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The genetics of hybrid male sterility between the allopatric species pair *Drosophila persimilis* and *D. pseudoobscura bogotana*: Dominant sterility alleles in collinear autosomal regions

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ABSTRACT

F_1 hybrid male sterility is thought to result from interactions between loci on the X chromosome and dominant-acting loci on the autosomes. While X-linked loci that contribute to hybrid male sterility have been finely localized in many animal taxa, their dominant autosomal interactors have been more difficult to localize precisely and/or have been shown to be of relatively smaller effect. Here, we identified and mapped at least four dominant autosomal factors contributing to hybrid male sterility in the allopatric species pair *Drosophila persimilis and D. pseudoobscura bogotana*. Using these results, we tested predictions of reduced recombination models of speciation. Consistent with these models, three of the four QTLs associated with hybrid male sterility occur in collinear (uninverted) regions of these genomes. Furthermore, these QTLs do not contribute significantly to hybrid male sterility in crosses between the sympatric species *D. persimilis* and *D. pseudoobscura pseudoobscura*. The autosomal loci identified in this study provide the basis for introgression mapping and, ultimately, for molecular cloning of interacting genes that contribute to F_1 hybrid sterility.

HALDANE's (1922) rule observes that, in general, when one sex of hybrids between species is sterile or inviable, it is more frequently the heterogametic sex. The causes of this rule have been studied extensively (see reviews by WU *et al.* 1996; LAURIE 1997; ORR 1997), and much of the pattern seems to be explained by the "dominance theory" (MULLER 1942; ORR 1993; TURELLI and ORR 1995), which posits that sterility or inviability in the heterogametic sex often results from deleterious interactions between loci on a hemizygous X chromosome and dominant-acting loci on the autosomes. While several X-linked loci contributing to hybrid male sterility have been precisely localized (e.g., GUENET *et al.* 1990; OKA *et al.* 2004; STORCHOVA *et al.* 2004), especially within Drosophila (e.g., CABOT *et al.* 1994; PEREZ and WU 1995; MacDONALD and GOLDSTEIN 1999; ORR and IRVING 2001), dominant autosomal effects have typically been crudely localized or of comparatively small effect (e.g., MOEHRING *et al*. 2006; but see SLOTMAN *et al*. 2004).

The genetics of hybrid male sterility has been studied in the *Drosophila pseudoobscura-D. persimilis* species pair for over 70 years. The USA subspecies of *D. pseudoobscura* (*D. p. pseudoobscura*) co-occurs with *D. persimilis* on the west coast of North America. The two species have been shown to hybridize in natural populations at very low levels (DOBZHANSKY 1973; POWELL 1983), and variable amounts of introgression have been detected across regions of their genomes (MACHADO *et al.* 2002; MACHADO and HEY 2003; HEY and NIELSEN 2004). The Bogota subspecies of *D. pseudoobscura*, *D. p. bogotana*, occurs allopatrically in South America. *D. pseudoobscura* and *D. persimilis* diverged between 0.5 and 1.0 million years ago

(AQUADRO *et al*. 1991; WANG *et al.* 1997; LEMAN *et al*. 2005) while *D. pseudoobscura* and *D. p. bogotana* diverged between 150,000 and 200,000 years ago (SCHAEFFER and MILLER 1991; WANG *et al*. 1997). Hybrid males between *D. persimilis* and either *D. pseudoobscura* subspecies are viable but sterile, while hybrid females are fertile, consistent with HALDANE's (1922) rule. Previous studies have mapped the underlying genetic factors that contribute to hybrid sterility between the sympatric species pair (DOBZHANSKY 1936; ORR 1987, 1989; NOOR *et al.* 2001). These factors are strongly associated with three inversions (two on the X and one on the 2nd chromosome) that are fixed or nearly fixed, differentiating *D. pseudoobscura* and *D. persimilis*: Because these sterility-conferring loci are associated with inversions, they have not been precisely localized.

Additionally, because *D. pseudoobscura* and *D. persimilis* differ by these inversions and show multiple forms of pre- and postzygotic isolation, they have been a suitable system in which to study the effect of recombination on the evolution and maintenance of reproductive isolation. Recombination between genomes can potentially prevent the evolution or persistence of co-adapted gene complexes that confer species-specific adaptations and/or reproductive isolation between species. Thus, suppressing such recombination can allow the persistence of species despite occasional gene flow. One means for suppressing recombination and for facilitating species persistence is through chromosomal rearrangements: crossover products are not recovered from heterozygotes (hybrids) for such rearrangements.

Several empirical studies (e.g., RIESEBERG *et al.* 1999; FEDER *et al*. 2003, PANITHANARAK *et al*. 2004, STUMP *et al*. 2005), including studies in the *D.*

pseudoobscura group (NOOR *et al.* 2001), support the role for chromosomal rearrangements and other regions of suppressed recombination (e.g., centromeric regions; STUMP *et al.* 2005) in hybrids in preserving gene complexes that confer reproductive isolation (see reviews in ORTIZ-BARRIENTOS *et al.* 2002; BUTLIN 2005). Furthermore, theoretical models show that chromosomal rearrangements can facilitate the accumulation of hybrid incompatibilities between parapatric populations (e.g., NAVARRO and BARTON 2003; KIRKPATRICK and BARTON 2006). In the *D. pseudoobscura* group, BROWN *et al*. (2004) show that genetic factors contributing to pre- and postzygotic isolation are associated with inverted regions of the genome in the sympatric species *D. pseudoobscura* and *D. persimilis* but are associated with both inverted and probably uninverted (i.e., collinear) regions in the allopatric species *D. p. bogotana* and *D. persimilis*. The reduced recombination model of speciation directly predicts this association. However, these putative collinear-region effects were not mapped and may have been complicated by an additional fixed inversion difference between *D. p. bogotana* and *D. persimilis* on the third chromosome.

Here, we build on the results of previous studies in two significant ways. First, we use 26 microsatellite markers to demonstrate that hybrid male sterility between the allopatric species *D. p. bogotana* and *D. persimilis* maps to dominant-acting autosomal regions of the genome outside of the inversions that distinguish these species. We show that at least some of these regions do not confer hybrid sterility between the sympatric species *D. pseudoobscura* and *D. persimilis*, as predicted by the reduced recombination models of speciation. Second, we localize these dominant autosomal genetic factors to regions of the 2nd, 3rd, and 4th chromosomes using a large-scale backcross analysis. These

factors are among the first dominant autosomal factors contributing to hybrid male sterility to be precisely mapped, and this study thus provides the basis for introgression mapping and ultimately, molecular cloning, of interacting genes that contribute to F_1 hybrid sterility.

MATERIALS AND METHODS

Fly stocks and crosses: *Drosophila pseudoobscura bogotana* females carrying a *white* eye mutation (hereafter bogw) were collected as virgins and maintained for seven days. On day eight, bogw were crossed to *D. persimilis* MSH 1993 (hereafter per) males. F_1 females were backcrossed to bogw males to generate backcross males (hereafter BCbogw males) for fertility assays. Only male progeny bearing the *white* mutation were scored. The bogw strain is a subculture of the *D. p. bogotana* El Recreo line collected in 1978 (provided by H. A. ORR). The per line was derived from females collected at Mt. St. Helena, California in 1993 (NOOR 1995). All crosses were performed on standard sugar/yeast/agar medium at 20 ± 1^{o} C and 85% relative humidity.

Fertility assays of BCbogw males: BCbogw males were collected as virgins and maintained for seven days in vials containing 10 to 20 males. On day eight, the fertility of each backcross male was assessed by dissection of the testes in Ringer's solution following the method of COYNE (1984). A male was scored "fertile" if at least one motile sperm was observed and "sterile" if no motile sperm were observed. Treating fertility as a binary trait has been shown to be conservative (CAMPBELL and NOOR

2001), though other methods of scoring fertility exist (e.g., WHITE-COOPER 2004). All dissected BCbogw males were labeled and stored at –20^{o} C.

Microsatellite genotyping of BCbogw males: DNA was extracted from all dissected BCbogw males following the protocol of GLOOR and ENGELS (1992). Microsatellite genotyping was performed in two steps. First, all 4853 BCbogw males were genotyped for markers associated with each inversion that distinguishes *D. pseudoobscura* (and *D. p. bogotana*) and *D. persimilis*. The markers used for this initial screen were: DPSX002 (chromosome arm XL); DPSX030 (XR); and *bicoid* (bcd; 2) (ORTIZ-BARRIENTOS *et al.* 2006). These markers identified the inversion arrangement on these chromosome arms. Second, because we were interested in localizing sterility-conferring alleles that map outside the inverted regions between these species, only those 1102 BCbogw males that were hemi- or homozygous for the *D. p. bogotana* allele at the three inversion markers (hereafter BCbogwLim males) were further genotyped. This procedure thus would identify dominant *D. persimilis* alleles that interact with a predominantly *D. p. bogotana* genetic background. Surveys of other markers along the X-chromosome showed that this procedure also essentially selected for an almost complete *D. p. bogotana* X-chromosome as well as much of the second chromosome.

BCbogwLim males were genotyped for 23 microsatellite markers distributed evenly on the 2nd, 3rd, and 4th chromosomes. Primer sequences for all markers used in this study are available in the Appendix/online supplementary information. PCR amplification followed a touchdown protocol: 95° for 1 min; 3 cycles of 94° for 30 sec,

56° for 30s, 72° for 30 sec; 3 cycles of 94° for 30 sec, 56° for 30 sec, 72° for 30 sec; and 30 cycles of 94° for 30 sec, 50° for 30 sec, 72° for 30 sec. PCRs were visualized on acrylamide gels on LiCor 4200 DNA sequencer/analyzers.

Mapping hybrid male sterility: QTL mapping was first performed with composite interval mapping (CIM) (ZENG 1994) using Windows QTL Cartographer V. 2.5 (WANG *et al*. 2006). We focus on our CIM results rather than our other analysis (see below) because of the longer history of confirmation of effects initially mapped using CIM. Fertility was treated as a binary trait (the presence or absence of sperm, see above). Though this violates the assumption of normality in CIM, a previous study (MOEHRING *et al*. 2004) has shown that this treatment gives essentially the same result as when a trait is continuous, if CIM is based on logistic regression (e.g., XU and ATCHLEY 1996). Thresholds for significance were set by permutations (experiment-wise $P = 0.05$ and N = 1000).

Nonetheless, because our dataset does violate an assumption of the CIM procedure, the QTLs detected using CIM were further confirmed using the new binary multiple interval mapping (bMIM) (LI *et al.* 2006) procedure in Windows QTL Cartographer V.2.5 (WANG *et al.* 2006). Results from both forward and backward regression methods on markers are reported.

F_1 hybrid male sterility is thought to result from epistatic interactions between recessive X-linked and dominant autosomal loci (MULLER 1942; ORR 1993; TURELLI and ORR 1995). We do not explicitly test for epistasis in this study because we have limited the dataset to just those males bearing an X-chromosome from *D. p. bogotana*,

hence identifying dominant autosomal loci contributing to sterility derived from *D. persimilis*. While there may be epistasis among autosomal loci, bMIM currently does not include a test for epistasis (S. WANG, personal communication), and such a test is beyond the scope of the hypotheses we examine in this study.

We evaluated whether the same QTLs are associated with hybrid male sterility in backcross hybrids between per and *D. pseudoobscura* (hereafter, ps) vs. between per and *D. p. bogotana* (hereafter, bog) using a three-way contingency test based on a log-linear model (SOKAL and ROHLF 1995). For the per-ps hybridization, we used the raw data from our previous mapping study (NOOR *et al.* 2001), limited the dataset to those backcross hybrid males bearing the three inversion-associated markers from *D. pseudoobscura*, and examined the effects of markers closest to the ones surveyed in our per-bog backcross. Markers DPS2003 and DPS3001 were surveyed in both crosses and their associations with hybrid male sterility were compared in this manner. On the 4th chromosome, we did not have data for markers immediately adjacent to the per-bog sterility QTL in the per-ps backcross. Thus, for the 4th chromosome QTL, we performed a more conservative three-way contingency test using a marker even further from the sterility QTL in per-bog (DPS4G1e) than the nearest marker surveyed in per-ps (*Adh*).

RESULTS

Controlling for the effects of 3 microsatellite markers (DPSX002, DPSX030, and *bcd*; see MATERIALS AND METHODS) associated with the inversion differences

between *D. persimilis* and *D. p. bogotana* allowed us to detect QTLs that confer hybrid male sterility occurring outside the chromosomal rearrangements that distinguish these species. Using 26 microsatellite markers, we mapped using CIM at least four autosomal dominant QTLs with large effects on hybrid male sterility that interact with a predominantly *D. p. bogotana* genetic background. Furthermore, we were able to localize two of these four QTLs to relatively small regions: On the 2nd chromosome, a QTL was localized to an interval of about 840 Kb between markers DPS2-390p and DPS2-534j (Fig. 1A). The annotated part of this region contained 104 genes, based on sequence homology to *D. melanogaster* (GILBERT 2005; see Supplementary Table 1). The second QTL on this chromosome is associated with marker DPS2-1206e; we could not localize the size of this genomic region due to a lack of markers beyond DPS2-1206e, which lies about 17 Kb from the centromeric end of the 2nd chromosome sequence assembly. On the 4th chromosome, a QTL was localized to an interval of about 1.2 Mb between markers DPS4G1h and DPS4033b (Fig. 1B) and is closely associated with marker DPS4G1a. The annotated part of this region contained 136 genes (GILBERT 2005; see Supplementary Table 1). We were unable to refine the location of the 3rd chromosome QTL further because of an inversion difference between *D. persimilis* and *D. p. bogotana*. The biological and molecular functions of the 239 genes contained in the two smaller QTL intervals were evaluated using PANTHER (MI et al. 2005) software and are included in Supplementary Table 2. Figure 2 shows the relative effects of these QTLs individually and in combination on hybrid male sterility.

We further confirmed the presence and location of these QTLs using bMIM. Table 1 shows the additive effects associated with the three major QTLs, as well as the

microsatellite markers flanking each QTL, for both regression forward and backward selection on markers. The results from bMIM (Table 1) and those from CIM (Figure 1) were highly comparable, aside from minor movements of the exact peak location. Using a backward regression method in bMIM also detected the fourth QTL near the centromeric end of chromosome 2 (data not shown), as did CIM.

Previously, BROWN *et al.* (2004) showed that genes likely associated with collinear regions significantly decreased fertility in hybrids between *D. persimilis* and *D. p. bogotana* but do not have detectable effects on fertility in hybrids between *D. persimilis* and *D. pseudoobscura*. This analysis was based on a comparison between the proportion of sterile males from a *D. p. bogotana* backcross and the proportion of sterile males from a *D. pseudoobscura* backcross. Here, we directly evaluate whether the same collinear-region QTLs are associated with hybrid male sterility in hybrids between the two hybridizations by using a three-way contingency test based on a log-linear model (SOKAL and ROHLF 1995). In backcross hybrids, the major QTLs on the 2^{nd}, 3^{rd}, and 4^{th} chromosomes all contribute significantly to hybrid male sterility in hybrids between *D. persimilis* and *D. p. bogotana* but do not contribute significantly to sterility between *D. persimilis* and *D. pseudoobscura,* and the differences in effect are all statistically significant ($G^2 = 94.05$, $P < 0.001$; $G^2 = 159.97$, $P < 0.001$; and $G^2 = 61.72$, $P < 0.001$, respectively; Supplementary Table 1). Thus, these genomic regions contribute significantly more to hybrid male sterility in hybridizations of the allopatric species than in hybridizations of the sympatric species, as predicted by the reduced recombination model of speciation.

DISCUSSION

In this study, we fine-map at least three *Drosophila persimilis* autosomal dominant QTLs that confer hybrid male sterility in a *D. p. bogotana* genetic background. These QTLs are located outside the chromosomal inversions that differentiate the two arms of the X-chromosome and on the 2nd chromosome in *D. persimilis* and *D. p. bogotana*. Furthermore, we demonstrate that the effects of these QTLs on hybrid male sterility are greater in crosses between *D. persimilis* and *D. p. bogotana* than between *D. persimilis* and *D. pseudoobscura*.

These results confirm a prediction of the reduced recombination model of speciation: in allopatric species where the potential for gene flow does not exist, genetic factors associated with reproductive isolation should reside both within and outside genomic regions experiencing reduced recombination (in this case, within and outside chromosomal inversions); in sympatric species, factors associated with reproductive isolation should reside preferentially within genomic regions experiencing reduced recombination (within inversions). Thus, in the *D. pseudoobscura* species group, chromosomal rearrangements appear to contribute to the maintenance of species persistence by restricting recombination between genomic regions that contain genetic factors underlying reproductive isolation, while gene flow has homogenized collinear regions.

Chromosomal rearrangements have also been proposed to contribute directly to reproductive isolation via strong underdominance (WHITE 1969) resulting from meiotic difficulties. This type of chromosomal speciation model is distinct from the

recombination-reduction models in that hybrid sterility results from the chromosomal rearrangements directly, not from effects of loci captured within the rearrangement. Although bearing some recent support (DELNERI *et al.* 2003), this model remains controversial (COYNE and ORR 2004). In contrast, our results directly support a prediction of the reduced recombination chromosomal speciation models.

A previous study by BROWN *et al.* (2004) showed that almost all hybrid males between *D. persimilis* and *D. p. pseudoobscura* homozygous or hemizygous for the three inverted regions were fertile. In contrast, only about one-third of the male hybrids between *D. persimilis* and *D. p. bogotana* homozygous or hemizygous for the three inverted regions were sterile, suggesting that other factors conferring sterility likely occurs in collinear regions. By selecting only those *D. p. bogotana* backcross hybrid males that were homozygous or heterozygous for the three inversions, we built on this work by directly localizing these additional hybrid male sterility loci to small regions on the 2nd and 4th chromosomes and a region of the 3rd chromosome. Our findings are also consistent with DNA sequence surveys of these species, which indicate extensive introgression in collinear regions between *D. pseudoobscura* and *D. persimilis* but probably not between *D. persimilis* and *D. p. bogotana* (MACHADO and HEY 2003).

The sterility effects of these three autosomal regions are dominant, as hybrid males heterozygous (i.e., carrying both the *D. persimilis* and the *D. p. bogotana* allele) for these loci are more likely to be sterile. The dominance theory proposed to explain Haldane's rule suggests that F_1 hybrid male sterility often results from a deleterious interaction between a hemizygous (recessive) sex-chromosome effect and dominant autosomal effects. While many studies have precisely localized the recessive X-chromosomal

effects (GUENET *et al.* 1990; CABOT *et al.* 1994; PEREZ and WU 1995; MacDONALD and GOLDSTEIN 1999; ORR and IRVING 2001; OKA et al. 2004; STORCHOVA *et al.* 2004; MOEHRING *et al*. 2006), most have failed to pinpoint the locations of individually significant dominant autosomal effects with high confidence (but see SLOTMAN *et al.* 2004).

Although we identify at least three such dominant autosomal regions, the effect of each individual allele appears to be weak as well (Figure 2): no single factor caused complete or nearly complete sterility. Figure 2 shows that the effect on the sterility phenotype increases with the addition of each QTL, though they must interact with *D. p. bogotana* factors that were not identified in this study (such as the X-chromosome). As expected, the highest proportion of hybrid male sterility (83%) occurred in those hybrid males that were heterozygous for all four of the QTLs.

To attempt to examine whether genetic introgression has occurred between *D. persimilis* and *D. p. bogotana* in the major QTL intervals on the 2nd and 4th chromosomes, two 900-bp regions (one within each interval) were randomly selected for amplification and sequencing. The region on the 2nd chromosome contains coding DNA while the region on the 4th chromosome contains only non-coding DNA. These sequences were obtained for two *D. persimilis* strains, two *D. p. bogotana* strains, two *D. pseudoobscura* strains, and one *D. miranda* (outgroup) strain. Sequences obtained are available in GenBank (accession numbers EF392818 to EF392831). No fixed differences were detected between *D. persimilis* and *D. p. bogotana* or between *D. persimilis* and *D. pseudoobscura* in the ~1800-bp amplified so it appears that the differentiation associated with sterility between these species may be rather localized. We refrained for further

analysis of our sequence data because of the presently still coarse scale of our QTL mapping.

Because the QTLs identified in this study were mapped only in a single line of *D. persimilis* (MSH 1993) and in a single line of *D. p. bogotana* (El Recreo), it is also possible that the alleles detected in these hybrids could reflect intraspecific polymorphism for alleles associated with sterility. However, BROWN *et al.* (2004) did not observe dramatic differences in hybrid male sterility among three backcross lineages of *D. pseudoobscura* or among three backcross lineages of *D. p. bogotana*, suggesting that our mapping results may be representative of much of these species. Further, because precision-mapping of any phenotypic trait typically involves scoring hundreds, if not thousands, of individuals for that trait, this caveat applies to almost all QTL mapping studies published to date. Because this study localizes the QTLs to relatively small autosomal regions, it does provide a basis for future introgression studies of these factors that cause hybrid male sterility between *D. persimilis* and *D. p. bogotana* and for the eventual cloning of "sterility genes."

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Table 1. Location and additive effect of the three major QTLs detected using binary multiple-interval mapping (bMIM) (LI *et al*. 2006).

Forward regression

QTL	Chromosome	Additivity	Flanking Markers
1	2	2.634	DPS2-390p ; DPS2-534j
2	3	2.049	DPS3001 ; DPS3026
3	4	1.784	DPS4G1a ; DPS4033b

Backward regression

QTL	Chromosome	Additivity	Flanking Markers
1	2	2.047	DPS2-390p ; DPS2-534j
2	3	2.008	DPS3001 ; DPS3026
3	4	1.756	DPS4G1h ; DPS4G1a

Figure Legends

Figure 1—QTLs associated with hybrid male sterility on the (A) 2nd and (B) 4th chromosomes. Plots are the likelihood-ratio (LR) test statistic as determined by composite interval mapping on the y-axis. The significance thresholds were determined by permutation testing to be approximately LR = 7.8 and are indicated by horizontal dashed lines. Marker locations are represented by black triangles on the x-axis of the QTL plot. The markers flanking the significant QTLs are indicated by name. Above the QTL plots are diagrams of the chromosomes with the physical location of relevant markers indicated by hatch marks. An inversion is represented by the presence of an open oval. A bracket indicates the area magnified in the QTL plot below. Scale bars (representing either recombinational or physical distance) are given.

Figure 2—Relative effects of each QTL on hybrid male sterility. Hybrid male genotypes are given on the y-axis and sterility (as determined by sperm motility) is given on the x-axis. Heterozygous QTLs (hybrid males carry both the *D. persimilis* and *D. p. bogotana* alleles) are indicated by grey rectangles and homozygous QTLs (hybrid males carry only the *D. p. bogotana* allele) are indicated by white rectangles. A marker

associated with each QTL is used to determine the average sterility of males hetero- or homozygous for that QTL. N is the number of males of a given genotype.

Figure 1A

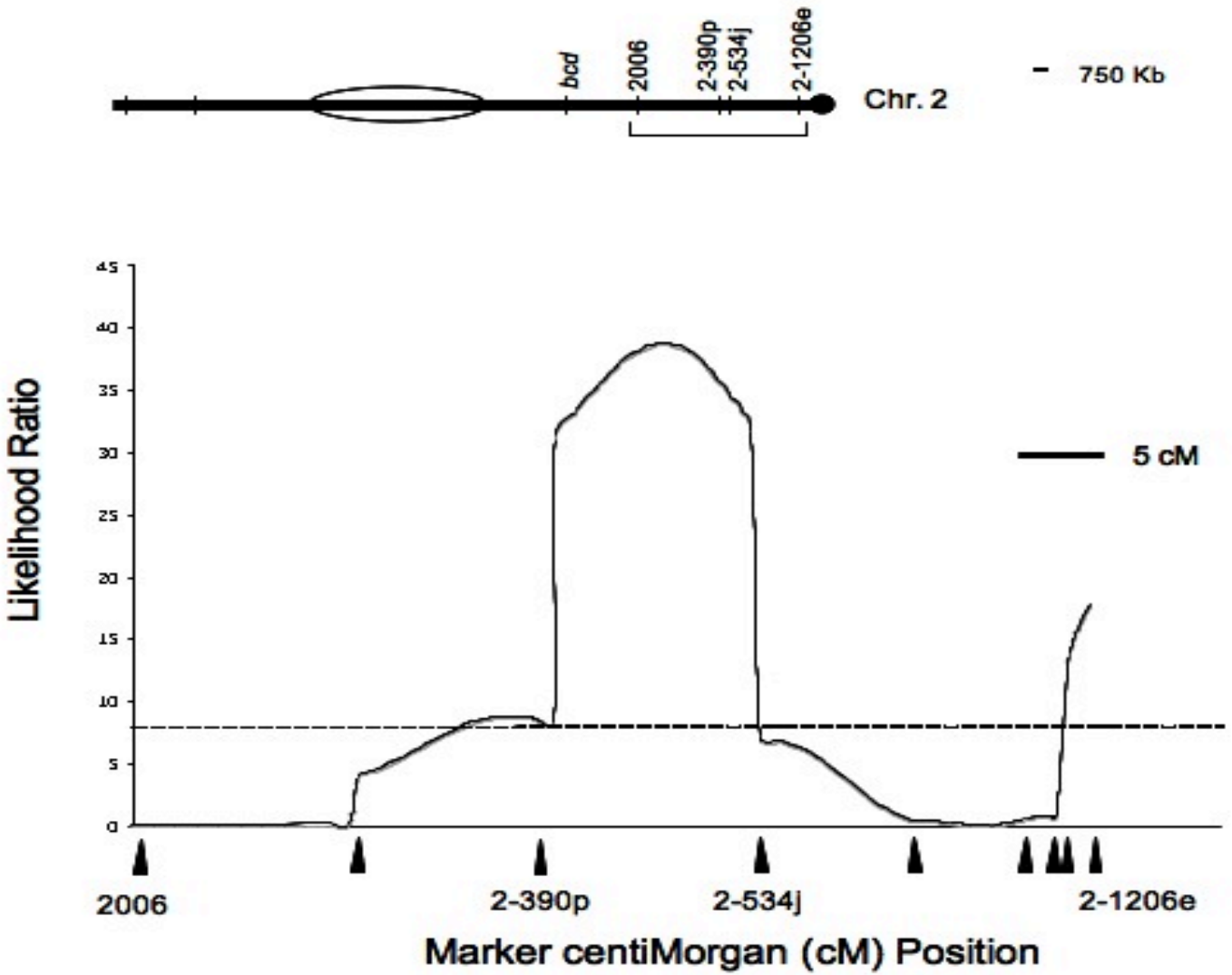

bcd
2006
2-390p
2-534j
2-1206e
Chr. 2
750 Kb
Likelihood Ratio
45
40
35
30
25
20
15
10
5
0
5 cM
2006
2-390p
2-534j
2-1206e
Marker centiMorgan (cM) Position

Figure 1B.

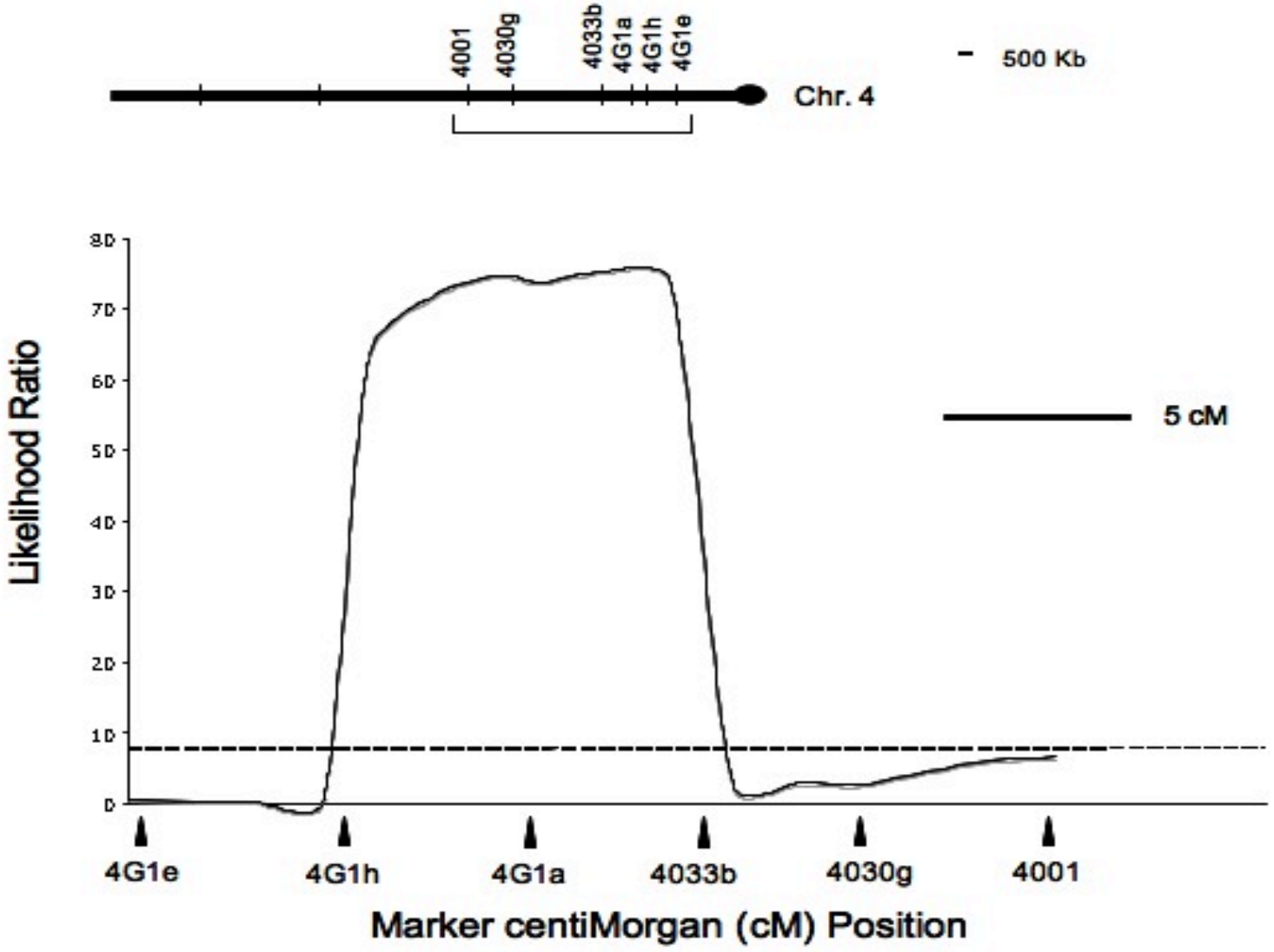
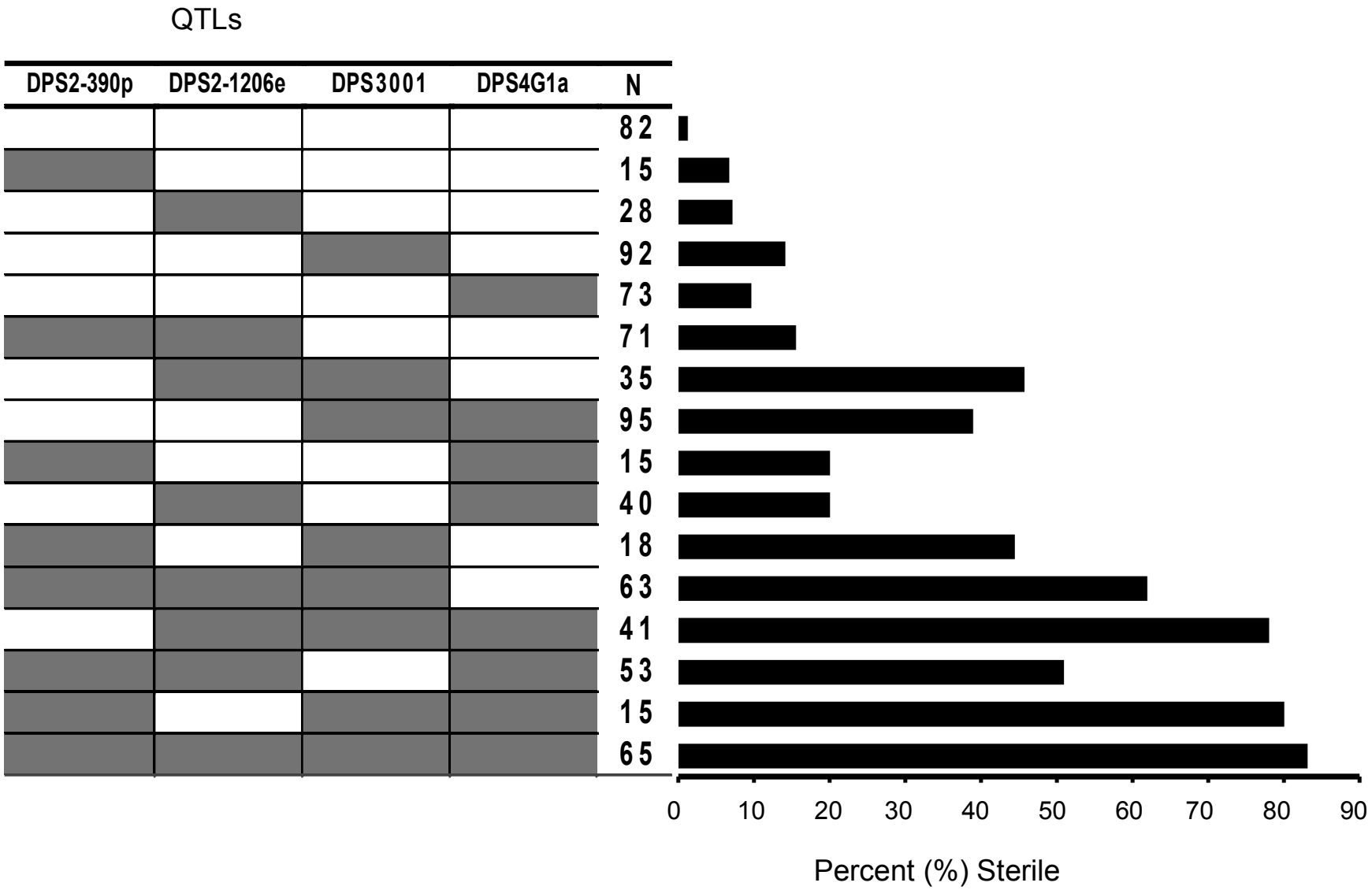

4001
4030g
4033b
4G1a
4G1h
4G1e
Chr. 4
500 Kb
Likelihood Ratio
80
70
60
50
40
30
20
10
0
5 cM
4G1e
4G1h
4G1a
4033b
4030g
4001
Marker centiMorgan (cM) Position

Figure 2.


QTLs
DPS2-390p
DPS2-1206e
DPS3001
DPS4G1a
N
82
15
28
92
73
71
35
95
15
40
18
63
41
53
15
65
0
10
20
30
40
50
60
70
80
90
Percent (%) Sterile