Original Contributions

Incorporating Mouse Genome
Mammalian Genome
Genes and Phenotypes

Genetic analysis of X-linked hybrid sterility in the house mouse

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Abstract

Hybrid sterility is a common postzygotic reproductive isolation mechanism that appears in the early stages of speciation of various organisms. *Mus musculus musculus* and *Mus musculus domesticus* represent two recently separated mouse subspecies particularly suitable for genetic studies of hybrid sterility. Here we show that the introgression of Chr X of *M. m. musculus* origin (PWD/Ph inbred strain, henceforth PWD) into the genetic background of the C57BL/6J (henceforth B6) inbred strain (predominantly of *M. m. domesticus* origin) causes male sterility. The X-linked hybrid sterility is associated with reduced testes weight, lower sperm count, and morphological abnormalities of sperm heads. The analysis of recombinant Chr Xs in sterile and fertile males as well as quantitative trait locus (QTL) analysis of several fertility parameters revealed an oligogenic nature of the X-linked hybrid sterility. The *Hstx1* locus responsible for male sterility was mapped near *DXMit119* in the central part of Chr X. To ensure full sterility, the PWD allele of *Hstx1* has to be supported with the PWD allelic form of loci in at least one proximal and/or one distal region of Chr X. Mapping and cloning of *Hstx1* and other genes responsible for sterility of B6–$X^{PWD}Y^{B6}$ males could help to elucidate the special role of Chr X in hybrid sterility and consequently in speciation.

Hybrid sterility is defined as a situation when two related taxa, each of which is fertile *inter se*, give rise to a hybrid that is sterile. The sterility typically affects the heterogametic (heterozygous) sex, thus obeying Haldane's rule (Haldane 1922). Although genetic analysis of hybrid sterility has been pursued, mostly in the *Drosophila* species, for over 70 years its genetic architecture, molecular mechanism, and preferential occurrence in heterogametic sex are poorly understood. Several lines of experimental evidence point to a special role of the X chromosome (Chr X) in these phenomena. A disproportionate effect of Chr X genes on hybrid sterility was observed in *Drosophila* interspecies backcrosses, a phenomenon designated ''large X-effect'' by evolutionary biologists (Coyne and Orr 1989). To explain Haldane's rule, Muller's dominance theory pointed out that recessive X-linked mutations can disrupt gametogenesis in heterogametic (XY) but not in homogametic (XX) sex (Muller 1940; Turelli and Orr 1995).

The role of Chr X in postzygotic reproductive isolation was also documented in mice, in experimental crosses between inbred strains originated from various mouse (sub) species. One of the few segregating hybrid sterility loci in (B6 × *M. spretus*) × B6 backcross was mapped on Chr X by Guenet and colleagues (1990). A more detailed study of X-linked hybrid sterility was performed by genetic analysis of a congenic strain B6.SPRET- $Hprt^{a}$, where a 17-cM segment of *M. spretus* Chr X was dissected in a series of subcongenics (Elliott et al. 2001).

When choosing a model system to analyze the genetic control of hybrid sterility, it is important to select a pair of species as closely related as possible, otherwise the identified genetic variants could represent a consequence rather than the cause of speciation. In the house mouse, *M. m. domesticus* and *M. m. musculus* represent two closely related subspecies, which separated from their last common ancestor between 350,000 (She et al. 1990) and 1,000,000 (Silver 1995) years ago, but which still

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display an incomplete reproductive barrier in their hybrid zone (Boursot et al. 1996; Munclinger et al. 2003; Sage et al. 1993). Hybrid sterility between *M. m. musculus* and *M. m. domesticus* was first described in the laboratory crosses between trapped wild mice of the *M. m. musculus* subspecies and the laboratory inbred strain C57BL/10, predominantly of *M. m. domesticus* origin (Forejt and Ivanyi 1974). The first gene engaged in hybrid sterility of the *M. m. musculus* × C57BL/10 strain (hybrid sterility 1, *Hst1*) was mapped on Chr 17 in a region of 0.5 cM or 350 kb (Forejt and Ivanyi 1974; Forejt et al. 1991; Gregorova et al. 1996; Trachtulec et al. 1997).

In this study we report on the genetic analysis of the hybrid sterility phenomenon caused by the introgression of Chr X of *M. m. musculus* origin (PWD inbred strain) into the B6 background (~80% of *domesticus* origin). The resulting consomic B6–$X^{PWD}X^{B6}$ females are viable and fully fertile, but B6–$X^{PWD}Y^{B6}$ males are almost completely sterile, in accordance with Haldane's rule.

Materials and methods

Mice. The PWD strain derived from wild mice *M. m. musculus* (Gregorova and Forejt 2000) has been established and maintained at the Institute of Molecular Genetics (IMG, Academy of Science, Czech Republic). The B6 strain was purchased from Jackson Laboratories (Bar Harbor, ME, USA) and the BALB/c strain was purchased from Velaz s.r.o. (Lysolaje, Czech Republic). To construct the B6–X^{PWD} consomic strain, (B6 × PWD)F_1 females were repeatedly backcrossed to B6 males. In each backcross generation, females with nonrecombinant Chr X of PWD origin were selected by genotyping with SSLP markers distributed along Chr X.

The principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) as well as specific Czech Law No. 246/1992 Sb were observed.

Genotyping. The following SSLP markers (Dietrich et al. 1994) with indicated absolute positions on the Chr X were used for genotyping: *DXMit55* (4 Mbp), *DXMit89* (8 Mbp), *DXMit81* (23 Mbp), *DXMit49* (24 Mbp), *DXMit166* (38 Mbp), *DXMit140* (44 Mbp), *DXMit92* (45 Mbp), *DXMit144* (48 Mbp), *DXMit87* (51 Mbp), *DXMit143* (53 Mbp), *DXMit142* (54 Mbp), *DXMit25* (56 Mbp), *DXMit119* (57 Mbp), *DXMit60* (63 Mbp), *DXMit93* (72 Mbp), *DXMit128* (78 Mbp), *DXMit114* (83 Mbp), *DXMit16* (84 Mbp), *DXMit170* (88 Mbp), *DXMit173* (112 Mbp), *DXMit234* (124 Mbp), *DXMit31* (144 Mbp). The marker positions were determined according to the Ensembl Mouse Genome Server (http://www.ensembl.org/Mus_musculus/) based on the NCBI Mouse Genome build 30. Genomic DNA for genotyping was obtained from the mouse tail as follows: 2 mm of a tail were added to 600 µl of 50 mM NaOH, heated to 95°C for 90 min, vortexed, and neutralized with 50 µl of 1 M Tris (pH 8). Amplification of microsatellite markers was performed in a PTC 225 thermal cycler (MJ Research) under the following conditions: 95°C for 2 min (94°C for 30 sec, 55°C for 40 sec, 72°C for 40 sec) for 36 cycles, then 72°C for 5 min. PCR in a 15-µl volume contained: 1× PCR buffer comprising 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P40, and 1.5 mM $MgCl_2$ (Fermentas); 0.2 mM of each dNTP (Fermentas); 0.33 µM of each primer (Invitrogen), 0.3 U Taq polymerase, and 0.5 µl DNA prepared as described above. For amplification of SSLP markers that did not amplify under these conditions, we used PCR buffer with $(NH_4)_2SO_4$ (Fermentas) and annealing temperature 51°C. PCR products were separated on a 4% agarose gel and visualized with ethidium bromide.

Analysis of male fertility. To analyze male fertility, adult males (2–6 months old) were mated for 2–3 months with three BALB/C females. The number of offspring was counted as the average number of sired offspring per mating unit, OMU (1 OMU = one male caged with one female for 30 days). After mating, the males were sacrificed, testes weighed, and the number of spermatozoa in cauda epididymides was counted.

Morphological analysis of spermatozoa. Spermatozoa obtained from the cauda epididymides were washed twice with phosphate-buffered saline (PBS), pH 7.2, followed by centrifugation for 10 min at 600 *g*, and then fixed with methanol:acetic acid (3:1) and loaded on glass slides. Preparations of spermatozoa were observed under a light microscope.

Acrosomal status tested by monoclonal antibody. Monoclonal antibody against boar acrosin (ACR.2), which cross-reacts with mouse epididymal spermatozoa, was used for determination of the acrosome state of mouse spermatozoa by the immunohistochemical method as described (Peknicova et al. 2002; Peknicova and Moos 1990).

Histology of testis. Testes were fixed in Bouin solution, embedded in paraffin, sectioned to 5-µm slices, and stained with hematoxylin–eosin according to standard procedures.

Table 1. SSLP (MIT) markers used to distinguish B6 and PWD allelic forms. Current position based on analysis of 346 backcross animals

Marker	*MGI position (cM)*	*Current position (cM)*	*Ensembl position (Mbp)*
DXMit55	1.4	(1.4)	3.5
DXMit81	9.3	11.8	22.6
DXMit49	13	13.8	23.8
DXMit166	15.5	23.9	38.2
DXMit140	19	29.1	43.7
DXMit119	29.5	34.1	56.6
DXMit16	37	40.4	84.2
DXMit170	41.5	44.5	87.9
DXMit173	50.5	51.4	112.2
DXMit234	60	64.1	123.8
DXMit31	72	71.9	143.9

QTL analysis. Interval mapping generating likelihood ratio statistics (LRSs) was done with the aid of computer software MapManager QTX, version b19, freely available at http://www.mapmanager.org/ (Manly et al. 2001). The permutation test available in MapManager QTX was used to calculate LRS thresholds for suggestive, significant, and highly significant linkage. In the permutation test, 1000 permutations in 1-cM steps were generated and the additive regression model was fitted for the permuted data. Confidence intervals for particular QTLs were estimated by bootstrap analysis (Walling et al. 1998) available in the MapManager QTX. The same software was also used to search for interaction effects between pairs of QTL loci.

Results

Introgression of the M. m. musculus Chr X from the PWD strain into the B6 genetic background. To construct the B6–X^{PWD} consomic strain (also designated as a chromosome substitution strain), Chr X from the PWD inbred strain was introgressed by repeated backcrossing into the B6 genetic background, predominantly of *M. m. domesticus* descent. Eleven SSLP markers (Dietrich et al. 1994) distributed across the X chromosome were used to select the backcross animals carrying the nonrecombinant X^{PWD} chromosome (Table 1). The most proximal and the most distal markers were situated at positions 3.5 Mbp (*DXMit55*) and 143.9 Mbp (*DXMit31*), respectively (Hubbard et al. 2002) (http://www.ensembl.org/Mus_musculus/ ENSEMBL v19.30.1). Because of the lack of suitable markers, we could not monitor the allelic forms of the terminal 6.1-Mbp segment, including the pseudoautosomal region.

Fertility of males changed in the course of repeated backcrosses. The (PWD × B6)F_1 hybrid males were completely sterile with small testes, early meiotic arrest, and no sperm in the ductus epididymis, as described (Gregorova and Forejt 2000). At the early BC generations, some males with the nonrecombinant X^{PWD} chromosome became fertile. From the BC_5 generation further on, all nonrecombinant B6–$X^{PWD}Y^{B6}$ males were fully sterile. In contrast to F_1 hybrids, the sterile B6–$X^{PWD}Y^{B6}$ males displayed a complete meiotic cycle with the sperm present in ductus epididymides (see below). All B6–$X^{PWD}X^{B6}$ females were viable and fertile.

Uneven distribution of recombination events along Chr X. The SSLP (MIT) markers were selected according to the consensus genetic map (MGI 2.96, http://www.informatics.jax.org) (Blake et al. 2003) with a 12-cM maximum distance between neighboring loci (Table 1). However, the actual recombination frequencies as measured in our backcrosses were significantly different from the consensus map, with a 4.05-fold enhancement in the *DXMit49–DXMit166* interval and a 0.47-fold depression ($p < 0.01$) in the *DXMit140– DXMit*119 region. This variation could be explained by a PWD-specific cold spot and hot spot of recombination or by small structural rearrangement(s), such as inversion(s), causing recombination suppression and adjacent compensatory enhancement. A closer inspection of the MGI database showed that most if not all genetic mapping data came from interspecies crosses involving *M. spretus*. Thus, the interpretation of the observed cold and hot spots of recombination could be biased by possible structural variations between Chr Xs of *M. m. musculus*, *M. m. domesticus*, and *M. spretus*. Therefore, we projected the genetic map in centimorgans directly onto the physical map of the chromosome. The total genetic length of 70.6 cM in MGI and 70.5 cM in our present map corresponds to 140.4 Mbp on the physical map, the resulting average value of 0.5 cM/Mbp is in good agreement with the expected frequency of 0.6 cM/Mbp for the whole mouse genome (Waterston et al. 2002). Five out of ten measured intervals showed significantly enhanced or suppressed recombination

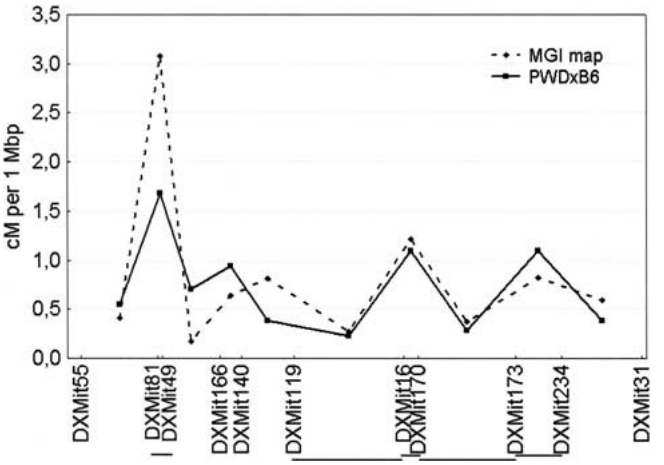

Fig. 1. Relation between genetic and physical distances in ten intervals of Chr X of the backcross (B6–X^{PWD} X^{B6} × B6) (n = 346). The physical distances between SSLP markers on the *X* axis are in scale. Five out often measured intervals show significantly enhanced or suppressed recombination (p < 0.05 with Bonferroni correction, marked by horizontal bars). The MGI consensus map is presented for comparison.

p < 0.05 with Bonferroni correction, see Fig. 1). The most prominent cold spot was seen in the *DXMit119– DXMit16* interval, which carries the main QTL locus for the X-linked hybrid sterility, as is shown below. Note that the variation in recombination frequency, measured as cM/Mbp, is entirely different from the results based on comparisons of the two genetic maps.

Fertility assessment of the (B6–X^{PWD} X^{B6} × B6) backcross males. Starting from BC_5, all tested B6–$X^{PWD}Y^{B6}$ males with the nonrecombinant X^{PWD} chromosome were completely sterile, and the X^{PWD} chromosome had to be transmitted solely through the fertile B6–$X^{PWD}Y^{B6}$ females. The males with recombinant Chr X displayed a range of variation in fertility impairment. To assess the fertility quantitatively, the following traits were scored: (i) number of offspring sired by one male with one female in one month (OMU), (ii) wet weight of paired testes, (iii) number of spermatozoa in cauda epidydimides, and (iv) percentage of morphologically abnormal spermatozoa. Besides these traits, we checked the integrity of acrosome in sperm heads and examined the seminal vesicle weight. The sterile B6–$X^{PWD}Y^{B6}$ males had a significantly lower testes weight and sperm count in comparison to their fertile littermates with nonrecombinant B6-derived Chr X (referred to here as B6–$X^{B6}Y^{B6}$) (p < 0.0001; Table 2). The seminal vesicle weight, reflecting the sensitivity to and the level of serum testosterone, did not differ between sterile and fertile males.

The majority of sperm heads of B6–$X^{PWD}Y^{B6}$ males were morphologically abnormal (Fig. 2). We observed sperm heads with slightly shortened hooks, sperm heads with shortened hooks and/or reduced distal parts, and, as an extreme, spermatozoa with fan-shaped or rhomb-shaped heads. Some of these spermatozoa also had an abnormal midpiece attachment site and some other displayed rare anomalies (Table 3). The stained acrosome did not differ between sterile and fertile males. The cauda epidydimis of sterile males contained some round spermatids not observed in fertile controls.

Histological sections from testes of sterile B6–$X^{PWD}Y^{B6}$ males showed disrupted integrity of the germ cell layer and premature release of germ cells into the lumen of seminiferous tubules (Fig. 3). These abnormalities were observed in 43% of the cross sections of seminiferous tubules in sterile males.

Towards mapping of the X-linked hybrid sterility genes. The fertility parameters were recorded in 163 BC_5-BC_8 male offspring from the backcrosses (B6–$X^{PWD}X^{B6}BC_{4–7}$ × B6). The number of offspring per mating unit (OMU) is an ultimate measure of infertility, yet most distant from the causative gene product. Based on the distribution pattern of OMU among the recombinant males, we arbitrarily selected OMU ≤ 2 as the full sterility borderline. For a more detailed mapping, an additional 11 SSLPs with distinct PWD and B6 alleles were used.

Mapping the Full Sterility Phenotype. Scatter plots of two classes of the Chr X single recombinants, PWD/B6 and B6/PWD, show dependency of the OMU and percentage of normal spermatozoa on the location of a PWD congenic segment in rec-

Table 2. Fertility parameters of B6–$X^{PWD}Y^{B6}$ consomic males

Genotype	*Testes weight (mg)*	*Sperm count × 10^{-6}*	*Seminal vesicle (mg)*
B6–$X^{PWD}Y^{B6}$	149 ± 20 (n = 21)	12.0 ± 3.0 (n = 9)	231 ± 79 (n = 12)
B6–$X^{B6}Y^{B6}$	221 ± 13 (n = 8)	21.8 ± 2.5 (n = 5)	275 ± 27 (n = 5)

The differences between sterile B6–$X^{PWD}Y^{B6}$ and fertile B6–X^{B6} Y^{B6} males in testes weight and sperm count are highly significant (p < 0.0001).

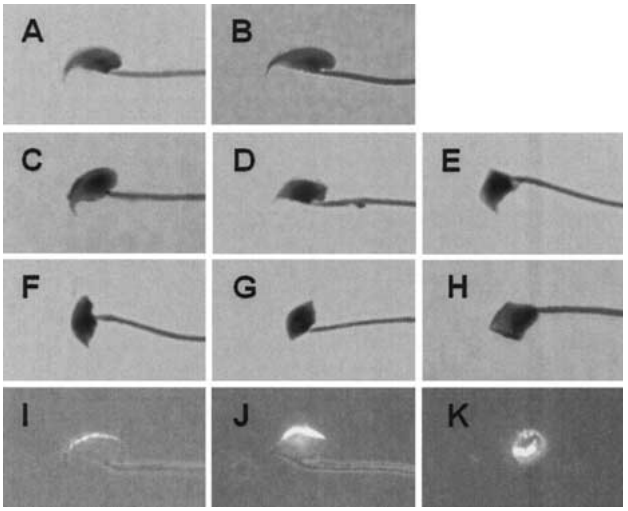

Fig. 2. Examples of morphologically abnormal sperm heads. (**A, B**) Sperm heads of control parental strains C57BL/6J (**A**) and PWD (**B**). Sperm heads of sterile B6–$X^{PWD}Y^{B6}$ males show various abnormalities: a slightly shortened hook (**C**), a shortened hook and/or a reduced distal part (**D–F**), fan-shaped (**G**) or rhomb-shaped (**H**) heads. Spermatozoa (**I, J**) and a round spermatid (**K**) with the acrosome labeled by antiacrosin antibody ACR.2.

ombinant Chr X (Fig. 4). The PWD/B6 recombinants with the PWD segment encompassing the centromere– *DXMit144* locus were fertile. However, when the recombination point moved 8 Mbp distally, behind *DXMit119*, the males became sterile, with two exceptions.

The reciprocal single recombinants, B6/PWD, were sterile when the PWD part of the chromosome included the telomeric end up *to DXMit119* or more proximally. When the recombination point was distal to *DXMit119*, the males became fertile (Fig. 4). Thus, the PWD allele of a locus designated hereafter hybrid sterility-X1, *Hstx1*, and located within the *DXMit119 region*, seems to be an indispensable component of hybrid sterility genetic control. However, the analysis of double recombinants clearly showed that the PWD allele of *Hstx1* is a necessary but not sufficient condition for sterility. At least one region proximal and or one region distal to the region encompassing the *Hstx1* gene must carry the PWD allelic form to keep the males sterile (data not shown). Mapping the recombination breakpoints in double recombinants was not always consistent with fertility parameters. To map additional loci more precisely, it will be necessary to analyze the phenotype of each recombinant Chr X in more than one male.

QTL Analysis of Fertility Impairment. The MapManager QTX software (version b19) (Manly et al. 2001) was used to treat the fertility parameters as quantitative traits to localize the respective QTLs on the Chr X map. For the OMU parameter, 86 Mbp encompassing the *DXMit119* and *Hstx1* showed the LRS > 14.9, which was the threshold for highly significant linkage. The highest LRS peak mapping the *Hstx1* QTL reached the value 112.7 (n =117; Fig. 5a) with a 99% confidence interval, estimated by bootstrap analysis as well as by 2-LOD support interval between *DXMit119* and *DXMit60* markers (57–63 Mbp).

The highest LRS for abnormal sperm head morphology (n = 160, LRS = 69.7; Fig. 5b) was also close to the *DXMit119* marker, but, in this case, unexpectedly all Chr X markers displayed a highly significant QTL linkage with LRS > 14.5. The testis weight and sperm count showed a major peak (n = 163, maximum LRS = 73.6; Fig. 5c and n = 121, maximum LRS = 30; Fig. 5d) in the same region as the *Hstxl* locus; however, a second major peak of a highly significant linkage was near the telomeric end between *DXMit173* and *DXMit234* markers.

The analysis of QTL–QTL epistatic effects revealed significant interactions between central (*DXMit87–DXMit119*) and proximal (*DXMit55–DXMit49*) parts in the case of abnormal sperm head morphology (interaction LRS = 35.2). For the testes weight, the interactions between central and distal (*DXMit170–DXMit31*) parts (interaction LRS = 10.3) were observed.

Discussion

The genome of a classical laboratory mouse strain such as B6 is a mosaic, which originated mainly

Table 3. Percentages of sperm-head abnormalities in B6–$X^{PWD}Y^{B6}$ consomic males and their parental controls

	Normal	*Shorter hook and/or reduced distal part*	*Fan-shaped or rhomb-shaped heads*	*Round spermatids in epididymis*	*Other abnormalities*
B6–$X^{PWD}Y^{B6}$	13.7	58.7	24.1	1.4	2.1
B6–$X^{B6}Y^{B6}$	75.4	15.4	5.5	0	3.7
B6	90.8	5.0	2.0	0	2.2
PWD	89.5	5.5	2.2	0	2.8

At least two animals of each genotype were analyzed. A minimum of 300 sperm were evaluated per animal.

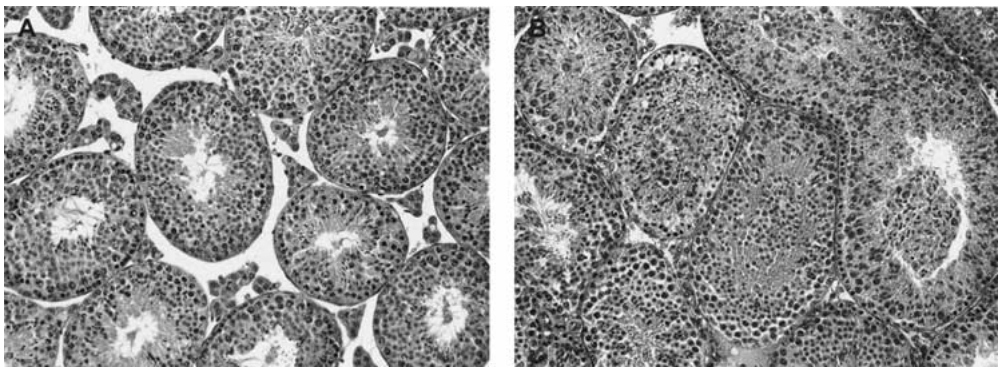

Fig. 3. Testicular cross-sections stained with hematoxylin–eosin. (**A**) Fertile B6–X^{B6} Y^{B6} male. (**B**) Sterile B6–$X^{PWD}Y^{B6}$ male. Seminiferous tubules of the sterile B6–$X^{PWD}Y^{B6}$ male show disrupted integrity of the germ cell layer and premature release of germ cells into the lumen of seminiferous tubules.

from the *M. m. domesticus* subspecies and about 20% from the *M. m. musculus* subspecies component (Wade et al. 2002; Yonekawa et al. 1980). The PWD inbred strain is 100% of *M. m. musculus* origin (Gregorova and Forejt 2000). Male but not female (PWD × B6)F_1 hybrids are sterile, obeying the classical Haldane rule of hybrid sterility. Thus, in spite of their artificial nature, B6 and PWD inbred strains and their hybrids can be utilized to assemble a laboratory model of hybrid sterility to get insight of the early event of speciation (Forejt 1996; Orr and Presgraves 2000; Wu and Ting 2004). In this study we investigated the role of Chr X in hybrid sterility, using the B6–$X^{PWD}Y^{B6}$ consomic male as a model. The male-limited hybrid sterility was caused by incompatibilities between Chr X of PWD (*M. m. musculus*) origin and the gene(s) in the genetic background of the B6 inbred strain, representing the *M. m. domesticus* subspecies. Our unpublished data show that the gene or genes incompatible with PWD Chr X do not lie on Chr Y. This result is not surprising since Chr Y of the B6 strain is apparently of Asian *M. m. musculus* origin (Tucker et al. 1992b). The consomic males had virtually no offspring, displayed significantly lower testes weight and sperm count, and their spermatozoa showed various degrees of morphological abnormalities. Disrupted integrity of the germ cell layer and premature release of germ cells into the lumen of seminiferous tubules indicated impaired interactions between Sertoli cells and germ cells.

Mapping of sterility genes on Chr X revealed, rather unexpectedly, the complex oligogenic nature of infertility. With 163 backcross males, all sharing the B6 genetic background, the expected resolution of the cross was 0.61 cM per backcross animal. However, such precision was not achieved because of the apparent multigenic nature of sterility and because of the occurence of ''exceptions.'' Seven males out of 163 tested appeared to be the exceptions, with their phenotype not matching their genotype in a sensible way. Such irregularity was not seen in the previous mapping of the *Hst1* gene (Gregorova et al. 1996), where none of the 183 genotyped backcross males behaved as an ''exception.''

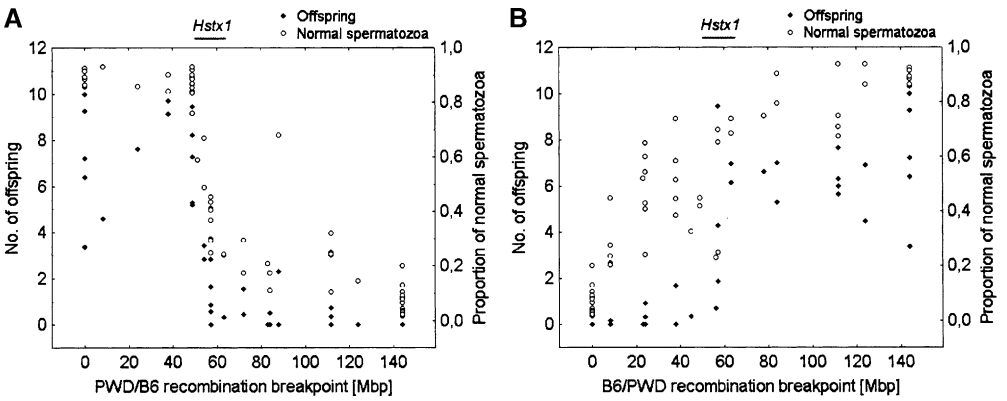

Fig. 4. Scatter plots of the two classes of the X chromosome single recombinants, PWD/B6 (**A**) and B6/PWD (**B**). The fertility decline in males with recombination breakpoint near the marker *DXMit119* (57 Mbp) indicates that this region encompasses gene/genes involved in the X-linked hybrid sterility.

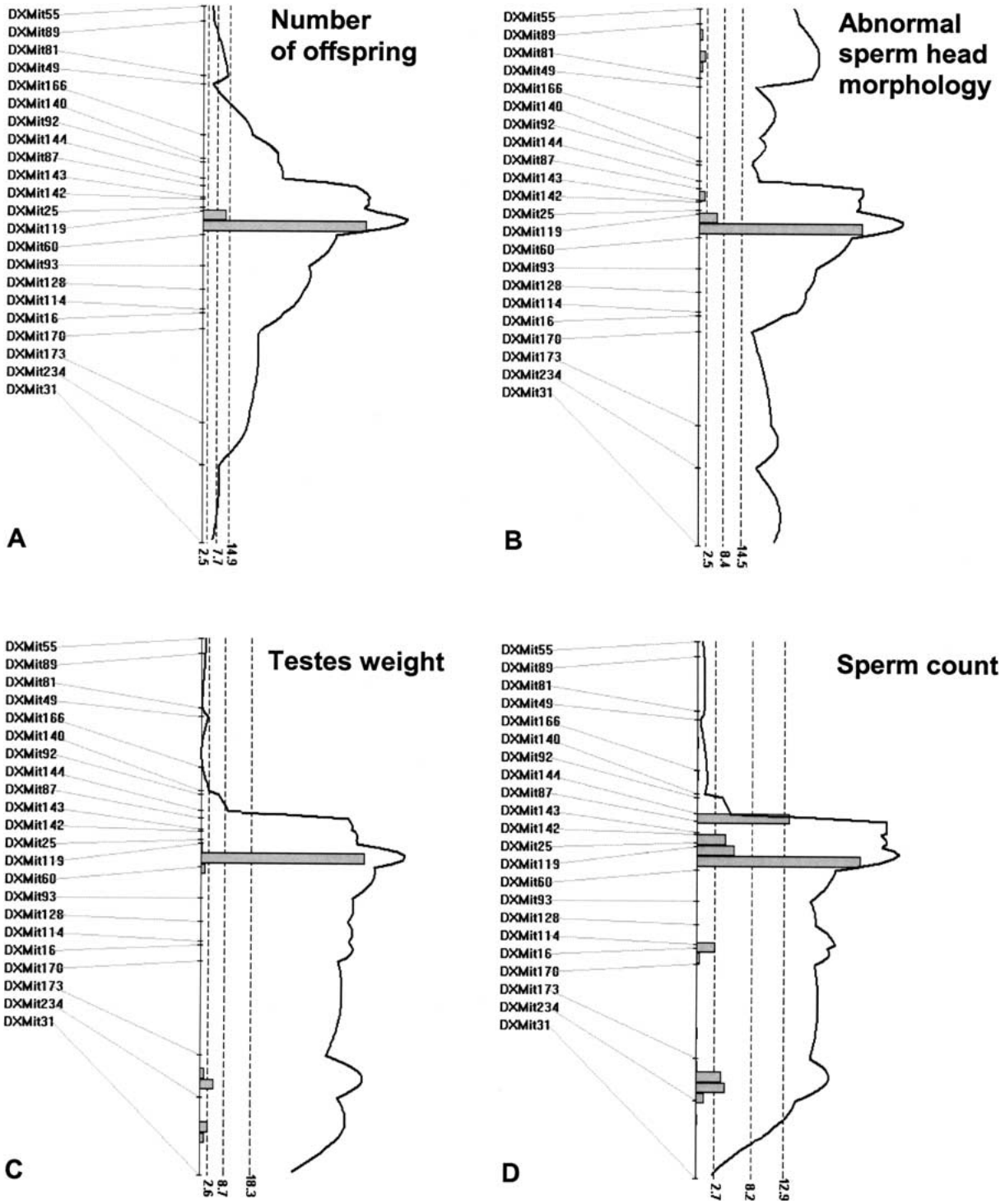

Fig. 5. LRS (likelihood ratio statistic) curves for the number of offspring (**A**), abnormal sperm head morphology (**B**), testes weight (**C**) and sperm count (**D**) created by MapManager QTX software. Broken lines indicate LRS thresholds for suggestive, significant, and highly significant linkage corresponding to the α values 0.63, 0.05, and 0.001, respectively. Histograms calculated by the bootstrap test show the confidence intervals for the particular QTLs on the chromosome.

Inspection of the Chr X recombination patterns, as well as QTL analysis, identified a new locus, designated *Hstx1*, as a major QTL for the OMU parameter. The data clearly showed that the *Hstx1* locus is necessary but not sufficient for the X-linked sterility.

In an attempt to construct a consomic strain carrying Chr X of *M. m. molossinus* and *M. m.*

castaneus on the genetic background of a mixed laboratory strain, the laboratory of Toshihiko Shiroishi (Takagi et al. 1994) reported male-limited infertility of their consomics. Preliminary mapping of a gene responsible for abnormal sperm heads was 8 cM proximal to *Tabby* in the case of *M. m. molossinus* Chr X. Although tentative, this map position fits well with our location of the *Hstx1* gene.

M. spretus is a mouse species which separated from a common ancestor of the *M. musculus* group about 3 millions years ago, much earlier than *M. m. musculus* and *M. m. domesticus* subspecies (Silver 1995). Elliott and colleagues (2001) prepared a hybrid congenic strain AT24 carrying 17 cM of Chr X from *M. spretus* on a B6 background. The males were subfertile and their testis weight was reduced to one half compared with the B6 parental strain. The *Ihtw1* locus controling testis weight was located proximal to *DXMit140* [MGI consensus map position 18.0 cM (Blake et al. 2003)]. The present data situated the *Hstx1* locus approximately 12 cM distal from *DXMit140*, closer to *DXMit119* on the MGI consensus map. Although the allelism of both loci seems unlikely, the ultimate assessment must await direct testing. The *Hstx1* locus is very closely linked to another *M. spretus–M. musculus* incompatibility locus, interspecific hybrid placental dysplasia (*Ihpd*) (Zechner et al. 1996), at position 32 cM. This locus is responsible for hyperplasia of placentae in hybrids where the female parent is of *M. spretus*, *M. macedonicus*, or *M. spicilegus* species and the male parent is *M. musculus*. Another formal similarity is that *Ihpd* and *Hstx1* loci appear to be members of families of multiple loci scattered on Chr X and acting synergistically to produce the abnormal phenotype (Hemberger et al. 1999; Montagutelli et al. 1996). QTL analysis revealed linkage of a large part of Chr X with placental dysplasia similar to how a major portion of Chr X showed linkage with OMU and abnormal sperm in the present study.

An alternative hypothesis explaining the involvement of an unusually large part of Chr X in the hybrid sterility of B6–$X^{PWD}Y^{B6}$ males is that rather than multiple hybrid sterility loci on Chr X, some epigenetic changes, such as abnormal chromatin structure of Chr X, could be responsible for sterility. A large body of mostly indirect evidence suggests that Chr X is transcriptionally silenced in primary spermatocytes (Forejt 1982; Lifschytz and Lindsley 1972; Turner et al. 2002). If the silencing and subsequent activation of Chr X chromatin depends on interaction with autosomal gene(s), then the size of the PWD portion of a recombinant Chr X could determine the extent of genetic misregulation. This model could explain some peculiarities of both X-linked hybrid sterility and placental dysplasia (imprinted X silencing in spongiotrophoblast). However, many untested premises should be verified prior to further elaboration of such an epigenetic model. Moreover, X silencing could not explain a similar autosomal phenomenon—correlation between the length of a *M. m. molossinus*-derived congenic segment of Chr 19 and the frequency of testicular germ cell tumors in the 129.MOLF/Chr19 consomic strain (Youngren et al. 2003).

In *Drosophila* interspecies crosses, the hybrid sterility loci sometime map to fixed inverted regions (Noor et al. 2001). There are two cold spots and three hot spots of recombination on Chr X when the present data are compared with the ENSEMBL physical map. The stongest cold spot maps to the interval harbored by the *Hstx1* locus and could indicate the presence of an inversion or another structural chromosomal abnormality.

A specific approach used by evolutionary biologists to identify the parts of the genome that play a role in reproductive isolation of closely related species is to study the degree of introgession of species-specific alleles and/or genomic markers across the hybrid zone. The regions with reduced introgression are likely to carry genes governing the reproductive isolation. Such studies on the hybrid zone of *M. m. domesticus* and *M. m. musculus* species with a limited number of Chr X markers indeed indicated reproductive isolation gene(s) in the proximal part of Chr X (Dod et al. 1993; Tucker et al. 1992a). Recently, Nachman and colleagues (manuscript submitted) evaluated the patterns of introgression across the hybrid zone of *M. m. domesticus* and *M. m. musculus* species using a panel of 13 diagnostic X-linked markers. They identified a locus corresponding to 34 cM on the MGI consensus map within the interval of the possible location of the *Hstx1* gene described here. If both loci prove to be identical, then the present laboratory model of hybrid sterility will enable mapping and cloning of a gene that acts in natural populations, preventing the gene flow between *M. m. musculus* and *M. m. domesticus* subspecies. Thus, the *Hstx1* gene could become a clue to elucidation of the old questions about the X chromosomal effects in hybrid sterility and in speciation.

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