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. In this study, we show that a newly evolved sex chromosome harbours 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 harbours loci for male courtship display traits that contribute to behavioural isolation, while 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 suggest that sex chromosome turnover might play a greater role in speciation than 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 (i.e. beneficial in one sex and detrimental in the other sex) and the sex-determination locus, resulting in a reduction of recombination between sex chromosomes[1]. 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 males[2]. 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 play an important 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 consistent[6].

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 is particularly striking in fishes[9,10]. Many independent groups of fishes show evidence for rapid evolution of sex chromosomes through several different mechanisms, including the transposition of an existing male-determination locus to an autosome[11], the evolution of a new male-determination locus on an autosome[12], and fusions between an autosome and an existing Y-chromosome[13,14]. It has been suggested that rapid turnover of sex chromosomes driven by sexual conflict might also play 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 stickleback[20] 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 (LG) 19 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 analyzed (Fig. 1a). These data suggested 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 Japan Sea males (n = 4) from two different populations have an odd number of chromosomes (2n = 41), with one large unpaired chromosome that hybridized to the LG19 and LG9 probes, providing evidence for the LG9-Y fusion (Fig. 1b; Supplementary Fig. 1). By contrast, 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; 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 system[13], 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 likely 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; 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; Supplementary Discussion). We previously demonstrated that behavioural isolation and hybrid male sterility are asymmetric, but act in complementary directions[19]. Pacific Ocean females mate exclusively with Pacific Ocean males, while the Japan Sea females mate with both types of males in laboratory mate choice trials[19]. Although Japan Sea females do mate with Pacific Ocean males, hybrid males resulting from this cross have severely reduced fertility, while reciprocal hybrid males and all hybrid females are fertile[19].

In order 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 analyzed 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, $n = 59$) are smaller than Pacific Ocean males (standard length = 76.17 ± 0.47 mm, $n = 45$; 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% CI = 0.624-0.992) of choosing a conspecific male (Fig. 3a), suggesting that additional factors play a role 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 serve as a way for the male to display his dorsal spines to the female[23,24]. In Japan Sea males, the dorsal pricking display is greatly exaggerated, and the male pushes the female upwards during dorsal pricking (Fig. 3b)[19]. In addition, dorsal spine length is sexually dimorphic (males have longer spines than females) in the Japan Sea form[25]. By 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, while the Japan Sea females did not escape from males after dorsal pricking (Fig. 3b).

Genetic mapping of isolating barriers

In order to investigate the chromosomal locations of the isolating barriers between the two forms, we backcrossed F1 hybrid (Japan Sea female x 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 additional microsatellite markers that together span the stickleback genome. Hybrid male sterility and

male body size mapped to LG19 (Fig. 4; Supplementary Fig. 4; 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; 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)/446.6 Mb = 4.5% for each LG in the stickleback genome assembly; BROAD S1, Feb 2006). Because mapping in a backcross is likely to overestimate the effects of the hemizygous X[26], 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; Supplementary Table 2).

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 debated[5], 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 on hybrid male sterility. It may be that the relative age and/or levels of degeneration of sex chromosomes are an important factor 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 forms[25] and maps to the ancestral X. By contrast, first dorsal spine length is only sexually dimorphic in the Japan Sea form[25], and the dorsal pricking display is exaggerated in the Japan Sea males; these traits both map to the neo-X. 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 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 population[7]. Alternatively, these male beneficial traits may have accumulated on the neo-X chromosome[3] 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.

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 where 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 Bankei) 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 #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 described[22]. For population genetic analysis, 969 fish were genotyped with twelve neutral microsatellite markers (Supplementary Table 4). Mate choice experiments

were conducted as previously described[19]. For QTL mapping, a Japan Sea female and a Pacific Ocean male were crossed to obtain an F1 hybrid family (J_1 x 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 x 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 previously described[19]; 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[33], and QTL analyses were performed in MapQTL 4.0[34] and R/qtl[35].

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Supplementary Information including detailed methods, a supplementary discussion, seven figures, five tables, and associated references accompanies the paper on www.nature.com/nature.

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Author Contributions J.K., S.M., and C.L.P. conceived and designed the study. F.C.J., Y.F.C., D.M.A., J.G., J.S., R.M.M., and D.M.K. contributed new reagents and carried out the SNP genotyping experiments for genome-wide linkage mapping. J.K, J.A.R., S.M., M.K., and C.L.P. performed all other experiments and analyzed the data. J.K. and C.L.P. wrote the manuscript.

Author Information All SNP information has been deposited at http://www.ncbi.nlm.nih.gov/projects/SNP/. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.L.P. (e-mail: cpeichel@fhcrc.org).

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

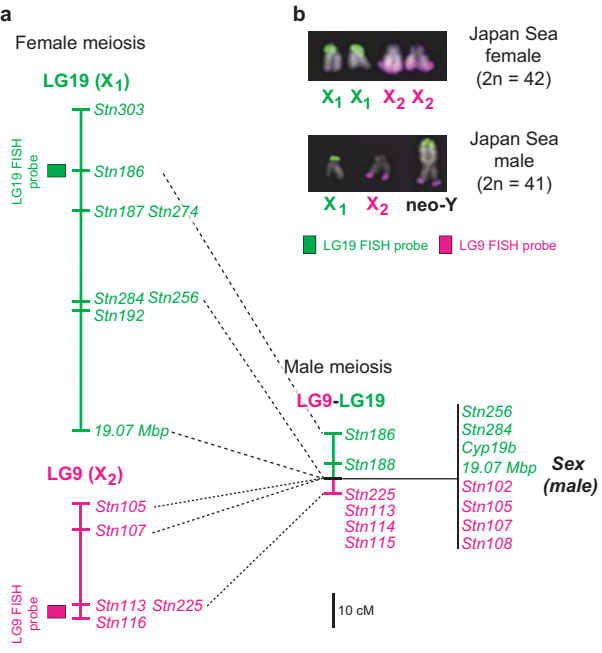
(X-Y recombination) of LG9/19 (neo-Y) is to the right. LG19 (X_1) is in green and LG9 (X_2) is in magenta. Genetic distances between the markers are drawn to scale (scale bar = 10 centimorgans (cM)). Several LG9 and LG19 markers do not recombine with each other or with the sex-determination locus (*Sex*) 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 lower 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 represented in red, and the Pacific Ocean cluster represented 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, while 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.

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 axis indicates the body size difference in mm between males (conspecific male standard length minus heterospecific male standard length). Each symbol indicates a mate choice trial, where 1 indicates the female chose a conspecific male, while 0 indicates the female chose a heterospecific male. Trials were conducted with lab-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 upper 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 lower panel shows the percentage of trials in which a female escaped after dorsal pricking by either a Pacific Ocean or 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 Bonferonni correction).

Figure 4 | Genetic mapping of isolating barriers. a, Genetic linkage maps of LG19 (X_1; green) and LG9 (X_2; magenta) in the backcross. The locations of FISH probes are indicated to the left of the map, while the names of genetic markers are indicated to the right of the map. 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 panel 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 analyzed 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 $\pm$ s.e.m.) are indicated for genotypes at the marker closest to the QTL peak; i.e *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 ANCOVA (dorsal spine length).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Female meiosis
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LG19 FISH probe
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Stn284 Stn256
Stn192
19.07 Mbp
LG9 (X2)
Stn105
Stn107
LG9 FISH probe
Stn113 Stn225
Stn116
Male meiosis
LG9-LG19
Stn186
Stn188
Stn225
Stn113
Stn114
Stn115
Stn256
Stn284
Cyp19b
19.07 Mbp
Stn102
Stn105
Stn107
Stn108
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Japan Sea male (2n = 41)
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LG19 FISH probe
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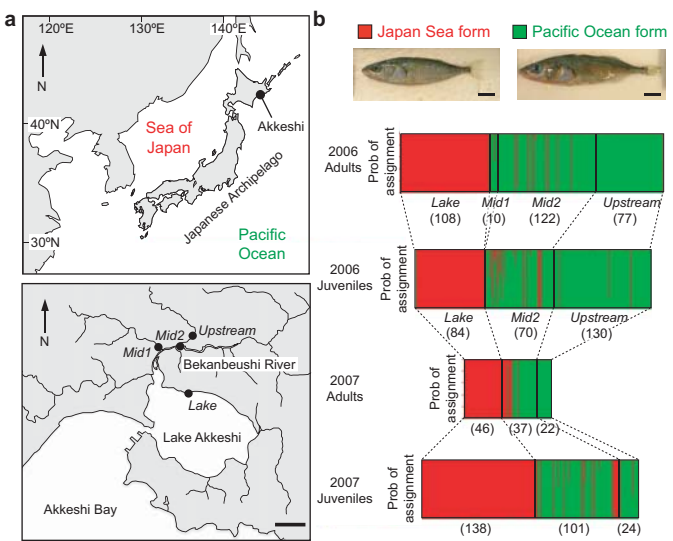
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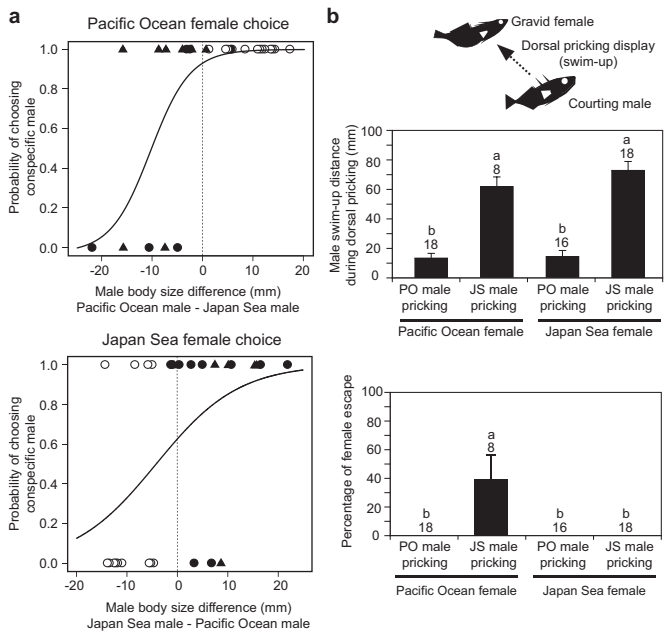
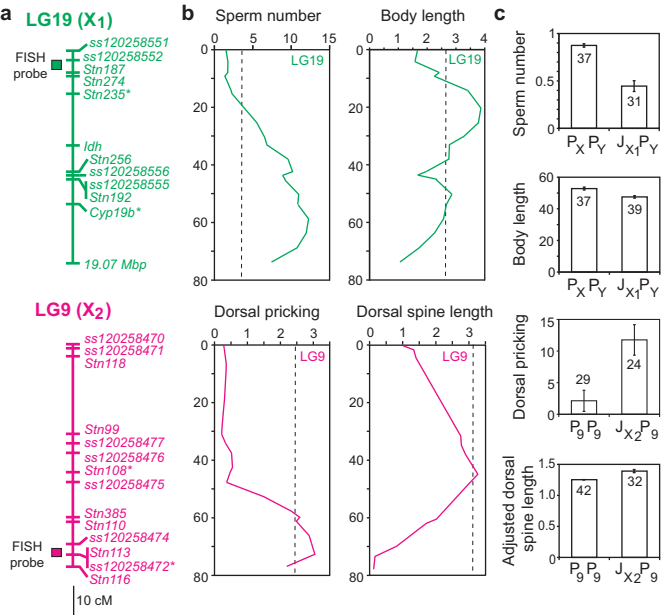
Pacific Ocean form
2006 Adults
Prob of assignment
Lake (108)
Mid1 (10)
Mid2 (122)
Upstream (77)
2006 Juveniles
Prob of assignment
Lake (84)
Mid2 (70)
Upstream (130)
2007 Adults
Prob of assignment
(46)
(37)
(22)
2007 Juveniles
Prob of assignment
(138)
(101)
(24)


a
Pacific Ocean female choice
Probability of choosing conspecific male
1.0
0.8
0.6
0.4
0.2
0.0
-20
-10
0
10
20
Male body size difference (mm)
Pacific Ocean male - Japan Sea male
Japan Sea female choice
Probability of choosing conspecific male
Male body size difference (mm)
Japan Sea male - Pacific Ocean male
b
Gravid female
Dorsal pricking display
(swim-up)
Courting male
Male swim-up distance during dorsal pricking (mm)
100
80
60
40
20
0
b
18
a
8
b
16
a
18
PO male pricking
JS male pricking
PO male pricking
JS male pricking
Pacific Ocean female
Japan Sea female
Percentage of female escape
b
18
a
8
b
16
b
18


a
LG19 (X1)
FISH probe
ss120258551
ss120258552
Stn187
Stn274
Stn235*
Idh
Stn256
ss120258556
ss120258555
Stn192
Cyp19b*
19.07 Mbp
LG9 (X2)
ss120258470
ss120258471
Stn118
Stn99
ss120258477
ss120258476
Stn108*
ss120258475
Stn385
Stn110
ss120258474
Stn113
ss120258472*
Stn116
FISH probe
10 cM
b
Sperm number
Body length
Dorsal pricking
Dorsal spine length
LG19
LG9
c
Sperm number
Body length
Dorsal pricking
Adjusted dorsal spine length
37
31
37
39
29
24
42
32
PX PY
JX1 PY
P9 P9
JX2 P9