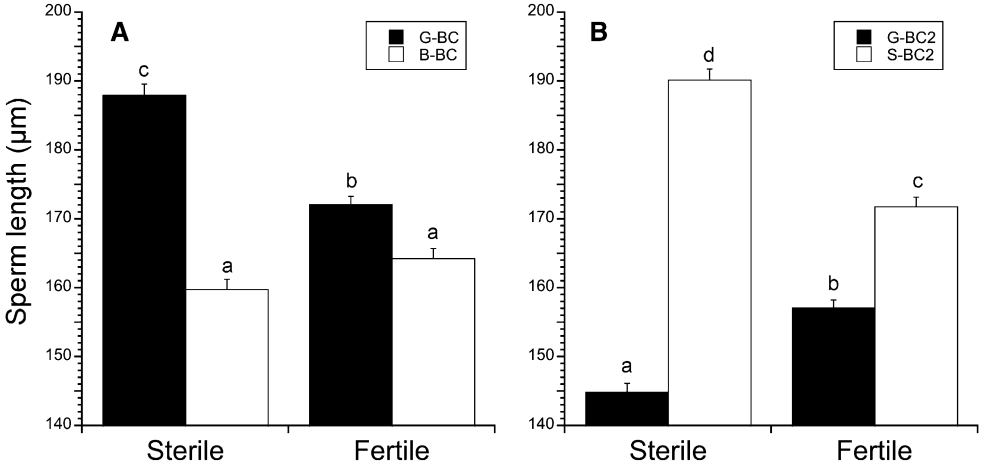


**Figure 3** Least-squares mean (±1 SE) sperm lengths for sterile and fertile males in (A) the two directions of the B-G cross and (B) the two directions of the S-G cross (cf. Figure 1). Letters indicate differences among means within A and B according to post hoc Student's t-tests with $P < 0.05$.

chromosome 2 explained 2.4% of the variation in G-BC males. G-BC males carrying a B allele at ms392 on chromosome 1 or *crc* on the X had more female-biased sex ratios while those with a B allele at ms39 had more male-biased sex ratios (Figure 4E). B-BC males carrying a G allele at *crc* had more male-biased sex ratios while those carrying a G allele at ms392 had more female-biased sex ratios. Thus, an X-linked B region near *crc* acts as a driver. This cryptic X drive system appears to be suppressed by a factor near ms392 on C1. In contrast, male-biased sex ratios appear to be suppressed in G males by a region on chromosome 2.

***Sperm length (B-G crosses):*** Almost all of the genetic effects on sperm length were caused by a major QTL on the X chromosome (Figure 4C) in the B-G crosses. The QTL between ms395 and *crc* was detected in both directions of the cross and explained 31.2% of the variation in sperm length among G-BC males and 5.8% of the variation among B-BC males. A QTL near ms174 on chromosome 1 explained 2.5% of the variation in G-BC males and 1.7% in B-BC males. A foreign allele at *crc* had strongly nonadditive effects on sperm length. G-BC males that carried a B allele at *crc* had much longer sperm while B-BC males with a G allele had shorter sperm than males carrying the paternal X-linked allele (Figure 4F) consistent with the phenotypic patterns described above (*cf*. Figure 3a).

***Sterility (S-G crosses):*** In the S-G crosses, male sterility was influenced by three autosomal and two X-linked regions (Figure 5A). On chromosome 1 a QTL at ms402a explained 7.9% of the variation in sterility in the G-BC2 males while a QTL at ms223 explained 4% among the G-BC2 males and 9.0% among the S-BC2 males. On chromosome 2, a QTL at ms39 explained 14.7% of the variation in sterility in the S-BC2 males. Finally, QTL on the X at ms125 and ms71 explained 5.7 and 6.8%, respectively, among the G-BC2 males. Inspection of the proportion of sterile males produced by each of the four possible genotypes in the crosses revealed that the presence of a foreign allele causes sterility to increase by 30–50% at each QTL, with the two X-linked regions having the largest phenotypic effect. The presence of an S allele at either X-linked marker resulted in nearly complete sterility in the G-BC2 males (Figure 5D). Absence of X-linked QTL in S-BC2 males is likely due to reduced recombination given that only 22 of 261 males carried a G allele at any X-linked marker. Interestingly, all of these cases were recombinants for loci between ms395 and ms71 and 21 were sterile, consistent with a major X-linked effect on sterility.

***Sex ratio (S-G crosses):*** Brood sex ratios in the S-G crosses were influenced by two QTL on chromosome 1 and one marginally significant QTL on chromosome 2 (Figure 5B). On chromosome 1 a QTL at ms223 explained 7.4% of the variation in G-BC2 male sex ratios while a QTL near ms262z explained 27.6% of the variation in S-BC2 males. On chromosome 2 the QTL at ms39 explained 5.4% of the variation in G-BC2 male sex ratios. Foreign alleles at each of these QTL increased sex-ratio bias, indicating that these regions contain drive suppressors (Figure 5E). G-BC2 males heterozygous for ms223 or ms39 had more male-biased broods if they carried an S allele. In contrast, S-BC2 males carrying a G allele at ms262z produced more female-biased broods. No QTL were detected for brood sex ratio on the X because, as mentioned above, the presence of any foreign X-linked alleles caused nearly complete sterility, *i.e.*, 159 of 165 fertile males in the G backcross had only G alleles at X-linked loci and 117 of 117 fertile males in the S backcross had only S alleles at X-linked loci. Thus, interactions between autosomal modifiers and an unidentified region of the X (or Y) chromosome (or both) are responsible for the overall female-bias in males with X-linked S alleles and the overall male-bias in males with X-linked G alleles (Figure 5E).

***Sperm length (S-G crosses):*** All three chromosomes contained factors that influence the length of mature sperm bundles in the S-G crosses. On chromosome 1, ms402a explained 7.9%, ms223 explained 6.3%, and ms262z explained 3.4% of the variation in G-BC2 male sperm length while ms392 explained 7.2% of the variation in S-BC2 male sperm length (Figure 5C). On chromosome 2 ms39 explained 29.2% of the variation in S-BC2 male sperm length. Finally, on the X, ms70 explained 7.4% and ms71 explained 3.9% of

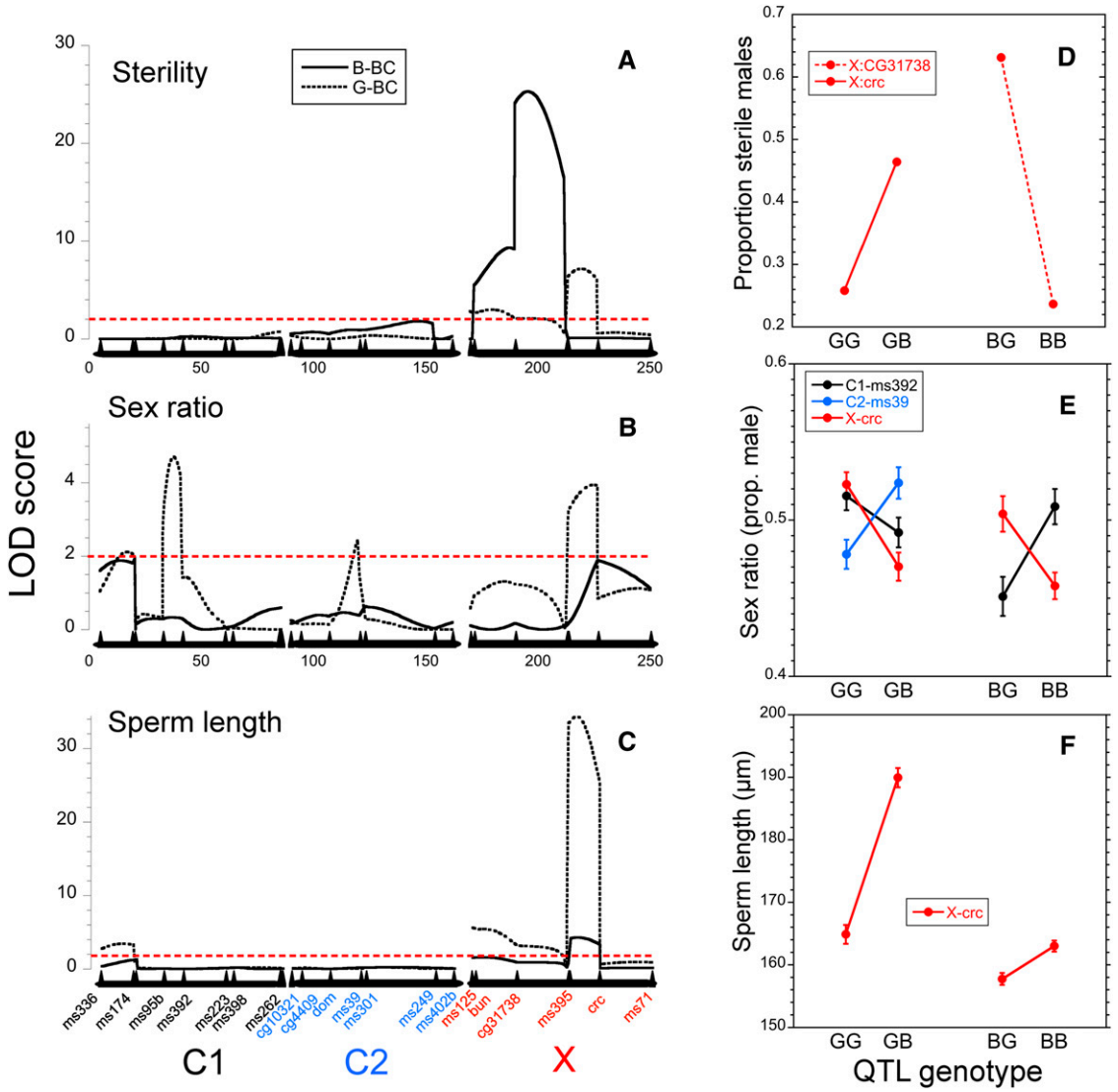


**Figure 4** Mapping results for the B-G crosses (cf. Figure 1). Solid black lines indicate LOD scores obtained by multiple QTL mapping (MQM, see *Materials and Methods*) for B-BC males while dashed black lines are for G-BC males and are plotted against chromosome position (centimorgans) for (A) male sterility, (B) brood sex ratio, and (C) sperm length with marker loci indicated by triangles. The red dashed line shows the genome-wide threshold of QTL detection for a probability of 0.05 based on 1000 permutations. Marker loci labels are color coded by chromosome: black, C1; blue, C2; red, X. Trait phenotype is plotted against genotype for (D) proportion of sterile males, (E) mean (±1 SE) sex ratio expressed as proportion males in the brood, and (F) mean (±1 SE) sperm length at the marker locus nearest a significant QTL in each of the two B-G crosses. The second letter of the genotype label denotes the origin of the nonpaternal allele for autosomal markers or the haploid allele for X-linked markers.

the variation in G-BC2 males while regions near ms125 and *crc* explained 17.7 and 4.1%, respectively, of the variation in S-BC2 male sperm length. In general, major QTL have opposing effects on sperm length. For example, G-BC2 males carrying an S allele at ms125 have longer sperm but S-BC2 males with a G allele at ms125 have shorter sperm (Figure 5F). In addition, G-BC2 males heterozygous at marker ms223 have shorter sperm while S-BC2 males heterozygous at ms39 have longer sperm (Figure 5F).

### *Trait associations within and between crosses*

Comparison of QTL locations across traits in the two sets of crosses (Figure 4 and Figure 5) reveals the presence of several genomic regions that appear to influence more than one trait in each set of crosses. In the B-G crosses an X-linked region between ms395 and *crc* influences sterility, sex ratio, and sperm length. Similarly, in the S-G crosses, regions near ms223 on chromosome 1 and ms39 on chromosome 2 are associated with all three traits. Randomization tests in which QTL are scored present or absent at marker locations indicate that shared QTL occur more frequently than expected for sterility and sperm length in both the B-G (three QTL shared, $P = 0.006$) and S-G (four QTL shared, $P = 0.038$) crosses. Sperm length and sex ratio QTL also co-occurred on autosomes more often than expected in the S-G crosses (3 QTL shared, $P = 0.020$) although not significantly

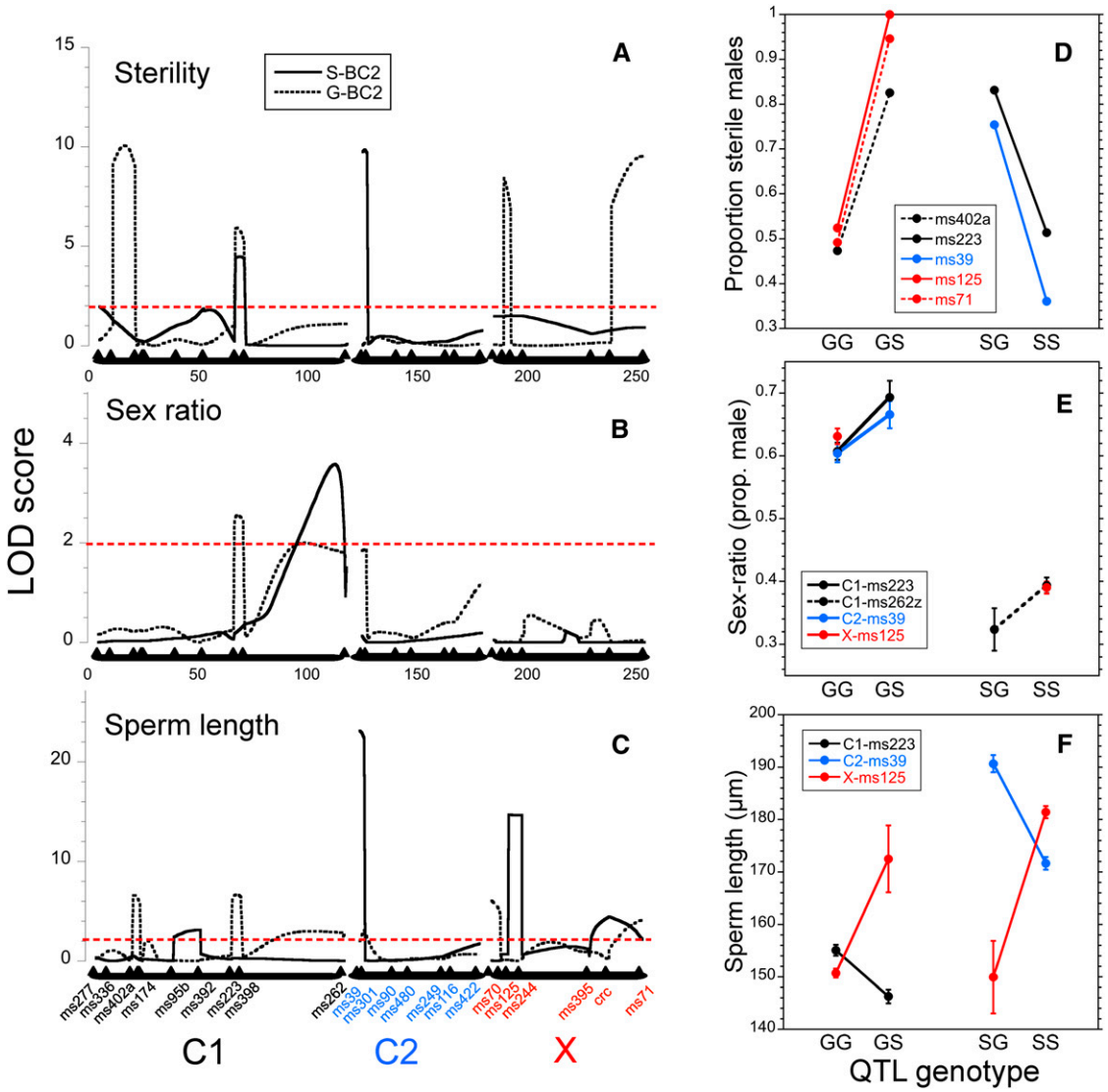


**Figure 5** Mapping results for the S-G crosses (cf. Figure 1). MQM LOD scores plotted against chromosome position (centimorgans) for (A) male sterility, (B) brood sex ratio, and (C) sperm bundle length with marker loci indicated by triangles. Solid black lines indicate LOD scores for S-BC2 males while dashed black lines are for G-BC2 males. Trait phenotype is plotted against genotype for (D) proportion of sterile males, (E) mean (±1 SE) sex ratio expressed as proportion males in the brood, and (F) mean (±1 SE) sperm length at the marker locus nearest a significant QTL in each of the two S-G backcrosses. See Figure 4 legend for additional information.

so across all chromosomes in the B-G crosses (two QTL shared, $P = 0.197$). Sterility and sex ratio shared one X-linked QTL in the B-G crosses ($P = 0.576$) but two autosomal QTL in the S-G crosses, which approaches significance ($P = 0.093$). If, however, either of the X-linked sterility QTL in the S-G crosses also influenced sex ratio, then the probability would be 0.0167. Given that in this cross an X-linked QTL is shared for sterility and sperm length, three other QTL are shared between sperm length and sex ratio, and there is a major X-linked effect on sex ratio, this scenario is not implausible, but unfortunately cannot be confirmed due to our inability to map sex ratio on the X in the S-G crosses.

In contrast, the only QTL that appears to be shared for any trait across the two sets of crosses is ms39 on chromosome 2 for progeny sex ratio. The probability of one shared autosomal QTL for sex ratio between the crosses is 0.699. Thus, associations between causal genomic regions for different traits within the same cross are stronger than associations between genomic regions for the same trait between crosses.

## Discussion

The majority of studies that have investigated a possible relationship between sex chromosome drive and hybrid sterility have used species or populations where drive is masked by one or more suppressing factors in extant populations (McDermott and Noor 2010; but see McDermott and Noor 2012). Consequently, in most cases selection on the drive system may be weak or absent until a new modifier evolves (Hall 2004). In *T. dalmanni*, males that produce

90% or more female offspring occur at frequencies of 5–30% in the wild (Wilkinson *et al.* 2003; Cotton *et al.* 2014; K. Paczolt and J. Reinhardt, personal communication), which should generate strong selection as a consequence of biased sex ratios (Lande and Wilkinson 1999). The drive polymorphism appears to be stabilized through a combination of frequency-dependent selection operating via male fertility (Wilkinson and Fry 2001) and balancing selection on female fecundity (Wilkinson *et al.* 2006). To determine how genes elsewhere in the genome interact with and potentially control driving X chromosomes, we conducted crosses between flies from a population in peninsular Malaysia (G) and two different allopatric populations in Sumatra (S and B). These experiments produced a rich variety of results indicating that genes that interact with the SR drive system have diverged among these populations to cause unusual variation in progeny sex ratios and sperm length, and as a consequence contribute to male hybrid sterility.

### *Cryptic drive and extraordinary sex ratios*

Perhaps our most surprising result is the remarkable variation in sex ratio that we uncovered in both sets of crosses, none of which can be ascribed to the extant $X^{SR}$ chromosome. The existence of both female- and male-biased sex ratios in male progeny from crosses in which the paternal male showed no sex-ratio bias indicates the presence of cryptic drive, which is currently masked in the S, B, and G populations by modifier loci that suppress driving X (or Y) chromosomes. The biased sex ratios we observed must, therefore, be the result of an older drive system that is currently suppressed in local populations. While weakly male-biased broods have previously been reported in *T. dalmanni* (Presgraves *et al.* 1997), the bias observed was far less than that found in this study, where multiple males produced only male offspring (*i.e.*, contrast Figure 2 with Figure S1).

While the two different population crosses generated males that produced both female- and male-biased sex ratios, QTL mapping indicates that genetic control of the variation in brood sex ratios differs between the populations. In the S-G crosses the X chromosome from the S population exhibits partial drive, *i.e.*, male carriers have 10–30% sons, in the absence of a suppressor on chromosome 1. In addition, the Y chromosome from the G population appears to exhibit drive, *i.e.*, male carriers have 60–100% sons, when a region on chromosome 1 and one on chromosome 2 fail to suppress it. This apparent Y drive is unaffected by the presence of an extant $X^{SR}$ haplotype, which has no discernable effect on brood sex ratios in the S-G crosses (*cf*. Figure S2).

The sex-ratio phenotypes exhibited in the B-G crosses differ from those from the S-G crosses in several ways. Extreme drive, in which males produce >95% daughters or sons, was nearly absent in any B-G cross but was present in both directions of the S-G cross. The B-G crosses did not differ in direction of sex-ratio bias, while the S-G crosses did. Finally, a significant QTL on the X for sex ratio was detected only in the B-G crosses, albeit we cannot determine if this region is also important in the S-G crosses because fertile males lacked segregating X-linked markers. Interestingly, the X-linked QTL was between ms395 and *crc*. Not only did this region also associate with sterility and sperm length (see below), but ms395 was also recently reported to be the best predictor of strong X drive in field-caught flies from the Gombak valley (Cotton *et al.* 2014). This result suggests that ancestral and extant X drive factors may be located in the same region but have different sets of modifiers.

### *Haldane's rule and meiotic drive*

Sex chromosome drive is expected to favor rapid evolution of autosomal or Y-linked suppressors. The drive hypothesis for Haldane's rule predicts that if modifiers evolve independently in isolated populations and are important for spermatogenesis, then mismatches between drive and suppressor alleles will cause male sterility (Frank 1991; Hurst and Pomiankowski 1991). Thus, one piece of evidence in support of this hypothesis is the presence of cryptic drive in hybrid males due to the absence of coevolved suppressor alleles. Given that we identified autosomal suppressors in both sets of crosses, our study adds another example to the growing list of cases in which cryptic drive has been detected in such crosses (Hauschteck-Jungen 1990; Mercot *et al.* 1995; Dermitizakis *et al.* 2000; Tao *et al.* 2001; Fishman and Willis 2005; Orr and Irving 2005). Furthermore, even though all crosses used flies from the G population, the location of autosomal modifiers of drive largely differed between crosses (*cf*. 4B and 5B). The potential exception was a QTL for sex ratio at ms39 on chromosome 2, which had a LOD score that exceeded significance in one of the B-G crosses but only approached significance in one of the S-G crosses. Nevertheless, these results suggest that some of the factors that cause and suppress X (or Y) drive are not in the same chromosomal locations in the S and B populations. Given that these two populations last shared a common ancestor <100,000 years ago (Swallow *et al.* 2005), their drive systems appear to have evolved rapidly and independently.

A second line of evidence in support of the drive hypothesis is that suppressor loci should also influence sterility when alleles from one population are combined with cryptic drive X chromosomes from a recently diverged population. Demonstrating such an association between sterility and biased sex ratios is challenging because sterile males cannot be scored for sex ratio. Nevertheless, results from both sets of crosses indicate that some genomic regions influencing these two traits are not independent. In the B-G cross, a B allele near *crc* on the X jointly increased sterility, frequency of female-biased sex ratios, and sperm length. As noted above, this region is also near a marker of the extant $X^{SR}$ drive system. In the S-G cross, an S allele at two QTL, one near ms223 on chromosome 1, and one near ms39 on chromosome 2 jointly influenced sterility, sex ratio, and sperm length. In addition, even though we were unable to obtain sex ratios for any males from this cross with

recombinant X chromosomes, foreign alleles at two different genomic regions on the X caused nearly complete sterility. Thus, in the S-G cross, regions of the X chromosome have major effects on both biased sex ratios and sterility. Because we used relatively few genomic markers, these results are not sufficient to distinguish pleiotropy from linkage. Thus, it is possible that the genes that influence fertility exhibit linkage to genes that influence sex ratios.

The X chromosome in *T. dalmanni* contains ~15% of the genes in the genome (Baker and Wilkinson 2010). In the B-G cross, both of the QTL detected for sterility were on the X chromosome, whereas in the S-G cross, 40% (2 of 5) of the QTL for sterility were on the X suggesting that factors influencing sterility tend to evolve more rapidly on the X than on the autosomes. Evidence for rapid evolution on the X chromosome has been found in other studies of hybrid sterility (Presgraves 2010), most of which have been conducted in *Drosophila*. Diopsid stalk-eyed flies are, however, notably different in that their X chromosome is Muller element B (Baker and Wilkinson 2010). When Muller element B first became an X is unknown, although it may have preceded the origin of the diopsids. Nevertheless, novel sex chromosomes have been hypothesized to be prone to exhibit drive because genes elsewhere in the genome may not have had sufficient time to evolve suppression (Burt and Trivers 2006). Thus, it may not be coincidental that two of three examples of drive–sterility associations (McDermott and Noor 2010) involve the *obscura* group of *Drosophila* in which the X has fused to an autosome to create a neo-X chromosome (Schaeffer *et al.* 2008).

### *Mechanism of association between drive and sterility*

While the genes affecting both drive and sterility have been identified in *Drosophila simulans* (Tao *et al.* 2001, 2007a,b) and *D. pseudoobscura* (Phadnis and Orr 2009), the mechanism by which these genes might exert pleiotropic effects is not clear. One possibility is that chromatin alteration influences X chromosome silencing during spermatogenesis (McDermott and Noor 2010). *Overdrive* encodes a protein with a Myb/SANT-like domain in an *Adf-1* (MADF) DNA-binding domain. *Adf-1* is a transcription factor known to regulate gene expression during development (England *et al.* 1992). The suppressor *Tmy* has also been hypothesized to influence chromatin remodeling (Tao *et al.* 2007a,b). Without identifying the genes associated with each QTL, we cannot determine whether changes in chromatin influence drive and sterility in *Teleopsis*. However, because we also measured sperm length, we know that genomic regions that influence sterility also influence sperm length and sex ratio, which may provide an alternative explanation for how sterility and sex ratio are linked.

If genes that influence sterility also influence sperm elongation, then sperm length could become more variable. Previous work shows that sperm length is influenced by X-linked genes (Johns and Wilkinson 2007), evolves rapidly, and exhibits correlated evolution with female reproductive tract dimensions in diopsids (Presgraves *et al.* 1999). Sperm from males that have longer or shorter sperm than is typical for their population, either fail to get stored or fail to move through the reproductive tract to the location where they can fertilize an egg (Rose *et al.* 2014). Changes in sperm length could, therefore, provide a simple mechanism for causing sterility in males that produced sperm. Notably, in both sets of crosses, sterile males had significantly longer or shorter sperm than fertile males, although the population effect on sperm length was reversed in the two sets of crosses (*cf.* Figure 3). These results are consistent with our mapping data, which show that the presence of a foreign X-linked allele causes sperm to increase or decrease in length in opposite directions relative to the phenotype associated with the paternal population allele (*cf.* Figure 4F and Figure 5F). In addition, some of the variation in sperm length in the B-G cross could be attributed to a maternal effect, suggesting a potential effect of mitochondria. Further study is needed, therefore, to determine if conflict between the mitochondrial and nuclear genomes (Dowling *et al.* 2008) also contributes to hybrid dysfunction in these flies.

### *Conclusions*

Stalk-eyed flies in the family Diopsidae have become a model for studies of sexual selection due to the dramatic sexual dimorphism in head shape exhibited by many species (Burkhardt and de la Motte 1985; Wilkinson and Dodson 1997; David *et al.* 2000; Baker and Wilkinson 2001; Cotton *et al.* 2004). High frequencies of sex chromosome drive (Wilkinson *et al.* 2003; Cotton *et al.* 2014), reproductively isolated populations (Christianson *et al.* 2005; Swallow *et al.* 2005; Rose *et al.* 2014), and increasing availability of genomic resources (Baker *et al.* 2009; Baker and Wilkinson 2010; Reinhardt *et al.* 2014) make them also a tractable system for studying the genetic bases of reproductive isolation, such as those causing hybrid sterility. Our mapping results from two sets of interpopulation crosses suggest that genomic regions that influence sperm length also influence sterility and sex ratio. However, distinguishing between pleiotropy and linkage as possible causes for these effects will require higher-resolution mapping and gene manipulation studies. Nevertheless, even with a relatively coarse linkage map these results provide compelling evidence that genes influencing meiotic drive, sterility, and sperm length differ between populations that diverged within the past 100,000 years (Swallow *et al.* 2005), consistent with rapid evolution of the drive system and sperm length. These results suggest that sperm morphology could play a key role in other cases of Haldane's rule for organisms, such as flies, where sperm must enter an egg to effect fertilization (Karr 1991), but also in mammals, when sperm head morphology is disrupted in hybrids (*cf.* Campbell and Nachman 2014).

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GENETICS

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# Haldane's Rule Is Linked to Extraordinary Sex Ratios and Sperm Length in Stalk-Eyed Flies

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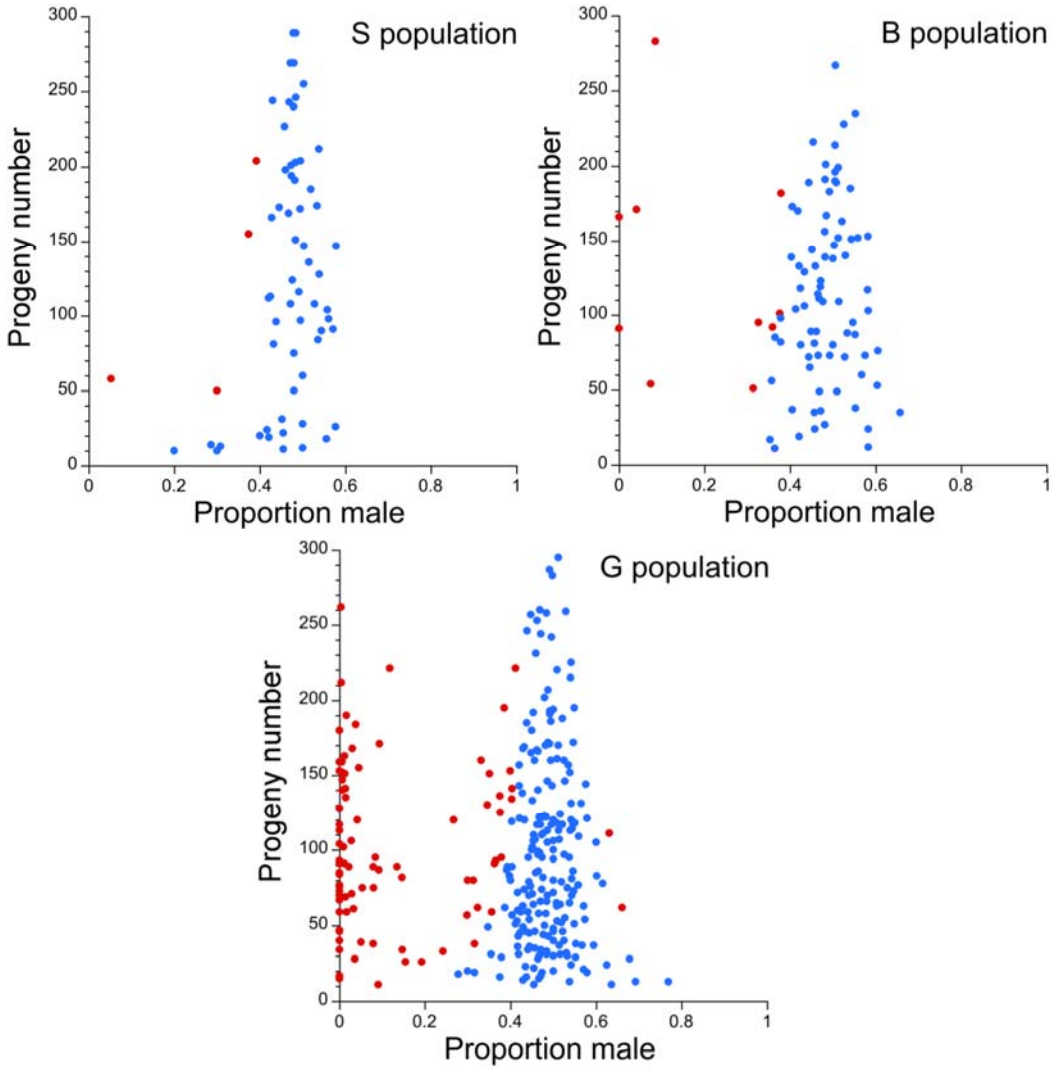


**Figure S1** Scatterplots of progeny number on brood sex ratio (proportion male) for outbred males producing more than 10 progeny for each of the three populations used in the study. Red indicates males producing brood sex ratios significantly different from 50% male according to chi-square analysis after adjusting for multiple testing as described in the text. Data are from Wilkinson et al. (2003; 2006) and previously unpublished for Gombak males bred within three generations after capture in 2012.

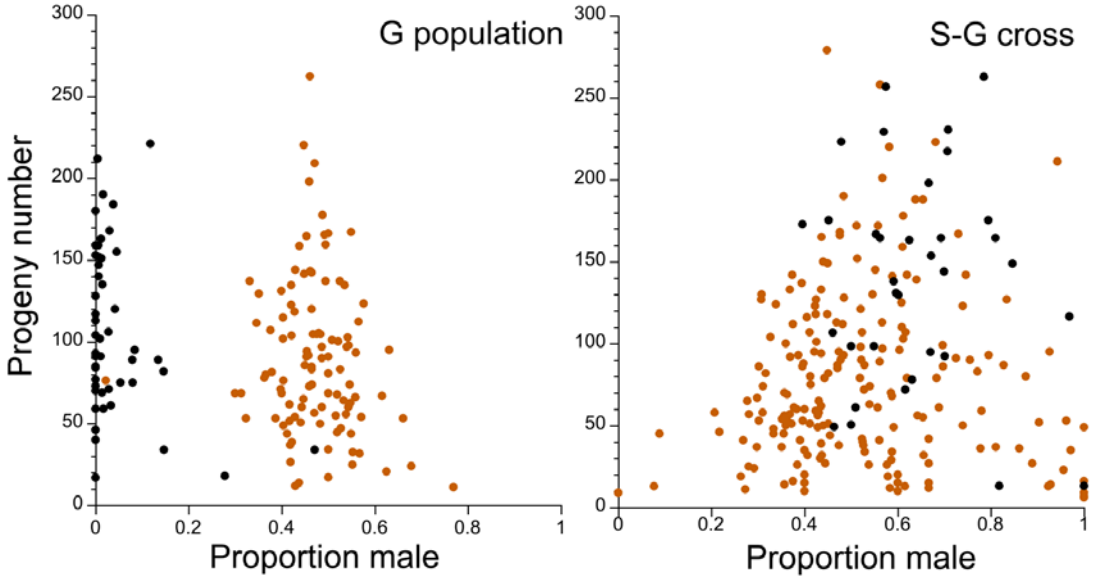


**Figure S2** Scatterplots of progeny number on brood sex ratio (proportion male) for males producing more than 10 progeny from the G population or the S-G cross. Black indicates males carrying X-linked microsatellite alleles, e.g. ms395 > 220 bp, characteristic of the extant Gombak $X^{SR}$ chromosome (cf. Wilkinson et al. 2006; Cotton et al. 2014). Data are from Wilkinson et al. (2006) and unpublished for Gombak males bred within three generations after capture in 2012 and genotyped at multiple X-linked markers. In outbred flies, the association between drive and haplotype is strong although not perfect, i.e. one G male with the SR haplotype had an unbiased sex ratio and one G male with an extremely female-biased sex ratio lacked the $X^{SR}$ haplotype. In contrast, the $X^{SR}$ haplotype exhibits no association with brood sex ratio in the males produced in the S-G cross.

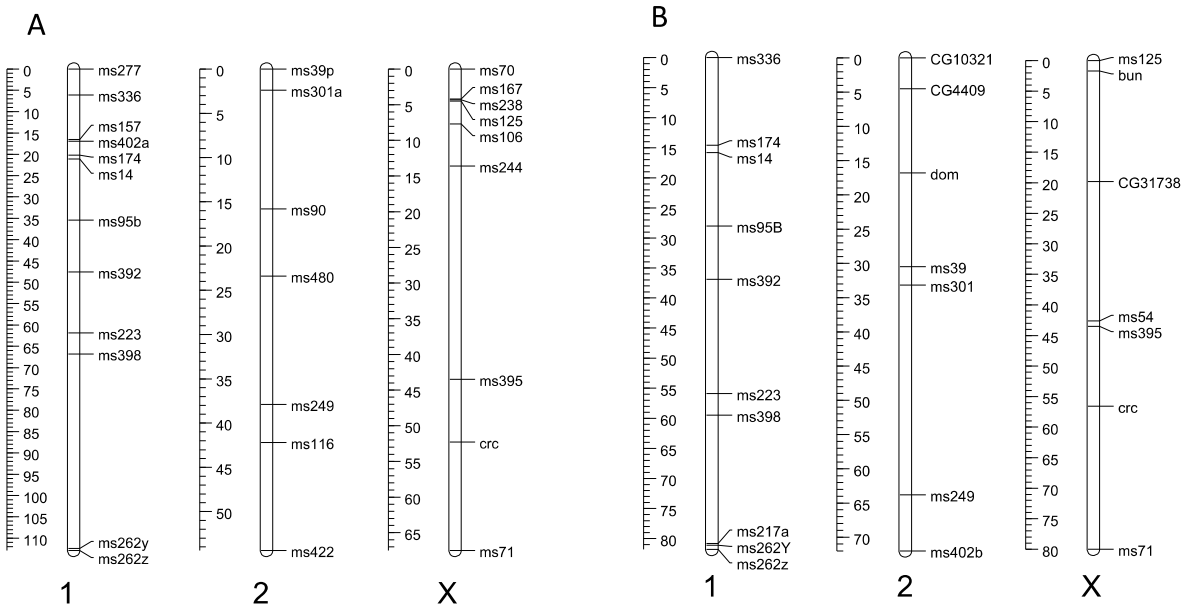


**Figure S3** Combined linkage maps for A) the S-G cross and B) the B-G cross estimated using a multipoint maximum likelihood algorithm in JoinMap v. 4.0 on genotypes of the male progeny from each set of backcrosses. Genetic markers were selected to span each chromosome and be informative in both sets of crosses whenever possible. Note that the marker order between linkage maps estimated for the two sets of crosses was highly congruent with two exceptions. First, three markers were typed on the proximal end of chromosome 2 in the B-G cross, but not in the S-G cross, which extended the mappable region on that chromosome in the the B-G cross. Second, several X-linked markers in the S-G cross appear to segregate together, indicating the presence of a small inversion that reduces recombination between the S and G X chromosomes.

**Table S1 Number of male offspring measured for each phenotypic trait within each family of each cross**

| Cross (m x f) | Paternal rep | Maternal rep | Fertility (N) | Genotype (N) | Sperm (N) | Sex-ratio (N) |
|---|---|---|---|---|---|---|
| S x S($G^{SR}$S) | A | 1 | 15 | 18 | 16 | 6 |
| S x S($G^{SR}$S) | B | 1 | 33 | 33 | 16 | 8 |
| S x S($G^{SR}$S) | B | 2 | 32 | 32 | 27 | 22 |
| S x S($G^{SR}$S) | B | 3 | 13 | 13 | 12 | 6 |
| S x S($G^{SR}$S) | C | 1 | 1 | 1 | 0 | 0 |
| S x S($G^{SR}$S) | C | 2 | 2 | 15 | 4 | 0 |
| S x S(GS) | A | 1 | 94 | 106 | 90 | 52 |
| S x S(GS) | B | 1 | 1 | 1 | 1 | 0 |
| S x S(GS) | B | 2 | 27 | 51 | 19 | 0 |
| S x S(GS) | B | 3 | 20 | 28 | 20 | 3 |
| S x S(GS) | C | 1 | 23 | 56 | 18 | 2 |
| G x G($G^{SR}$S) | A | 1 | 19 | 19 | 19 | 15 |
| G x G($G^{SR}$S) | A | 2 | 41 | 41 | 38 | 31 |
| G x G($G^{SR}$S) | A | 3 | 1 | 1 | 0 | 0 |
| G x G($G^{SR}$S) | B | 1 | 20 | 23 | 13 | 4 |
| G x G($G^{SR}$S) | B | 2 | 13 | 25 | 11 | 4 |
| G x G($G^{SR}$S) | B | 3 | 26 | 31 | 10 | 5 |
| G x G($G^{SR}$S) | C | 1 | 7 | 7 | 7 | 5 |
| G x G($G^{SR}$S) | C | 2 | 33 | 33 | 30 | 13 |
| G x G($G^{SR}$S) | C | 3 | 17 | 17 | 17 | 5 |
| G x G($G^{SR}$S) | C | 4 | 41 | 41 | 35 | 24 |
| G x G(GS) | A | 1 | 41 | 38 | 12 | 4 |
| G x G(GS) | A | 2 | 14 | 19 | 18 | 11 |
| G x G(GS) | A | 3 | 52 | 50 | 20 | 11 |
| G x G(GS) | B | 1 | 8 | 8 | 3 | 1 |
| G x G(GS) | B | 2 | 45 | 51 | 13 | 1 |
| G x G(GS) | B | 3 | 15 | 23 | 15 | 3 |
| G x G(GS) | C | 1 | 20 | 20 | 11 | 4 |
| G x G(GS) | C | 2 | 9 | 11 | 10 | 7 |
| G x G(GS) | C | 3 | 16 | 27 | 25 | 6 |
| B x BG | A | 1 | 16 | 15 | 8 | 4 |
| B x BG | A | 2 | 5 | 4 | 2 | 2 |
| B x BG | A | 3 | 2 | 1 | 0 | 0 |
| B x BG | B | 1 | 9 | 7 | 7 | 3 |
| B x BG | B | 2 | 6 | 6 | 6 | 3 |
| B x BG | B | 3 | 2 | 2 | 2 | 0 |
| B x BG | C | 1 | 76 | 72 | 8 | 59 |
| B x BG | D | 1 | 50 | 45 | 0 | 31 |
| B x BG | E | 1 | 8 | 5 | 6 | 2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| B x BG | E | 2 | 1 | 1 | 0 | 0 |
| B x BG | E | 3 | 13 | 10 | 9 | 2 |
| B x BG | G | 1 | 29 | 6 | 0 | 5 |
| B x BG | F | 1 | 12 | 10 | 10 | 5 |
| B x BG | F | 2 | 24 | 20 | 8 | 5 |
| B x BG | F | 3 | 16 | 13 | 10 | 2 |
| B x GB | A | 1 | 2 | 1 | 0 | 0 |
| B x GB | A | 2 | 3 | 3 | 2 | 2 |
| B x GB | A | 3 | 13 | 8 | 5 | 4 |
| B x GB | B | 1 | 111 | 106 | 91 | 56 |
| B x GB | C | 1 | 129 | 120 | 97 | 69 |
| B x GB | D | 1 | 12 | 11 | 8 | 2 |
| B x GB | D | 2 | 4 | 3 | 3 | 0 |
| B x GB | E | 1 | 7 | 6 | 7 | 2 |
| B x GB | F | 1 | 1 | 1 | 1 | 1 |
| B x GB | F | 2 | 10 | 12 | 7 | 3 |
| B x GB | F | 3 | 4 | 4 | 4 | 1 |
| G x BG | A | 1 | 14 | 12 | 7 | 4 |
| G x BG | A | 2 | 52 | 45 | 15 | 11 |
| G x BG | A | 3 | 25 | 22 | 15 | 9 |
| G x BG | B | 1 | 11 | 3 | 0 | 6 |
| G x BG | C | 1 | 12 | 12 | 10 | 0 |
| G x BG | C | 2 | 11 | 9 | 6 | 4 |
| G x BG | D | 1 | 27 | 10 | 0 | 7 |
| G x BG | E | 1 | 167 | 163 | 15 | 118 |
| G x BG | F | 1 | 27 | 14 | 12 | 11 |
| G x BG | F | 2 | 47 | 48 | 13 | 23 |
| G x BG | G | 1 | 10 | 3 | 0 | 4 |
| G x BG | H | 1 | 2 | 3 | 2 | 1 |
| G x BG | H | 2 | 3 | 1 | 1 | 2 |
| G x BG | H | 3 | 26 | 22 | 12 | 9 |
| G x GB | A | 1 | 2 | 1 | 1 | 0 |
| G x GB | A | 2 | 6 | 4 | 5 | 1 |
| G x GB | A | 3 | 11 | 10 | 8 | 5 |
| G x GB | B | 1 | 27 | 0 | 0 | 2 |
| G x GB | C | 1 | 124 | 119 | 110 | 86 |
| G x GB | D | 1 | 89 | 84 | 69 | 57 |
| G x GB | E | 1 | 2 | 1 | 1 | 1 |
| G x GB | E | 2 | 16 | 15 | 12 | 6 |
| G x GB | E | 3 | 9 | 7 | 7 | 5 |
| G x GB | F | 1 | 9 | 10 | 5 | 5 |
| G x GB | F | 2 | 10 | 9 | 6 | 4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| G x GB | F | 3 | 1 | 1 | 0 | 0 |
| G x GB | G | 1 | 34 | 12 | 0 | 11 |
| G x GB | H | 1 | 12 | 11 | 9 | 5 |
| G x GB | H | 2 | 15 | 14 | 13 | 4 |

For each family the male parent came from an inbred line with different individuals denoted by letter under Paternal Rep. Superscript "SR" refers to a male carrying a $X^{SR}$ chromosome as illustrated in Figure S2. Female parent identity is denoted by number under Maternal Rep, but females mated to the same male were full-sib sisters.

**Table S2 General linear model results for sperm length by cross**

| **B-G cross** | | 2-parameter model | | 3-parameter model | | **4-parameter model** | | 5-parameter model | | 6-parameter model | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Source | df | L-R Chi-square | P | L-R Chi-square | P | L-R Chi-square | P | L-R Chi-square | P | L-R Chi-square | P |
| Full Model | 2-6 | 174.18 | <0.0001 | 199.58 | <0.0001 | **258.92** | **<0.0001** | 260.30 | <0.0001 | 261.63 | <0.0001 |
| Chi-square improvement | 1 | | | 25.40 | <0.0001 | **59.34** | **<0.0001** | 1.38 | > 0.05 | 1.34 | > 0.05 |
| Paternal population | 1 | 109.17 | <0.0001 | 120.62 | <0.0001 | **161.57** | **<0.0001** | 161.32 | <0.0001 | 146.47 | <0.0001 |
| Female hybrid type (BG or GB) | 1 | 66.03 | <0.0001 | 51.86 | <0.0001 | **54.96** | **<0.0001** | 55.98 | <0.0001 | 53.13 | <0.0001 |
| Fertility | 1 | | | 25.40 | <0.0001 | **24.44** | **<0.0001** | 25.13 | <0.0001 | 25.70 | <0.0001 |
| PatPop*Fertility | 1 | | | | | **59.34** | **<0.0001** | 57.84 | <0.0001 | 52.48 | <0.0001 |
| Female*Fertility | 1 | | | | | | | 1.38 | 0.2402 | 1.66 | 0.1974 |
| Female*PatPop | 1 | | | | | | | | | 1.34 | 0.2475 |

| **S-G cross** | | 2-parameter model | | 3-parameter model | | **4-parameter model** | | 5-parameter model | | 6-parameter model | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Source | df | L-R Chi-square | P | L-R Chi-square | P | L-R Chi-square | P | L-R Chi-square | P | L-R Chi-square | P |
| Full Model | 2-6 | 312.99 | <0.0001 | 313.02 | <0.0001 | **426.47** | **<0.0001** | 429.49 | <0.0001 | 429.83 | <0.0001 |
| Chi-square improvement | 1 | | | 0.03 | > 0.05 | **113.45** | **<0.0001** | 3.01 | > 0.05 | 0.34 | > 0.05 |
| Paternal population | 1 | 246.16 | <0.0001 | 246.18 | <0.0001 | **325.42** | **<0.0001** | 328.22 | <0.0001 | 328.42 | <0.0001 |
| Female hybrid type ($G^{SR}S$ or GS) | 1 | 39.97 | <0.0001 | 39.43 | <0.0001 | **54.45** | **<0.0001** | 48.69 | <0.0001 | 46.92 | <0.0001 |
| Fertility | 1 | | | 0.03 | 0.8616 | **5.34** | **0.0209** | 5.26 | 0.0218 | 5.31 | 0.0212 |
| PatPop*Fertility | 1 | | | | | **113.45** | **<0.0001** | 115.99 | <0.0001 | 112.94 | <0.0001 |
| Female*Fertility | 1 | | | | | | | | | 0.34 | 0.5594 |
| Female*PatPop | 1 | | | | | | | 3.01 | 0.0826 | 3.05 | 0.0805 |

Distribution is normal, with ML estimation