Mamm Genome (2011) 22:648–660
DOI 10.1007/s00335-011-9353-3

Genetic dissection of testis weight in mice: quantitative trait locus analysis using F_2 intercrosses between strains with extreme testis weight, and association study using Y-consomic strains

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Received: 31 March 2011 / Accepted: 16 July 2011 / Published online: 21 August 2011

Abstract In the present study, dissection of genetic bases of testis weight in mice was performed. Autosomes and the X chromosome were searched using traditional quantitative trait locus (QTL) scans, and the Y chromosome was searched by association studies of Y-consomic strains. QTL analysis was performed in ♀DDD × ♂CBA F_2 mice; the inbred mouse DDD has the heaviest testes, whereas the inbred mouse CBA has the lightest testes. Two significant testis weight QTLs were identified on chromosomes 1 and X. A DDD allele was associated with increased and decreased testis weight at the locus on chromosomes 1 and X, respectively. In the reciprocal cross ♀CBA × ♂DDD F_2 mice, QTL on chromosome 1, and not on chromosome X, had a significant effect on testis weight. The DDD allele at the X-linked locus could not sustain testis weight in combination with the Y chromosome of the CBA strain. The Y chromosome per se had a significant effect on testis weight, i.e., DH-Chr Y^{DDD} had significantly heavier testes than DH-Chr Y^{CBA}. On the basis of the results of Y-chromosome-wide association studies using 17 Y-consomic strains, variations in *Uty*, *Usp9y*, and *Sry* were significantly associated with testis weight. Thus, testis weight is a complex quantitative phenotype controlled by multiple genes on autosomes and sex chromosomes and their interactions.

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Introduction

Testis weight is a physiologically important reproductive phenotype because of its direct connection with male fertility, i.e., spermatogenic ability. Indeed, the sperm production rate depends on testis weight, e.g., testis weight in polygamous males is higher than that in monogamous males in primates (Harcourt et al. 1981). A seasonal change in testis weight is reported in wild animals (seasonal breeders); they tend to have a higher testis weight during their breeding season (Leader-Williams 1979).

Testis weight is a representative quantitative phenotype that varies widely among inbred mouse strains. For example, at age 80 days, the average paired testis weight in the DDD/Sgn strain is 296.5 mg, whereas that in the CBA/N strain is only 105.2 mg (Suto 2008; unpublished data). This difference is not due to a difference in body weight because body weight does not vary significantly between these two strains, suggesting the existence of genes that primarily determine testis weight. Several investigators have examined the genetic basis of testis weight by quantitative trait locus (QTL) analysis, and many testis weight QTLs have been identified on mouse chromosomes (Zídek et al. 1998; Le Roy et al. 2001; Elliott et al. 2004; Oka et al. 2004; Storchová et al. 2004; Bolor et al. 2006; L'Hôte et al. 2007; Good et al. 2008; Vyskočilová et al. 2009; Otsuka et al. 2010). Analysis of a series of mouse mutants has also provided direct evidence for the presence of genes associated with testis weight (Mouse Genome Informatics [MGI], http://www.informatics.jax.org). Most of these genes or loci are autosomal or X-linked. Genes on the Y chromosome have also been implicated in the control of testis weight, although this result is controversial to some extent (Hayward and Shire 1974; Herrick and Wolfe 1977; Hunt and Mittwoch 1987; Chubb 1992; Le Roy et al.

2001). Hayward and Shire (1974), Hunt and Mittwoch (1987), and Le Roy et al. (2001) reported results supporting a Y chromosome effect. In contrast, Herrick and Wolfe (1977) and Chubb (1992) claimed that the Y chromosome is unlikely to have a substantial effect. To resolve this controversy, comprehensive analyses of testis weight were performed in Y-consomic strains in which the Y chromosome from various inbred mouse strains was inserted into an inbred DH/Sgn strain background (Suto 2008). My findings support the role of Y-linked genes in the control of testis weight because statistically significant differences were observed among the Y-consomic strains.

The first objective of this study was to identify additional autosomal and/or X-linked QTLs for testis weight. Novel testis weight QTLs could be identified by analyzing F_2 intercrosses produced using the above-mentioned DDD and CBA strains. The second objective was to identify the Y-linked gene or genes involved in the control of testis weight. Genes that control testis weight in Y-consomic mouse strains might be identified by searching for associations between gene polymorphisms and testis weight.

Materials and methods

Mice

Inbred mouse strains DH/Sgn (hereafter referred to as DH), DDD/Sgn (DDD), CF1/Sgn (CF1), RR/Sgn (RR), and SS/Sgn (SS) were maintained at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). Inbred strains A/J (A), CAST/EiJ (CAST), AKR/J (AKR), RF/J (RF), SJL/J (SJL), and SWR/J (SWR) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Inbred strains BALB/cA (BALB), C3H/HeJ (C3H), C57BL/6 J (B6), DBA/2 J (DBA), and KK/Ta (KK) were purchased from Clea Japan (Tokyo, Japan), and the inbred strain CBA/N (CBA) was purchased from Japan SLC (Hamamatsu, Japan).

The Y chromosome of these strains was successively backcrossed to the DH strain for at least 15 generations. The following Y-consomic strains were assessed: DH-Chr Y^{A}, DH-Chr Y^{AKR}, DH-Chr Y^{B6}, DH-Chr Y^{BALB}, DH-Chr Y^{C3H}, DH-Chr Y^{CAST}, DH-Chr Y^{CBA}, DH-Chr Y^{CF1}, DH-Chr Y^{DBA}, DH-Chr Y^{DDD}, DH-Chr Y^{DH} (this is identical to DH), DH-Chr Y^{KK}, DH-Chr Y^{RF}, DH-Chr Y^{RR}, DH-Chr Y^{SJL}, DH-Chr Y^{SS}, and DH-Chr Y^{SWR}.

All mice were maintained in a specific-pathogen-free facility with a regular light cycle and controlled temperature and humidity. Food (CRF-1, Oriental Yeast Co. Ltd., Tokyo) and water were freely available throughout the experimental period. All animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee of NIAS.

Phenotyping

At age 80 days, the mice were weighed on an electric balance to the nearest 0.01 g. They were then killed, and their testes were removed and placed in physiological saline. After being rinsed and wiped using wet chromatography paper, the paired testes were weighed on the electric balance to the nearest 1 mg. Both "absolute (paired) testis weight" and "relative testis weight (testis weight relative to body weight)" were assessed. The relative testis weight was calculated by dividing testis weight (mg) by body weight (g). Spleen weight was similarly determined for QTL analysis because it served as a reference for a parenchymatous organ. Trait names were abbreviated as follows: Tw for absolute testis weight, rTw for relative testis weight, Sw for absolute spleen weight (mg), and rSw for relative spleen weight [Sw (mg)/body weight (g)]. Tw and rTw and Sw and rSw were simultaneously referred as "testis weight" and "spleen weight," respectively.

Genotyping of microsatellite markers, single nucleotide polymorphisms (SNPs), and *Sry*

Microsatellite sequence length polymorphisms were identified after PCR amplification of genomic DNA by 10% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. SNP genotyping was performed by direct sequencing of the PCR product of the genomic region containing the SNP site. The nucleotide sequence of *Sry* was also determined by direct sequencing of the PCR product. *Sry* has nine known polymorphisms that differ between Y^{Mus} and Y^{Dom} (Eicher 1994). These include polymorphisms in the number of CAG repeats in *Sry*.

QTL analysis

As mentioned above, DDD is a mouse strain having the heaviest testes, whereas CBA is a strain having the lightest testes. In this study, QTL analysis was performed in F_2 mice produced by crossing DDD and CBA strains.

A total of 96 selected ♀DDD × ♂CBA F_2 mice, including 35 mice with the highest Tw, 33 mice with the lowest Tw, 33 mice with the highest rTw, and 39 mice with the lowest rTw, were genotyped for the following 72 microsatellite marker loci: *D1Mit316*, *D1Mit214*, *D1Mit334*, *D1Mit390*, *D1Mit33*, *D1Mit362*, *D2Mit312*, *D2Mit297*, *D2Mit75*, *D2Mit274*, *D2Mit346*, *D3Mit46*, *D3Mit217*, *D3Mit45*, *D4Mit149*, *D4Mit214*, *D4Mit26*, *D4Mit190*, *D5Mit200*, *D5Mit239*, *D5Mit30*, *D5Mit223*, *D6Mit116*, *D6Mit188*, *D6Mit111*, *D7Mit76*, *D7Mit253*, *D7Mit15*, *D8Mit64*, *D8Mit248*, *D8Mit215*, *D9Mit90*, *D9Mit229*, *D9Mit212*, *D9Mit1003*, *D10Mit188*, *D10Mit183*, *D10Mit261*, *D11Mit231*,

D11Mit242, *D11Mit214*, *D12Mit109*, *D12Mit158*, *D12Mit141*, *D13Mit207*, *D13Mit64*, *D13Mit233*, *D13Mit130*, *D13Mit171*, *D14Mit11*, *D14Mit120*, *D14Mit64*, *D14Mit194*, *D14Mit165*, *D15Mit175*, *D15Mit63*, *D15Mit159*, *D16Mit32*, *D16Mit4*, *D16Mit139*, *D17Mit176*, *D17Mit180*, *D17Mit93*, *D18Mit123*, *D18Mit142*, *D18Mit25*, *D19Mit46*, *D19Mit91*, *D19Mit71*, *DXMit141*, *DXMit172*, and *DXMit239*. Genome-wide scans were performed on ♀DDD × ♂CBA F_2 mice, and some of the results were confirmed in ♀CBA × ♂DDD F_2 mice. All ♀DDD × ♂CBA F_2 mice ($n = 253$) were genotyped for the underlined microsatellite markers. All ♀CBA × ♂DDD F_2 mice ($n = 113$) were genotyped for microsatellite markers *D1Mit33*, *DXMit141*, *DXMit172*, and *DXMit239*.

Normality of trait data distribution in F_2 mice was tested using the Shapiro–Wilk W test (JMP 8.0.2, SAS Institute Japan, Tokyo). If the trait values did not follow a normal distribution, they were appropriately transformed.

A genome-wide scan was performed in ♀DDD × ♂CBA F_2 mice for single QTLs using the R/qtl (Broman et al. 2003; Broman and Sen 2009). Threshold LOD scores for suggestive ($P < 0.63$) and significant ($P < 0.05$) linkages were determined by performing 1,000 permutations for each trait. In particular, since selective genotyping was used in this study, stratified permutation tests were applied to the data set as recommended. Furthermore, X-chromosome-specific threshold LOD scores were independently determined. After single QTL scans, pairwise evaluations of the potential interaction between marker loci were performed. At this stage, threshold LOD scores were based strictly on the recommended ones according to "A brief tour of R/qtl" by Broman (http://www.rqtl.org).

Next, data on ♀DDD × ♂CBA F_2 mice and ♀CBA × ♂DDD F_2 mice were combined and analyzed. To assess evidence for the interaction between X-linked QTLs and the Y chromosome, QTL × covariate interaction was tested with the cross-direction (pgm) included as an interactive covariate.

Y-chromosome-wide association studies

Y-linked genetic variations controlling testis weight were identified using the following three-step approach. First, the effects of genes on autosomes and X chromosomes were eliminated using Y-consomic strains, and the net phenotypic effects of Y-linked genes were assessed. Second, whether a trait was indeed Y-linked was determined using Dunnett's multiple-comparison tests with the background DH strain as a reference. Third, data from all strains were assembled on the basis of SNP genotypes, and the statistical significance of differences was assessed. Two groups partitioned by genotype were compared using Student's or Welch's *t*-test, and three groups were compared by one-way ANOVA. On the basis of the number of SNP loci (n) genotyped, the significant threshold P value was determined as $0.05/n$ by Bonferroni correction.

Other statistics

Statistical differences between two and three groups were analyzed using Student's or Welch's *t*-test and one-way ANOVA. Multiple comparisons were performed using Tukey–Kramer HSD tests. $P < 0.05$ was considered statistically significant.

Broad-sense heritability was determined according to the method of Massett et al. (2009). Briefly, heritability (H^2) was calculated with the formula: $H^2 = [V_{F2} - (1/2V_{F1} + 1/4V_{P1} + 1/4V_{P2})]/V_{F2}$, where V_{P1} and V_{P2} are the variances of the parental strains, V_{F1} is the variance of the F_1 generation, and V_{F2} is the variance of the F_2 generation.

Results

Testis weight in parental and Y-consomic strains and in F_1 and F_2 mice

Figure 1a shows scatterplots of Tw in parental DDD ($n = 25$), CBA ($n = 10$), DDD × CBA F_1 ($n = 9$), CBA × DDD F_1 ($n = 14$), DDD × CBA F_2 ($n = 253$), CBA × DDD F_2 ($n = 113$), DH-Chr Y^{DDD} ($n = 41$), and DH-Chr Y^{CBA} ($n = 21$) mice.

In parental mice, DDD mice had significantly higher Tw than CBA mice (average 296.5 mg vs. 105.2 mg, $P < 6.4 \times 10^{-24}$). In F_1 mice, CBA × DDD F_1 mice had significantly higher Tw than DDD × CBA F_1 mice (232.5 mg vs. 192.2 mg, $P < 0.00003$). In F_2 mice, Tw in CBA × DDD F_2 mice had significantly higher Tw than DDD × CBA F_2 mice (198.9 mg vs. 176.9 mg, $P < 2.3 \times 10^{-10}$). In Y-consomic mice, DH-Chr Y^{DDD} mice had significantly higher Tw than DH-Chr Y^{CBA} mice (204.5 mg vs. 191.6 mg, $P < 0.00004$).

A broad-sense heritability of Tw was 0.76 in DDD × CBA F_2 mice and 0.77 in CBA × DDD F_2 mice.

QTL analysis in DDD × CBA F_2 mice

Figure 1b shows a histogram of the distribution of Tw in DDD × CBA F_2 mice. Trait values of Tw, and not of rTw, Sw, and rSw, were normally distributed (data not shown); therefore, Box–Cox transformation was applied to these data to achieve a normal distribution.

Table 1 shows the results of single QTL scans. Two significant and six suggestive QTLs were identified for Tw. A significant QTL on chromosome 1 was designated *Twdq2* because *Twdq1* was previously assigned to QTL on chromosome 9 (Fig. 2a) (Suto 2008). At *Twdq2*, the DDD

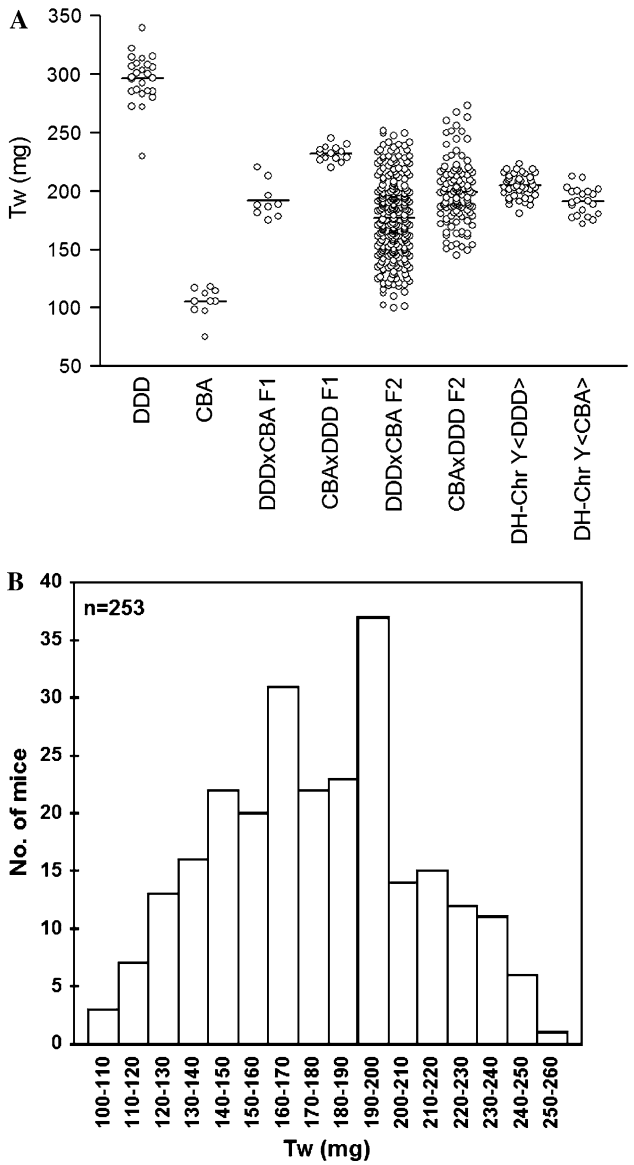

Fig. 1 **a** Scatterplots of paired testis weight in parental DDD and CBA strains, Y-consomic strains, and reciprocal F_1 and F_2 mice. Each point represents the testis weight of an individual mouse. *Horizontal bar* indicates the average for the strain. **b** A *histogram* showing distribution of paired testis weight in DDD × CBA F_2 mice. The mean ± standard error is 176.9 ± 2.1 mg

allele was associated with increased Tw (Table 2). A significant QTL on the X chromosome was designated *Twdq3* (Fig. 2a). At *Twdq3*, the CBA allele was associated with increased Tw. The distal end of 95% CI could not be defined because its position extended distally and no available microsatellite markers were found distal to *DXMit239*. A suggestive QTL on chromosome 9 was coincidental with *Twdq1*; therefore, the same gene symbol was assigned to this locus. Except for a locus on chromosome 2, the DDD allele was associated with increased Tw at all suggestive loci. Two significant and three suggestive QTLs were identified for rTw. A significant QTL on chromosome 1 was designated *Rtwdq3* because *Rtwdq1* and *Rtwdq2* were previously assigned to chromosomes 14 and 17, respectively (Fig. 2a) (Suto 2008). At *Rtwdq3*, the DDD allele was associated with increased rTw (Table 2). A significant QTL on the X chromosome was designated *Rtwdq4* (Fig. 2a). At *Rtwdq4*, the CBA allele was associated with increased rTw. The distal end of 95% CI could not be defined again. Except for a locus on chromosome 6, the DDD allele was associated with increased rTw at all suggestive loci.

Coincidental QTLs for testis weight and spleen weight were unexpectedly identified on distal X chromosome (Table 1). Highly significant QTLs for Sw and rSw were designated *Swdq2* and *Rswdq1*, respectively (Fig. 2b) [*Swdq1* was previously assigned to QTL on chromosome 11 (Suto 2008)]. At *Swdq2* and *Rswdq1*, the DDD allele was associated with increased spleen weight (Table 2).

Analysis in reciprocal CBA × DDD F_2 mice

To confirm the results obtained in DDD × CBA F_2 mice, all individuals were genotyped for microsatellite markers *D1Mit33*, *DXMit141*, *DXMit172*, and *DXMit239*. One-way ANOVA showed that *D1Mit33* had significant effects on both Tw and rTw, and DDD alleles at these loci were associated with increased testis weight (Table 2). In contrast, *DXMit239* had no significant effects on Tw and rTw, and the DDD allele tended to be associated with increased testis weight. *DXMit239* had significant effects on both Sw and rSw, and the DDD allele was associated with increased spleen weight.

When the mean trait values from four F_2 subgroups, $DXMit239^{DDD}Y^{DDD}$, $DXMit239^{DDD}Y^{CBA}$, $DXMit239^{CBA}Y^{DDD}$, and $DXMit239^{CBA}Y^{CBA}$, were simultaneously compared using a Tukey–Kramer HSD test, $DXMit239^{DDD}Y^{CBA}$ showed significantly lower testis weight than the other three F_2 subgroups (Table 3) (a significant difference between $DXMit239^{DDD}Y^{DDD}$ and $DXMit239^{CBA}Y^{CBA}$ was also observed for Tw).

To assess evidence for the interaction between X-linked QTL and Y chromosome, both F_2 data sets were combined and QTL × covariate interaction was tested with the cross-direction (pgm) included as an interactive covariate. As summarized in Table 4, QTL × pgm interaction was significant for QTL on X chromosome, but was not for QTL on chromosome 1 with regard to both traits.

Y-chromosome-wide association studies

Of the Y-consomic strains, DH-Chr Y^A, DH-Chr Y^{B6}, DH-Chr Y^{BALB}, DH-Chr Y^{C3H}, DH-Chr Y^{CBA}, DH-Chr Y^{CF1}, DH-Chr Y^{DBA}, DH-Chr Y^{DH}, DH-Chr Y^{KK}, DH-Chr Y^{RR},

Table 1 Identification of testis and spleen weight loci by a single QTL scan

Traits	Chr[a]	Location (cM)[b]	95% CI (cM)[c]	Max LOD[d] (% variance)[e]	Nearest marker (Mb position)	High allele[f]	Name[g]
Tw	1	65	54–85	5.15* (5.0)	*D1Mit33* (160)	DDD	*Twdq2*
	2	40		3.37 (2.0)	*D2Mit75* (80)	CBA	
	8	44		2.93 (3.2)	*D8Mit248* (95)	DDD	
	9	20		2.29 (1.9)	*D9Mit90* (32)	DDD	*Twdq1*
	11	22		2.57 (4.3)	*D11Mit231* (35)	DDD	
	13	54		2.61 (1.9)	*D13Mit130* (111)	DDD	
	14	38		2.30 (1.9)	*D14Mit194* (94)	DDD	
	X	54	32–[h]	8.39* (11.5)	*DXMit239* (na)	CBA	*Twdq3*
rTw	1	63	48–89	4.48* (6.8)	*D1Mit33* (160)	DDD	*Rtwdq3*
	6	21		2.03 (4.2)	*D6Mit116* (25)	CBA	
	8	52		2.68 (3.1)	*D8Mit248* (95)	DDD	
	13	44		2.41 (2.9)	*D13Mit233* (83)	DDD	
	X	61	51–[h]	7.44* (9.1)	*DXMit239* (na)	CBA	*Rtwdq4*
Sw	X	56	52–59	40.24* (50.5)	*DXMit239* (na)	DDD	*Swdq2*
rSw	12	54		2.71 (2.2)	*D12Mit141* (111)	CBA	
	X	54	50–58	44.42* (52.1)	*DXMit239* (na)	DDD	*Rswdq1*

na not available

Threshold LOD scores for suggestive and significant linkage for Tw were 2.27 and 3.73 for the autosomes and 1.58 and 2.81 for the X chromosome. Threshold LOD scores for suggestive and significant linkage for rTw were 2.02 and 3.28 for autosomes and 1.56 and 2.91 for X chromosome

[a] Chromosome

[b] Location is a chromosomal position showing a peak LOD score in cM units

[c] 95% CI is defined by a 1.5-LOD support interval and is determined only for significant QTLs

[d] Maximum LOD score for QTL. Significant QTLs are indicated by * (suggestive QTLs shown without an *asterisk*)

[e] Total variance explained by QTL at this locus is expressed as percent

[f] Allele that is associated with higher trait values

[g] Assigning a QTL name was limited to significant QTLs

[h] Distal end of CI could not be defined because it extends distally

and DH-Chr Y^{SS} possess the *Mus musculus musculus* Y chromosome (Y^{Mus}), whereas DH-Chr Y^{AKR}, DH-Chr Y^{DDD}, DH-Chr Y^{RF}, DH-Chr Y^{SJL}, and DH-Chr Y^{SWR} possess the *Mus musculus domesticus* Y chromosome (Y^{Dom}). The strains were classified on the basis of the following criteria: (1) a C-to-T transitional substitution at nucleotide position (nt) 8491 in the high-mobility group (HMG) box of *Sry* (nucleotide numbering throughout this article is based on the GenBank entry X67204) (Y^{Mus} had T and Y^{Dom} had C) (Kunieda and Toyoda 1992); and (2) the presence of a C-to-T change that created a TAG termination codon at nt 9006 in the third major CAG repeat starting at nt 8985 in Y^{Dom} (Coward et al. 1994). This was absent in Y^{Mus} (mouse *Sry* has four major sites consisting of about 10 CAG repeats). *Sry* in DH-Chr Y^{CAST} had C at nt 8491, but it did not possess TAG at nt 9006. Therefore, Y^{CAST} was not classified.

Figure 3 shows a histogram of the distribution of Tw in 472 mice from among the 17 Y-consomic strains. Tw and rTw showed bell-shaped distribution curves (data not shown); however, they did not follow normal distribution in a strict statistical sense. Therefore, Box–Cox transformation was applied before subsequent analyses.

The raw phenotypic data on Tw was compared for each Y-consomic strain (Fig. 4). DH-Chr Y^{C3H}, DH-Chr Y^{CBA}, DH-Chr Y^{DBA}, DH-Chr Y^{KK}, DH-Chr Y^{RR}, and DH-Chr Y^{SJL} had significantly lower Tw than DH-Chr Y^{DH}. The results of the comparison of rTw are summarized in Table 5. DH-Chr Y^{DBA}, DH-Chr Y^{C3H}, and DH-Chr Y^{SS} had significantly lower rTw than DH-Chr Y^{DH}, whereas DH-Chr Y^{KK} had a significantly higher rTw than DH-Chr Y^{DH}.

Table 6 lists 30 SNPs and 9 *Sry* polymorphisms identified in the 16 Y-consomic strains (DH-Chr Y^{DH} was excluded). These SNP loci were selected on the basis of SNP data retrieved from the Mouse Phenome Database (MPD, http://phenome.jax.org). A high-density strain set, comprising 18 inbred strains, had 18 SNPs associated with

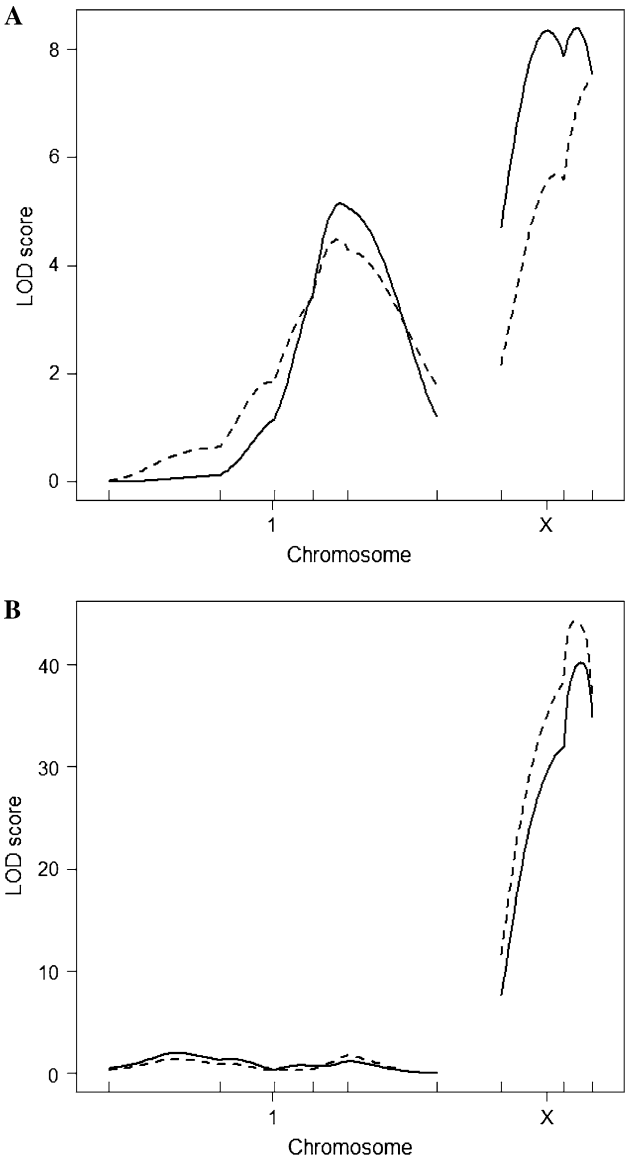

Fig. 2 LOD score plots for testis weight QTLs on chromosomes 1 and X (**a**) and for spleen weight QTLs on chromosomes 1 and X (**b**). *X* axis represents the microsatellite marker position and *Y* axis represents the LOD score. LOD score plots for Tw and Sw are indicated by a *solid line*, while those for rTw and rSw are shown by a *dashed line*

nonsynonymous amino acid changes. One of them, rs51394161, which was located on exon 5 of *Zfy2*, could not be determined; therefore, 17 nonsynonymous SNPs were typed (nonsynonymous SNPs were preferentially selected based on the assumption that coding SNPs must be the causal variants). MPD search also yielded 25 synonymous SNPs, 13 of which were genotyped. *Sry* polymorphisms included nucleotide substitutions at seven sites and a number of major CAG repeats at two sites (Eicher 1994). Polymorphic patterns are color-coded as yellow, blue, and purple to facilitate discrimination. No polymorphisms were observed at 11 SNP loci; these cells are not color-coded (see online version for color codings).

Table 7 summarizes the results of statistical analyses. Mice were divided into two or three groups according to SNP or polymorphism in *Sry*. Statistically significant differences in mean values between or among groups were then tested. Because 39 polymorphisms were examined, the significance threshold at $\alpha = 0.05$ was 0.0013. Variation in the ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome (*Uty*) gene (rs46947134) was significantly associated with Tw and rTw. DH-Chr Y^{C3H}, DH-Chr Y^{CBA}, DH-Chr Y^{DBA}, and DH-Chr Y^{RR} had a G allele, which was associated with decreased Tw and rTw. rs46947134 was a nonsynonymous SNP and was accompanied by an Asp-to-His amino acid change. Variation in the gene for ubiquitin-specific peptidase 9 Y chromosome (*Usp9y*) (rs51766109) was also significantly associated with rTw. DH-Chr Y^{C3H}, DH-Chr Y^{CBA}, DH-Chr Y^{DBA}, DH-Chr Y^{RR}, DH-Chr Y^{CAST}, and all DH-Chr Y^{Dom} strains had the T allele, which was associated with decreased rTw. rs51766109 was a nonsynonymous SNP and was accompanied by a Gly-to-Glu amino acid change.

Twenty-five polymorphisms (color-coded green and red in Table 6) approximated the classification of Y^{Mus} and Y^{Dom}. These SNPs were significantly associated with Tw and rTw, i.e., Y^{Dom} had a significantly higher Tw and rTw than Y^{Mus} (Table 7). Strains with Y^{Dom} had significantly higher Tw and rTw than strains with Y^{Mus}.

The number of first major CAG repeats, starting at nt 8733 of *Sry*, was significantly associated with Tw and rTw; strains with 12 CAG repeats (DH-Chr Y^{C3H}, DH-Chr Y^{CBA}, DH-Chr Y^{DBA}, and DH-Chr Y^{RR}) had significantly lower testis weight than those with 9 or 11 repeats (Table 7). The number of second major CAG repeats, starting at nt 8811 of *Sry*, was also significantly associated with Tw and rTw; strains with 10 CAG repeats (DH-Chr Y^{C3H}, DH-Chr Y^{CBA}, DH-Chr Y^{DBA}, and DH-Chr Y^{RR}) had significantly lower testis weight than those with 12 or 13 repeats. These polymorphisms resulted in differences in the length of the polyglutamine stretch.

Discussion

The DDD allele was associated with increased Tw and rTw at *Twdq2*/*Rtwdq3* on chromosome 1, whereas the CBA allele was associated with increased Tw and rTw at *Twdq3*/*Rtwdq4* on the X chromosome. The low-testis-weight strain-derived allele was associated with increased testis weight. Zídek et al. (1998) identified one significant and two suggestive testis weight QTLs using recombinant inbred strains derived from C57BL/6J and DBA/2J. At two of these three QTLs, including a significant one, a

Table 2 Allele effects of QTL on the reciprocal F_2 intercross between CBA females and DDD males

F_2 mice	Traits	QTL	Nearest marker	Means ± SEM of the trait values by genotype			Nominal *P* value
				CBA/CBA (CBA)	CBA/DDD	DDD/DDD (DDD)	
♀DDD × ♂CBA	Tw (mg)	*Twdq2*	*D1Mit33*	160.7 ± 3.9 ($n = 67$)	180.7 ± 2.8 ($n = 122$)	186.5 ± 4.3 ($n = 64$)	9.89×10^{-6}
		Twdq3	*DXMit239*	189.3 ± 2.8 ($n = 123$)		165.1 ± 2.9 ($n = 130$)	4.61×10^{-9}
	rTw	*Rtwdq3*	*D1Mit33*	4.53 ± 0.10	4.99 ± 0.07	5.17 ± 0.12	0.000042
		Rtwdq4	*DXMit239*	5.24 ± 0.08		4.61 ± 0.07	5.00×10^{-9}
	Sw (mg)	*Swdq2*	*DXMit239*	54.8 ± 1.0		77.1 ± 1.1	9.95×10^{-37}
	rSw	*Rswdq1*	*DXMit239*	1.51 ± 0.02		2.17 ± 0.04	6.98×10^{-38}
♀CBA × ♂DDD	Tw (mg)	*Twdq2*	*D1Mit33*	184.8 ± 3.8 ($n = 20$)	200.5 ± 3.5 ($n = 64$)	205.1 ± 5.5 ($n = 29$)	0.037
		Twdq3	*DXMit239*	195.7 ± 3.5 ($n = 53$)		201.8 ± 3.7 ($n = 60$)	0.26
	rTw	*Rtwdq3*	*D1Mit33*	4.64 ± 0.13	5.06 ± 0.10	5.38 ± 0.16	0.0081
		Rtwdq4	*DXMit239*	4.98 ± 0.11		5.15 ± 0.11	0.29
	Sw (mg)	*Swdq2*	*DXMit239*	60.4 ± 1.5		84.6 ± 1.8	1.59×10^{-18}
	rSw	*Rswdq1*	*DXMit239*	1.53 ± 0.04		2.15 ± 0.05	1.94×10^{-17}

Although the data are presented as raw trait values, statistical analyses were performed on normally transformed data

Table 3 Comparison of testis weight among four F_2 subgroups partitioned by the genotypes of *DXMit239* and Y chromosome

Tw				
		DXMit239		
		Y		
DXMit239	Y	CBA DDD	DDD CBA	DDD DDD
CBA	CBA	NS	$P < 0.0001$	$P < 0.05$
CBA	DDD		$P < 0.0001$	NS
DDD	CBA			$P < 0.0000$[a]
rTw				
		DXMit239		
		Y		
DXMit239	Y	CBA DDD	DDD CBA	DDD DDD
CBA	CBA	NS	$P < 0.0001$	NS
CBA	DDD		$P < 0.03$	NS
DDD	CBA			$P < 0.0002$

Multiple comparisons were performed using Tukey–Kramer HSD tests

[a] A lower limit could not be defined

low-testis-weight C57BL/6J strain-derived allele was associated with increased testis weight. The discrepancy was considered to be appropriately explained in this study because the CBA allele was associated with increased testis weight at the locus showing the highest LOD score. Several explanations account for the discrepancy. First, the DDD allele was associated with increased Tw at all suggestive loci except for a locus on chromosome 2, and the DDD allele was associated with increased rTw at all suggestive loci except for a locus on chromosome 6. Thus, cumulative contribution conferred by these suggestive loci should not be underestimated. In particular, suggestive loci on chromosome 9 for Tw and chromosome 14 for rTw were identified as significant QTLs in a previous study involving DDD × DH F_2 mice (Suto 2008). Second, the DDD allele may not be able to sustain testis weight when combined with the Y chromosome from the CBA strain. Table 3 shows the results of comparisons of testis weight among mice with four possible genotypes with regard to a combination between X and Y chromosomes. It can be concluded that F_2 mice with $DXMit239^{DDD}Y^{CBA}$ have significantly lower values than mice with other genotypes. Furthermore, QTLs on X chromosome were shown to interact with cross-direction, i.e., Y chromosome (Table 4). This does not preclude the possibility that influences of mitochondrial or other genes cause the discrepancy.

Another question to be addressed is whether X-linked QTLs for testis weight and spleen weight are allelic because 95% CIs for both QTLs overlap. At a glance, the allele effect was in opposite directions between the two QTLs, i.e., the DDD allele at *Twdq3*/*Rtwdq4* was associated with decreased testis weight, whereas the DDD allele at *Swdq2*/*Rswdq1* was associated with increased spleen weight. This suggests that they are unlikely to be allelic. However, this is not conclusive because they involve different traits. Indeed, one gene can produce such opposite effects on the weight of different organs. For example, the A^y allele at the agouti locus significantly increases the spleen weight but reduces the testis weight in mice (Suto 2009). The spleen weight QTL is probably due to the

Table 4 Summary of analyses of the interaction between X and Y chromosomes in combined F_2 data sets

Chr	Trait	LOD score		
		pgm as additive covariate (LOD_a)[a]	pgm as interactive covariate (LOD_f)[b]	LOD_i(LOD_f–LOD_c)[c]
1	Tw	5.91 (65)	6.16 (65)	0.54 (29)
	rTw	5.62 (63)	5.99 (63)	0.83 (2)
X	Tw	6.85 (45)	9.41 (45)	4.13 (61)
	rTw	4.55 (55)	7.93 (61)	3.89 (61)

Peak position (cM) is given in parentheses

[a] Significant threshold LOD scores for Tw and rTw were 3.53 and 3.75 for autosomes and 2.83 and 2.88 for X chromosome

[b] Significant threshold LOD scores for Tw and rTw were 5.81 and 4.30 for autosomes and 3.50 and 3.40 for X chromosome

[c] LOD_i is the difference between the LOD score with pgm as an interactive covariate (LOD_f) and the LOD score with pgm as an additive covariate (LOD_a). It concerns the test of the QTL × pgm interaction. Significant threshold LOD scores for Tw and rTw were 3.63 and 1.78 for autosomes and 3.25 and 3.08 for X chromosome

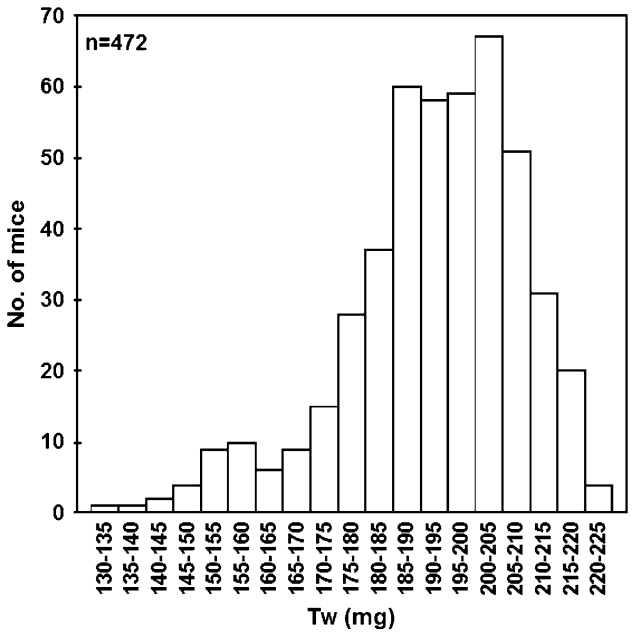

Fig. 3 Histograms showing distributions of Tw in all mice from among the 17 Y-consomic strains ($n = 472$)

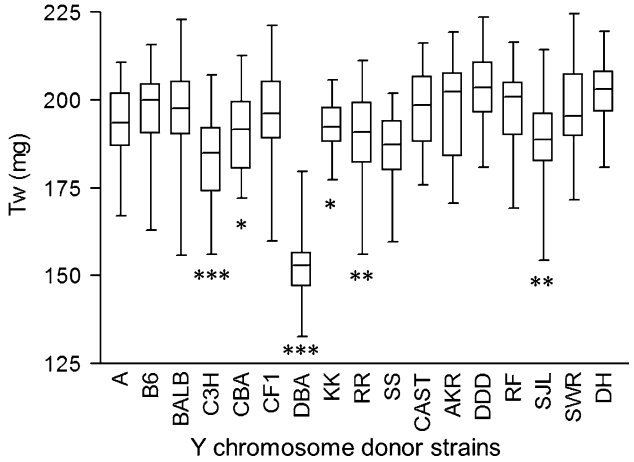

Fig. 4 *Box* (median value ± one quartile) and *whisker* (range between maximum and minimum values) plots for Tw for each Y-consomic strain are shown. Significant deviations, after Box–Cox transformation, are indicated by *asterisks*. Significance levels: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. The strain names on the *X* axis apply only to the Y chromosome constitution in an otherwise DH background

Bruton agammaglobulinemia tyrosine kinase (*Btk*, formerly designated as *xid*) mutation that specifically occurs in the CBA/N strain (Scher et al. 1975; Rawlings et al. 1993; Thomas et al. 1993). According to Marquis et al. (1985), the average spleen weight was significantly higher in the CBA/Ca than in the CBA/N strain. Thus, low spleen weight in CBA/N is probably due to the *Btk* mutation. The *Btk* locus is located at the 56.18-cM position on the X chromosome, and this is within 95% CI for *Swdq2*/*Rswdq1*. In contrast, low testis weight appears to be common among CBA substrains (Shire and Bartke 1972; Hayward and Shire 1974; Hunt and Mittwoch 1987; Le Roy et al. 2001). On the basis of these facts, the X-linked testis weight QTL (*Twdq3*/*Rtwdq4*) is unlikely to be allelic with X-linked spleen weight QTL (*Swdq2*/*Rswdq1*).

The distal end of the 95% CI for *Twdq3*/*Rtwdq4* could not be defined. This 95% CI may extend to the pseudoautosomal region (PAR). PAR is a chromosomal region of sequence identity between the X and Y chromosomes. X–Y pairing has been suggested to play an important role in male fertility (Matsuda et al. 1991). Irrespective of the location of *Twdq3*/*Rtwdq4*, the reduction in testis weight can be hypothesized to be the consequence of incompatibility between genes on the X and Y chromosomes.

For QTLs identified in this study, coincidental QTLs have been reported. Otsuka et al. (2010) identified a highly significant QTL that is associated with decreased testis weight in MRL mice for *Twdq2*/*Rtwdq3* on chromosome 1.

Table 5 Relative testis weight in Y-consomic strains

Y chromosome donor strain	Relative testis weight (mean ± SD)[a]	*P* value vs. DH	Sample size
DBA	0.89 ± 0.07	<0.0001	24
C3H	1.27 ± 0.06	<0.0001	40
SS	1.56 ± 0.07	0.0008	22
CBA	1.91 ± 0.11	NS	21
B6	1.92 ± 0.06	NS	32
RR	2.00 ± 0.08	NS	26
CAST	2.06 ± 0.08	NS	26
BALB	2.07 ± 0.11	NS	24
AKR	2.11 ± 0.09	NS	37
DH	2.12 ± 0.11	NS	19
A	2.15 ± 0.08	NS	27
SWR	2.16 ± 0.09	NS	27
SJL	2.16 ± 0.10	NS	29
RF	2.17 ± 0.07	NS	32
CF1	2.22 ± 0.08	NS	21
DDD	2.35 ± 0.07	NS	41
KK	2.85 ± 0.08	<0.0001	24

[a] Trait values were transformed to achieve normal distribution

The 95% CI overlaps with that of *Twdq2*/*Rtwdq3*. For X-linked QTLs, 95% CIs for QTLs reported by Le Roy et al. (2001) and Bolor et al. (2006) overlap those for *Twdq3*/*Rtwdq4*. At these QTLs, the allele from the high-testis-weight strain was associated with increased testis weight. Elliott et al. (2004), Oka et al. (2004), Storchová et al. (2004), Good et al. (2008), and Vyskočilová et al. (2009) also reported X-linked testis weight QTLs as a part of their investigations on hybrid male sterility.

As mentioned above, the Y chromosome is a potential source of testis weight genes, and indeed, DH-Chr Y^{DDD} had significantly higher Tw and rTw than DH-Chr Y^{CBA}. Further in-depth studies were performed to identify Y-linked genetic variations that control testis weight. A three-step approach was developed for the following reasons: (1) to precisely assess the net phenotypic effects of Y-linked genes using Y-consomic strains to eliminate the effects of genes on autosomes and X chromosomes. Indeed, because major testis weight determinants are autosomal and X-linked (Suto 2008), comparison of the phenotypic effects of Y-linked genes is possible only when the Y chromosomes are isolated, i.e., Y-consomic strains; (2) to determine whether a trait was indeed Y-linked for which Dunnett's multiple-comparison test was used, with the background DH (DH-Chr Y^{DH}) strain as a reference. Using this approach, Tw and rTw were found to be controlled by Y-linked genes. (3) Data from all strains were assembled on the basis of SNP genotypes and tested for the statistical significance of differences. Unlike with autosomes and X chromosomes, genetic mapping was not applicable to the Y chromosome; therefore, this appeared to be the only way to evaluate phenotypic effects. Genome-wide association studies (GWAS) to map genes linked to complex disease-related traits have been attempted in inbred mice (Grupe et al. 2001; Liu et al. 2006; Guo et al. 2007). However, unlike in humans, GWAS in inbred mice revealed spurious associations (Su et al. 2010). This is because of the intricate population genetic structure of laboratory mice (Su et al. 2010). Therefore, controversial arguments exist over the pertinence of performing GWAS in inbred mice (Darvasi 2001; Su et al. 2010). However, GWAS can be a powerful tool in mice when performed in combination with linkage analysis (Park et al. 2003; DiPetrillo et al. 2004; Cervino et al. 2005). In the present study, whether the trait was linked to the Y chromosome was initially determined. This was facilitated by the fact that the Y chromosome range was extremely narrow, and most Y-linked genes described in this study were located within a 2-Mbp region. These experimental steps corresponded to linkage mapping of autosomes. Using the above procedures, several significant associations between gene polymorphisms and testis weight were identified.

Uty and *Usp9y* encode distinct epitopes of the histocompatibility Y (HY) antigen (Greenfield et al. 1996; Vogt et al. 2000a, b; Graves 2010). In other words, the HY antigen is a mixture of epitopes encoded by different Y-linked genes, and *Uty* and *Usp9y* represent such HY-coding genes in mice and humans. Each HY-coding gene is ubiquitously expressed, but their physiological functions are largely unknown. The results of this study suggest possible roles for these genes. In humans, *USP9Y* has been implicated in infertility associated with oligospermia and azoospermia because of its localization (Sun et al. 1999; Krausz et al. 2006). Although, according to a recent study, *USP9Y* is not essential for normal spermatogenesis in humans (Luddi et al. 2009), it might play a role in testis weight determination.

Strains with Y^{Dom} had significantly higher testis weight and relative testis weight than those with Y^{Mus}. The classification between Y^{Dom} and Y^{Mus} is phylogenetically determined; therefore, polymorphisms that are well correlated with this classification (e.g., rs48685451 and rs48834187 in *Kdm5d*, Table 5) might be similar. However, *Kdm5d*, formerly called *Smcy* or *jarid1d*, was also known as an HY-coding gene (Scott et al. 1995; Wang et al. 1995).

Sry encodes a transcription factor that is a member of the HMG-box family of DNA-binding proteins. The HMG-box region is well conserved among species. In mice, *Sry* also contains a large CAG trinucleotide repeat region that encodes a glutamine-rich domain. Four major CAG repeat regions exist that contain about 10 CAG repeats.

Table 6 Identification of genotypes at 30 SNP loci and 9 *Sry* polymorphisms

Gene (exon)	SNP	A	B6	BALB	C3H	CBA	CF1	DBA	KK	RR	SS	CAST	AKR	DDD	RF	SJL	SWR
Zfy1 (3)	rs47359684	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G
Zfy1 (5)	rs47900677	C	C	C	C	C	C	C	C	C	C	C	-	T	T	T	T
Zfy1 (5)	rs46080695	C	C	C	C	C	C	C	C	C	C	C	-	T	T	T	T
Zfy1 (5)	rs52139814	A	A	A	A	A	A	A	A	A	A	A	-	C	C	C	C
Zfy1 (9)	rs45850354	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Ube1y1 (4)	rs48064925	T	T	T	T	T	T	T	T	T	T	-	-	T	T	T	T
Ube1y1 (5)	rs51995337	G	G	G	G	G	G	G	G	G	G	-	-	G	G	G	G
Ube1y1 (11)	rs51133250	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Ube1y1 (11)	rs50647790	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Ube1y1 (12)	rs51277152	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Kdm5d (9)	rs48685451	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A
Kdm5d (9)	rs48834187	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A
Uty (12)	rs46947134	C	C	C	G	G	C	G	C	G	C	C	C	C	C	C	C
Uty (15)	rs51756947	C	C	C	C	C	C	C	C	C	C	C	T	T	T	T	T
Usp9y (5)	rs48554025	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Usp9y (5)	rs47574660	A	A	A	A	A	A	A	A	A	A	A	C	C	C	C	C
Usp9y (11)	rs51766109	C	C	C	T	T	C	T	C	T	C	T	T	T	T	T	T
Usp9y (11)	rs49468864	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G
Usp9y (15)	rs49623242	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C
Usp9y (16)	rs51230091	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C
UsP9y (31)	rs49614307	C	C	C	C	C	C	C	C	C	C	C	T	T	T	T	T
Usp9y (34)	rs48926479	T	T	T	T	T	T	T	T	T	T	T	G	G	G	G	G
Usp9y (46)	rs51025923	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G
Usp9y (46)	rs48512209	T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C
Zfy2 (3)	rs47293184	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Zfy2 (3)	rs51685350	C	C	C	C	C	C	C	C	C	C	C	T	T	T	T	T
Zfy2 (6)	rs47616691	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Zfy2 (6)	rs51560704	A	A	A	A	A	A	A	A	A	A	A	C	C	C	C	C
Zfy2 (8)	rs46643293	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Zfy2 (8)	rs51529727	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Sry[a)]																	
Nucleotide at nt 8491		T	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C
Nucleotide at nt 8701		G	G	G	G	G	G	G	G	G	G	G	T	T	T	T	T
Nucleotide at nt 8711		T	T	T	T	T	T	T	T	T	T	C	C	C	C	C	C
Nucleotide at nt 8731		T	T	T	T	T	T	T	T	T	T	T	C	C	C	C	C
No. of first CAG repeats starting at nt 8733		11	11	11	12	12	11	12	11	12	11	11	9	9	9	9	9
No. of second CAG repeats starting at nt 8811		12	12	12	10	10	12	10	12	10	12	12	13	12	13	12	12
Nucleotide at nt 8930		C	C	C	C	C	C	C	C	C	C	C	G	G	G	G	G
Nucleotide at nt 8934		G	G	G	G	G	G	G	G	G	G	G	C	C	C	C	C
Nucleotide at nt 9006		C	C	C	C	C	C	C	C	C	C	C	T	T	T	T	T

[a] Nucleotide position (*nt*) is based on the numbering of GenBank entry X67204

The C-to-T substitution at nt 9006 creates a premature TAG termination codon in the third major CAG repeat region of Y^{Dom} (Table 6). Accordingly, polymorphisms in the first and second CAG repeat regions were investigated. A polymorphic stretch of CAG repeats in *Sry* is believed to be correlated with an increased incidence of B6.Y^{Dom} sex

Table 7 Effects of gene polymorphisms on trait values

SNP/gene	Polymorphism	Trait	Trait values (mean ± SE)[a]			*P* value
rs46947134/(*Uty*)	Nonsynonymous substitution		C	G		
			($n = 342$)	($n = 111$)		
		Tw	53.6 ± 0.7	39.6 ± 1.4		7.68×10^{-17}
		rTw	2.16 ± 0.03	1.48 ± 0.06		1.15×10^{-24}
rs51766109/(*Usp9y*)	Nonsynonymous substitution		C	T		
			($n = 150$)	($n = 303$)		
		Tw	51.3 ± 1.0	49.7 ± 0.9		0.31
		rTw	2.12 ± 0.04	1.92 ± 0.04		0.00096
Plural SNP loci[b]	Y^{Mus} vs. Y^{Dom}		Y^{Mus}	Y^{Dom}		
			($n = 261$)	($n = 166$)		
		Tw	46.3 ± 0.9	55.6 ± 1.1		4.53×10^{-10}
		rTw	1.85 ± 0.04	2.20 ± 0.04		5.76×10^{-10}
Sry	Nonsynonymous substitution at nt 8491		T	C		
			($n = 261$)	($n = 192$)		
		Tw	46.3 ± 0.9	55.5 ± 1.0		9.29×10^{-11}
		rTw	1.85 ± 0.04	2.18 ± 0.03		8.88×10^{-10}
Sry	No. of CAG repeats starting at nt 8733		9	11	12	
			($n = 166$)	($n = 176$)	($n = 111$)	
		Tw	55.6 ± 1.1	51.8 ± 1.0	39.6 ± 1.4	1.04×10^{-18}
		rTw	2.20 ± 0.04	2.11 ± 0.04	1.48 ± 0.06	5.07×10^{-27}
Sry	No. of CAG repeats starting at nt 8811		10	12	13	
			($n = 111$)	($n = 273$)	($n = 69$)	
		Tw	39.6 ± 1.4	53.3 ± 0.8	55.0 ± 1.6	1.63×10^{-17}
		rTw	1.48 ± 0.06	2.16 ± 0.03	2.14 ± 0.06	1.51×10^{-26}

[a] Trait values were transformed to achieve normal distribution

[b] See Table 6

reversal (Eicher et al. 1983; Coward et al. 1994). Intriguingly, Coward et al. (1994) postulated that variations in the polyglutamine amino acid sequence that result from trinucleotide repeats are associated with the degree of sex reversal/hermaphrodism, i.e., Y^{Dom} that causes complete (severe) sex reversal has 11 repeats, Y^{Dom} that causes partial (fetal) sex reversal has 13, and Y^{Dom} that does not cause sex reversal has 12 repeats. This hypothesis has since been rejected by the same group (Carlisle et al. 1996): the severity of sex reversal is not completely correlated with the number of CAG repeats. In contrast, testis weight in this study was completely correlated with the number of CAG repeats. Testis weight decreased as the number of CAG repeats increased, starting at nt 8733, and increased as the number of CAG repeats increased, starting at nt 8811 (Table 6). Of note, the number of CAG repeats in *Sry* was not limited to the above-mentioned three classes (Miller et al. 1995).

In conclusion, testis weight is a complex quantitative phenotype controlled by multiple genes on autosomes and sex chromosomes and their interactions.

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Nos. 15500305 and 19500373).

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