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Molecular Cytogenetic Evidence of Rearrangements on the Y Chromosome of the Threespine Stickleback Fish

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ABSTRACT

To identify the processes shaping vertebrate sex chromosomes during the early stages of their evolution, it is necessary to study systems in which genetic sex determination was recently acquired. Previous cytogenetic studies suggested that threespine stickleback fish (*Gasterosteus aculeatus*) do not have a heteromorphic sex chromosome pair, although recent genetic studies found evidence of an XY genetic sex-determination system. Using fluorescence *in situ* hybridization (FISH), we report that the threespine stickleback Y chromosome is heteromorphic and has suffered both inversions and deletion. Using the FISH data, we reconstruct the rearrangements that have led to the current physical state of the threespine stickleback Y chromosome. These data demonstrate that the threespine Y is more degenerate than previously thought, suggesting that the process of sex chromosome evolution can occur rapidly following acquisition of a sex-determining region.

SIMPLE genetic sex determination (GSD), in which a single master sex-determination locus (*SEX*) initiates sexual development, is often associated with the presence of a visible size difference (heteromorphy) in one chromosome pair. The chromosome containing *SEX*, the Y in a male heterogametic (XY/XX) system or the W in a female heterogametic (ZW/ZZ) system, is the sex chromosome. The association between GSD and heteromorphic sex chromosomes was initially based on empirical data (Wilson 1905), and the correspondence of phenotypic traits to distinct sex chromosomes was later used to support the chromosomal theory of heredity (Morgan 1910; Bridges 1916). The association of GSD and heteromorphy has been suggested to result initially from selection for reduced recombination between linked sex-determination loci, followed by selection for reduced recombination between *SEX* and linked genes with sexually antagonistic alleles (Charlesworth and Charlesworth 1978; Bull 1983; Rice 1987b; Charlesworth *et al.* 2005). The reduction of recombination around sex-determination loci, seen in a plethora of taxa (Fraser and Heitman 2005), allows for a degenerative process that involves the accumulation of mutations and mobile sequence elements, intrachromosomal inversions, and deletions (Rice 1987a; Jablonka and Lamb 1990; Charlesworth and Charlesworth 2000; Charlesworth *et al.* 2005; Steinemann and Steinemann 2005; Graves 2006). This onslaught can drastically alter the amount of genetic material comprising the sex chromosome.

Sequence-based characterizations of the human Y chromosome have provided a striking example of the outcome of this degenerative process. During the ~166–300 million years that the mammalian Y has been diverging from the X (Lahn and Page 1999; Veyrunes *et al.* 2008), the Y has experienced intrachromosomal inversions (Tilford *et al.* 2001; Skaletsky *et al.* 2003) and deletions (Kuroda-Kawaguchi *et al.* 2001; Repping *et al.* 2002; Noordam and Repping 2006) leading to the loss of function of most coding regions on the Y (reviewed in Ross *et al.* 2006). Deletions are likely the predominant cause of human sex chromosome heteromorphy, as the Y contains one-third as much DNA as the X (Skaletsky *et al.* 2003; Ross *et al.* 2005). Despite this rich literature in mammalian sex chromosome evolution, this work has not led to identification of the mechanisms underlying the initial degenerative process on a vertebrate sex chromosome, in part because sex chromosome degeneration eventually erases the molecular signatures of earlier events. Thus, to study the early steps in the evolution of vertebrate sex chromosomes, it is preferable to study a species in which GSD has arisen recently. Fish are attractive organisms in which to study this process, because even closely related species can use different sex-determination mechanisms (Devlin and Nagahama 2002). Such species are likely to have sex chromosomes in the early stages of heteromorphy.

We previously demonstrated that threespine stickleback fish (*Gasterosteus aculeatus*) use a single Mendelian locus, found in a nonrecombining region of the male-

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specific Y chromosome, to initiate male sexual development (Peichel *et al.* 2004). Earlier cytogenetic studies had found no evidence for the presence of a heteromorphic sex chromosome in *G. aculeatus* (Chen and Reisman 1970; Klinkhardt and Buuk 1990; Cuñado *et al.* 2002), while a heteromorphic XY pair was found in a closely related species, *G. wheatlandi* (Chen and Reisman 1970). The same study found evidence for a ZW sex chromosome system in a more distantly related stickleback species, *Apeltes quadracus* (Chen and Reisman 1970). In contrast, no heteromorphic sex chromosome pairs have been reported in the outgroup family Syngnathidae (Vitturi *et al.* 1998; Libertini *et al.* 2006). The existence of different sex chromosome systems within the stickleback family, along with the availability of genetic resources and the complete genome sequence of the female threespine stickleback (Peichel *et al.* 2001; Kingsley *et al.* 2004; Kingsley and Peichel 2007) make this fish a compelling vertebrate system in which to study sex chromosome evolution.

Our previous analysis comparing a few hundred thousand base pairs of sequence from the nonrecombining region of the Y and the homologous region from the X showed that the Y chromosome has accumulated many sequence characteristics of a sex chromosome, including an elevated transposable element content and small intrachromosomal duplications and inversions (Peichel *et al.* 2004). These findings raised the possibility that more extensive rearrangements might have occurred on the Y chromosome. In the present study, we use fluorescence *in situ* hybridization (FISH) with bacterial artificial chromosome (BAC) probes to demonstrate that the Y chromosome of the threespine stickleback is indeed heteromorphic, with gross physical differences between the X and Y due both to deletion and inversions on the Y. On the basis of our FISH-based cytogenetic maps of the X and Y, we propose a model for the rearrangements that led to the present structure of the Y.

MATERIALS AND METHODS

X chromosome sequence assembly: The sequence of the X chromosome, linkage group (LG) 19, from a single threespine stickleback female has been assembled into three supercontigs (SC): SC85 (1–529,649 bp) + SC34 (1–3,292,649 bp) + SC3 (1–16,416,407 bp). The order of supercontigs in this public assembly was determined by a threespine stickleback LG19 genetic map (Broad Institute and Stanford University Center of Excellence in Genomic Sciences, unpublished data). We confirmed the relative orientation of supercontigs by BLASTing (Altschul *et al.* 1990) the sequences of mapped genetic markers (Peichel *et al.* 2004) against the public genome assembly, which was produced at the Broad Institute and is accessible via the Ensembl and University of California Santa Cruz genome browsers (http://www.ensembl.org/Gasterosteus_aculeatus/index.html; http://genome.ucsc.edu/cgi-bin/hgGateway) and found that the orientation of SC3 is inverted in the X assembly with respect to our genetic map (Peichel *et al.* 2004). To produce an assembly of X chromosome supercontigs both ordered and oriented by our genetic map, we reversed the sequence of SC3 and then joined the three supercontigs. Our X assembly comprises SC85 (1–529,649) + SC34 (1–3,292,604) + SC3 (16,416,407–1) (Figure 1).

Genetic mapping: We genetically mapped several microsatellite markers using previously described methods and crosses (Peichel *et al.* 2004) to characterize the extent of a putative deletion on the Y chromosome. The five primer pairs, named for their positions on our X chromosome sequence assembly, were 17.16 Mbp 5′-TTGGAGAGTAATGCATTCATGG-3′ and 5′-GGGCTGTTCTCAAACACAGG-3′; 18.10 Mbp 5′-GGGCCTGGTATAAGCTCTGC-3′ and 5′-ACGGCACAGATTGTGAGTGG-3′; 18.41 Mbp 5′-CTGTTGTAACTCGGGAGAAGG-3′ and 5′-CAGGGAGAGATTCGTGTTGG-3′; 18.73 Mbp 5′-GCGTCCGTTCTCTACATGG-3′ and 5′-AGGAGGGTTCATCTTCATGC-3′; 19.68 Mbp 5′-GGCAGCCATTACTTGAGAGG-3′ and 5′-CTTTAGTACGAGCAGTTCTTCC-3′.

Identification of FISH probes: Threespine stickleback BAC clones from the CHORI-213 library (Kingsley *et al.* 2004) used as FISH probes (appendix) were identified using one of two methods. In the first, overgo probes designed to LG19 markers were used to screen BAC library filters as in Peichel *et al.* (2004). Primers used to generate overgo probes were *Stn191* 5′-CCTTTTTTTTGTTCCTTACCTGTCCG-3′ and 5′-GACAAGGAGATCCATTGACGGACAGG-3′; *Stn192* 5′-AGCAAACAACGCCACACGTAACTG-3′ and 5′-CCAACAAGACGTGAACCAGTTACG-3′; *Stn194* 5′-ACCAGCTCCCAGATACTCGCTGT-3′ and 5′-CTGGGTCCTGAGATAACAGCGAG-3′.

We sequenced the ends of BACs identified in the library screens as follows: 160 ng of isolated BAC DNA in 10 mm TRIS pH 7.4 was combined with 10 pmol of sequencing primer, 3 μl Big Dye Terminator v3.1 (Applied Biosystems), and 5× sequencing buffer (Applied Biosystems) to yield 1× final concentration. The reactions were then cycle sequenced (94° for 4 min; 100 cycles of 94° for 10 sec, 50° for 10 sec, and 60° for 4 min, stored at 4°) and run on an ABI 3100 Genetic Analyzer (Applied Biosystems). Sequencing primers were CHORI T7.29 (5′-GCCGCTAATACGACTCACTATAGGGAGAG-3′) and gSP6 (5′-GTTTTTTGCGATCTGCCGTTTC-3′). We used Phred (Ewing and Green 1998; Ewing *et al.* 1998) to call bases using a trim cutoff value of 0.001. The BAC end sequences were BLASTed (Altschul *et al.* 1990) against the stickleback genome to verify their positions on the LG19 supercontigs (appendix).

We also used publicly available paired BAC end sequences to identify clones spanning additional positions of interest on LG19. T7 and SP6 reads from CHORI-213 BAC clones obtained from the threespine stickleback genome survey sequence (Kingsley and Peichel 2007) were RepeatMasked (http://www.repeatmasker.org) and then BLASTed (Altschul *et al.* 1990) against the stickleback genome. Clones whose paired end sequences met four criteria [full-length matches to LG19, opposing orientation, appropriate separation for a CH213 BAC insert size (average 190 kbp, Kingsley *et al.* 2004), and flanking the LG19 BLAST position of genes or markers of interest (appendix)] were used as FISH probes. Each FISH-probe clone is identified here by the name of the genetic marker or sequence feature that it contains or to which it is nearest (appendix).

Cytogenetic techniques: Metaphase spreads were prepared from primary stickleback tissue. Ten μl of 1% colchicine in phosphate-buffered saline was intraperitoneally injected into an adult male and an adult female Pacific Ocean threespine stickleback collected from the Bekanbeushi River (Hokkaido Island, Japan). After 16 hr of incubation in an aquarium, the fish were anesthetized with tricaine methanesulfonate (MS-222, Fisher), sex was confirmed by gonad morphology, and spleens removed into 0.56% KCl on ice. The spleens were Dounce homogenized into a single-cell suspension, diluted in

0.56% KCl until barely turbid, and incubated on ice for 45 min. Cells remaining in suspension were then fixed three times by pelleting in a centrifuge and resuspending the pellet in 3:1 methanol:glacial acetic acid. The fixed cells were resuspended in fresh fixative and dropped onto glass slides that were then cured for at least 18 hr at room temperature prior to hybridization. These procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol no. 1575).

FISH was carried out as previously described (Trask 1999) with the following modifications. BAC DNA was isolated using an Autogen 740 automated system (Autogen) and quantified by gel electrophoresis. One microgram of each BAC clone was labeled with either ChromaTide Alexa Fluor 488-5-dUTP or 568-5-dUTP (Invitrogen) using the Vysis nick translation kit (Abbott Labs). Two hundred nanograms of each labeled clone were ethanol precipitated together with 10 μg salmon sperm DNA. Hybridization was performed over 2–3 nights at 37°. Washed slides were mounted and counterstained in DAPI with AntiFade (Vector Labs) and viewed with a 100× objective on a Nikon Eclipse 80i microscope with an automated filter turret using Chroma filters 31000v2, 41001, and 41004. Images were captured with a Photometrics Coolsnap ES2 camera using Nikon Elements software and pseudocolored white (DAPI), green (Alexa 488), and purple (Alexa 568) using Adobe Photoshop.

RESULTS

Comparison between the X chromosome genetic map and sequence assembly: Before comparing the X and the Y to look for differences (heteromorphy), we first verified that the published genetic map (Peichel *et al.* 2004) and the public assembly of the X agreed on the arrangement of markers. Indeed, the order of markers within each supercontig corresponded to their genetic order. However, in the public assembly of X supercontigs, marker *Stn192* (at 53 cM) is located between *Stn186* (27.3 cM) and *Stn187* (35.6 cM), inconsistent with the genetic order of these markers. We resolved this discrepancy between the public X assembly and our genetic map by reversing the sequence of SC3 to create our own X chromosome sequence assembly (see materials and methods) in which the order of markers in the sequence map is congruent with their order in the genetic map (Figure 1).

The Y chromosome is heteromorphic: It is impossible to use genetic mapping to determine the order of many markers on the Y chromosome because many X chromosome marker alleles do not recombine with the Y in males (Peichel *et al.* 2004). We therefore performed FISH using threespine stickleback BAC clones as probes (appendix) to compare the locations of markers on the X and the Y chromosomes.

The first evidence of sex chromosome heteromorphy was obtained using a BAC probe containing the *Idh* gene. In males, the probe hybridizes to two chromosomes at different chromosomal locations (Figure 2a), whereas it hybridizes to an identical location on the q (long) arm of two visibly similar submetacentric chromosomes in females (Figure 2b). In males, one labeled chromosome is similar in shape and probe location to those labeled in females, defining it as the X. The second chromosome's male-specific hybridization pattern defines it as the Y. Although the X and Y are similar in size, the Y is metacentric and carries the *Idh* signal

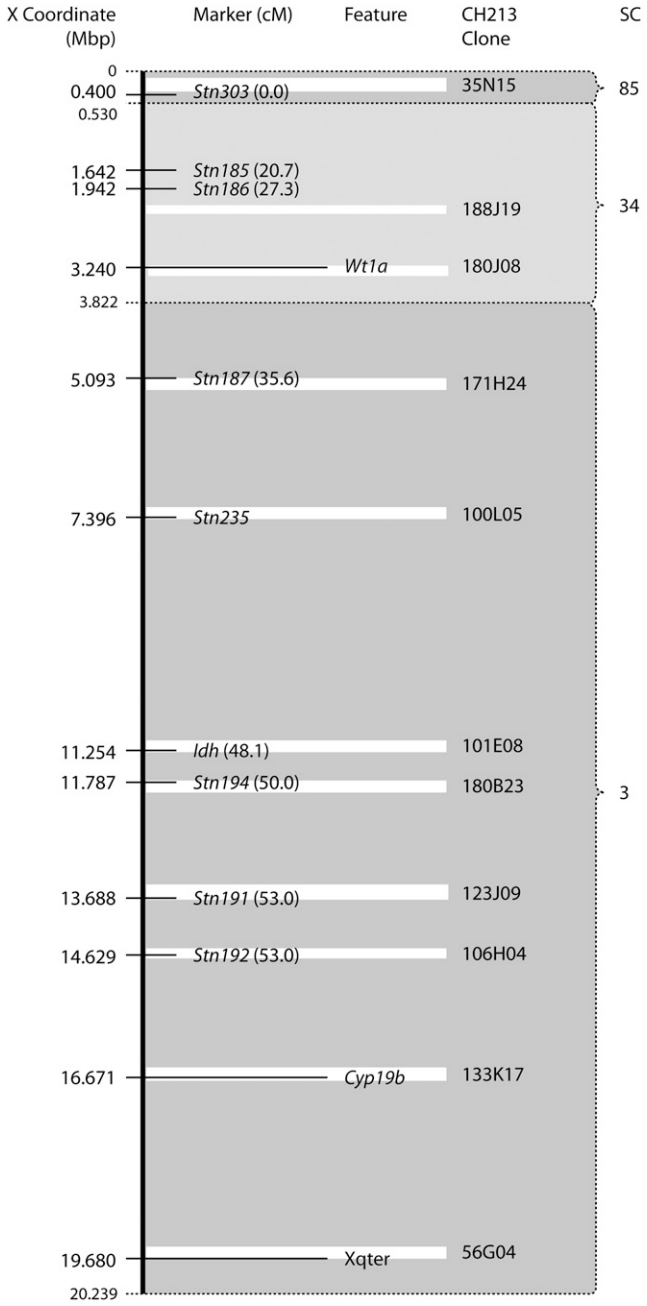

Figure 1.—Correspondence between genetic and sequence maps of the threespine stickleback X chromosome. The three supercontigs (SC, shaded regions) are oriented by marker order on the genetic map (Peichel *et al.* 2004). The sequence coordinates of markers and features (solid horizontal lines) are given on the left. Coordinates in smaller text give the sequence coordinates of the SC boundaries (dotted lines). Positions of BAC clones used as FISH probes (open horizontal bars) encompassing markers or genes of interest are shown to scale. The genetic positions of markers are from Peichel *et al.* (2004). *Stn235* has not been genetically mapped; its position was determined by BLAST (Altschul *et al.* 1990).

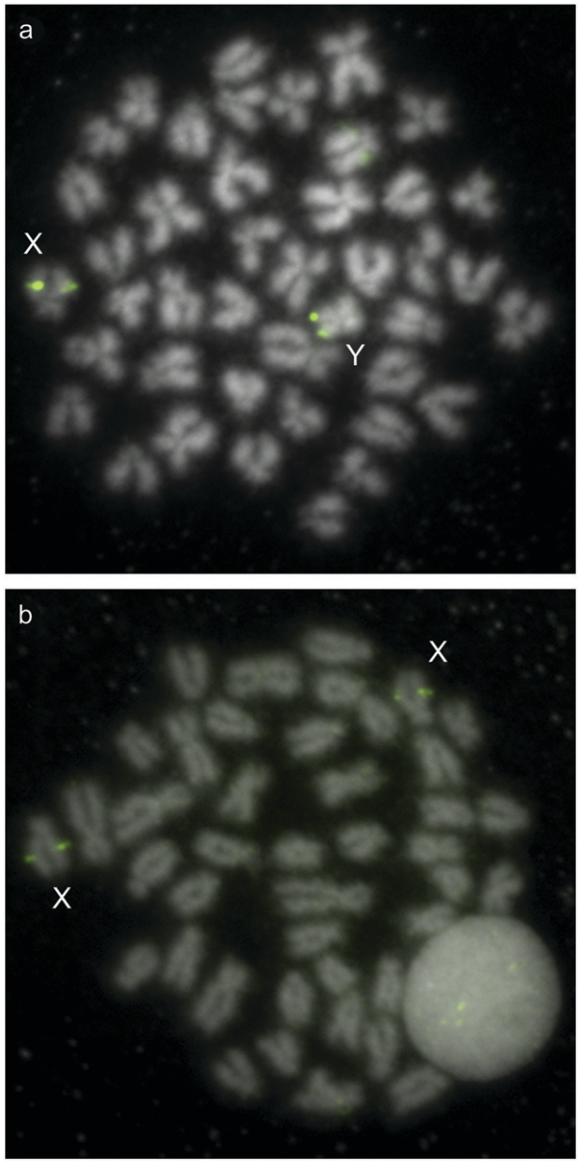

FIGURE 2.—The Y chromosome is heteromorphic. (a) *Idh* probe (green) hybridized to a male (XY) threespine stickleback metaphase spread ($2n = 42$). The hybridized submetacentric chromosome is identical to the X chromosomes in Figure 2b. The other chromosome with terminal hybridization signal is metacentric, identifying it as the Y chromosome. (b) *Idh* probe (green) hybridized to a female (XX) threespine stickleback metaphase spread ($2n = 42$). Hybridization signals identify the two submetacentric X chromosomes.

near one telomere. By virtue of *Idh* being located on Xq, we will refer to the arm of the Y containing *Idh* as Yq. This X–Y dimorphism in location of *Idh* and the position of the centromere are apparent in all metaphase chromosome spreads from the Japanese Pacific Ocean male threespine stickleback used in this study and in eight additional *G. aculeatus* males from populations in Lake Washington and Conner Creek, Washington and the Little Campbell River, British Columbia, Canada (data not shown). A karyogram from a male *G. aculeatus* metaphase spread is shown in Figure 3; the metaphase spread used to create this karyogram is provided in supplemental Figure 1.

The Y chromosome has experienced deletion: Because *Idh* appears to be terminal on the Y, we tested whether sequences telomeric to *Idh* on the X are present on the Y. In each analysis of male metaphase spreads, we used the *Idh* BAC to distinguish the X and Y. A BAC containing *Cyp19b*, which is located at 16.7 Mbp in the X assembly (Figure 1, APPENDIX), hybridizes only to the X and not to the Y nor to any other location in the genome (Figure 4), suggesting that part of the Y chromosome has been deleted. This conclusion is supported by the presence on the X but not on the Y of other probes around *Cyp19b* (Figure 5 shows the *Stn191*, *Stn192*, and Xqter probes). Moreover, we previously showed that microsatellite markers *Stn191* and *Stn192* can be PCR-amplified from X chromosome alleles but no products are produced from the Y (PEICHEL *et al.* 2004). Five new microsatellite markers within this putative deletion at 17.16, 18.10, 18.41, 18.73, and 19.68 Mbp are also Y null (data not shown). Taken together, these data suggest that the interval bounded by *Stn191* and Xqter, spanning 6 Mbp on the X, has been deleted from the Y.

The Y chromosome has experienced inversion: The difference in centromere position in the X and Y could be due to a pericentric inversion. We performed a three-probe FISH experiment to test for such an inversion (Figure 6). The order of probe signals is *Wt1a*-CEN-*Stn187*-*Idh* on the X, but *Stn187*-CEN-*Wt1a*-*Idh* on the Y. This result is consistent with an inversion of at least 1.7 Mbp encompassing *Wt1a*, the centromere, and *Stn187*.

FISH-based cytogenetic maps of the X and Y chromosomes: To identify additional rearrangements of the Y, we conducted multiple FISH experiments and constructed cytogenetic maps of the stickleback X and Y chromosomes. In each experiment, performed on a male metaphase spread, we cohybridized the *Idh* probe with another BAC containing a sex chromosome genetic marker or gene of interest (Figure 5; APPENDIX). All BACs were found to hybridize to a single locus on the X and/or Y but not to any other chromosome.

The cytogenetic map of the X chromosome is congruent with our genetic map and sequence assembly. However, the cytogenetic map of the Y chromosome (Figure 5) is very different from that of the X outside of the ~3.2 Mbp region that freely recombines between the X and the Y (containing *Stn303* and *Stn186*). In addition to the aforementioned deletion and inversion, we find the *Stn194* probe in the *Idh*-qTEL interval on the X but in the pTEL-CEN interval on the Y.

We established the relative orders of *Wt1a* and *Stn235* within the Y chromosome CEN-*Idh* interval and of *Stn194* and *Stn187* within the Y chromosome pTEL-CEN interval by FISH experiments with the *Idh* probe and the two probes whose order was to be determined. On the Y, the order is CEN-*Wt1a*-*Stn235*-*Idh* (supplemental Figure 2a) and *Stn194*-*Stn187*-CEN-*Idh* (supplemental

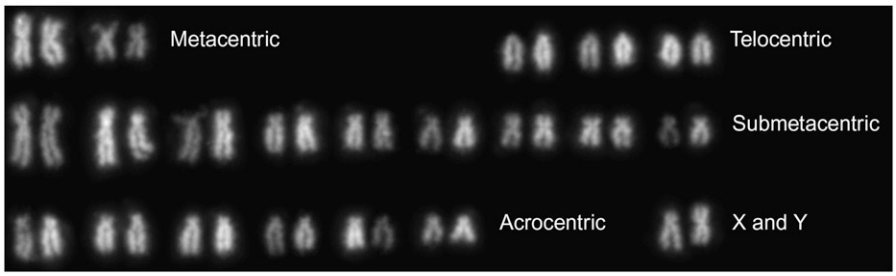

Figure 3.—Threespine stickleback male karyogram. The karyogram was produced from a male (XY) metaphase spread ($2n = 42$) shown in supplemental Figure 1. The chromosomes are aligned vertically by centromere position and ordered from largest (left) to smallest (right) within each class. There are two metacentric pairs, three telocentric pairs, nine submetacentric pairs, six acrocentric pairs, and the heteromorphic X (submetacentric) and Y (metacentric) pair.

Figure 2b). In contrast, the order is *Wt1a*-CEN-*Stn235*-*Idh* and CEN-*Stn187*-*Idh*-*Stn194* on the X, in agreement with the X sequence and genetic maps. The most parsimonious model of intrachromosomal rearrangements that accounts for the physical order of FISH markers on the Y is shown in Figure 7.

DISCUSSION

In this report, we used FISH to compare the physical structures of the threespine stickleback X and Y chromosomes and found that the Y chromosome of *G. aculeatus* is heteromorphic, despite prior reports (Chen and Reisman 1970; Klinkhardt and Buuk 1990; Cuñado *et al.* 2002), implying that the threespine Y is more degenerate than previously expected. Although there is a large (6 Mbp) deletion on the Y equivalent to 30% of the sequence content of the X chromosome, the X and Y appear similar in size at metaphase. Without molecular cytogenetics, heteromorphy is apparent only by a change in the position of the centromere, reinforcing the point that a heteromorphic sex chromosome need not be visibly smaller than its homolog, especially during the early stages of its evolution (reviewed in Ming and Moore 2007).

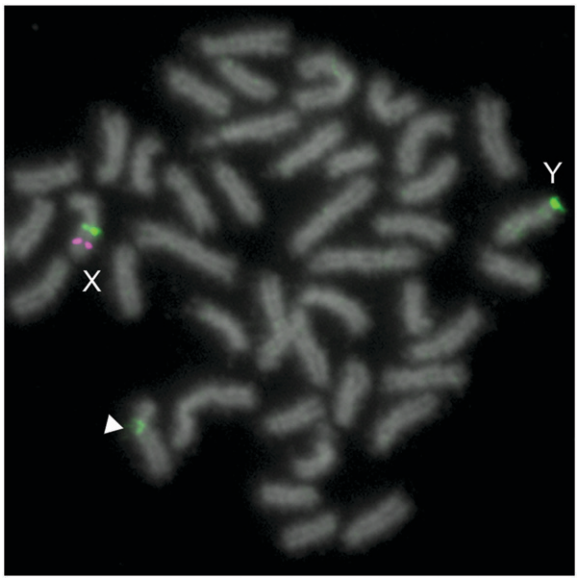

Figure 4.—Deletion on the Y chromosome. *Idh* probe (green) and *Cyp19b* probe (purple) hybridized to a male (XY) threespine stickleback metaphase spread. The hybridized submetacentric chromosome at left exhibits the internal *Idh* hybridization signal of the X chromosome; *Cyp19b* signal is near the q arm telomere. The hybridized metacentric chromosome at right exhibits the terminal *Idh* hybridization signal of the Y chromosome; no *Cyp19b* staining is seen. In most metaphase spreads hybridized with the *Idh* probe, a diffuse signal can also be seen at the centromere of one submetacentric chromosome (arrowhead).

This similarity in size might explain why heteromorphy was not identified previously in the threespine stickleback. Although prior studies did not use molecular cytogenetic techniques to search for heteromorphy, we cannot rule out the possibility that the Y chromosomes in the Atlantic threespine stickleback populations used in previous studies (Chen and Reisman 1970; Klinkhardt and Buuk 1990; Cuñado *et al.* 2002) had not experienced the same repertoire of rearrangements that produced sex chromosome heteromorphy in the Pacific Ocean-derived populations that we have studied. Identical X–Y dimorphism in *Idh* location and centromere position is seen in threespine males from multiple populations (data not shown), suggesting that the heteromorphic X–Y pair described here is shared at least among Pacific Ocean threespine sticklebacks. We suspect that detailed molecular cytogenetic analyses, such as those performed here, will reveal heteromorphic sex chromosomes in many more species, especially in fishes, in which sex-determination mechanisms and sex chromosomes are remarkably labile (Devlin and Nagahama 2002).

To render X and Y chromosomes of similar size at metaphase, the deletion on the threespine Y chromosome might be compensated by accumulation of repetitive DNA (Graves 1995; Steinemann and Steinemann 2000). In support of this argument, our sequence comparison of the X and the Y chromosomes showed that accumulation of mobile DNA has expanded the Y by over 38% in the region analyzed around the *Idh* locus (Peichel *et al.* 2004). The addition of repetitive DNA might even outpace the attrition of the Y chromosome caused by deletion (Graves 1995). We note, however, that coarse size measurements of the X and Y at metaphase might not correlate with their sequence content.

Our model for the evolution of the stickleback Y chromosome (Figure 7) explains the change in position

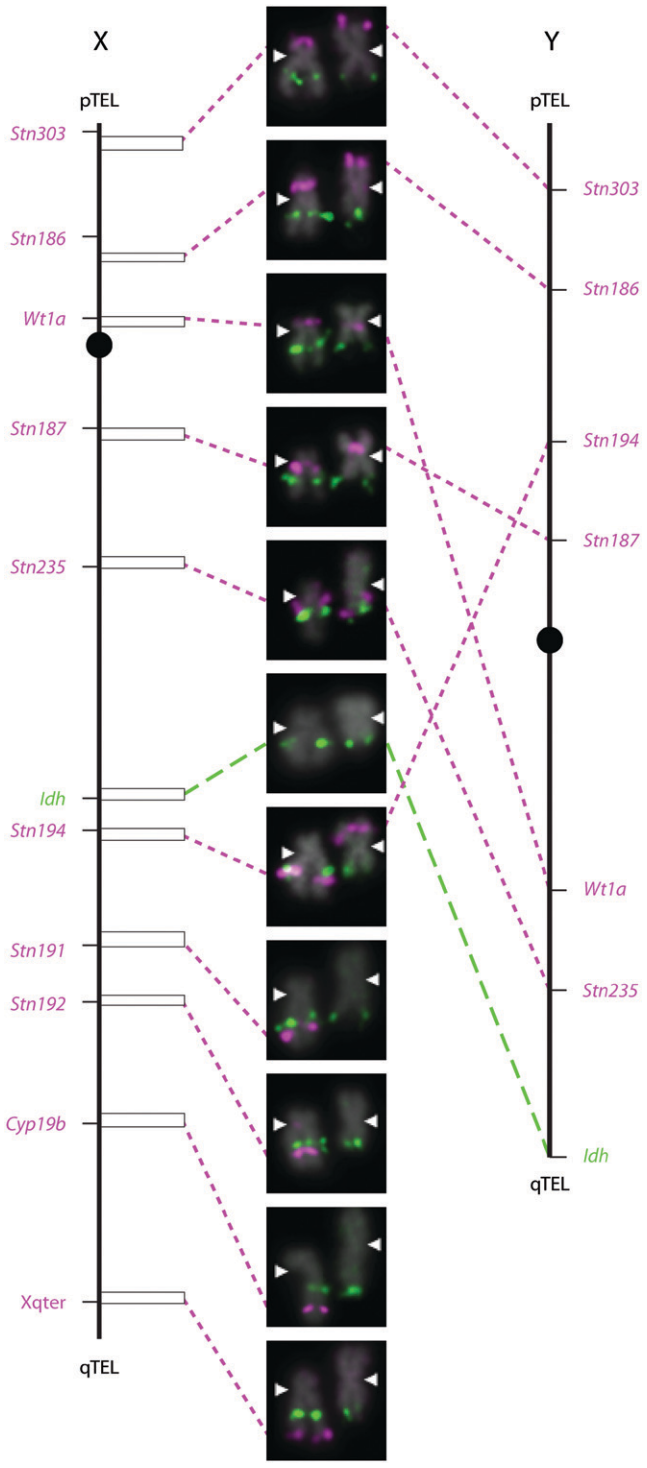

Figure 5.—FISH-based cytogenetic maps of the threespine stickleback X and Y chromosomes. The sequence map of the X (left) is shown to scale with horizontal black lines representing positions of markers and features and horizontal white bars representing BAC clones used as probes (for clone identities, see Figure 1). The black circles represent the positions of the centromeres. Each FISH section (center) contains the X (left) and Y (right) from a single metaphase spread to which the *Idh* probe (green) and one additional probe (purple) were hybridized. The X and Y are vertically aligned by the position of *Idh*, and the position of the centromere is identified with a white arrowhead. Dashed lines joining the X map to the FISH sections indicate the BAC probe used in each section. Dashed lines leading from the FISH data to the Y cytogenetic map (right, not to scale) indicate the physical interval (pTEL-CEN or CEN-Idh) to which each FISH probe hybridizes.

of the centromere from the submetacentric X chromosome to the metacentric Y due to pericentric inversions. Our cytogenetic mapping of the X centromere between *Wt1a* and *Stn187* is supported by the X sequence map. This interval contains a gap between SC34 and SC3. We expect the centromere to be composed of repetitive sequences and refractory to sequence assembly (Schueler *et al.* 2001; reviewed in Henikoff 2002); thus, this gap in the sequence assembly might indicate the position of the centromere.

Our model predicts that multiple inversions have occurred on the stickleback Y chromosome, yet Y alleles of some markers involved in these inversions were previously found to recombine with the X (Peichel *et al.* 2004). Thus, the expectation that inversions on the Y will cause loss of recombination with the X raises the possibility that the nonrecombinant interval on the Y containing *SEX* may be larger than previously determined. Given these results, we now believe that the rare X–Y recombination events reported previously are likely phenotypic sex revertants or due to mistaken genotypes or phenotypes; however, DNA from the key recombinant fish are no longer available to test this hypothesis (Peichel *et al.* 2004). On the basis of our current genetic and cytogenetic maps, we now conclude that the nonrecombining *SEX* interval on the threespine stickleback Y extends at least from *Wt1a* to Xqter, a physical region equivalent to 16 Mbp on the X. On the Y, the nonrecombining interval around *SEX* may be as large as 10 Mbp, given that at least one deletion of 6 Mbp has occurred. This value is only an estimate, as the Y might have experienced additional deletions and/or accumulated mobile sequence elements in the nonrecombining region. We also conclude that the physical size of the X–Y homologous region, in which all recombination events between the X and Y occur, is <3.2 Mbp, extending from pTEL to an inversion breakpoint between it and *Wt1a* (Figure 7).

Both the *Cyp19b* and *Wt1a* genes are present on the threespine stickleback X chromosome; *Cyp19a* and *Wt1b* are found on the sex chromosome of the Nile tilapia (*Oreochromis niloticus*) (Lee and Kocher 2007), suggesting that the sex chromosomes of Nile tilapia and threespine stickleback evolved from homeologous chromosomes produced during an ancient genome duplication event in fishes (Amores *et al.* 1998). Nevertheless, it is unlikely that a single ancestral autosome became the sex chromosomes in all fish employing genetic sex determination, because the linkage groups containing *SEX* in different species are not syntenic. The sex-determination locus mapped in the pufferfish *Takifugu rubripes* lies in a region without synteny either to the

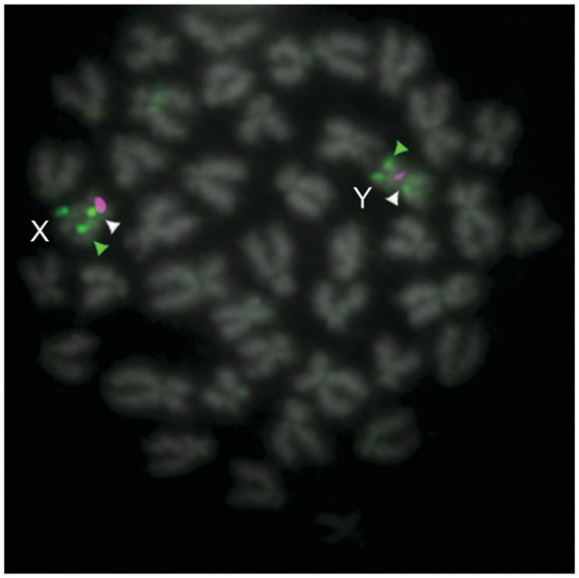

Figure 6.—Pericentric inversion on the Y chromosome. *Idh* probe (green), *Stn187* probe (green), and *Wt1a* probe (purple) hybridized to a male (XY) threespine stickleback metaphase spread ($2n = 42$). While the X marker order is *Wt1a* (purple), centromere (white arrowhead), *Stn187* (green), *Idh* (green; green arrowhead), the order is changed on the Y: *Stn187* (green), centromere (white arrowhead), *Wt1a* (purple), and *Idh* (green; green arrowhead).

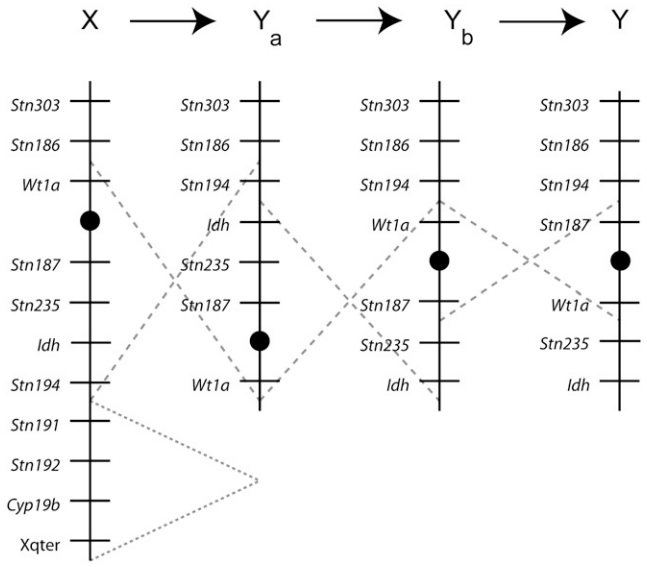

Figure 7.—Parsimony model for the evolution of the threespine stickleback Y. This model is the most parsimonious way to use inversions and deletions to arrive at the physical order of markers on the Y (right) having started with the order on the X (left). We hypothesize that three inversions (crossing dashed lines) containing the centromere (solid circle) and one deletion (dotted lines that meet to the right) gave rise to the extant Y. Theoretical intermediate Y chromosome states are labeled Y_a and Y_b, although the order of inversions in this model is arbitrary. The relative timing of the deletion is also arbitrary and not necessarily concomitant with an inversion.

stickleback or to the medaka (*Oryzias latipes*) sex chromosomes (Kikuchi *et al.* 2007). It is perhaps not surprising that fish species of different taxonomic orders have sex chromosomes derived from different autosomes, as divergence in sex chromosome systems have been reported for closely related species of the Oryzias genus (Takehana *et al.* 2007a; Takehana *et al.* 2007b; Tanaka *et al.* 2007), the tilapia genus Oreochromis (Lee *et al.* 2003; Lee *et al.* 2004; Cnaani *et al.* 2008), the poeciliid fishes (Volff and Schartl 2001), and salmonids (Phillips *et al.* 2001; Woram *et al.* 2003).

The lone sex-determination gene known in fish, *DMY* (Matsuda *et al.* 2002) or *DMRT1Y* (Nanda *et al.* 2002) in the medaka, arose as a result of an interchromosomal duplication onto a chromosome (LG1) syntenic to human chromosome 4 (Kondo *et al.* 2006). In contrast, part of stickleback LG19 is syntenic to human chromosome 15 (Peichel *et al.* 2004), again reflecting the independent evolutionary histories of the medaka and stickleback sex chromosomes. While the medaka Y chromosome is young, having arisen ~10 million years ago (Kondo *et al.* 2004; Kondo *et al.* 2006), the degenerate Y-specific region has no homologous sequence on the X; thus, the basis for heteromorphy and lack of recombination around *DMY* is quite clearly due to its hemizygous status (Schartl 2004).

Our analysis finds that the threespine stickleback Y has already experienced many aspects of sex chromosome degeneration, despite being chronologically younger than the mammalian Y. Like evolving sex chromosomes in a number of species (Jablonka and Lamb 1990; Charlesworth and Charlesworth 2000; Charlesworth *et al.* 2005; Graves 2006), the stickleback Y has accumulated repetitive DNA in its nonrecombining region (Peichel *et al.* 2004). The present study also demonstrates the existence of inversions on a sex chromosome in a region that lacks recombination. This situation is very similar to the *Silene latifolia* Y chromosome, which is believed to be 10–20 million years old (Nicolas *et al.* 2005; Bergero *et al.* 2007). The *S. latifolia* Y is heteromorphic and larger than the X (Westergaard 1958; Vyskot and Hobza 2004), possibly due to the accumulation of repetitive elements on the Y (Hobza *et al.* 2006; Kejnovsky *et al.* 2006; Marais *et al.* 2008). The *S. latifolia* Y has also experienced at least one pericentric and one paracentric inversion (Hobza *et al.* 2007). However, a recent study concluded that inversions were not involved in cessation of X–Y recombination in *S. latifolia* (Bergero *et al.* 2008). The presence of multiple pericentric inversions on the *G. aculeatus* Y (Figure 7) is consistent with Ohno's prediction that a pericentric inversion could be used to establish sex chromosome heteromorphy (Ohno 1967). Additional analysis of the levels of X–Y divergence across the stickleback sex chromosome pair may identify "evolutionary strata" similar to those on the *S. latifolia* Y chromosome (Filatov 2005; Nicolas *et al.* 2005; Bergero *et al.* 2007; Marais

et al. 2008) and allow us to test for the association between these pericentric inversions and the suppression of recombination.

Sex chromosome heteromorphy has been reported in the black-spotted stickleback *G. wheatlandi* (XY) and in the fourspine stickleback *A. quadracus* (ZW) (Chen and Reisman 1970), both of which likely diverged from the threespine stickleback within the past 20 million years. Thus, comparative studies of sex chromosomes within the stickleback family promise to yield insights into the evolution of sex chromosome systems. With the molecular, genetic, and genomic tools available and being developed for the threespine stickleback (Peichel *et al.* 2001; Kingsley *et al.* 2004; Kingsley and Peichel 2007), including the molecular cytogenetic tools used in this study to provide the first report of FISH in sticklebacks, comparative analyses of sex chromosome evolution in stickleback fishes may help us understand the process of transition between XY and ZW systems of genetic sex determination in closely related species.

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APPENDIX

Sequence and clone sources

Feature	Clone end	X position (Mbp)	GenBank accession	Identification method	Probe alias
SC85		0.000	—		
CH213-35N15	T7	0.098	CL642751	*In silico*	*Stn303*
CH213-35N15	SP6	0.318	CL642750	*In silico*	*Stn303*
Stn303		0.400	BV154586		
SC85/SC34		0.530	—		
Stn185		1.642	G72214		
Stn186		1.942	G72215		
CH213-188J19	SP6	2.220	CL648631	*In silico*	*Stn186*
CH213-188J19	T7	2.355	CL648632	*In silico*	*Stn186*
CH213-180J08	SP6	3.213	CL648481	*In silico*	*Wt1a*
Wt1a		3.240	NM_001104701	*In silico*	
CH213-180J08	T7	3.393	CL648482	*In silico*	*Wt1a*
SC34/SC3		3.822	—		
CH213-171H24	T7	5.091	CL648304	*In silico*	*Stn187*
Stn187		5.093	G72216		
CH213-171H24	SP6	5.269	CL648303	*In silico*	*Stn187*
CH213-100L05	SP6	7.209	CL645741	*In silico*	*Stn235*
Stn235		7.396	BV678166		
CH213-100L05	T7	7.421	CL645742	*In silico*	*Stn235*
CH213-101E08	T7	11.073	AC144485	Peichel *et al.* (2004)	*Idh*
Idh		11.254	—	Peichel *et al.* (2004)	
CH213-101E08	SP6	11.277	AC144485	Peichel *et al.* (2004)	*Idh*
CH213-180B23	T7	11.752		Library screen	*Stn194*
Stn194		11.787	G72220		
CH213-180B23	SP6	11.946		Library screen	*Stn194*
CH213-123J09	T7	13.494		Library screen	*Stn191*
Stn191		13.688	G72218		
CH213-123J09	SP6	13.706		Library screen	*Stn191*
CH213-106H04	SP6	14.518		Library screen	*Stn192*
Stn192		14.629	G72319		
CH213-106H04	T7	14.703		Library screen	*Stn192*
CH213-133K17	T7	16.511	CL647204	*In silico*	*Cyp19b*
Cyp19b		16.671	AF183908	*In silico*	
CH213-133K17	SP6	16.734	CL647203	*In silico*	*Cyp19b*
CH213-56G04	SP6	19.470	CL643820	*In silico*	Xqter
CH213-56G04	T7	19.674	CL643821	*In silico*	Xqter
Xqter		19.680	—		
SC3		20.239	—		

BAC clone, marker, gene, and X chromosome assembly features are listed in the first column. For BAC clone sequences, the ends (T7 and SP6) are given in the second column. All features are sorted in ascending order in the third column, which gives the BLAST positions of the features to our X chromosome assembly. The accession numbers of previously published sequences are given in the fourth column; the accession numbers listed for genes *Wt1a* and *Cyp19b* refer to the query sequences used to identify the positions of those genes on the stickleback X by BLAST. The fifth column indicates whether the clone or gene was identified by our *in silico* method or library screen (see MATERIALS AND METHODS) or in published work. The sixth column lists the aliases used in this article to refer to BAC clones. Xqter is arbitrarily defined as position 19.68 Mbp on the X assembly; clone 56G04 (aliased as "Xqter") is the most terminal clone hybridizing to the q arm of the X chromosome.