

A role for a neo-sex chromosome in stickleback speciation

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Sexual antagonism, or conflict between the sexes, has been proposed as a driving force in both sex-chromosome turnover and speciation. Although closely related species often have different sex-chromosome systems, it is unknown whether sex-chromosome turnover contributes to the evolution of reproductive isolation between species. Here we show that a newly evolved sex chromosome contains genes that contribute to speciation in threespine stickleback fish (*Gasterosteus aculeatus*). We first identified a neo-sex chromosome system found only in one member of a sympatric species pair in Japan. We then performed genetic linkage mapping of male-specific traits important for reproductive isolation between the Japanese species pair. The neo-X chromosome contains loci for male courtship display traits that contribute to behavioural isolation, whereas the ancestral X chromosome contains loci for both behavioural isolation and hybrid male sterility. Our work not only provides strong evidence for a large X-effect on reproductive isolation in a vertebrate system, but also provides direct evidence that a young neo-X chromosome contributes to reproductive isolation between closely related species. Our data indicate that sex-chromosome turnover might have a greater role in speciation than was previously appreciated.

Sexually antagonistic selection has been proposed as a major driving force in the evolution of sex chromosomes. Specifically, natural selection is expected to favour linkage between genes with sexually antagonistic effects (that is, beneficial in one sex and detrimental in the other) and the sex-determination locus, resulting in a reduction of recombination between sex chromosomes¹. In an XY sex-chromosome system, a reduction in recombination ultimately leads to the degeneration of the Y chromosome, thereby exposing alleles on the hemizygous X chromosome to selection in males². Thus, male-beneficial alleles, manifested as sexually dimorphic and/or sexually selected traits, are predicted to accumulate on the X chromosome^{3,4}. When these male-beneficial traits or alleles are important for reproductive isolation between species, the X chromosome is also predicted to have a key role in speciation. A disproportionately large effect of the X chromosome has been demonstrated for hybrid male sterility^{5,6}, although the data supporting a large X-effect for other isolating barriers has been less consistent⁶.

Sexually antagonistic selection is also predicted to drive the divergence of sex-chromosome systems between closely related species^{7,8}. Sex-chromosome turnover has been observed across many taxa, but it is particularly striking in fishes^{9,10}. Many independent groups of fishes show evidence for the rapid evolution of sex chromosomes through several different mechanisms, including the transposition of an existing male-determination locus to an autosome¹¹, the evolution of a new male-determination locus on an autosome¹², and fusions between an autosome and an existing Y chromosome^{13,14}. It has been suggested that the rapid turnover of sex chromosomes driven by sexual conflict might also have a role in the high speciation rates seen in some groups of fishes^{15–17}. However, a direct role for sex-chromosome turnover in speciation has not been empirically investigated in any system.

Here we demonstrate that there is a newly formed sex-chromosome system in a threespine stickleback population found in Japan. This Japan Sea form diverged from the Pacific Ocean threespine stickleback during periods of geographical isolation between the Sea of Japan and the Pacific Ocean about 1.5–2 million years ago^{18,19}. Because we can cross the derived Japan Sea form to the ancestral Pacific Ocean form, we have been able to take advantage of the genetic tools available for the threespine stickleback²⁰ to investigate whether the evolution of a neo-sex chromosome has contributed to the evolution of male traits that act as isolating barriers between the derived Japan Sea and the ancestral Pacific Ocean populations.

Japan Sea stickleback neo-sex chromosome

In all threespine stickleback populations examined previously, including the Pacific Ocean form, linkage group 19 (LG19) is the sex chromosome and LG9 is an autosome^{14,21,22}. However, in the course of making a linkage map from a Japan Sea cross, we noticed that several LG9 markers co-segregated with LG19 markers previously found to be tightly linked to the sex-determination locus in a region of reduced recombination and rearrangements on the Y chromosome^{21,22}. This association was observed when male meioses, but not female meioses, were analysed (Fig. 1a). These data indicated that one copy of LG9 might be fused to one copy of LG19 (the Pacific Ocean Y chromosome), forming a neo-Y chromosome in Japan Sea sticklebacks. Therefore, we performed fluorescence *in situ* hybridization (FISH) with LG19 and LG9 probes on metaphase chromosome spreads from two different populations of the Japan Sea form. We found that the Japan Sea males ($n = 4$) from two different populations have an odd number of chromosomes ($2n = 41$), with one large

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unpaired chromosome that hybridized to the LG19 and LG9 probes, providing evidence for the LG9–Y chromosome fusion (Fig. 1b and Supplementary Fig. 1). In contrast, the Japan Sea females ($n = 4$) from both populations have an even number of chromosomes ($2n = 42$), and the LG9 and LG19 probes hybridize to separate chromosome pairs (Fig. 1b and Supplementary Fig. 1). Because the fused copy of LG9 segregates with the Y chromosome in Japan Sea males, the other copy segregates as an X chromosome; this neo-sex chromosome system is defined as an X_1X_2Y system¹³, in which X_1 is the ancestral X chromosome (LG19) and X_2 is the neo-X chromosome (LG9). Thus, the Japan Sea form has a unique neo-sex chromosome system, which has probably evolved within the past 1.5–2 million years of isolation between the Pacific Ocean and Japan Sea sticklebacks^{18,19}.

Components of reproductive isolation

We have the opportunity to test the role of this neo-sex chromosome in reproductive isolation between the Japan Sea and the Pacific Ocean forms because both are anadromous and migrate into Lake Akkeshi and the Bekanbeushi marsh during the breeding season. In this location, they co-occur, along with hybrid adults and juveniles, within a hybrid zone in the Bekanbeushi River (Mid2 in Fig. 2, and Supplementary Fig. 2). We found that temporal isolation, behavioural isolation and hybrid male sterility contribute to reproductive isolation between the forms in the hybrid zone (Supplementary Fig. 3 and Supplementary Discussion). We previously demonstrated that behavioural isolation and hybrid male sterility are asymmetric, but act in complementary directions¹⁹. Pacific Ocean females mate exclusively with Pacific Ocean males, whereas the Japan Sea females mate

with both types of males in laboratory mate-choice trials¹⁹. Although Japan Sea females do mate with Pacific Ocean males, hybrid males resulting from this cross have severely reduced fertility, whereas reciprocal hybrid males and all hybrid females are fertile¹⁹.

To test whether reproductive isolation is linked to sex-chromosome divergence, we first investigated which male mating traits contribute to asymmetric behavioural isolation. First, we analysed the relationship between final female choice and differences in male body size and found that both types of females tend to choose larger males (Fig. 3a; Pacific Ocean females, $n = 30$, coefficient estimate = 0.254, $Z = 2.276$, $P = 0.0228$, logistic regression; Japan Sea females, $n = 29$, coefficient estimate = 0.122, $Z = 2.349$, $P = 0.0188$, logistic regression). Because Japan Sea males (standard length = 63.44 ± 0.37 mm (mean \pm s.e.m.), $n = 59$) are smaller than Pacific Ocean males (standard length = 76.17 ± 0.47 mm, $n = 45$; analysis of variance (ANOVA), $F_{1,102} = 475$, $P < 10^{-15}$), divergence in body size is one of the factors that contributes to asymmetric behavioural isolation. However, even in the absence of body size divergence, Pacific Ocean females still have a 93.5% probability (95% confidence interval = 0.624–0.992) of choosing a conspecific male (Fig. 3a), suggesting that other factors are involved in Pacific Ocean female choice.

We found that a difference in male dorsal pricking behaviour also contributes to asymmetric behavioural isolation. Dorsal pricking is a component of male mating behaviour specific to threespine sticklebacks in which the male raises his dorsal spines and pricks the female. This behaviour might help the male assess the female, provide tactile stimulation to induce female spawning, or act as a way for the male to display his dorsal spines to the female^{23,24}. In Japan Sea males, the

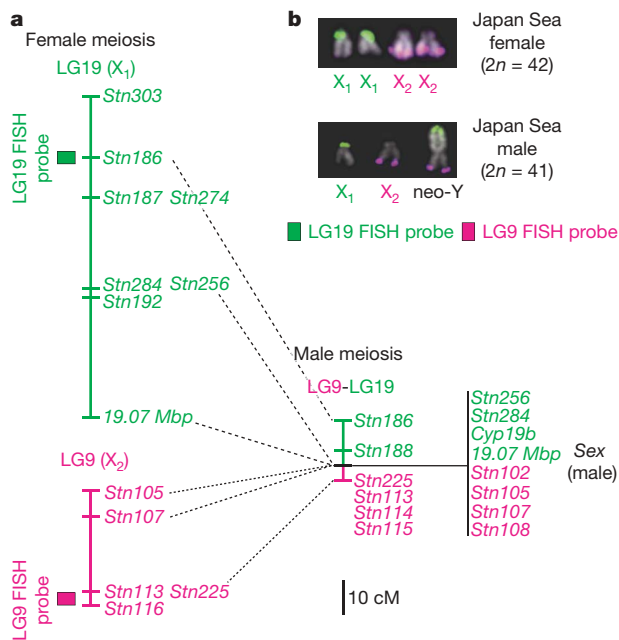


Figure 1 | Genetic and cytogenetic evidence of a fusion between one copy of LG9 and the Y chromosome in Japan Sea males. **a**, A cross between Japan Sea individuals was used to create a linkage map. The female meiotic maps (X–X recombination) of LG19 (X_1) and LG9 (X_2) are to the left, and the male meiotic map (X–Y recombination) of LG9/19 (neo-Y) is to the right. LG19 (X_1) is in green and LG9 (X_2) is in magenta. Genetic distances between the markers are drawn to scale. Scale bar, 10 centimorgans (cM). Several LG9 and LG19 markers do not recombine with each other or with the sex-determination locus (Sex) in males. The coloured bars to the left of the map indicate the relative position of the FISH probes. **b**, The X_1 and X_2 chromosomes from a representative Akkeshi Japan Sea female metaphase spread and the X_1 , X_2 and neo-Y chromosomes from a representative Akkeshi Japan Sea male metaphase spread are shown. A LG9 BAC (magenta) and a LG19 BAC (green) were used as probes for FISH.

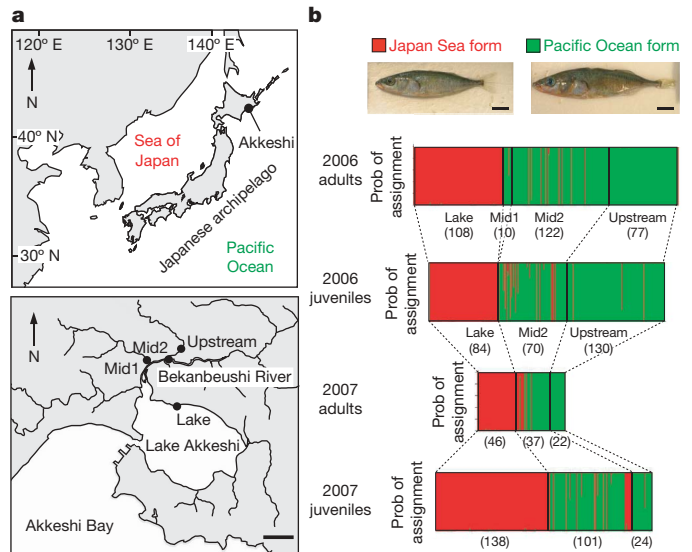


Figure 2 | Distribution of the Japanese threespine stickleback species pair in a region of sympatry. **a**, The top panel shows a map of the Japanese archipelago, indicating the location of Akkeshi in eastern Hokkaido, Japan. The bottom panel shows a map of our four collection sites (Lake, Mid1, Mid2 and Upstream) in Lake Akkeshi and the Bekanbeushi marsh. Scale bar, 2 km. **b**, The top panels show representative photos of a Japan Sea male and a Pacific Ocean male. Scale bar, 10 mm. Genetic analysis of fish collected in 2006 ($n = 601$) and 2007 ($n = 368$) was performed using STRUCTURE. There are two genetic clusters in Akkeshi (Supplementary Fig. 2), with the Japan Sea cluster shown in red, and the Pacific Ocean cluster shown in green. In the STRUCTURE plots, each individual is indicated by a single vertical line. The probability of assignment to the Japan Sea cluster or the Pacific Ocean cluster is indicated by the extent of the coloured bar. Fish collected from the same location are grouped together, with locations separated by thick black lines. The number of individuals per location is indicated in parenthesis. The Japan Sea form is mostly found in the lake, whereas the Pacific Ocean form is mostly found in the upstream region of the Bekanbeushi River. The midstream region (Mid2) is a hybrid zone that contains both parental forms and hybrids.

dorsal pricking display is greatly exaggerated, and the male pushes the female upwards during dorsal pricking (Fig. 3b)¹⁹. Furthermore, dorsal spine length is sexually dimorphic (males have longer spines than females) in the Japan Sea form²⁵. In contrast, in the Pacific Ocean form, the dorsal pricking display is weak (Fig. 3b), and dorsal spine length is not sexually dimorphic^{19,25}. We found that the Pacific Ocean females frequently escaped and did not resume mating after they encountered the aggressive dorsal pricking of Japan Sea males, whereas the Japan Sea females did not escape from males after dorsal pricking (Fig. 3b).

Genetic mapping of isolating barriers

To investigate the chromosomal locations of the isolating barriers between the two forms, we backcrossed F₁ hybrid (Japan Sea female × Pacific Ocean male) females to Pacific Ocean males and conducted quantitative trait locus (QTL) mapping of male dorsal pricking, male dorsal spine length, male body size, and hybrid male sterility. Individuals were genotyped with 90 single nucleotide polymorphism (SNP) markers and 13 other microsatellite markers that together span the stickleback genome. Hybrid male sterility and male body size mapped to LG19 (Fig. 4, Supplementary Fig. 4 and Supplementary Table 1), which is the ancestral X chromosome shared by the Japan Sea form and Pacific Ocean form. Dorsal pricking and first dorsal spine length mapped to distinct locations on LG9 (Fig. 4, Supplementary

Fig. 4 and Supplementary Table 1), which is the neo-X chromosome in the Japan Sea form. A genome-wide scan for epistatic interactions identified a significant conspecific interaction between loci on LG19 for hybrid male sterility (log likelihood ratio of linkage (LOD) comparing the full model with interaction to the additive model = 5.08; genome-wide significance threshold = 3.99 ($\alpha = 0.05$); Supplementary Fig. 5). No significant epistatic interactions were found for any other traits or loci examined.

Our data demonstrate that loci important for both prezygotic and postzygotic isolation map to the X chromosomes in this natural vertebrate system. This large X-effect is unlikely to result from an over-representation of these chromosomes in the stickleback genome, as LG9 and LG19 comprise just 9.0% of the stickleback genome (20.2 megabases (Mb) each of 446.6 Mb total—4.5% for each linkage group in the stickleback genome assembly; BROAD S1, Feb 2006). Because mapping in a backcross probably overestimates the effects of the hemizygous X chromosome²⁶, we also performed QTL mapping of the same traits in an independent F₂ intercross to ensure that the observed large X-effect was not simply the result of our backcross mapping strategy. We still detect QTL for dorsal pricking and first dorsal spine length on LG9 and QTL for male sterility and body size on LG19, with the addition of a single QTL for testis size on LG1 (Supplementary Fig. 6 and Supplementary Table 2).

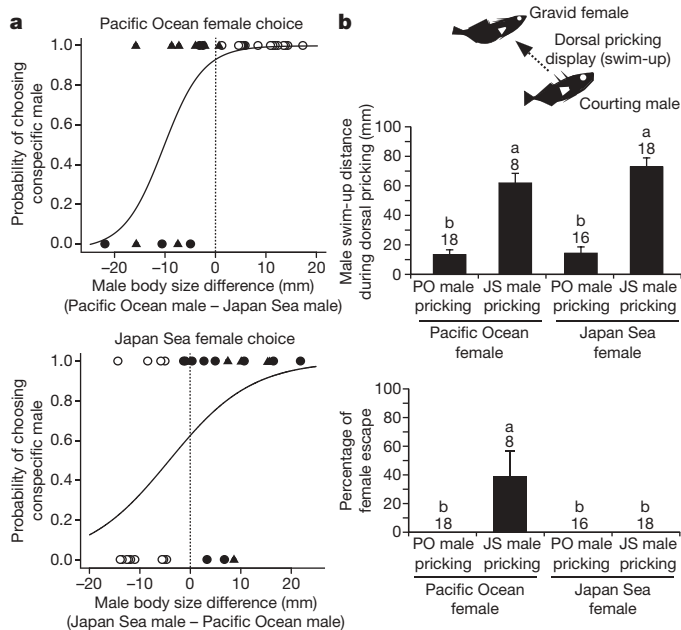


Figure 3 | Behavioural isolation results from divergence in male body size and male dorsal pricking behaviour. **a**, The top panel indicates the preferences of Pacific Ocean females ($n = 30$), and the bottom panel indicates the preferences of Japan Sea females ($n = 29$) as a function of male body size divergence. The horizontal axes indicate the body size difference in mm between males (conspecific male standard length minus heterospecific male standard length). Each symbol indicates a mate choice trial, in which 1 indicates the female chose a conspecific male, and 0 indicates the female chose a heterospecific male. Trials were conducted with laboratory-reared Pacific Ocean males (solid circles), resident freshwater Pacific males (solid triangles), or without size manipulation (open circles). The logistic regression curves indicate the probability of a female choosing a conspecific male for a given difference in male body size. **b**, The top panel shows the distance a Pacific Ocean (PO) or Japan Sea (JS) male moved upwards during dorsal pricking with Pacific Ocean or Japan Sea females. The bottom panel shows the percentage of trials in which a female escaped after dorsal pricking by either a Pacific Ocean or a Japan Sea male. The sample size for each mating pair is shown above each column. Lower case letters above the bars represent pairs that are significantly different from each other (pairwise Mann–Whitney U -test, $P < 0.05$ after Bonferroni correction).

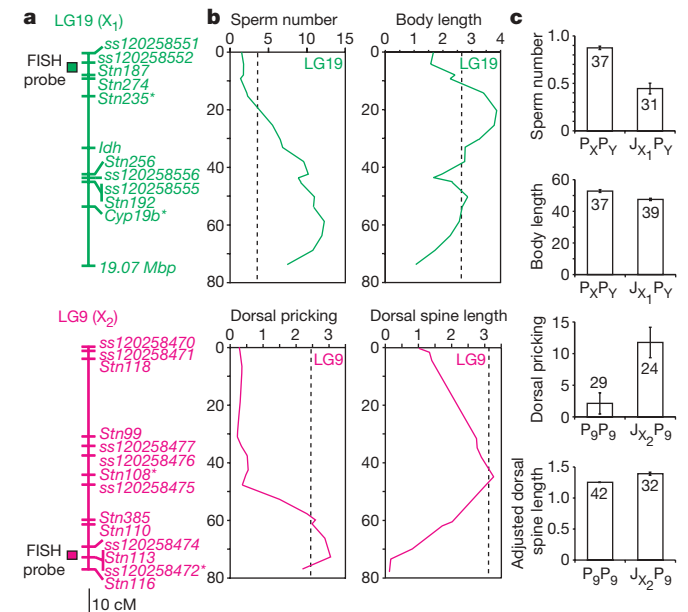


Figure 4 | Genetic mapping of isolating barriers. **a**, Genetic linkage maps of LG19 (X₁; green) and LG9 (X₂; magenta) in the backcross. The locations of FISH probes are indicated to the left of the map, whereas the names of genetic markers are indicated to the right. The asterisk indicates the marker closest to the QTL peak. **b**, For each QTL, the LOD score is indicated across the top and is plotted relative to the positions of the markers indicated in **a**, with distance in cM indicated. The hybrid male sterility QTL is represented by sperm number, the body size QTL is represented by body length, and the dorsal pricking QTL is represented by mean dorsal pricking. The dorsal spine length QTL is represented by first dorsal spine length, which was analysed with body length as an interacting covariate. Dashed lines indicate the genome-wide significance thresholds determined by permutation tests ($\alpha = 0.05$). **c**, For each trait, the phenotypic values (mean ± s.e.m.) are indicated for genotypes at the marker closest to the QTL peak; that is, *Cyp19b* for sperm number, *Stn235* for body length, SNP *ss120258472* for mean dorsal pricking, and *Stn108* for dorsal spine length. Sample sizes for each genotypic class are shown in the graph. These were the only genomic regions with significant phenotype-genotype associations after Bonferroni correction ($P < 0.0001$), detected either by a Kruskal–Wallis test (sperm number, body length, and dorsal pricking) or by analysis of covariance (ANCOVA) (dorsal spine length).

Discussion

Mapping of hybrid male sterility to the ancestral X chromosome (LG19) is consistent with previous studies on hybrid male sterility in other systems^{5,6}, suggesting that the large X-effect on hybrid male sterility is common across diverse taxa. Although the reasons for the large X-effect on hybrid sterility are debated⁵, in some cases it results from genetic conflict in the form of sex ratio meiotic drive^{27,28}. However, we find no evidence for sex ratio distortion in the weakly fertile F₁ hybrid males resulting from a cross between Japan Sea females and Pacific Ocean males (Supplementary Table 3). We do, however, find evidence for conspecific epistasis between X-linked loci, which is commonly observed for hybrid male sterility in *Drosophila*^{29,30}. Despite this finding of a common large X-effect across widely separated taxa, we found no evidence for any effect of the neo-X chromosome on hybrid male sterility. It may be that the relative age and/or levels of degeneration of sex chromosomes are important factors in determining whether the X chromosome contributes to the evolution of hybrid male sterility.

Unlike male sterility, male courtship display traits conferring behavioural isolation between the Japan Sea and Pacific Ocean forms map to both the ancestral X chromosome and the neo-X chromosome. Interestingly, male body size is sexually dimorphic in both the Japan Sea and Pacific Ocean forms²⁵ and maps to the ancestral X chromosome. In contrast, first dorsal spine length is only sexually dimorphic in the Japan Sea form²⁵, and the dorsal pricking display is exaggerated in the Japan Sea males; these traits both map to the neo-X chromosome. Thus, these display traits might have evolved as a result of differential fitness effects between males and females specifically in the Japan Sea lineage. Although our cross design did not allow us to directly test whether these traits mapped to the neo-Y chromosome as predicted by theory^{7,8}, it is possible that selection for linkage between male beneficial traits, such as dorsal pricking and dorsal spine length, and the sex-determination locus actually promoted the spread of the fusion between LG9 and the Y chromosome in the Japan Sea population⁷. Alternatively, these male beneficial traits may have accumulated on the neo-X chromosome³ after the formation of the neo-Y chromosome in the last 1.5–2 million years. In either case, our data provide direct empirical evidence linking sex chromosome turnover and reproductive isolation between closely related species.

The turnover of sex chromosomes between species is common in many groups of animals, where closely related species often differ in sex-determination and sex-chromosome systems^{9,10}. Although it has been suggested that sex-chromosome turnover might drive rapid speciation in cichlid fishes in which a female colour trait is linked to an invading ZW sex-chromosome system^{15–17}, this hypothesis has not been directly tested. Given the potential role of sexual antagonism in driving both sex-chromosome evolution^{1,3,7,8} and speciation^{31,32}, we suggest that sex-chromosome divergence between closely related species should be given further consideration as an important mechanism contributing to the evolution of reproductive isolation.

METHODS SUMMARY

Threespine sticklebacks of the Pacific Ocean and the Japan Sea form were collected with seine nets and minnow traps in Lake Akkeshi and the Beukanbeushi River in May–July of 2003–2008. Japan Sea fish were also collected from an allopatric site (Cape of Benkei) on the west coast of Hokkaido in 2008. For cytogenetic and behavioural studies, live fish were transported to the Fred Hutchinson Cancer Research Center; all experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC number 1575). Cytogenetics and FISH were performed on Japan Sea males ($n = 2$) and a female ($n = 1$) from Akkeshi, as well as Japan Sea males ($n = 2$) and females ($n = 3$) from the allopatric site, using fluorescently labelled bacterial artificial chromosome (BAC) clones as previously described²². For population genetic analysis, 969 fish were genotyped with 12 neutral microsatellite markers (Supplementary Table 4). Mate-choice experiments were conducted as previously described¹⁹. For QTL mapping, a Japan Sea female and a Pacific Ocean

male were crossed to obtain an F₁ hybrid family ($J_1 \times P_1$). These F₁ females were crossed with males resulting from a cross between a Pacific Ocean female and another Pacific Ocean male ($P_2 \times P_3$) to generate backcross progeny. At maturity, 76 backcross males were phenotyped for traits related to body size, fertility and dorsal pricking behaviour. These males were genotyped with LG9 and LG19 microsatellites, as well as a panel of SNP markers distributed across the stickleback genome (Supplementary Table 5). All DNA isolation and microsatellite genotyping were conducted as previously described¹⁹; SNP genotyping was performed using Illumina Golden Gate arrays. The genotypes of 90 SNPs and 14 microsatellites were used to create a linkage map in JoinMap 3.0 (ref. 33), and QTL analyses were performed in MapQTL 4.0 (ref. 34) and R/qtl³⁵.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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