The Evolution of Sex-Chromosome Systems in Stickleback Fishes

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Abstract

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The genetical and physical degeneration of sex chromosomes in many diverse taxa has been described, yet the mechanisms initiating the degenerative process have not been characterized extensively by studying young sex-chromosome systems. Following the genetic identification of an XY pair in threespine sticklebacks, *Gasterosteus aculeatus*, I conducted sequence analyses and a cytogenetic characterization of the Y chromosome. I found that the chronologically young threespine Y has a nonrecombining region that exhibits sequence characteristics expected of evolved sex chromosomes, including an accumulation of repetitive sequence elements, reduced homology with the X, and the presence of inversions and deletions. These findings fulfill predictions that repetitive DNA and inversions might initiate loss of recombination on a sex chromosome. Efforts to clone and sequence the threespine Y for further analysis have thus far yielded 1.9 Mbp of sequence. My comparative cytogenetic studies have identified the presence of three previously unknown heteromorphic sex-chromosome systems in stickleback species. The identification of two independent X_1X_2Y/XX systems having relationships to XY sex-chromosome systems in sister taxa along with the presence of a ZW pair in stickleback species diverged less than twenty million years have stimulated research addressing existing theories of sex chromosome-autosome fusions and will permit the characterization of the transition between sex-chromosome systems and the genetic evaluation of the role of chromosomal rearrangements in speciation. These findings reflect the lability of sex-determination mechanisms and sex-chromosome systems in fish and strengthen the stickleback family of fish as a model system in which to study the evolution of sex determination and sex chromosomes.

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PREFACE

It has been exciting and a pleasure to have helped develop stickleback fish as model systems for studying the dynamic and unpredictable path that sex chromosomes tread and to have been a part of the development of sticklebacks as model genetic and genomic organisms. The process of refining and improving the techniques for cloning a sex chromosome, creating custom computational tools for analyzing sequence data, and developing FISH techniques for sticklebacks were particularly rewarding by allowing me to explore diverse disciplines.

I feel particularly blessed to have developed a project that I was excited daily to engage because I knew I could discover something exciting and novel at any point. While I hate to leave behind so many questions, the answers to which seem to be just around the corner, it is with great pride and satisfaction that I do so because it is as a result of my research that these questions are possible for another generation in the Peichel lab to ask.

Joseph A. Ross
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Seattle, Washington

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DEDICATION

This work is dedicated to the memories of my grandfather

Marden Ross

(1916–1991)

and grandmother

Bonnie Merlin

(1921–1992)

without whom none of this would have been possible.

Chapter 1. Background

As long ago as in ancient Greece, the mechanism of how an individual becomes male or female was pondered. Parmenides suggested that the side of the womb an embryo developed in defined its sex; Anaxagoras later countered that the side of the father's testis was the determining factor (Mittwoch 2005). Although we now know that sex is determined by a single gene that resides on the male-restricted Y chromosome present in most mammals, recent findings have raised new questions to ponder.

The impact of sex determination on development is huge, causing an organism to adopt a suite of sex-specific characteristics encompassing morphology, physiology, and behavior. Thus, one might reasonably expect that sex-determination pathways would be among the most highly conserved developmental processes. Yet, despite the broad tendency for developmental genetic pathways to be conserved (Carroll 2000), genes initiating sex determination in some taxa are evolutionarily labile, such that closely-related species appear to use different sex-determination triggers.

When a gene initiating male or female development comes into existence, the chromosome bearing the sex-determining locus (a "sex chromosome") must be restricted to one sex, because a male- or female-determining locus, by definition, only exists in members of a single sex. In many species, the chromosome containing a sex-determining locus does not appear physically similar to its homologous chromosome. The physical changes that take place on a sex chromosome are thought to be deleterious, but the widespread presence of sex chromosomes suggests that benefits must exist to counteract the costs of sex-chromosome "degeneration".

Thus, some of the key questions in the field of sex chromosome evolution at present include how rapidly sex chromosomes degenerate, what physical mechanisms cause degeneration, whether sex chromosomes become larger or smaller than their homologs as a result of degeneration, and whether certain chromosomes are destined to be the site of evolution of new sex-determination loci. Determining whether laws of sex chromosome evolution can be defined will require the study of these questions in many disparate taxa to identify trends in the evolution of sex chromosomes; my dissertation research addresses these questions.

Mechanisms of Sex Determination

In general, the sex-determination pathway is initiated by a trigger, the sex-determination factor, that ultimately activates a sex-specific developmental pathway. Broadly, sex-determination systems have been classified into two categories based on the type of sex-determining factor: genetic sex determination (GSD) and environmental sex determination (ESD). Dioecious species use either GSD or ESD, or a combination of the two ("polyfactorial"), to affect sex determination. In a polyfactorial system, as was reported recently to exist in the bearded dragon lizard *Pogona vitticeps* (Quinn *et al.* 2007), one system (ESD or GSD) supercedes the other: one system is effective under certain conditions and the other system determines sex under other conditions. In ESD, a cue such as temperature, pH, or social interaction establishes sex-specific development in an embryo.

In species employing simple GSD, the presence of a single sex-determining locus defines the heterogametic sex, the sex that produces two types of gamete. One gamete type carries the sex-determining locus and produces offspring of the heterogametic sex, and one type causes offspring to develop as the homogametic sex. In male heterogamety, the male sex-determining locus (*SEX*) is on a chromosome designated Y. The homologous chromosome, called X, can be nearly identical to the Y except that it must, by defini-

tion, lack *SEX*. Diploid males have a sex-chromosome constitution of XY and females of XX. In an evolved sex-chromosome pair like in humans, the X and Y share only a small region of homology called a pseudoautosomal region, in which obligate recombination events occur.

In the case of a male-heterogametic GSD system, two genetic properties distinguish males (XY) and females (XX): males have a Y while females do not; males also have a single X chromosome while females have two. When *SEX* acts in a dominant manner, the Y carries a male sex-determining factor that is absent from the X. Thus, presence of an intact Y is sufficient to produce males. When *SEX* acts in a recessive manner, the sex-determining locus might consist of a gene that has been mutated or deleted on the Y, such that it is no longer active. Hence, sex is determined by the absence of a locus on the Y. Males have a single active copy of this locus on the X, while females have two copies, one on each of their X chromosomes. In a recessive XY system, the number of X chromosomes directly or indirectly determines sex, as in the case of Drosophila. In a female-heterogametic system, a female sex-determining locus resides on a sex chromosome designated W; its homolog is called Z; thus ZW organisms are female and ZZ are male.

Identifying the Presence of GSD

Wilson was the first to note that the segregation pattern of a heteromorphic (visibly different in size or structure) chromosome pair in Hemipteran insects, such as the beetle Tenebrio, corresponded with sexual phenotype in offspring (Wilson 1905). This observation established that presence of a heteromorphic chromosome pair is evidence of a genetic sex-determination system. Since 1905, many heteromorphic sex chromosome pairs have been identified in diverse species. Visual differences that have been used to distinguish the X and Y, or the Z and W, in some species include difference in relative

chromosome length at metaphase, relative difference in centromere position, or difference in heterochromatin content. In humans, for example, the Y is much smaller than the X (Ross *et al.* 2005; Ross *et al.* 2006), while in the plant *Silene latifolia*, the Y is larger than the X (Westergaard 1958; Vyskot and Hobza 2004).

One limitation of the visual, cytogenetic, approach to identifying the presence of a GSD system is that not all sex-chromosome pairs are grossly heteromorphic. In medaka fish, *Oryzias latipes*, which use an XY GSD system that evolved recently, the X and Y are not grossly visibly different at metaphase (Matsuda *et al.* 1998). Such findings have suggested that a GSD system evolves prior to the onset of sex-chromosome heteromorphy. Furthermore, a heteromorphic pair does not predict the presence of a dominant sex determining locus on the sex chromosome, as the Y chromosome in flies of the Drosophila genus is heteromorphic compared to the X, yet the Y plays no role in sex determination (Hackstein *et al.* 1996; Carvalho 2002).

Aside from cytogenetic identification of GSD by heteromorphy, another approach to identifying GSD is to genetically map sex-determining loci. By establishing a cross between a male and a female, the segregation pattern of genetic marker alleles with sexual phenotype can reveal whether alleles from a region on a male-specific chromosome (Y) or female-specific chromosome (W) are inherited only by sons or daughters, respectively. The utility of this approach is that it might identify GSD in situations where a heteromorphic pair has not yet evolved.

Vertebrate Sex-chromosome Systems

Most male mammals have an XY sex-chromosome pair (Graves 2006). Reinforcing the assumption that sex-determination pathways should be highly conserved, a single GSD system, on the order of 166-300 million years old (MYO) (Lahn and Page 1999; Veyrunes *et al.* 2008), is common to almost all mammals. As the identity of a mamma-

lian sex-determining factor is known, it has been possible to test species for the presence of this gene. The gene *SRY* (Sex-determining Region of the Y) (Gubbay *et al.* 1990a; Sinclair *et al.* 1990) was identified as a Y-limited gene in mice sufficient to cause male sexual development; it is absent from XY mice that develop as females (Gubbay *et al.* 1990a; Gubbay *et al.* 1990b). Further studies in mice of the role of *SRY* described sex-reversed XY females carrying *de novo* mutations in *SRY* (Berta *et al.* 1990; Jager *et al.* 1990). Supplying chromosomally female mice with mouse *SRY* leads to their development into males (Koopman *et al.* 1991).

Although a heteromorphic XY pair carrying *SRY* is present in almost all mammals, a few species interdigitated through the mammalian phylogeny do not seem to carry *SRY*. It is likely that loss of *SRY*, perhaps accompanied by transitions to new sex-determination systems, occurred in these species. In the wood lemming *Myopus schisticolor* (Fredga *et al.* 1976), the mole vole *Ellobius lutescens* (Just *et al.* 2002), Japanese spinous country rats *Tokudaia osimensis osimensis* (Honda *et al.* 1977; Honda *et al.* 1978), and akodon rodents (Hoekstra and Hoekstra 2001), the presence of XY females suggests that the Y no longer controls male sex determination (Hoekstra and Edwards 2000).

Molecular evidence suggests that the XY system of mammals is 300 MYO (Lahn and Page 1999), while phylogenetic evidence has suggested a more recent age of 166 MY. Metatherians (marsupial mammals) diverged from eutherians (placental mammals) approximately 130 million years ago (MYA) (Just *et al.* 2002) and have *SRY* (Foster *et al.* 1992). However, prototherians (egg-laying mammals, comprising the duck-billed platypus, *Ornithorhynchus anatinus*, and a few species of echidna), diverged about 210 MYA (Woodburne *et al.* 2003; Grutzner *et al.* 2004a) but do not have *SRY* (Wallis *et al.* 2007; Waters *et al.* 2007). These findings support the assertion that *SRY* arose less than 210 MYA, after the divergence of monotremes. The platypus has a heteromorphic sex-chromosome system comprising ten chromosomes (Rens *et al.* 2004), some of which have

homology to both the eutherian Y and the bird W (Grutzner *et al.* 2004a). This unusual configuration raises the question of whether the sex-chromosome system of monotremes is evidence of an ancestral relationship between the XY system in mammals and the ZW system in birds (Veyrunes *et al.* 2008).

Aside from mammals, heteromorphic sex-chromosome pairs are also widely found in birds, reptiles, and amphibians (Schartl 2004b). In birds, the W is small and heterochromatic and shares a small homologous region with the Z, much like the pseudoautosomal regions of the human X and Y (Smith and Sinclair 2004). However, the chicken and human sex chromosomes do not share a common set of genes and hence are not syntenic (Nanda *et al.* 1999), and *SRY* is not present in birds (Mizuno *et al.* 2002). These results suggest the independence of the bird and mammal GSD systems and the convergent evolution of characteristics of their sex chromosomes. While the bird sex-determining factor has not been identified, a candidate gene approach identified the presence of *DMRT1* only on the Z chromosome, raising the possibility that a dosage-sensitive GSD system might be at work (Nanda *et al.* 1999; Shetty *et al.* 2002). However, it is still unknown whether the bird sex-determination locus acts in a dominant or recessive manner.

Some species of lizards and amphibians use XY or ZW GSD. Of particular interest is the Japanese frog *Rana rugosa*, in which XY and ZW systems exist in distinct populations of the same species (Miura *et al.* 1998). Many snakes also have a heteromorphic ZW pair, as birds do. Because of the close relationship of snakes and birds in the vertebrate phylogeny (Smith and Sinclair 2004), it was thought that the bird and snake ZW pairs were related. The same ZW pair is present in birds (Shetty *et al.* 1999), but a different ZW pair is shared by snakes (Matsubara *et al.* 2006). Because the sex-determining mechanisms within mammals, birds, and snakes are conserved, these are not systems in which the evolution of sex determination is easily studied.

In contrast, sex-determination systems in fishes are very labile (Devlin and Nagahama 2002) and comprise the broadest range of systems found in vertebrates, including ESD and GSD. Many independent XY and ZW systems exist in fishes. Occasionally, both are found in closely related species, indicating that one or both systems recently evolved. Several instances of X_1X_2Y systems, in which two chromosomes (the X_1 and X_2) segregate opposite the Y, have also been reported, as have XY_1Y_2 and W_1W_2Z systems (references in (Devlin and Nagahama 2002)).

Polygenic GSD systems, in which multiple genes appear to contribute to sex determination, also exist in fish. Notable model fishes for studying polygenic GSD include tilapia from the genus Oreochromis and the platyfish, *Xiphophorus maculatus*. In two species of tilapia, both XY and ZW systems appear to operate (Lee *et al.* 2004; Cnaani *et al.* 2008); in some populations of the platyfish, X, W, and Y chromosomes are present (Volff and Schartl 2001). Because sex-determination systems in fishes are so varied and because they arise frequently (Mank *et al.* 2006), fish make excellent model systems in which to study genetics of sex determination and the evolution of sex chromosomes.

However, the most established fish model systems for developmental biology and genomics are also the least beneficial for studying sex determination. In the zebrafish, *Danio rerio*, no environmental or genetic sex-determination factors have yet been identified, and no heteromorphic sex-chromosome pair has been found (Daga *et al.* 1996; Sola and Gornung 2001). A sex-determination locus was recently mapped in one species of pufferfish, *Takifugu rubripes* (Kikuchi *et al.* 2007), but not in another, *Tetraodon nigroviridis* (Li *et al.* 2002), and a lack of cytogenetic tools for pufferfish has prevented visual assessment of a heteromorphic pair (Grützner *et al.* 1999; Fischer *et al.* 2000).

Evolution of Sex-determining Factors

How does GSD initially evolve? How do genes take on the role of initiating sex determination? What kinds of genes can become sex-determining factors? Common features of GSD pathways have become apparent through the study of GSD in fruit flies of the genus Drosophila, in the worm *Caenorhabditis elegans*, and in mammals and fish. The sex-determining pathway of *Drosophila melanogaster*, a species with XY GSD, is initiated by the splicing factor Sex-lethal (*Sxl*), which controls sex-specific splicing of transcription factors such as Transformer 2 (*Tra2*) and Doublesex (*dsx*) (Lucchesi and Skripsky 1981; Penalva and Sanchez 2003). *C. elegans* uses an X-counting XX/XO system, in which the number of X chromosomes determines sex (XO individuals being male and XX being hermaphrodite). In this species, the transcription factor XO-lethal (*Xol*) initiates the male sex-determining pathway upstream of transcription factors *Tra2* and male-abnormal 3 (*mab3*) and also plays a role in dosage compensation (Miller *et al.* 1988).

From these findings, patterns have emerged. *Xol* and *SRY* are both transcription factors, raising the question of whether transcription factors are commonly used to initiate sex determination. Also, while worms and flies use different sex-determining genes, parts of the pathways downstream of *Xol* and *Sxl* are conserved (Figure 1). Both genes act upstream of *Tra2*, and *dsx* and *mab3* are members of the same gene family, whose distinguishing characteristic is the presence of a DM (*Dsx* and *Mab3*) domain. Further, *SRY* in mammals acts upstream of the transcription factor *SOX9* and the gene *DMRT1* (*Dsx* and *Mab3*-Related Transcript) and evolved from the transcription factor *SOX3*, a gene in the same family as *SOX9* (Foster and Graves 1994). Upstream lability in these sex-determination cascades is juxtaposed with downstream stability of the factors affecting sex determination across a broad range of taxa, from insects to vertebrates.

Aside from *SRY*, one other sex-determining factor in a vertebrate is known: *DMY* (DM-domain on the Y) in the medaka fish *O. latipes* (Matsuda *et al.* 2002; Nanda *et al.* 2002). *DMY* originated as a duplicate copy of the *DMRT1* gene and retained its function as a transcription factor while taking on the novel function of initiating male sexual development. While *SRY* arose by divergence and *DMY* by duplication, both are transcription factors that evolved from genes downstream in the sex-determination cascade. These similarities support the hypothesis that sex-determining genes tend to be duplicate copies of downstream sex-determination pathway genes (Wilkins 1995). Such genes might already have appropriate tissue expression patterns and timing and the ability to regulate transcription of other downstream genes, allowing them to take on the role of initiating sexual development. This proposal has provided many hypotheses to test and has been

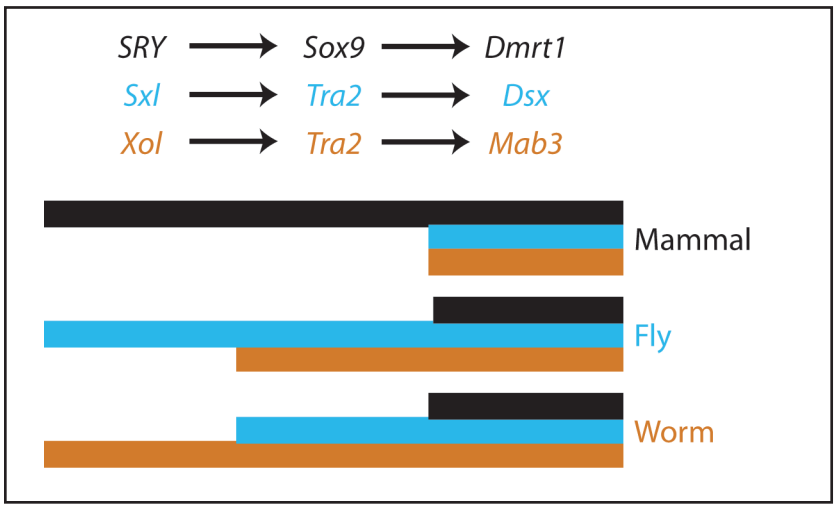


Figure 1. Conservation of genetic sex determination across taxa. Adapted from (Cotinot *et al*. 2002). Relative positions of genes in the sex determination pathways of humans (black), *Drosophila melanogaster* (blue) and *Caenorhabditis elegans* (red) are shown in the top panel. The bars below signify the extent of homology in pathways in the three clades. In "Mammal", the mammalian GSD pathway (black bar) converges on the fly and worm pathways at *Dmrt1*, which is homologous to *Dsx* (blue bar) and *Mab3* (red bar). The pathway in "Fly" (blue bar) shares homology with the worm pathway (red bar) starting at *Tra2*, and the same is true from the perspective of the "Worm" pathway (red bar). While none of the most upstream genes have homology to sex pathway genes in other species, the pathways all converge by using a *Doublesex* and *mab3*-related transcript (*Dmrt*) family gene.

the foundation for many ongoing attempts to characterize novel sex-determining genes in vertebrates to determine whether they are transcription factors related to downstream sex-determination pathway genes.

Sex-chromosome Evolution

Studies of the independent sex chromosomes of mammals and medaka suggest that before their sex-determination genes existed, the X and Y chromosomes were autosomal pairs. In mammals, the Y chromosome is much smaller than the X and has lost many of its coding regions, accumulated mobile sequence elements and repetitive DNA, and experienced numerous intrachromosomal rearrangements (Kuroda-Kawaguchi *et al.* 2001; Tilford *et al.* 2001; Repping *et al.* 2002; Skaletsky *et al.* 2003; Noordam and Repping 2006; Ross *et al.* 2006). The many physical differences between the extant X and Y have resulted in an almost complete lack of homology between the two, despite being referred to as homologous because of their ancestral relationship. This last vestiges of the relationship between the X and Y are evident in the pseudoautosomal regions (Graves 2006).

Determination of the DNA sequences of the human X (Ross *et al.* 2005) and Y (Skaletsky *et al.* 2003) allowed identification of a small number of genes in common, indicating that the X and Y were once truly homologous chromosomes. Reconstruction of the chromosomal rearrangements that led to the extant structures of the X and Y determined that the ancestral autosome pair that became the X and Y contained the *SOX3* gene. The X still contains a copy of *SOX3*, but the Y-borne allele diverged and became *SRY* (Foster and Graves 1994). Once *SRY* adopted a role initiating male sexual development, the autosome pair became an X and Y.

SRY arose around 166-300 MYA, but *DMY* in the medaka arose much more recently, about 10 MYA (Kondo *et al.* 2004), allowing a comparison of two vertebrate

sex chromosomes of different ages. Sequence analysis of the medaka Y revealed that *DMY* evolved from a duplicate copy of the *DMRT1* gene that is located in a 260 kbp block of DNA originating from an autosome (Naruse *et al.* 2004; Kondo *et al.* 2006). The duplicate *DMRT1* gene, apparently free from evolutionary constraint as has been suggested to be the case for duplicate genes (Lynch and Conery 2000; Lynch and Force 2000), acquired a role in initiating male sex determination. At that point, the chromosome harboring *DMY* became the Y. The duplicated block of DNA containing *DMY* harbors the only differences between the medaka X and Y (Schartl 2004a). While the medaka sex-chromosome pair is not grossly heteromorphic (Matsuda *et al.* 1998), the presence of the duplicated block can be distinguished using molecular cytogenetics (Nanda *et al.* 2002).

The correlation between GSD and sex-chromosome heteromorphy has prompted much effort to be directed toward understanding the consequences of carrying a sex-determination gene to a sex chromosome. Theoretical studies suggest that sex-determination loci (generically referred to as *SEX* from here; I will assume that *SEX* resides on a Y chromosome in the hypothetical examples to follow) arise near other genes with alleles having sexually antagonistic (SA) effects, where there is a benefit to males and cost to females of carrying one allele (Charlesworth and Charlesworth 1978; Bull 1983b; Rice 1987b; Charlesworth *et al.* 2005) (Figure 2A). Such genes are thought to be common in nature (Rice 1992).

For example, a SA gene might have an allele producing reproductive coloration necessary for males to acquire mates, but imposing a disadvantage by making them more prone to predation. In guppies, for example, 17 of 18 genes controlling coloration traits map within 10 cM of the sex-determining locus (Winge 1927). Such an allele could only be advantageous in males. If a female were to express the reproductive coloration allele, there would be no enhancement of her mating ability and she would still incur the cost of displaying attractive coloration, resulting in a net fitness loss. Evolutionary pressure to

prevent SA genes from entering the homogametic genome selects for reduction of recombination around *SEX* and nearby sexually-antagonistic genes. The complete linkage of a male-beneficial SA allele to *SEX* ensures that the entire region will be transmitted only to the heterogametic sex (Figure 2B).

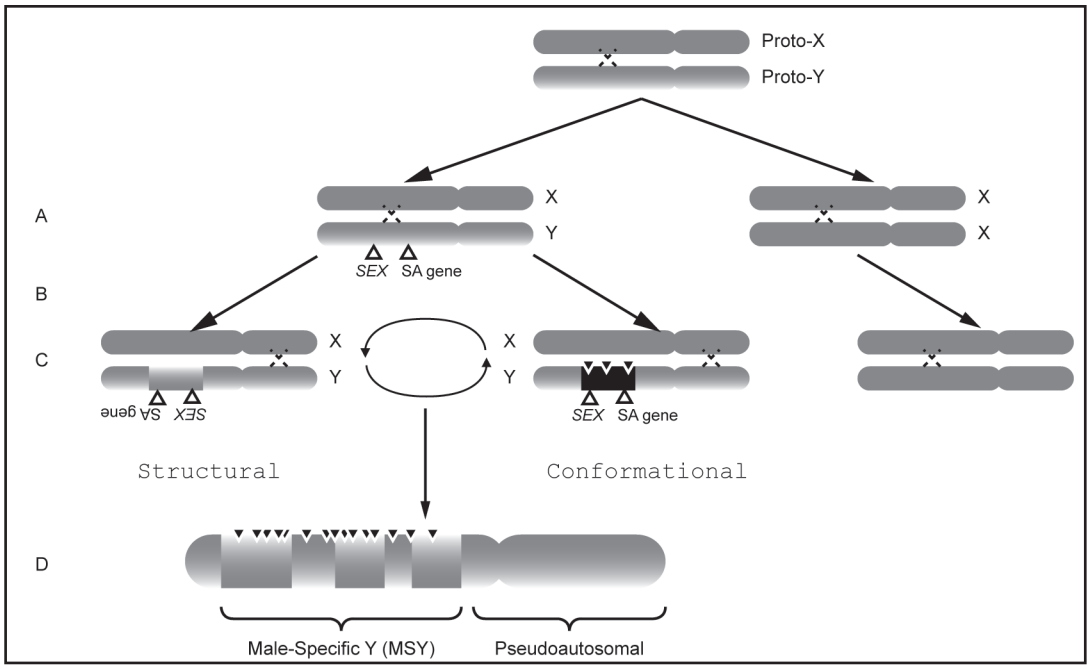


Figure 2. The evolution of degenerate sex chromosomes.
Recombination in an autosome pair (the proto-X and proto-Y) can occur across its length (represented by a dashed "X"). When a sex determining locus (*SEX*) arises (A, arrow to the left), it may do so near a gene with sexually antagonistic alleles (SA gene). The evolution of *SEX* defines the chromosome carrying it as the Y, and the homologous chromosome the X. At this point, males are XY and females are XX (A, arrow to the right). The benefit of maintaining the association between the male-determining gene and the nearby male-beneficial allele of the SA gene provides evolutionary pressure to reduce recombination between the two loci. The reduction of recombination is hypothesized first to occur by one of two mechanisms: structural (B, arrow to the left) or conformational (B, arrow to the right). In the structural mechanism, an inversion containing *SEX* and the SA gene eliminates the local homology of the inverted region to the X, reducing recombination in the inverted region of the Y. In the conformational mechanism, an accumulation of repetitive sequence elements (black triangles) triggers heterochromatin formation (black bar) around *SEX* and the SA gene, preventing recombination with the X. Recombination events between the X and Y are now restricted to other regions of the Y, while in females recombination still freely occurs across the X chromosome (C). After recombination is initially reduced in the region around *SEX*, additional inversions and repetitive sequences can accumulate in a cycle (C), ultimately resulting in a Y chromosome (D) containing repetitive sequence elements and multiple, perhaps nested, inversions. Recombination events between the X and Y are restricted to a region of the Y called a pseudoautosomal region.

Reduction of recombination around *SEX* has been identified in many taxa (Fraser and Heitman 2005), including the medaka fish (Kondo *et al.* 2001), and plants such as papaya, asparagus, and the black cottonwood (Ma *et al.* 2004; Telgmann-Rauber *et al.* 2007; Yin *et al.* 2008), suggesting its generality as a sex-chromosome characteristic. However, sex chromosomes never become completely free of recombination, as this would present serious consequences for chromosome segregation at cell division because each chromosome pair must recombine to segregate faithfully in meiosis. Instead, sex-chromosome recombination events tend to be restricted to pseudoautosomal regions, in which obligate recombination events occur (Figure 2C and 2D), except for in species lacking male recombination such as Drosophila.

The evolutionary benefit of sexual reproduction (recombination) is widely debated (Felsenstein 1974; Barton and Charlesworth 1998; Agrawal 2006). Although recombination can produce new beneficial combinations of alleles, it may also disrupt adaptive gene complexes. Thus, whether the increase in genetic variation that recombination affords is a benefit of sex is still an open question (Rice 2002); sexual reproduction in yeast has been shown to allow rapid adaptation to harsh environments (Goddard *et al.* 2005), suggesting a benefit of sex under selective conditions. One further benefit of recombination is that, following mutation in an individual, it can restore mutation-free combinations of alleles in subsequent generations. Given the apparent value of recombination in evolution, carrying a non-recombining region is detrimental to a sex chromosome. Lack of recombination facilitates the eventual degeneration of a sex chromosome into a heteromorphic state (Figure 2D).

Non-recombining regions tend to accumulate mutations (point mutations, mobile sequence elements, and deletions) through the processes of hitchhiking and Muller's ratchet (reviewed in (Charlesworth and Charlesworth 2000)). In hitchhiking, mutations occurring in linkage with a beneficial locus (as in a non-recombining region containing

SEX and sexually-antagonistic genes) will go to fixation as the beneficial locus sweeps through a population. Muller's ratchet states that, in a finite population of non-recombining Y chromosomes where each might contain any number of mutations, there is a possibility in each generation that the chromosome with the fewest mutations will be lost by drift. In the absence of recombination, the chromosome with the fewest mutations cannot be regained once lost. Thus, mutations, including mobile sequence elements, inevitably accumulate in nonrecombining regions.

The early process of gain of mobile sequence elements in a non-recombining region might cause an increase in sex-chromosome size relative to its homolog, while the later stages of degeneration, in which genetic material is lost through deletions mediated by the presence of a large amount of repetitive DNA, might cause the sex chromosome to decrease in size. This is the "addition/attrition" hypothesis (Graves 1995) and explains why the presence of a heteromorphic chromosome pair in a karyotype is sufficient to infer the presence of GSD. However, young sex chromosomes, such as the medaka Y, might not yet exhibit gross heteromorphy (Matsuda *et al.* 1998). In such species, genetic mapping is necessary to determine the presence of GSD. For this reason, a relatively poor idea of the extent of GSD among species exists, as many sex chromosomes studied to date were identified by virtue of sex chromosome heteromorphy.

Genes on the sex chromosome might eventually be lost through mutation or deletion with little consequence if dosage compensation evolves (Jablonka and Lamb 1990; Charlesworth 1996). The human Y has lost many of the genes it originally had; these genes are still present on the X. Thus, while males only have one copy of the genes on their X, females have two X chromosomes and twice the dosage of X-borne genes. Changes in gene dosage are often deleterious, as in the case of haploinsufficient genes, and it would likely have been disadvantageous for human males to have half the X gene dosage of females were it not for the evolution of dosage compensation. In humans, this

process entails transcriptional inactivation of one X chromosome during early development of females so that the expression level of X-borne genes is the same in females and in males (Lyon 1961; Plath *et al.* 2002). Other solutions to the issue of dosage imbalance caused by sex-chromosome degeneration or loss have been employed, such as hypertranscription of the single male X in Drosophila (Meller and Kuroda 2002) and repression of transcription by one-half of both X chromosomes in hermaphrodite *C. elegans* (Meyer and Casson 1986).

Mechanisms of Reduced Recombination

Reduction of recombination has been identified on many sex chromosomes around *SEX* loci, but determination of the mechanisms initiating this loss has been elusive. The first of two hypotheses as to how recombination might be reduced (Jablonka and Lamb 1990) (Figure 2C) suggests that an increase in heterochromatin around *SEX* (the conformational hypothesis), possibly due to accumulation of mobile sequence elements, could exclude the molecular machinery necessary for homologous recombination to occur. The second suggests that an accumulation of chromosomal inversions containing *SEX* (the structural hypothesis) causes the Y to be locally non-homologous to the X and eliminates the substrate for homologous recombination. Distinguishing these two possibilities has been difficult. In the case of the extensively characterized mammalian Y, which has existed for hundreds of millions of years since *SRY* arose (Lahn and Page 1999; Veyrunes *et al.* 2008), the degenerative process has eliminated the signatures of the earliest events on the Y chromosome that would tell us what processes occur on young vertebrate sex chromosomes.

Complicating matters, the conformational and structural hypotheses are not mutually exclusive, although one surely acts first. It is possible that repetitive sequences act both to generate an area of heterochromatin, causing a local reduction in X-Y recombina-

tion, and to facilitate ectopic intrachromosomal recombination events leading to an intrachromosomal inversion, which then prevents the larger region contained in the inversion from recombining with the X. In this larger region, more mobile elements would be free to accumulate via Muller's ratchet, perpetuating the cycle (Figure 2C). Thus, it is necessary to study newly evolved sex chromosomes to disentangle these two hypotheses, to define the early steps of vertebrate sex-chromosome degeneration, and to determine the processes that facilitate it.

Many molecular studies have shown an increase of mobile elements on sex chromosomes; cytogenetic or genetic evidence of inversions on sex chromosomes is less frequently reported. In species with young sex-chromosome systems, it is thought that Y-chromosome inversions arose subsequent to loss of X-Y recombination in *S. latifolia* (Bergero *et al.* 2008). In the medaka, the interchromosomal duplication of a block of DNA containing the *DMRT1* gene, which later evolved to become *DMY*, led to loss of recombination within the block (Schartl 2004a). Thus, structural changes caused loss of recombination around the sex-determination locus of the medaka but not in *S. latifolia*.

If an inversion suppresses X-Y recombination and allows accumulation of mutations, including additional inversions, then the older inversions on the Y may harbor alleles more diverged from the X than the Y alleles found in more recent inversions. In humans, these "evolutionary strata" were identified by comparing the positions and divergence of X-Y allele pairs, demonstrating that a series of inversions produced a stepwise loss of recombination between the X and Y (Lahn and Page 1999) and raising the distinct possibility that a nonrecombining region will increase in size over time. The mammalian Y is too old, however, to distinguish whether inversion was the first step in the decline of the Y.

Thus, to study the evolution of a young sex chromosome, it would be advantageous to use genetic mapping to identify a system in which GSD is present but where the

sex chromosome has not yet degenerated to the point of being heteromorphic. In such a system, it might be feasible to clone the nonrecombining region of a nascent sex chromosome to assess the genetic, sequence and physical characteristics of a young sex chromosome and to identify the sex-determination factor. In my dissertation research, I have used the threespine stickleback fish, *Gasterosteus aculeatus*, and closely related stickleback species to study the evolution of genetic sex determination and young sex chromosomes.

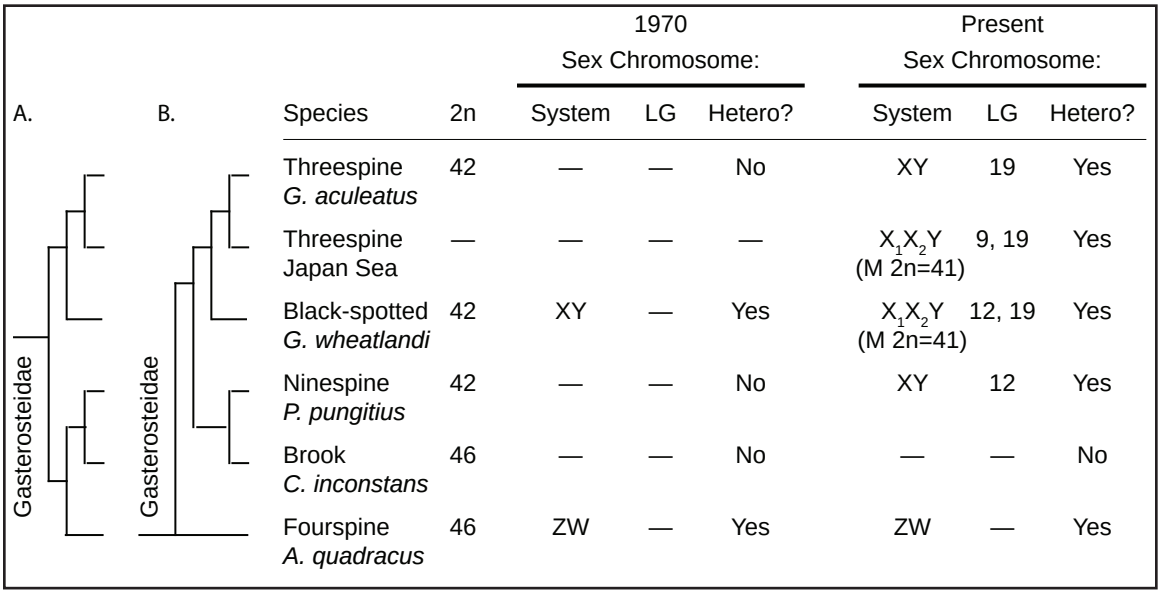
Sex Determination and Sex Chromosomes in Stickleback Fishes

The threespine stickleback is a small teleost fish found in freshwater, marine, and anadromous populations in temperate and sub-polar environments of the Northern hemisphere. The stickleback has been used as an ecological, behavioral, and evolutionary model system for years because of the variety of morphological and behavioral differences present in the many different populations that reside in a variety of environments (Bell 1994). Recently, many molecular and genetic tools for the stickleback have been developed, including genome-wide microsatellite genetic markers (Peichel *et al.* 2001), expressed sequence tag (EST) and bacterial artificial chromosome (BAC) libraries (Kingsley *et al.* 2004), transgenic tools (Hosemann *et al.* 2004), and a completed genome sequence of a female (XX) individual (The Broad Institute 2006).

Previous cytogenetic studies of stickleback species sought the presence of heteromorphic pairs. In the first survey of sticklebacks, Chen and Reisman concluded that threespine sticklebacks do not have a heteromorphic sex-chromosome pair (Chen and Reisman 1970). However, they found that the sister species to the threespine stickleback, the black-spotted stickleback (*G. wheatlandi*), has a heteromorphic XY pair; the threespine and black-spotted sticklebacks are about 10 MY diverged (Bell 1994). The ninespine stickleback, *Pungitius pungitius*, and brook stickleback, *Culaea inconstans*, were not found to have heteromorphic pairs; A ZW pair was reported in the fourspine stickleback,

Apeltes quadracus (Chen and Reisman 1970). The phylogenetic relationships among the stickleback species, as well as cytogenetic data on chromosome number and presence of heteromorphic pairs available at the point where I began my studies, is shown in the "1970" panel in Figure 3.

Although no visible sex chromosomes had been reported for threespine sticklebacks, sexually-dimorphic alleles were identified at the isocitrate dehydrogenase (*Idh)* locus and at nearby loci (Withler *et al.* 1986; Griffiths *et al.* 2000). Genetic mapping of sex determination in threespine sticklebacks (Peichel *et al.* 2004) revealed a non-recombining region of a male-specific chromosome harboring a male sex-determining locus, defining the threespine stickleback as having XY GSD and raising the possibility that the threespine and black-spotted sticklebacks share the same *SEX* locus. Fossil record



		1970 Sex Chromosome:			Present Sex Chromosome:		
Species	2n	System	LG	Hetero?	System	LG	Hetero?
Threespine *G. aculeatus*	42	—	—	No	XY	19	Yes
Threespine Japan Sea	—	—	—	—	X_1X_2Y (M 2n=41)	9, 19	Yes
Black-spotted *G. wheatlandi*	42	XY	—	Yes	X_1X_2Y (M 2n=41)	12, 19	Yes
Ninespine *P. pungitius*	42	—	—	No	XY	12	Yes
Brook *C. inconstans*	46	—	—	No	—	—	No
Fourspine *A. quadracus*	46	ZW	—	Yes	ZW	—	Yes

Figure 3. Phylogeny of North American stickleback fishes.
Phylogenetic topology (A) from (Mattern 2004) and (B) from (McLennan and Mattern 2001; Mattern and McLennan 2004)) and summary of previous cytogenetic findings in 1970 (data from (Chen and Reisman 1970)) and at present, incorporating (Peichel *et al*. 2004; Ross *et al*. in preparation; Shapiro *et al*. in preparation; Ross and Peichel in press). The common and scientific name of each species is given, along with the diploid number of chromosomes (2n), type of sex determination system, linkage group (LG) to which *SEX* maps, and presence of a heteromorphic pair. In the two X_1X_2Y species, females have the diploid chromosome number reported in 1970; the number of chromosomes in males (M) is listed. — indicates that no data are available.

evidence suggests that the threespine stickleback (XY) has been diverging from the fourspine stickleback (ZW) for only twenty million years (Bell 1994). The homomorphy of the threespine stickleback XY chromosome pair suggested that the XY system might have evolved recently enough that the threespine Y had not yet degenerated sufficiently to appear heteromorphic. Further, presence of XY and ZW GSD in two species less than 20 MY diverged suggests that at least one of those systems evolved in that time.

I have used the stickleback fish to study the sequence and cytogenetic characteristics of vertebrate sex chromosomes. In Chapter 2, I will describe the identification of XY GSD in the threespine stickleback by Catherine Peichel and my subsequent sequence analyses of their XY pair. These findings are followed in Chapter 3 by a description of my efforts, along with Amanda Bruner, to clone the threespine X and Y as a resource for sequencing the chromosomes and identifying the sex-determination locus. Chapter 4 contains my cytogenetic evaluation of the threespine Y, in which I showed that it exhibits many physical differences compared to the X. Chapter 5 describes my molecular cytogenetic experiments, informed by additional genetic mapping studies in sticklebacks by Catherine Peichel, Michael Shapiro and Jun Kitano, that probe the evolution of GSD and sex chromosomes in other stickleback species. Finally, Chapter 6 offers a summary of my findings and suggests future avenues of investigation that the data I report here have made possible. Altogether, my studies have provided empirical evidence to support key theoretical models of the field and have laid the groundwork for more extensive dissection of evolution of sex-determination systems and sex chromosomes in stickleback fishes.

Chapter 2. Characteristics of an XY Pair in Threespine Sticklebacks

Introduction

A genome-wide set of microsatellite markers for the threespine stickleback was developed to aid in the search for the genetic basis of adaptation in vertebrates (Peichel *et al.* 2001). Catherine Peichel also used these markers to look for a locus controlling male or female sexual development (XY or ZW GSD) in genetic crosses by genotyping and phenotyping the individuals in the crosses and then testing for linkage of a region of the stickleback genome with the phenotype of male or female sex. The resulting linkage analysis has been published previously (Peichel *et al.* 2004). In the same report, I conducted analyses of homologous sequences from the nonrecombining region of the Y and X to determine whether characteristics of more evolved sex chromosomes can be found on the threespine Y. This chapter summarizes Peichel's mapping study and then focuses on my contributions to this published work.

Materials and Methods

***Idh* genotyping**: PCR reactions consisted of: 1x PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM each dATP, dCTP, dGTP and dTTP, and 0.25 units AmpliTaq DNA polymerase (all from Applied Biosystems), 5 ng stickleback genomic DNA or 5 pg BAC DNA, and 10 pmol of each primer of a pair. The primer pair designed to the 3' UTR of *Idh* comprises: 5'-GGGACGAGCAAGATTTATTG-3' and 5'-TTATCGTTAGCCAGGAGATGG-3'. The cycle sequence protocol was: 95°C 2 min, 56°C 1 min, 72°C 2 min; five cycles of 94°C 1 min, 56°C 1 min, 72°C 2 min; 29 cycles of 90°C 1 min, 56°C 1 min, 72°C 2 min; 72°C 5 min; store at 4°C on MJ Research thermal cyclers. PCR products were analyzed on a 2%

agarose gel, stained with ethidium bromide, in 1x TBE buffer. A 302 bp *Idh* product band is produced from an X chromosome; a 270 bp band is produced from a Y chromosome.

Genetic mapping: The genetic markers, cross and techniques used have been previously published (Peichel *et al.* 2004; Ross and Peichel in press). Additionally, the marker *Ga1* (Griffiths *et al.* 2000) was mapped in this cross.

Sequence sources: BACs CH213-101E8, CH213-169J23, CH213-160O09 and CH213-119K16 were sequenced to completion at the Stanford Human Genome Center and are available from GenBank using accession numbers AC144485, AC144486, AC144487 and AC144488, respectively.

Assembly of BAC sequences: The region of overlap, containing the *Idh* gene, between a pair of Y chromosome BAC clones (CH213-119K16 and CH213-169J23) and a pair of X clones (CH213-101E08 and CH213-160O09) was identified by comparing the two members of each pair using the computer program cross_match (Green 1999). Once the orientation of both sequences in a pair was confirmed to be parallel, the region of overlap was removed from the sequence of one clone, and then the two clone sequences were combined into a single sequence representing both BAC clones originating from one chromosome (X or Y).

Production of a homologous pair of sequences from the X and Y: 5' or 3' sequence in the assembly from one chromosome lacking homologous sequence in the assembly of the other chromosome was identified by comparing the X and Y assemblies using cross_match (Green 1999). Any sequence at the 5' and 3' ends of both the X and Y assemblies that was not flanked on both sides by a region of at least 5000 bp homology to the other chromosome was removed.

Homology plots: The global pairwise alignment of the X and Y clone sequence assemblies was produced using the computer program VISTA (Mayor *et al.* 2000), with settings of 95% stringency and a 100-bp window size. The X and Y sequence assemblies

were subjected to pairwise analysis using the "compare" computer program and visualized in dot plot form using "dotplot" (Wisconsin package v. 10.2-UNIX) using the settings of 95% stringency and 100 bp window size.

Gene identification: Genes present on the assembly of *Idh* BAC sequences from the Y were identified by BLASTing (Altschul *et al.* 1990) the assembly against the GenBank non-redundant database. Representative mRNA of five genes from other species were aligned to the Y chromosome sequence using the program sim4 (Florea *et al.* 1998) and the exon positions of these gene are shown in Figure 6. The gene sequences used in the alignment were: human Semaphorin 4B (*Sema4B*; NM_020210.2), zebrafish C2H2 zinc finger protein (*Znf*; NM_199792.1), zebrafish NADP-dependent isocitrate dehydrogenase (*Idh*; NM_199564.1), human Ras protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf*; NM_002891.3) and rat Band 4.1-containing protein (*Band 4.1*; XM_230513.2).

Repeat analysis: Characterized multicopy sequence elements were identified using the computer program RepeatMasker (Smit *et al.* 2004). Multicopy elements unique to the threespine stickleback Y sequence assembly were identified by BLASTing (Altschul *et al.* 1990) the masked Y sequence against itself; the coordinates of multicopy sequences greater than 200 bp in length were then added to the Vista plot.

Results

Genetic mapping reveals an XY GSD system: In two families from a cross totaling 699 F2 fish, a single locus segregating with the phenotype of male sex (*SEX*) is associated with alleles inherited only from the male parent (Peichel *et al.* 2004). This is shown in the genetic map of linkage group (LG) 19 in male and female threespine sticklebacks (Figure 4) and defines the threespine stickleback as having an XY GSD system.

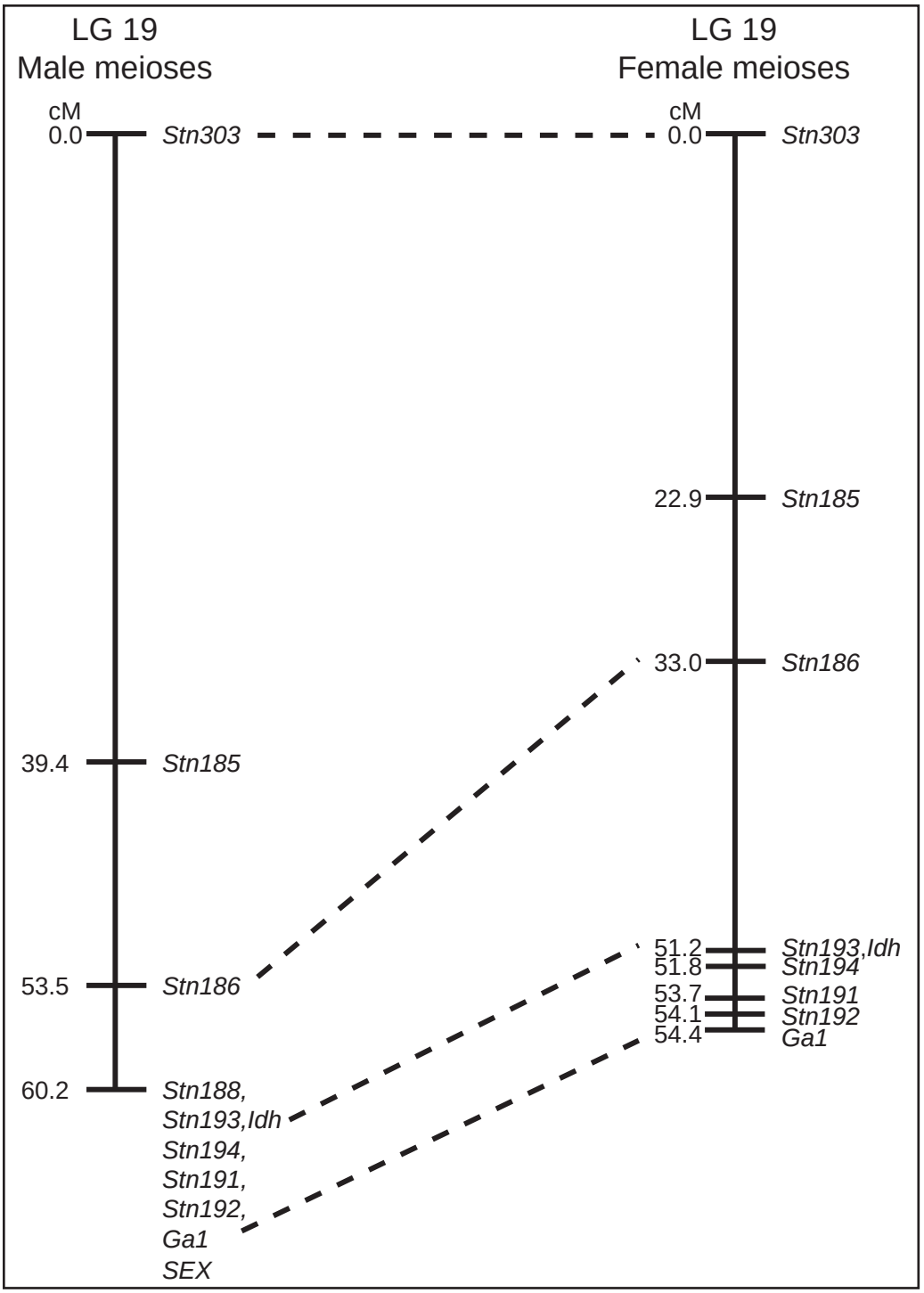


Figure 4. Linkage maps of the threespine stickleback X and Y chromosomes.
The maps are based on meiotic recombination events in males and females. The positions of genetic markers on the X and Y are given in centiMorgans (cM). *Stn188* is only polymorphic between the X and Y chromosomes, so it could not be mapped on the X. *SEX* is nonrecombinant with markers at the bottom of the male LG19 (Y).

The genetic distances between markers on the female LG19 (i.e., the X chromosome, right side of Figure 4) reflect X-X recombination events that occurred in the female parent of the cross, while the distances on the male LG19 (the Y chromosome, left side of Figure 4) are based on X-Y recombination events in the male parent. A difference in LG19 recombination rates between male and female meioses is particularly evident when comparing the genetic distances between *Stn303* and *Stn186*. In females, the genetic distance between these markers is 33.0 centimorgans (cM), while in males this region is 53.5 cM. However, this difference is not due to an overall increase in recombination rate in males, because the distance between another pair of loci on the same chromosome pair, *Stn192* and *Stn193*, is also sexually dimorphic, with the distance in females (2.9 cM) being larger than in males (0 cM).

This result demonstrates that some markers separated by recombination events on the X are nonrecombinant with each other on the Y. Recombination is increased at the top of the threespine Y linkage map (Figure 4), giving the appearance of a pseudoautosomal region to which X-Y recombination is restricted, and suppressed around *SEX*. Four F2 individuals were putative recombinants between sex phenotype and genotype, placing the *SEX* interval at the bottom of the linkage map of the Y. Later studies I performed (Chapter 4) called into question the validity of the genotypes or phenotypes of these four animals, raising the possibility that they were either sex-reversed or were mis-phenotyped. Thus, the position of *SEX* relative to the non-recombining region of the Y, the *Stn186–Ga1* interval, is unclear at present.

The Y allele of the *Idh* gene is nonrecombinant with *SEX* in the threespine Y genetic map; this agrees with prior findings that the *Idh* protein is sexually dimorphic in threespine sticklebacks (Withler *et al.* 1986). Cloning and sequencing of *Idh* cDNA from threespine sticklebacks led to the discovery of a 31-bp deletion from the 3' untranslated region (UTR) in the Y allele (Peichel *et al.* 2004). This discovery allowed the

development of a polymerase chain reaction (PCR) assay for genetic sex of threespine sticklebacks, in which XX females produce a single 302-bp band but males produce both the 302-bp band (X) and a 271-bp band (Y) that can be easily discerned by agarose gel electrophoresis.

Sequence assembly of the threespine stickleback *Idh* locus: To facilitate a comparison of sequence characteristics between the X and Y in a region of reduced recombination near *SEX*, four bacterial artificial chromosome (BAC) clones from the CHORI-213 (CH213) library (Kingsley *et al.* 2004) containing *Idh*, two with the X allele and two with the Y allele, were sequenced to completion by the Stanford Human Genome Center. The two X clones (CH213-101E08 and CH213-160O09) are minimally overlapping at *Idh*, as are the two Y clones (CH213-119K16 and CH213-169J23), providing sequence from homologous regions of the X and Y (Figure 5A). With these sequences, I determined whether X and Y chromosomes have diverged at the sequence level and whether repeti-

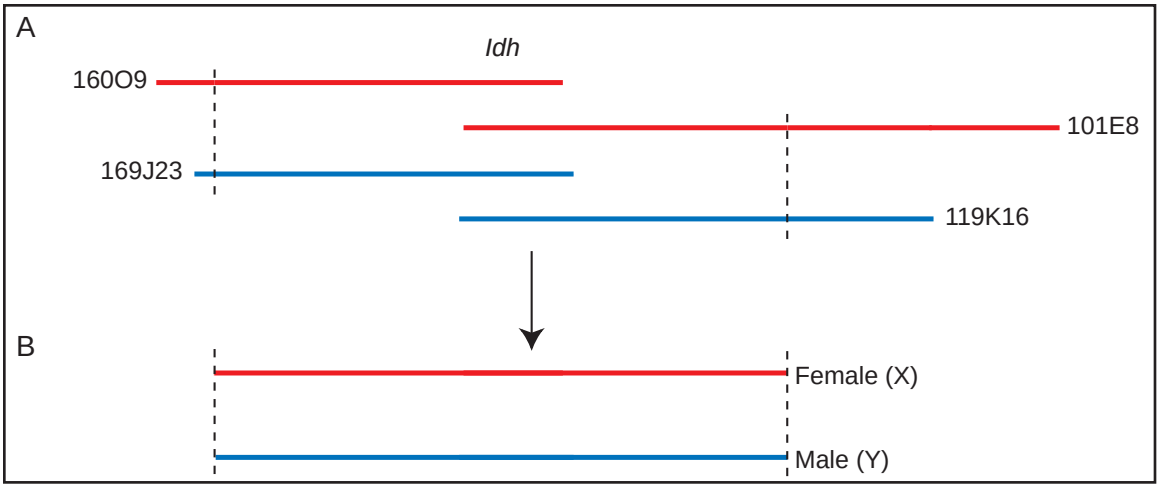


Figure 5. Assemblies of the X and Y chromosome *Idh* BAC sequences.
(A) Sequence of two CH213 X chromosome clones (red lines of arbitrary length) and Y clones (blue lines of arbitrary length) were obtained and the region of overlap of the two X clones and the two Y clones, and regions of homology at the ends of the X-Y pairs (vertical dashed lines), were identified. The X sequences and Y sequences were then trimmed and assembled (arrow) to produce two sequences from the homologous region of the X and Y (B).

tive DNA elements have accumulated on the Y. These characteristics are expected of degenerating sex chromosomes.

It was necessary to analyze only homologous sequence from each chromosome in order to directly compare of the numbers of repetitive sequence elements on the X and Y. To accomplish this, I first assembled the sequences of the two X BACs by removing the region of overlap, containing the *Idh* gene, found on both BAC sequences and then joining the two sequences together. I assembled the sequences of the two Y BACs in the same way. Then, to find the regions in these assemblies of homologous sequence from the X and Y, I identified homologous "anchor" sequences >5000 bp in length by aligning the X and Y assemblies using cross_match (Green 1999). The 5'-most and 3'-most X-Y homologous anchor sequences that contained no known repetitive DNA, ascertained by RepeatMasker (Smit *et al.* 2004), defined the 5' and 3' ends of the X and Y assemblies that I used in subsequent analyses. The resulting sequence assemblies were 229,012 bp (X) and 316,654 bp (Y), suggesting that the sequence surrounding the *Idh* locus on the Y is 38% larger than the homologous sequence on the X.

Global alignment plot of X-Y homology: The degree of homology between the X and Y sequences was displayed using the program Vista (Mayor *et al.* 2000). In the plot of a global pairwise alignment between the X and Y, the Y chromosome sequence coordinates are plotted along the X axis, and the percent identity of each 100 bp window of Y sequence to the X is plotted as the shaded area (Figure 6). I identified exon positions of genes on the Y by alignment of representative mRNA from other species to the Y assembly. A striking feature of this plot is that in some regions, particularly in areas containing coding regions, X-Y identity is near 100%, while there are many stretches of Y-chromosome sequence having no identity with the X greater than 50%. Overall, the X and Y have 63.7% identity in this alignment.

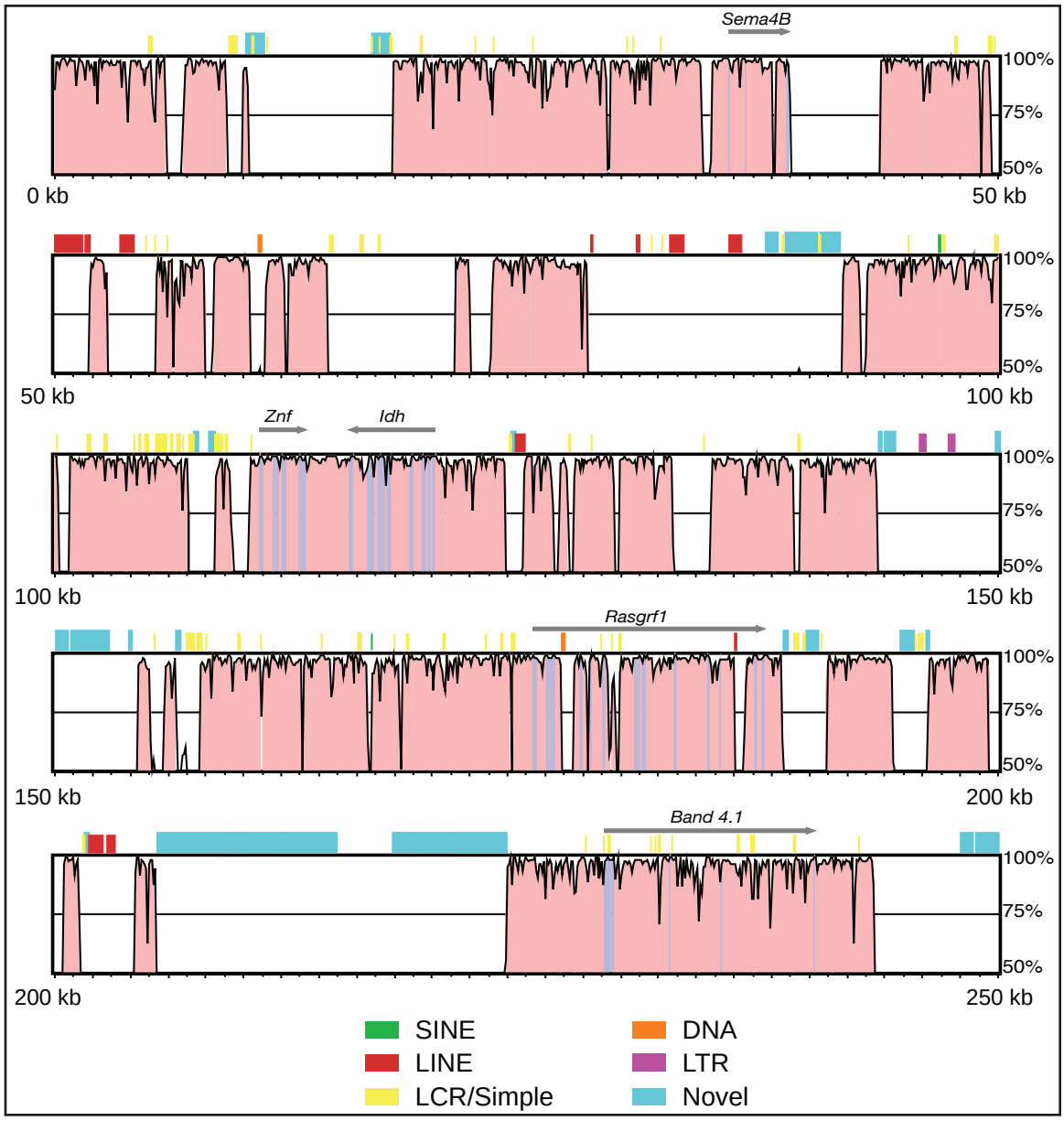


Figure 6. Vista plot of the X and Y *Idh* BAC sequence assemblies.
The Y chromosome coordinates are plotted on the horizontal axis, and the percent identity of the Y to the X is shown in the pink shaded area plotted on the vertical axis. Positions of genes are shown as horizontal arrows along the top of the plot, and exon positions are shown as vertical blue bars. Positions of repetitive sequence elements are shown as colored boxes along the top of the plot. "Novel" elements are those that I identified as being present in more than one location on the threespine stickleback *Idh* contig assembly Y sequence but not on the X sequence.

Table 1. Repeat content on the threespine X and Y at *Idh*.
The X and Y sequence assemblies at *Idh* were analyzed for the presence of mobile and repetitive DNA elements using RepeatMasker. For each assembly, the number of individual elements, the cumulative sequence length of that class of element, and the percentage of the sequence assembly that the class occupies is given.

	X chromosome sequence (229,012 bp)			Y chromosome sequence (316,654 bp)		
Repeat Type	**Number**	**Length (bp)**	**% of total sequence**	**Number**	**Length (bp)**	**% of total sequence**
SINE	0	0	0	4	210	0.07
LINE	0	0	0	13	12070	3.81
LTR	0	0	0	1	623	0.20
DNA	2	97	0.04	3	498	0.16
Simple Sequence	47	1884	0.82	60	3796	1.20
Total	49	1981	0.86	81	17197	5.44

Characterization of repetitive sequence elements: The Y chromosome sequence is 38% larger than the X sequence; insertions on the Y or deletions from the X could explain the interstitial losses of homology shown in the Vista plot as well as the change in homologous sequence length. My evidence suggests that insertions on the Y, not deletions from the X, are the cause of these characteristics. RepeatMasker (Smit *et al.* 2004) analysis of the Y chromosome sequence assembly identified many repetitive and mobile sequence elements on the Y not found on the X. These are quantitated in Table 1 and their positions are shown in colored boxes above the Vista plot in Figure 6.

As expected, the number of non-mobile repetitive sequence elements, such as simple sequence elements (microsatellites and low complexity regions), do not differ greatly between the X and Y, although the content of such regions is greater on the Y. However, the numbers of mobile sequence elements, the short and long interspersed nuclear elements (SINEs and LINEs) and retroviral elements with long terminal repeats (LTRs), are greater in the Y than in the homologous region of the X, in which no elements of these classes are found, supporting the conclusion that they are accumulating in the nonrecombining region of the threespine stickleback Y chromosome.

X and Y dot plots: To illustrate the difference in repetitive sequence content of the homologous regions of the Y and X, I produced dot plots (Figure 7). In these plots, the coordinates of the two sequences are plotted on the X and Y axes. Each 100 bp window along the X axis is compared with all 100 bp windows along the Y axis; when identity of at least 95% between a X and Y window exists, a dot is plotted on the graph.

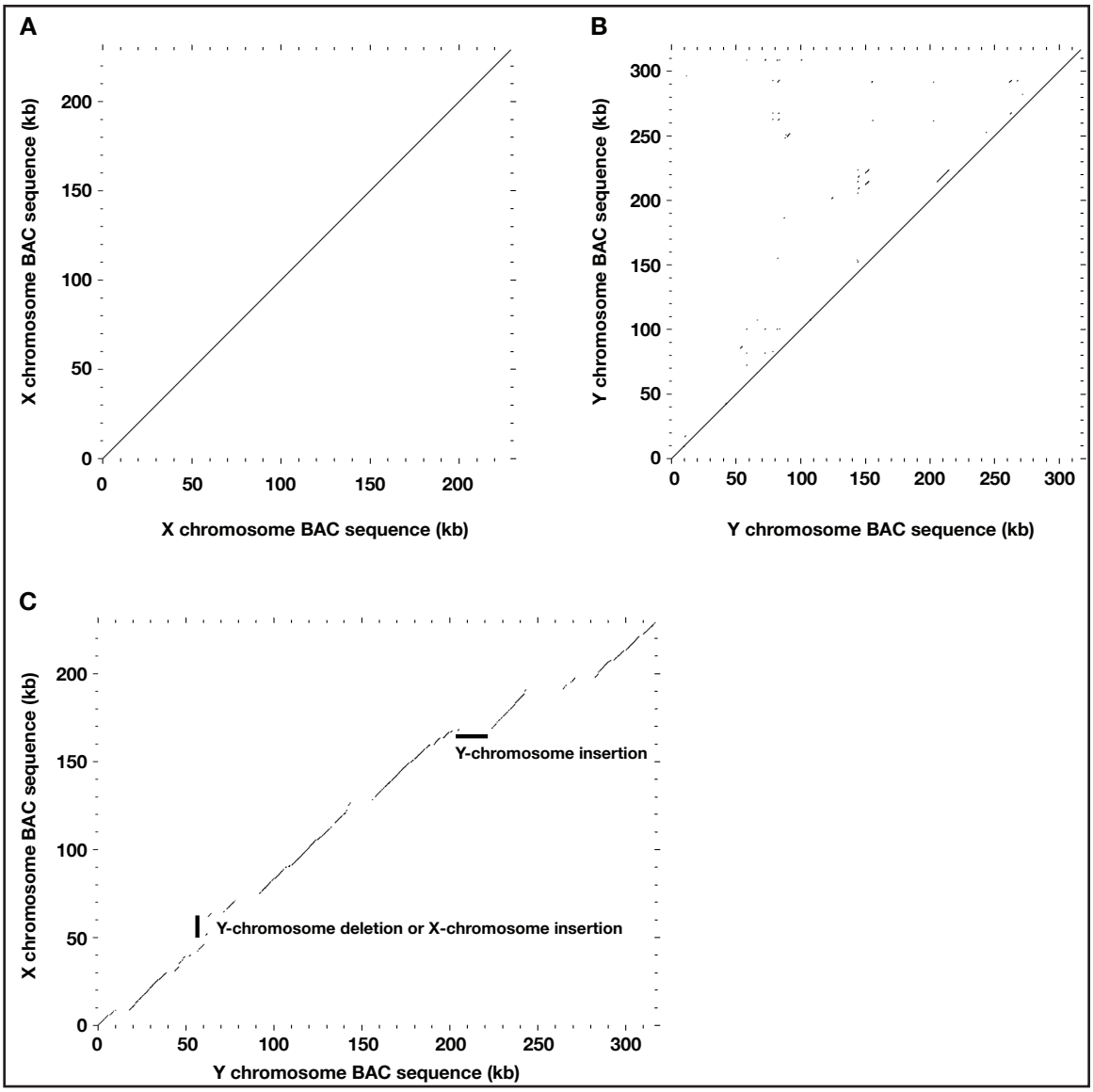


Figure 7. Dot plots of *Idh* sequence assemblies from the X and Y.
(A) Comparison of the X sequence to itself demonstrates no repetitive sequences. (B) Comparison of the Y sequence to itself demonstrates presence of repetitive sequences not present on the X. (C) Comparison of the X and Y sequences with positions of a putative Y deletion (vertical bar) and Y insertion (horizontal bar) indicated.

In Figure 7A, the X chromosome sequence is compared to itself. The solid diagonal line indicates that each sequence window on the X is identical to itself; any multicopy sequences would appear as off-diagonal dots or lines, depending on the size of the element.

Such multicopy elements are evident in Figure 7B, the comparison of the Y sequence to itself. The expected diagonal line indicates the identity of each window to itself; the off-diagonal dots and lines to the left of the diagonal indicate positions of multicopy elements at least 100 bp in size with at least 95% identity to each other. For example, the horizontal row of dots at about 290 kb indicates that one sequence is found twice around 80 kbp, once at 160, once at 210 and twice around 270 kbp. Also, the approximately 10 kb-long diagonal line at about 210 kbp indicates a large tandem duplication. The presence of off-diagonal dots only on the Y plot indicates the accumulation of multicopy elements on the Y chromosome but not the X chromosome.

By their nature, self dotplots like these can identify duplications but not deletions. To ascertain the presence of deletions or insertions on the Y relative to the X, I also produced an X-Y dot plot. In Figure 7C, the Y sequence is plotted along the horizontal axis, and the X sequence along the vertical axis. If the X and Y were completely homologous, a strong diagonal line would be seen. This plot illustrates that the 5' and 3' ends of the X and Y sequences are homologous to each other for at least 10 kbp, as I had required when the sequence assemblies were produced. Between these regions of homology, there are discontinuities. At no position on the Y sequence is there more than one homologous sequence on the X, that is, there are no off-diagonal lines in Figure 7C, because multicopy elements have accumulated only on the Y but not the X.

One cause for the discontinuity in the diagonal line is that, as mentioned earlier, the Y sequence is longer than the X sequence, necessitating gaps in this plot of homology. For example, a horizontal black bar in Figure 7C indicates a region where about 15 kbp of sequence present on the Y has no homologous sequence on the X, hence a Y-chromo-

some insertion. The coordinates of the tandem duplication in Figure 7B correspond to the position of this putative insertion, confirming that it is not a deletion from the X. A vertical black bar indicates the position on the X of sequence having no homology to the Y. While this might be a Y chromosome deletion, it is more difficult to say with certainty that it is not an insertion of sequence on the X. However, if it was an X insertion, it was not caused by a local duplication because the sequence is unique to the region of X sequence analyzed.

Discussion

Genetic mapping has revealed the presence of XY GSD in the threespine stickleback, and the genetic maps of the X and Y chromosomes illustrate differences expected of a sex chromosome and its homolog: restriction of recombination around *SEX* and an increase in recombination in a pseudoautosomal region of the Y (Figure 4). My analysis of 316 kbp of Y sequence containing the *Idh* gene and the homologous 229 kbp from the X has demonstrated that an increase of multicopy sequence elements on the Y has led to a reduction of X-Y homology (Figure 6) as well as an increase in size of the Y relative to the X around this locus (Figure 7C).

This last finding supports the addition-attrition hypothesis (Graves 1995), which addresses the dynamic nature of size differences between a sex chromosome and its homolog. A period of addition of multicopy elements in a nonrecombining region of a sex chromosome could cause it initially to increase in size relative to its homolog. However, because old sex chromosomes are typically smaller than their homologs, it is thought that increased multicopy element density eventually facilitates intrachromosomal rearrangements and deletions. Young sex-chromosome systems are not often identified and characterized, but this analysis of threespine Y sequence validates part of the addition-attrition

hypothesis that suffers from a dearth of empirical evidence: that a chronologically young sex chromosome can increase in size relative to its homolog in a nonrecombining region.

However, the dynamic nature of addition to and attrition of a sex chromosome makes it difficult to assert which occurred first and whether addition or attrition may be simultaneously occurring at other sex chromosome loci. The identification of an increase of mobile sequence elements in the nonrecombining region of a Y chromosome also supports the conformational hypothesis of reduced recombination (Jablonka and Lamb 1990). Nevertheless, a comparison of X and Y sequences at the *Idh* locus showed no evidence of inversions. The search for evidence of inversions on the threespine stickleback Y chromosome to support the structural hypothesis of reduced recombination awaited my development of molecular cytogenetic techniques for sticklebacks (Chapter 4).

Chapter 3. Cloning the Threespine Stickleback X and Y Chromosomes

Introduction

I endeavored to identify a minimally overlapping set of threespine Y-chromosome BAC clones spanning the nonrecombining *SEX* locus by chromosome walking in the CH213 BAC library (Kingsley *et al.* 2004). Such a "minimum tiling path" (MTP) of clones would be a useful resource for sequencing the male-specific region of the threespine stickleback Y chromosome in order to identify the sex-determining factor. At the same time, I also worked to identify a contig of BAC clones from the homologous region of the X to facilitate a more extensive sequence-based comparison of the X and Y than the one presented in Chapter 2. I generated the data in this Chapter with the assistance of Catherine Peichel and Amanda Bruner.

I used the following general process for cloning. I first identified the genetic markers most closely flanking the locus of interest (*SEX*); these markers defined the boundaries of the nonrecombining genetic interval to clone. Next, I probed a library of genomic clones with these flanking markers to identify sets of clones containing them. To identify the two minimally overlapping clones extending the farthest in each direction at each marker, I then identified how each clone in a set overlapped with the others. I accomplished this using PCR-based sequence-tagged site (STS) content mapping.

The rationale of STS content mapping is straightforward. An STS is any sequence unique in the genome; I developed stickleback STSs by designing PCR primer pairs to the end sequences of genomic clones identified during the cloning process. Such a primer pair should PCR-amplify the same clone when it is provided as a template; the insert of

any other BAC containing the same STS will also generate a PCR product. Using STSs from the ends of many overlapping clones to type all the clones in a set allowed me to identify the two clones extending the farthest in each direction from a probe (e.g. Figure 8). Such clones will have one STS that is present on most of the other clones; the other STS will be unique to the contig, defining it as the farthest-reaching clone end.

Once I determined the physical relationships among a set of clones at one locus (now a "contig" of clones) and identified the two least overlapping clones, the chromosome walking process began. New probes were designed to the STSs of the farthest-reaching clone ends and hybridized to the clone library to identify additional clones. The new sets of clones were STS-content mapped, new probes were made, and the walking process continued. Chromosome walking initially takes place in both directions from two markers because the direction to clone from one locus to reach the second is unknown. While walking in both directions from two markers flanking the region of interest, even-

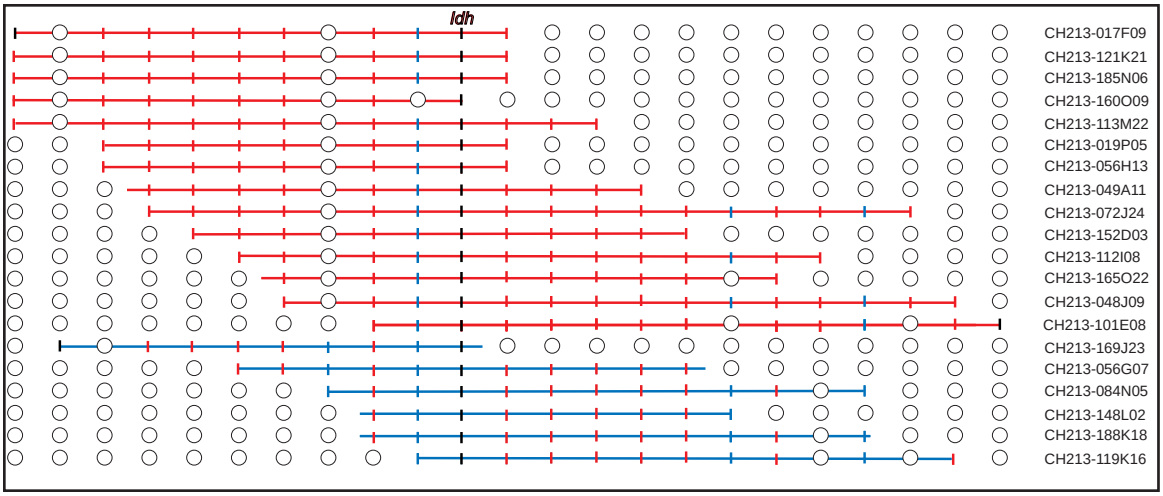


Figure 8. STS content map of the *Idh* contig of CH213 BAC clones.
Each clone (horizontal bar) was determined to be from the X (red) or Y (blue) based on *Idh* genotype. The vertical bar at each end of each clone represents an STS primer pair designed to each BAC end sequence. If no such vertical bar is present, then the sequence of that end was not determined and no primer was designed. Blue vertical bars indicate the amplification of a Y-clone STS using other clones as templates; red vertical bars indicate the amplification of an X clone STS using other clones as templates. Circles indicate positions where an STS was not present by PCR. Vertical black bars at the ends of 017F09, 169J23 and 101E08 indicate the farthest-reaching ends on the X and Y chromosome clone contigs that were later used as library probes. Data provided by Catherine Peichel.

tually one probe each from the two contigs will identify an overlapping set of clones, indicating that the region of interest has been crossed by clones. The presence of two genetic markers in the unified contig then orients the physical map of clones to the genetic linkage map of markers. Once a MTP across the nonrecombining interval is identified, the clones can be sequenced to search for candidate *SEX* loci. The clones can also be used in transgenic approaches to confirm the role of a candidate gene in sex determination (Chapter 6) and as fluorescence *in situ* hybridization (FISH) probes (Chapter 4).

In my approach, overgo probes (Ross *et al.* 1999; McPherson *et al.* 2001) were designed to sequences flanking the microsatellites at each marker; these sequences were obtained during the process of marker development (Peichel *et al.* 2001). The probes were labeled radioactively and hybridized to the CH213 library filter set. As the CH213 library provides 20x coverage of the stickleback genome and was produced from a pool of equal numbers of male and female sticklebacks (Kingsley *et al.* 2004), I expected to find roughly one Y chromosome BAC for every three X chromosome BACs in the library.

Materials and Methods

BAC library screening: Radiolabeled overgo probes designed to the 3' UTR of *Idh* and to the *Stn191, Stn192* and *Stn194* sequences were hybridized to CH213 BAC filter sets (CHORI). Two *Idh* overgo pairs were used: 5'-GGGACGAGCAAGATTTATTGGCAA-3' with 5'-GGACTGTCAAACGTATTTGCCCAAT-3', and 5'-GATAGTCGGAAAGACATGAGGTGG-3' with 5'-GTTGAGAGCTGTGCTTCCACCTCA-3'. The following primers were used to generate overgo probes for:
Stn191 (GenBank Accession G72218): 5'-CCTTTTTTTTGTTCCTTACCTGTCCG-3' and 5'-GACAAGGAGATCCATTGACGGACAGG-3', *Stn192* (GenBank Accession G72319) 5'-AGCAAACAACGCCACACGTAACTG-3' and 5'-CCAACAAGACGT-

GAACCAGTTACG-3', and *Stn194* (GenBank Accession G72220) 5'-ACCAGCTC-CCAGATACTCGCTGT-3' and 5'-CTGGGTCCTGAGATAACAGCGAG-3'. To generate overgo probes to hybridize to the control anchor spots on the library membranes, the following primers were used: 5'-GTTGCCAAATTCCGAGATCTTGGC-3' and 5'-AT-CATGTGGCTTCGTCGCCAAGAT-3'.

To label the probes, 10 pmol of each overgo primer of a pair were combined with water to total 5.5 µL. The overgo primer pair solutions were denatured at 80°C for five minutes and incubated at 37°C for ten minutes and then placed on ice. To a denatured overgo primer pair, 0.5 µL 2 mg/mL BSA, 5 µCi each ^{32}P-dATP and ^{32}P-dCTP, 1.5 units of Klenow fragment of DNA polymerase I (Invitrogen), and 2 µL overgo labeling buffer (0.25 M TRIS-HCl pH 8.0, 0.25 mM $MgCl_2$, 0.05 M beta-mercaptoethanol, 0.1 mM dTTP, 0.1 mM dGTP, 1 M HEPES-NaOH pH 6.6, 0.9 mM TRIS-HCl pH 7.4 and 0.06 mM EDTA) were added with water to total 10 µL. The labeling reaction was incubated at room temperature for sixty minutes.

As many as two BAC library filters, arranged back to back or sandwiched with nylon mesh, were prehybridized in a rotating hybridization bottle at 60°C in 25 mL prewarmed hybridization buffer (1 mM EDTA, 7% SDS, 0.5 M Na_2HPO_4) for at least two hours. All overgo probe reactions were combined and spun through a Sephadex Midi-Select-D G50 column in Tris-EDTA (IBI, Peosta IA): each column was packed in a 1.5 mL microcentrifuge tube at 14 krpm for two minutes. The 1.5 mL tube was then replaced, and the overgo reaction added to the top of the sepharose column. The reaction incubated on the column for two minutes and was then centrifuged at 14 krpm for two minutes. The purified probe mixture (the flow-through) was denatured at 95°C for ten minutes and then placed on slushy ice. The denatured overgo probe mixture was then added to 25 mL prewarmed hybridization buffer. The prehybridization buffer was decanted from the hybrid-

ization bottle and replaced with the hybridization mixture containing the overgo probes. Filters were hybridized with rotation at 60°C overnight.

Each hybridization bottle was then washed with 100 mL each: 60°C wash buffer B (1 mM EDTA, 1% SDS, 40 mM Na_2HPO_4) for thirty minutes, 60°C wash buffer 2 (1.5x SSC, 0.1% SDS) twice for twenty minutes, and 60°C wash buffer 3 (0.5x SSC, 0.1% SDS) for twenty minutes. The membranes were removed from the bottle and rinsed in 2xSSC. Each membrane was wrapped in plastic wrap and exposed to Kodak BioMax MR film in a cassette with intensifying screen at -80°C for at least overnight.

STS content mapping by PCR: BAC DNA was prepared as described in Chapter 4. PCR primers were designed using default settings for primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) with the following modifications: 18 to 22 bp oligonucleotides having 40% to 70% GC content and a 3 bp GC clamp. PCR was performed as for *Idh* genotyping described above, with the following changes: 0.25 ng BAC DNA and 10 pmol of each forward and reverse primer of an STS were supplied.

STS content mapping by dot blot: To generate BAC dot blots to perform STS content mapping by STS probe hybridization, BACs were miniprepped using the protocol in Chapter 4. BAC preps were denatured at 94°C for five minutes and then placed on ice. Approximately 20 ng of BAC DNA was spotted onto Hybond N+ nylon membrane (Amersham) in a gridded layout. In addition to CH213 or CH215 BAC clones to be assessed for STS content, clone 001A01 from the RPCI24 library was also added to two control anchor spots in the grid to facilitate orientation of the autoradiograph to the grid later. After spotting DNA, the membrane was allowed to air dry on blotting paper and was then crosslinked to the DNA with ultraviolet light using a Stratagene Stratalinker with its automatic crosslinking setting.

Primers used to generate RPCI24-001A01 probe were forward 5'-GGCAAAATCGAAAATCACATGG-3' and reverse 5'-CTTGAAAATCATGCCTTCTCC-3'. Probes

were generated by PCR using the *Idh* PCR protocol in Chapter 2 with the addition of 25 µCi of ^{32}P-dATP or ^{32}P-dCTP. Primers used were BAC end sequence STS primers designed for STS content mapping, and the PCR template for each probe PCR was the BAC the STS originated from. The radiolabeled probes were then column-purified and denatured using the overgo BAC library screening protocol above.

Dot blots were placed into glass hybridization bottles and the membranes wetted with 2x SSC. The SSC was replaced with 65°C Church buffer (1% BSA fraction V, 1 mM EDTA, 0.5M phosphate, 7% SDS) and the blots prehybridized at 65°C with rotation for two hours. Denatured probes were added to 25 mL aliquots of 65°C Church buffer, and the prehybridization buffer was replaced with this hybridization buffer. The probes were allowed to hybridize at 65°C with rotation for up to eighteen hours. Blots were then washed with 65°C 2x SSC for fifteen minutes and with 65°C 0.1x SSC/0.1% SDS for thirty minutes. Blots were then exposed to film using the overgo BAC library screening protocol.

Genotyping: BAC or genomic DNA was PCR-amplified using a fluorescently labeled forward *Stn194* primer 5'-6FAM-ACACTCTGCTCTCGCTCCG-3' and an unlabeled reverse primer: 5'-TGGAAAGGCTTACTGTTCCG-3'. Final PCR reactions consisted of: 1x PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM each dATP, dCTP, dGTP and dTTP, and 0.5 units AmpliTaq DNA polymerase (all from Applied Biosystems), 5 pg BAC DNA or 10 ng genomic DNA, and 2.5 pmol of each primer. The cycle sequence was the same as for *Idh* PCR genotyping above. Each reaction was diluted 1:10 in water, and 1/200th of the dilution analyzed on an Applied Biosystems 3100 Genetic Analyzer using POP-4 polymer and 36 cm capillary array. Genotyping data were analyzed using GeneMapper v. 3.7 software (Applied Biosystems).

Stickleback genomic DNA preparation: A single pectoral or caudal fin was placed into a microcentrifuge tube containing 600 µL of digest buffer (10 mM TRIS pH

8.0, 100 mM NaCl, 10 mM EDTA pH 8.0, and 0.5% SDS) and 0.2 µg proteinase K was added. The reaction was mixed and incubated at 55°C for 18 hours. Genomic DNA was isolated by phenol:chloroform extraction and ethanol precipitation and quantitated by spectrometry.

Results and Discussion

CH213 library initial screen: In the initial CH213 BAC library screen, performed by Catherine Peichel, four probes were used: *Idh*, *Stn194*, *Stn191* and *Stn192*. Despite the additional number of markers in the nonrecombining *SEX* region of the Y (Figure 4), the lack of evidence of X-Y heteromorphy supported the assertion that the genetic order of markers on the X and Y were the same and that chromosome walking in both directions from four marker loci would allow efficient cloning of the nonrecombining region.

Of the 21 CH213 BACs positive for the *Idh* probe, Peichel determined that fifteen contained the X allele and six the Y allele by *Idh* PCR (Figure 8); nineteen BACs were positive for the *Stn194* probe. Ideally, I desired to identify separate BAC contigs for the X and Y chromosomes at all loci. This is straightforward in situations where the members of a contig are able to be assigned to their chromosome of origin by their genotype at the marker probe locus, as was the case for clones positive for *Idh*.

I thus genotyped nine Salmon River (British Columbia) males and ten Salmon River females at *Stn194* to determine whether sex-specific alleles existed. The *Stn*-designated markers are microsatellite markers and typically consist of a long dinucleotide repeat. These markers are variable because of the propensity for replication errors adding or removing repeat units from the microsatellite tract; microsatellite genotyping relies on discrimination of the lengths of these tracts on the homologous chromosomes of an individual. The Salmon River fish were chosen because they represent the population from

which the CH213 BAC library was made and should have the same repertoire of alleles as those found on the BACs.

The allele sizes of *Stn194* in these individuals are shown in Table 2. In Salmon River males, all but one had an allele in the 113-118 bp range, where only two of the females had an allele in this range; restriction of this range of alleles to the Y chromosome had also been seen in genetic crosses genotyped by Catherine Peichel. As the concordance between phenotypic sex and allele size is not perfect, I was unable to use these data to assign a chromosome of origin to *Stn194*-positive BACs from CH213. However, clone 213-064F11 was found to have a 117-bp *Stn194* allele, where 213-180B23 has a 76-bp allele, making it likely that 213-064F11 is a Y clone and that 213-180B23 is an X clone.

Markers *Stn191* and *Stn192* are both Y-null in threespine crosses analyzed (Peichel *et al.* 2004). Markers might fail to amplify (be "null") using PCR for two reasons: deletion of the locus or polymorphism in the primer sites. Thirteen CH213 clones were positive for *Stn191* in the library screen, and five clones were positive for *Stn192*. Because these markers are both Y-null, it is not possible to determine whether any clones positive in these screens are from the Y chromosome by genotyping as had been done for clones containing *Idh* and *Stn194*.

CH213 Y cloning by STS content mapping: After the initial library screen for CH213 clones containing *Stn194*, *Stn192* and *Stn191*, I used the STS mapping approach to construct BAC contigs at these loci. Both ends of the genomic insert of each BAC clone were sequenced, generating two ~500-bp sequences from each clone (paired BAC end sequences). Occasionally, due to poor template quality or DNA composition refractory to sequencing, one or no end sequence was obtained from a clone. The name given to each BAC end sequence is composed of the name of the clone and the name of the vector primer (either T7 or SP6) used in the sequencing reaction; each BAC has one T7 end sequence and one SP6 end sequence. Once I obtained end sequences from the clones

Table 2. *Stn194* alleles in threespine sticklebacks.
Wild-caught males and females of the Salmon River population and Paxton Lake (British Columbia, Canada) benthic type were genotyped at microsatellite *Stn194* and the resulting allele sizes are given. Presence of a single allele size could be due to homozygosity or hemizygosity at the locus. Also, *Stn194* alleles of *Stn194*-positive CH215 BAC clones, which are derived from a Paxton benthic individual, are given.

Population	Template	Alleles
Salmon River	male 1	100, 107
	male 2	113, 118
	male 3	88, 116
	male 4	76, 116
	male 5	80, 116
	male 6	107, 115
	male 7	75, 116
	male 8	107, 113
	male 9	116
	female 1	79, 82
	female 2	77, 87
	female 3	111, 121
	female 4	85, 124
	female 5	83, 118
	female 6	85, 87
	female 7	93, 121
	female 8	97, 111
	female 9	89, 93
	female 10	79, 115
Paxton Benthic	male 1	90, 109
	male 2	103, 109
	male 3	96, 105
	female 1	90
	female 2	90
	female 3	90
	female 4	90
	female 5	90, 96
	CH215-021G06	90
	CH215-022E09	105
	CH215-031G12	90
	CH215-041M19	89
	CH215-043P20	90
	CH215-045C10	89

Table 2, continued.

	CH215-050F06	105
	CH215-054G05	105
	CH215-058I17	90
	CH215-059A18	90
	CH215-064H12	90
	CH215-070L23	90
	CH215-089N07	89

in a contig, I designed a PCR primer pair to each BAC end sequence (the STS). Then, each BAC from the contig was used as a template in PCR reactions with each primer pair from the contig.

For the *Idh* contig of 21 CH213 clones, 33 of 42 end sequences were obtained; a primer pair was designed for each. The STS content contig map of CH213 clones containing *Idh* is shown in Figure 8. Some STSs from Y clones amplified only other Y clones but not X clones, such as CH213-169J23T7, and some STSs of X clones only amplified other X clones, such as those from CH213-112I08. Finally, some X and Y clones shared a common STS, such as CH213-048J09SP6 and CH213-119K16T7.

At the *Idh* locus, chromosome walking was initiated on the X chromosome from STSs CH213-101E08T7 and CH213-017F09T7 (Figure 9). From 101E08T7, clone

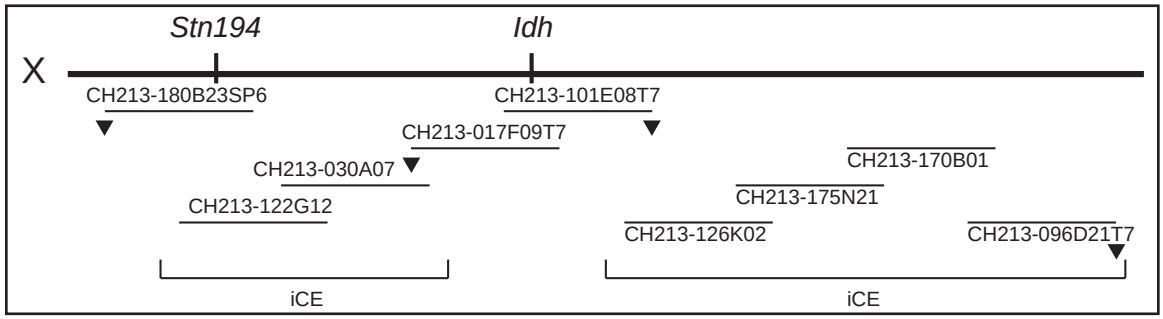


Figure 9. BAC minimum tiling path (MTP) from *Idh* X chromosome walk in CH213.
The positions of genetic markers are given along the schematic of the X (long horizontal bar). Relative positions of clones (short horizontal bars of arbitrary length) are shown. Black triangles indicate the clone end sequence STSs used to identify the overlapping clone in the MTP. Regions in which available BAC fingerprint maps (obtained by internet contig explorer, iCE) were used to extend the contig are indicated with brackets.

CH213-126K02 was identified. Using a restriction map-based set of BAC contigs defined for the CH213 library available *via* the internet contig explorer (iCE, available from Canada's Michael Smith Genome Sciences Center at http://www.bcgsc.ca), clones with overlapping restriction patterns to 126K02 were chosen and tested by PCR to confirm their overlap. This effort extended the *Idh*-X BAC contig with CH213-175N21, CH213-170B01, and CH213-096D21. An overgo probe was designed to the 96D21T7 STS, and clones containing that probe were identified.

From 17F09T7, clone CH213-030A07 was identified; it was present in an iCE contig with clone CH213-122G12, which was a clone identified in the initial library screen as containing *Stn194*. Thus, using a combination of chromosome walking and physical mapping data, I joined the *Idh* and *Stn194* contigs with an MTP of three BAC clones. I later learned, when the threespine stickleback female (XX) genome sequence was made available (The Broad Institute 2006), that the *Idh* and *Stn194* loci are about 500 kbp apart on the X, so the identification of a three-clone MTP was reasonable. 17F09 has the X allele of *Idh*; the chromosomal origin of 030A07 is unknown. 122G12 has a 95-bp allele of *Stn194*, which might be an X chromosome size (Table 2). I took an additional chromosome walking step from *Stn194* by probing the BAC library with probe CH213-180B23SP6, the most distant STS from *Stn194* in the direction opposite *Idh*; clones containing this STS were identified. As 180B23 has a 76 bp *Stn194* allele, it is possible that this clone originated from the X. In all, the MTP of putative X chromosome BACs in the contig containing *Idh* and *Stn194* comprises nine clones (Figure 9).

I initiated a chromosome walk at the *Idh* locus on the Y using CH213-169J23T7 but not CH213-119K16T7 (Figure 10) because 169J23T7 didn't PCR-amplify from X clones (Figure 8) and may be Y-specific; thus, its use as a probe was expected to identify only Y clones. From 169J23T7, a contig of clones containing CH213-061K15 was identified; the SP6 end of 61K15 was found to extend the farthest in the direction toward

Stn194 and so was used to probe the library again. This effort identified a contig containing CH213-044F19. 44F19SP6 was used to probe the library one more time, and clones positive for this probe have been identified. The present MTP of CH213 BAC clones at *Idh*, originating from a Y chromosome probe, comprises four clones (Figure 10). At *Stn194*, only three clones had a Y allele; no chromosome walking was conducted using STSs from these BACs. CH213-034P19SP6 was the *Stn194* BAC STS farthest reaching away from *Idh* on the Y and might be a good choice for continued cloning on the Y in the CH213 library.

At *Stn191* and *Stn192*, only one step in each direction from the initial STS contig of clones containing each probe has been accomplished (Figure 11 and Figure 12); the

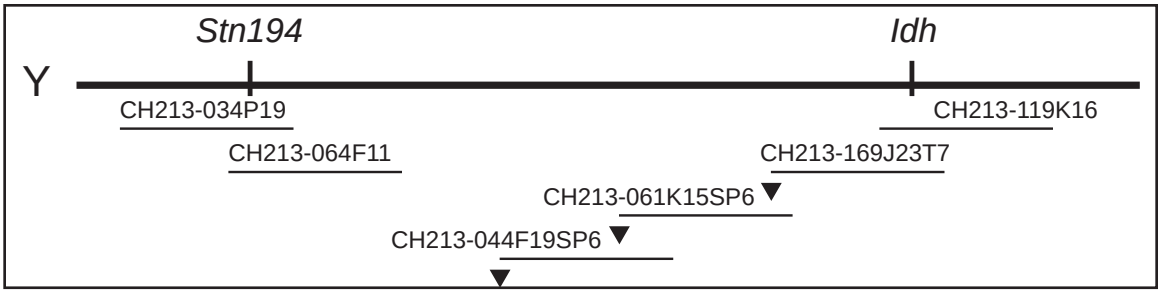


Figure 10. BAC MTP from *Idh* Y chromosome walk in CH213.
The positions of genetic markers are given along the schematic of the Y (long horizontal bar). Relative positions of clones (short horizontal bars of arbitrary length) are diagrammed. Black triangles indicate the clone end sequence STSs used to identify the overlapping clone in the MTP.

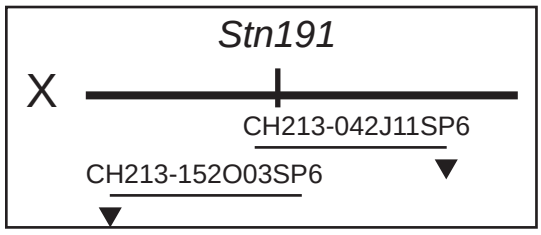


Figure 11. BAC MTP at *Stn191* in CH213.
The positions of genetic markers are given along the schematic of the X (long horizontal bar). Relative positions of clones (short horizontal bars of arbitrary length) are diagrammed. Black triangles indicate the clone end sequence STSs used to identify new clones in the chromosome walk.

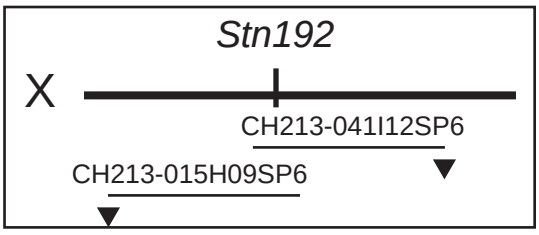


Figure 12. BAC MTP at *Stn192* in CH213.
The positions of genetic markers are given along the schematic of the X (long horizontal bar). Relative positions of clones (short horizontal bars of arbitrary length) are diagrammed. Black triangles indicate the clone end sequence STSs used to identify new clones in the chromosome walk.

clones identified in the screens have been identified but not yet STS content mapped. At *Stn191*, the MTP comprises clones CH213-042J11 and CH213-152O03, the SP6 ends of which were used in a library screen. At *Stn192*, the MTP comprises clones CH213-41I12 and CH213-015H09, the SP6 ends of which were used in a library screen.

STS content mapping complications: Several issues encountered when STS-mapping contigs from a sex chromosome and its homolog can generate false-positive or false-negative STS content data. By definition, an STS is unique in the genome and present in all individuals; this may rarely be the case for sex chromosome STSs, particularly when the X and Y chromosomes have diverged and when the Y might have accumulated repetitive DNA elements. As mentioned above, X-Y sequence divergence could cause an STS from one chromosome to appear null on the other. Additionally, in a BAC library such as CH213 (Kingsley *et al.* 2004), which was created from genomic DNA of several individuals, inter-individual polymorphism on the X and Y chromosomes could generate the same effect. Critically, STSs must be free of multicopy sequences.

In the process of chromosome walking on threespine stickleback LG19, many cases existed in which STSs did not appear to be common among the individuals used to create the BAC library or were not unique in the genome. I assembled contigs manually from STS content data with the goal of minimizing the number of false-positive and false-negative PCR reactions, but the contigs for each chromosome exhibited both issues. It was common to see one STS not amplify a template when flanking STSs could amplify from the same template; it was just as common to see one STS amplify from most of the templates despite flanking STSs amplifying only a few BAC insert templates from the contig. These complications made it very difficult to ascertain the relative order of STSs along the X and Y.

To address the issue of false-positive signals, I used RepeatMasker (Smit *et al.* 2004) to screen BAC end sequences for known repetitive and mobile DNA elements.

With these elements masked from BAC end sequences, it was more likely that STSs would be unique. However, the likelihood of uncharacterized, perhaps stickleback-specific, multicopy elements being present might have prevented this approach from being effective. To address this issue, I developed custom Perl scripts to identify portions of BAC end sequences that were found multiple times in other BAC end sequences from the STS contig mapping process. The recursive nature of the process of identifying which BAC end sequences contained repetitive DNA made it likely that I would identify an STS as multicopy after its use in STS content mapping or as a probe, reducing the feasibility of employing the process I developed. Therefore, use of this approach required constant rebuilding of all contigs upon receipt of each new batch of sequence information. Ultimately, the lack of efficiency of the chromosome-walking process reduced its utility. The process of STS content mapping on a sex chromosome would have benefited greatly from a computer program designed to integrate the process of building BAC contigs based on STS content data and also able to accept *post-hoc* data on the multicopy nature of existing STSs.

Not only is STS content mapping in a nonrecombining region fraught with perils, but so is positional cloning. Positional cloning is most efficient when the marker most tightly linked to the genomic region of interest is known and relies on recombination events to order genetic markers. As mentioned above, the relative order of *Stn191*, *Stn192*, *Stn194* and *Idh* has not been genetically determined for the Y chromosome, and while a MTP of putative X clones spans the *Idh* and *Stn194* markers (Figure 9), the contig of putative Y clones extending from *Idh* has not reached *Stn194* (Figure 10). Additionally, the *Stn191* and *Stn192* BAC contigs are not oriented to each other or to the *Idh*/*Stn194* contig, meaning that the physical order of markers in the nonrecombining portion Y has not been determined by chromosome walking. Finally, because *Stn191* and *Stn192* have

Y-null alleles, no BACs from these contigs have been assigned as originating from the X or Y by genotyping.

CH215 Y cloning by STS content mapping: One solution to the issue of inter-individual polymorphism and false negative STS content data was to clone the male-specific region of the threespine stickleback Y chromosome in the CHORI-215 (CH215) BAC library. Unlike CH213, the CH215 library was generated from the genomic DNA of a single individual, a Paxton Lake (British Columbia) male (Kingsley *et al.* 2004). The population of Paxton Lake sticklebacks has low allelic diversity, suggesting that it might be easier to identify single alleles common to the X or the Y.

The process of cloning the X and Y in the CH215 library began the same way as in the CH213 library: with the assistance of Amanda Bruner, I conducted a library screen using overgo probes for *Idh*, *Stn194*, *Stn191* and *Stn192*. Clones positive for these probes are given in Table 3. As in the Salmon River population used to make the CH213 library, the same *Idh* sexual dimorphism exists in Paxton fish, meaning that BACs containing *Idh* are able to be assigned to the X or Y by PCR. Five CH215 BACs were positive for *Idh*: four had the X allele and one the Y allele.

Thirteen CH215 clones contained *Stn194*. Given the expectation of lower allelic diversity than the CH213 Salmon River population, I determined whether X- and Y-specific allele sizes of *Stn194* could be found in the Paxton population. Genomic DNA from three Paxton males and five females were genotyped with the *Stn194* marker (Table 2). All of the Paxton females had alleles below 100 bp, and each male had at least one allele greater than 100 bp. I therefore concluded that >100 bp alleles of *Stn194* are Y-limited in this population. The thirteen BAC clones were then genotyped for *Stn194*, and three clones had 105 bp alleles, while the rest had 89 or 90 bp alleles. These allele sizes are similar to those seen in the Salmon River population but exhibit less diversity. I concluded that the 105-bp allele defines three CH215 clones as originating from the Y; the

Table 3. Initial CH215 BAC library screen clones.
The clones positive in screens for each of four LG19 genetic markers are given, as are their chromosomes of origin (X or Y), if they were possible to determine by genotyping.

Marker	Clone	Chromosome
Idh	002H23	X
	021M12	Y
	045E22	X
	066H11	X
	084C16	X
Stn191	018F11	–
	033G07	–
	061D15	–
	067E15	–
Stn192	031M11	–
	031M12	–
	044F06	–
	063E17	–
	080E21	–
	082G13	–
	089B09	–
	093P07	–
Stn194	021G06	X
	022E09	Y
	031G12	X
	041M19	X
	043P20	X
	045C10	X
	050F06	Y
	054G05	Y
	058I17	X
	059A18	X
	064H12	X
	070L23	X
	089N07	X

segregation of this allele size on the Y had also been seen in mapping crosses genotyped by Catherine Peichel.

Four CH215 clones positive for *Stn191* and eight clones for *Stn192* were identified. As these two markers are Y-null, the clones could not be assigned to the X or Y.

Due to the potential sensitivity of PCR to polymorphism, possibly causing false-negative STS content results, Amanda Bruner and I supplemented our PCR-based approach with a dot-blot approach to STS content mapping. For the CH215 library mapping, we first performed PCR-based STS content mapping as we did for CH213. To confirm the identities of the farthest-reaching BAC ends for each contig, we then arrayed all of the BAC templates from a contig onto nitrocellulose membranes and then PCR-amplified and labeled the STS PCR products of interest and hybridized each to a membrane containing the BACs from their contig of origin. As PCR probes are much larger than a typical overgo probe, I reasoned that their hybridization to BAC templates would be less sensitive to polymorphisms and perhaps give more robust STS content data.

For the *Idh* CH215 BAC contig (Figure 13A), STS mapping of the CH215 clones with their own STS markers as well as those developed for the *Idh* contig in CH213, in addition to dot blot hybridization, determined that CH215-021M12SP6 and CH215-002H23T7 were the *Stn194*-proximal ends of Y and X chromosome clones containing *Idh*, respectively.

For the *Stn194* contig (Figure 13A), CH215-043P20T7 extends the farthest away from *Idh* on the X; CH215-054G05T7 extends the farthest away from *Idh* on the Y. In the *Stn191* contig (Figure 13B), CH215-067E15SP6 and CH215-033G07T7 extend the farthest in either direction, and in the *Stn192* contig (Figure 13C), CH215-044F06T7 and CH215-031M11SP6 are the farthest-reaching clone ends.

Probes designed to the farthest reaching STSs in each of the CH213 and CH215 contigs for *Idh*, *Stn194*, *Stn191* and *Stn192* (CH213-169J23T7 (Y), CH213-017F09T7

(X), CH213-006M12SP6 (X), CH215-002H23T7 (X) and CH215-021M12SP6 (Y) from *Idh*; CH213-180B23SP6 (X), CH215-043P20T7 (X) and CH215-064H12SP6 (X) from *Stn194*; CH213-041I12SP6, CH213-039J03T7, CH215-044F06T7 and CH215-044F06SP6 from *Stn192*; CH213-042J11SP6, CH213-152O03SP6, CH215-067E15SP6 and CH215-033G07T7 from *Stn191*) were then screened in the CH215 library and the identities of positive clones assigned by PCR with the marker primers. The CH215-054G05T7 and CH215-064H12SP6 STSs had been determined to be identical by sequencing, as had the CH215-044F06SP6 and CH215-031M11SP6 STSs.

The dot blot STS content mapping approach succeeded in providing what a PCR-based assay lacked: binary data relating whether an STS is present on a BAC or not. The vagaries of PCR often resulted in weaker or stronger product bands on an agarose gel and

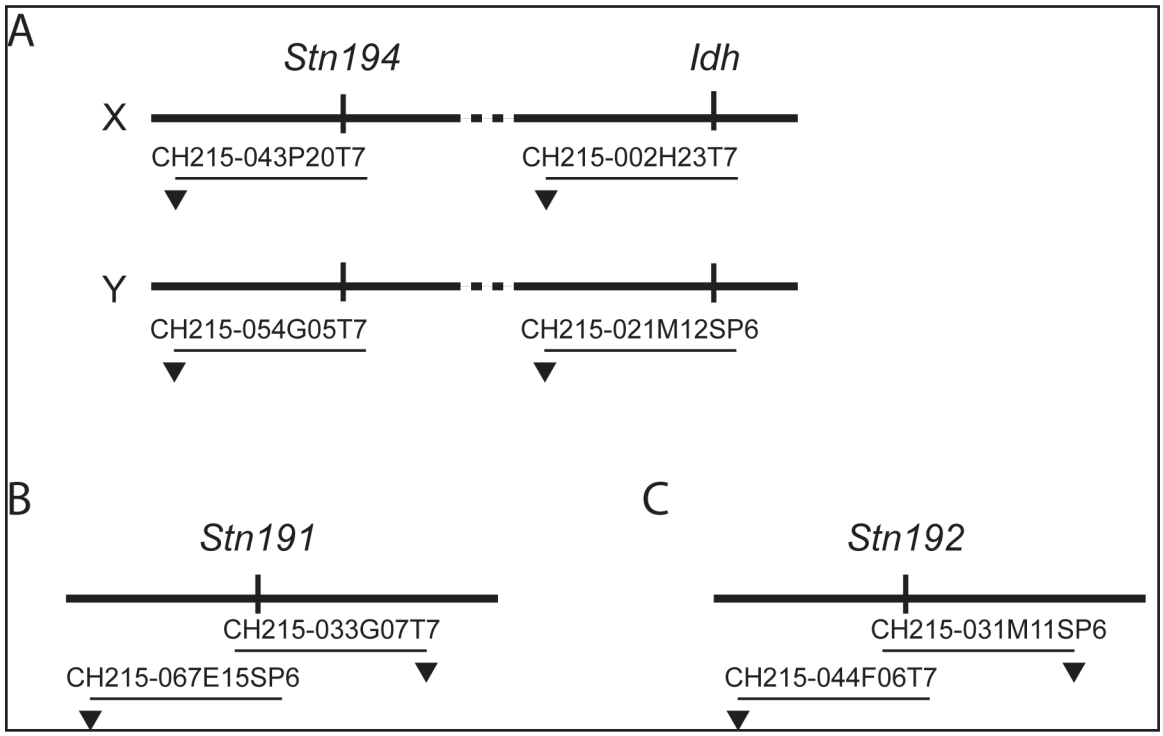


Figure 13. BAC MTPs from chromosome walks in CH215.
The positions of genetic markers are given along the schematic of the X and Y chromosomes (long horizontal bars). Dashed areas indicate that the relative orientation of the *Stn194* and *Idh* contigs are known but the area between the two contigs has not been cloned. Relative positions of clones (short horizontal bars of arbitrary length) are diagrammed. Black triangles indicate the clone end sequence STSs used to identify new clones in the chromosome walk.

in multiple bands in many cases, making results difficult to interpret; the dot blot hybridization assay typically produced either a strong hybridization signal or none at all.

While using the CH215 BAC library solved some problems encountered when STS content mapping a sex chromosome, one key deficit remained: the inability to efficiently identify the chromosome of origin of any clone of interest. In particular, no sex-specific polymorphisms were found in the *Stn191* and *Stn192* BAC contigs, preventing the identification of X- and Y-specific BACs in these contigs, although cytogenetic experiments later demonstrated that *Stn191* and *Stn192* are Y-null markers because of a large deletion on the Y (Chapter 4).

It was at this point in time that I received access to the genome sequence of a female (XX) threespine stickleback (The Broad Institute 2006), obviating the need to clone the X chromosome and allowing me to focus on cloning only the Y. My demonstration that the Y is 38% larger than the X around *Idh* (Peichel *et al.* 2004) raised the possibility that, while a grossly heteromorphic sex-chromosome pair had not been identified in earlier cytogenetic studies, it might be possible to use molecular cytogenetic techniques to identify subtle physical differences between the X and Y. Thus, I worked to develop the technique of FISH for stickleback fish to conduct a deliberate search for sex-chromosome heteromorphy. As I will explain in the next chapter, the combination of X chromosome sequence and the creation of a FISH-based physical map of the Y suggested that the nonrecombining *SEX* interval was indeed too large to efficiently clone by chromosome walking. The Peichel lab has since adopted new strategies for cloning and sequencing the threespine Y; these are discussed in Chapter 6.

Chapter 4. Cytogenetic Characterization of the Threespine Y Chromosome

Introduction

Simple 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 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 1983a; 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 approximately 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 despite the addition of much repetitive sequence element content (Skaletsky *et al.* 2003; Ross *et al.* 2005). The rich literature in mammalian sex-chromosome evolution, however, has not led to determination of the mechanisms that lead to the initial reduction in recombination around *SEX* on a vertebrate sex chromosome.

Two hypotheses suggest how such a reduction in recombination around a sex-determination locus might be achieved (Jablonka and Lamb 1990). The conformational hypothesis suggests that an increase in heterochromatin around *SEX* due to accumulation of mobile sequence elements would exclude the molecular machinery necessary for homologous recombination to occur. The structural hypothesis states that intrachromosomal inversions or translocations containing *SEX* produce a region of the Y that is locally not homologous to the X, thereby eliminating the ability to undergo homologous recombination. As the presence of mobile elements, inversions, deletions, and translocations on evolved sex chromosomes has been observed (Jablonka and Lamb 1990; Charlesworth and Charlesworth 2000; Charlesworth *et al.* 2005; Steinemann and Steinemann 2005; Graves 2006), and because these two hypotheses are not mutually exclusive, determining whether these mechanisms act coincidentally or sequentially during sex-chromosome evolution has been difficult.

Furthermore, because sex-chromosome degeneration eventually erases the molecular signatures of earlier events, it is not possible to identify the mechanisms underlying the initial degenerative process by studying highly evolved sex chromosomes such as the mammalian Y. Thus, to study the early steps in the evolution of vertebrate sex chro-

mosomes, 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.

My previous analysis (Chapter 2) 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 young 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.

Furthermore, a frequent concern throughout the Y chromosome cloning process was that the sizes of the X and Y chromosomes, and particularly the size of the nonrecombining Y region, were unknown. Thus, while working to clone the X and Y, equal focus was placed on estimating the physical size of the region to be cloned. After the cloning reported in Chapter 3 was accomplished, the genome sequence of a single female threespine stickleback (XX) was made available (The Broad Institute 2006). Analyses presented in this chapter involving this new resource suggested that the physical size of the *SEX* interval is too large to be cloned efficiently by chromosome walking and enabled the use of new approaches to assess the physical relationship between the X and Y. The necessity of understanding the physical properties of the sex-chromosome pair crystallized after I was first successful at conducting molecular cytogenetic experiments in the threespine stickleback.

In this chapter, which will be published as a separate manuscript (Ross and Peichel in press), I employ FISH with BAC probes to demonstrate that the chronologically young 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. These data suggest that rearrangements can occur early in the process of sex-chromosome evolution. Based on my FISH-based cytogenetic maps of the X and Y, I 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).

I 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 UCSC 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 a published genetic map (Peichel *et al.* 2004). To produce an assembly of X chromosome supercontigs both ordered and oriented by this genetic map, I reversed the sequence of SC3 and then joined the three supercontigs. My X assembly comprises SC85 (1–529,649) + SC34 (1–3,292,604) + SC3 (16,416,407–1) (Figure 14).

Genetic mapping: Catherine Peichel generated the genetic maps of the threespine X and Y chromosomes, based on recombination events in female and male meioses, by genotyping two families of the Paxton cross: family 4 (385 F2 individuals) and family 23 (314 F2 individuals) using markers and methods previously described (Peichel

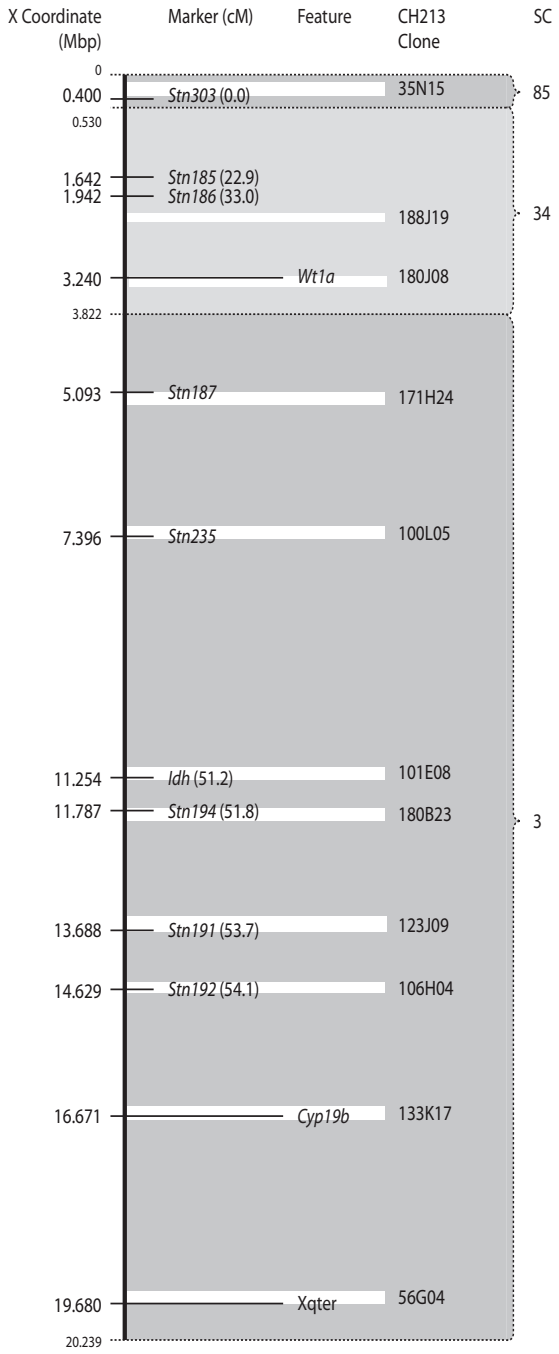


Figure 14. Genetic and sequence maps of the threespine X.
The three supercontigs (SC, shaded regions) are oriented by marker order on the genetic map (Peichel *et al*. 2004). The sequence coordinates of markers and features (black horizontal lines) are given on the left. Coordinates in smaller text give the sequence coordinates of the SC boundaries (dotted lines). Positions of BAC clones used as FISH probes (white horizontal bars) encompassing markers or genes of interest are shown to scale. The genetic positions of markers are from (Peichel *et al*. 2004). *Stn187* and *Stn235* had not been genetically mapped; I determined their positions on the X sequence by BLAST (Altschul *et al*. 1990).

et al. 2004). The original published X and Y linkage maps (Peichel *et al.* 2004) were based only on data from family 4. Peichel also genetically mapped several microsatellite markers using the same methods and crosses to characterize the extent of a putative deletion on the Y chromosome. The five primer pairs, named for their positions on my 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 (Table 4) 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'.

I 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 5x Sequencing Buffer (Applied Biosystems) to yield 1x final concentration. The reactions were then cycle sequenced (94°C for 4 min; 100 cycles of 94°C for 10 s, 50°C for 10 s, and 60°C for 4 min, store at 4°C) and run on an ABI 3100 Genetic Analyzer (Applied Biosystems). Sequenc-

Table 4. Sources of FISH probes.
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 my 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 my in silico method or library screen (see Materials and Methods, Chapter 4) or in published work. The sixth column lists the aliases used in this manuscript 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.

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*

Table 4, continued.

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	–		

ing primers were CHORI T7.29 5’-GCCGCTAATACGACTCACTATAGGGAGAG-3’ and

gSP6 5’-GTTTTTTGCGATCTGCCGTTTC-3’. I 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 (Table 4).

I 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 (Smit *et al.* 2004) 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 (Table 4)) 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 (Table 4).

Cytogenetic techniques: Metaphase spreads were prepared from primary stickleback tissue. 10 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 h 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 h at room temperature prior to hybridization. These procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol #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, Inc.) and quantified by gel electrophoresis. 1 µg 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). 200 ng of each labeled clone was ethanol precipitated together with 10 µg salmon sperm DNA. Hybridization was performed over 2-3 nights at 37°C. Washed slides were mounted and counterstained in DAPI with AntiFade (Vector Labs) and viewed with an 100x 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), I first verified that the genetic map of the X (Figure 4) 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 54.1 cM) is located between *Stn186* (33.0 cM) and *Stn193* (51.2 cM), inconsistent with the genetic order of these markers. I resolved this discrepancy between the public X assembly and the genetic map by reversing the sequence of supercontig 3 (SC3) to create a 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 14).

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). I therefore performed FISH using threespine stickleback BAC clones as probes (Table 4) in order to compare the locations of markers on the X and the Y chromosomes.

I obtained the first evidence of sex-chromosome heteromorphy using a BAC probe containing the *Idh* gene. In males, the probe hybridizes to two chromosomes at different chromosomal locations (Figure 15A), whereas it hybridizes to an identical location on the q (long) arm of two visibly similar submetacentric chromosomes in females (Figure 15B). In males, one hybridized 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 near one telomere. By virtue of *Idh* being located on Xq, I will refer to the arm of the Y containing *Idh* as Yq. This X-Y dimorphism in lo-

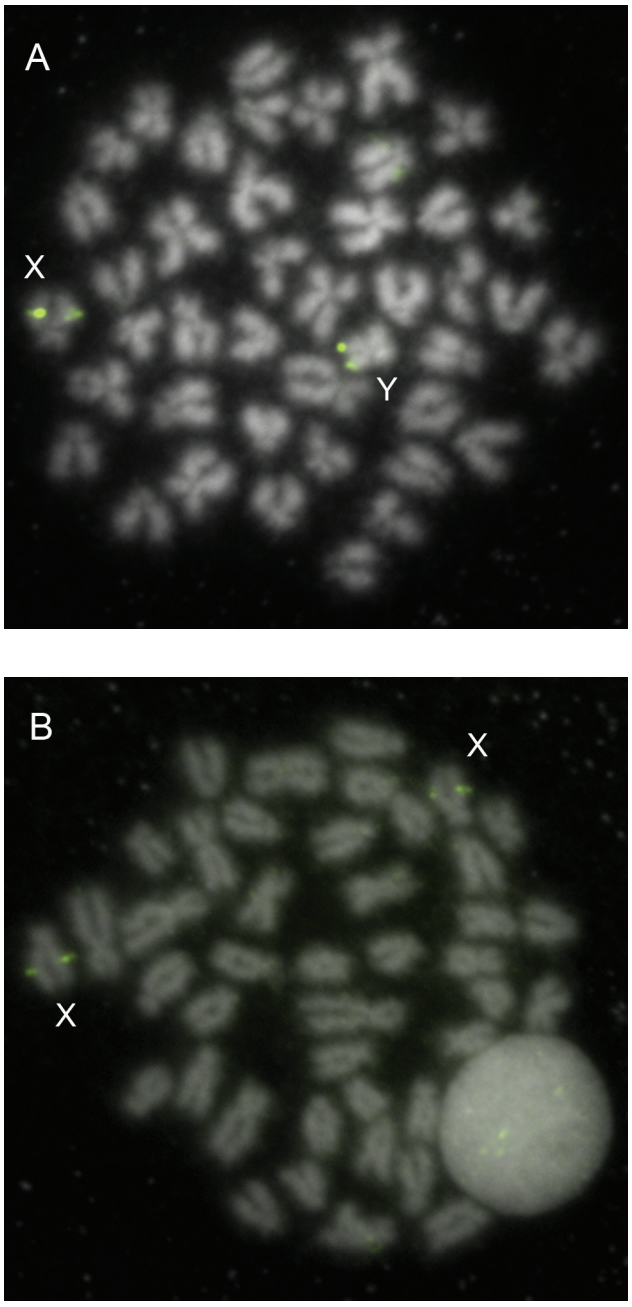


Figure 15. The threespine Y is heteromorphic.
(A) *Idh* probe (green) hybridized to a male (XY) threespine stickleback metaphase spread (2n=42). The hybridized submetacentric chromosome is identical to the X chromosomes in Figure 15B. The other chromosome with terminal hybridization signal is metacentric, 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.

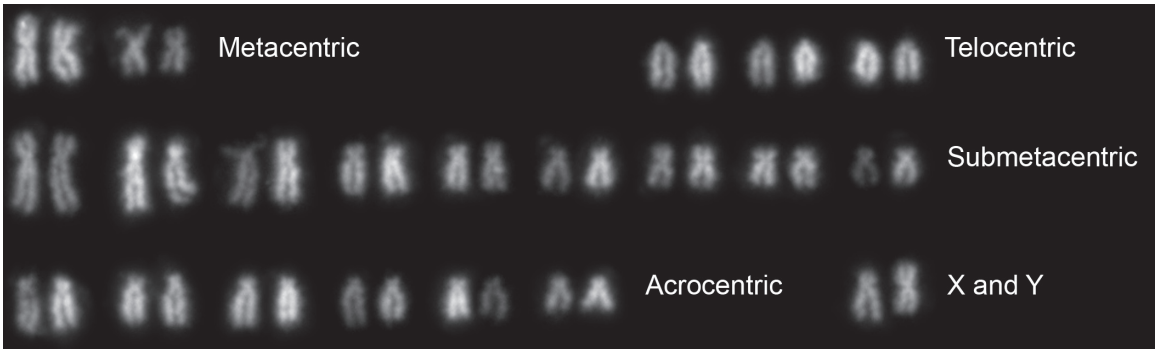


Figure 16. Threespine stickleback male karyogram.
The karyogram was produced from a male (XY) metaphase spread (2n=42). The chromosomes are aligned vertically by centromere position and ordered from largest (left) to smallest (right) within each class. There are two metacentric pairs, three telocentric pairs, nine submetacentric pairs, six acrocentric pairs, and the heteromorphic X (submetacentric) and Y (metacentric) pair.

cation of *Idh* and the position of the centromere is apparent in all metaphase chromosome spreads from the Japanese Pacific Ocean male threespine stickleback used in this study and in eight additional threespine males from populations in Lake Washington and Conner Creek, Washington, USA and the Little Campbell River, British Columbia, Canada (data not shown). A karyogram from a male threespine stickleback metaphase spread is shown in Figure 16.

The Y chromosome has experienced deletion: Because *Idh* appears to be terminal on the Y, I tested whether sequences telomeric to *Idh* on the X are present on the Y. In each analysis of male metaphase spreads, I 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 14 and Table 4), hybridizes only to the X and not to the Y nor to any other location in the genome (Figure 17), 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 18 shows the *Stn191, Stn192* and Xqter probes). Moreover, 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). Catherine Peichel identified five additional microsatellite markers within this putative deletion at 17.16, 18.10, 18.41,

18.73 and 19.68 Mbp that 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. I performed a three-probe FISH experiment to test for such an inversion (Figure 19). 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, I conducted multiple FISH experiments and constructed

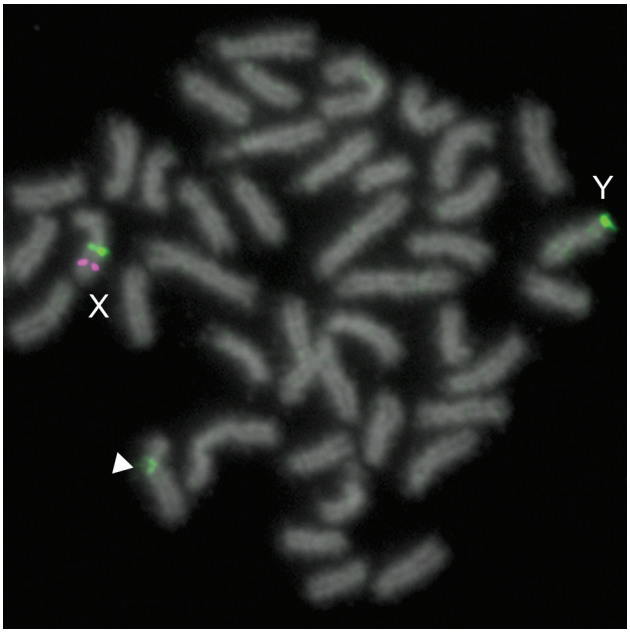


Figure 17. Deletion on the threespine Y.
Idh probe (green) and *Cyp19b* probe (purple) hybridized to a male (XY) threespine stickleback metaphase spread. The hybridized submetacentric chromosome at left exhibits the internal *Idh* hybridization signal of the X chromosome; *Cyp19b* signal is near the 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).

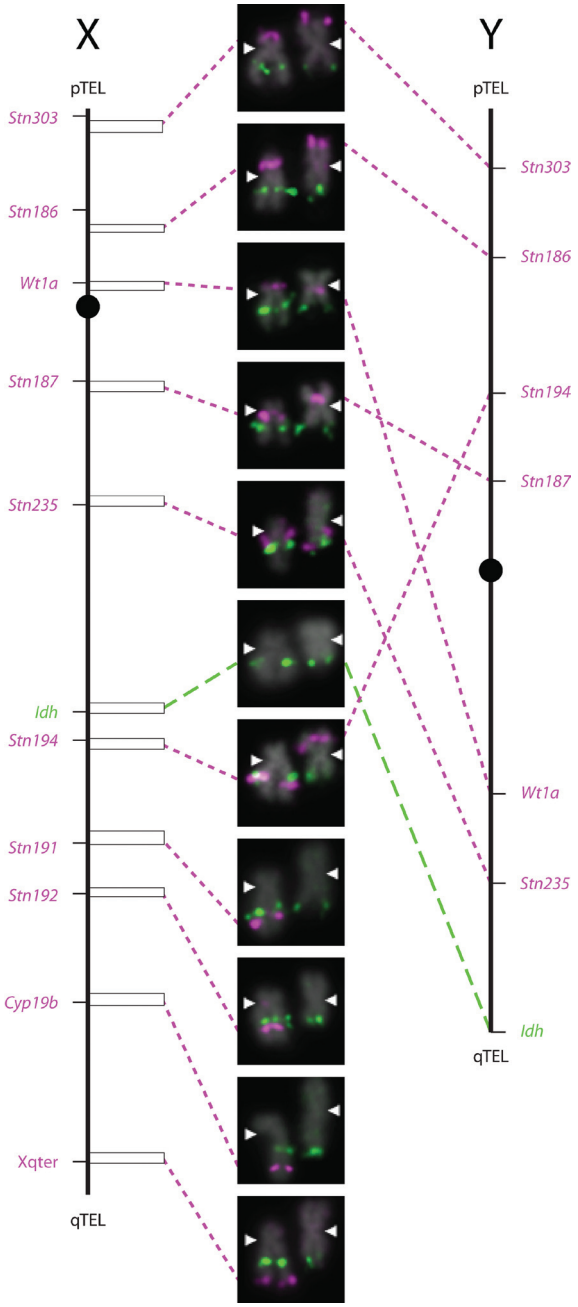


Figure 18. FISH-based cytogenetic maps of the threespine X and Y.
The sequence map of the X (left) is shown to scale with horizontal black lines representing positions of markers and features and horizontal white bars representing BAC clones used as probes (for clone identities, see Figure 14). The black circles represent the positions of the centromeres. Each FISH panel (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 panels indicate the BAC probe used in each panel. 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.

cytogenetic maps of the threespine stickleback X and Y chromosomes. In each experiment, performed on a male metaphase spread, I co-hybridized the *Idh* probe with another BAC containing a sex-chromosome genetic marker or gene of interest (Figure 18 and Table 4). 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 the genetic map and my X chromosome sequence assembly. However, the cytogenetic map of the Y chromosome (Figure 18) is very different from that of the X outside of the approximately 3.2 Mbp region that freely recombines between the X and the Y (spanning *Stn303* and *Stn186*). In addition to the aforementioned deletion and inversion, I find the *Stn194* probe in the *Idh*-qTEL interval on the X but in the pTEL-CEN interval on the Y.

I 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 in-

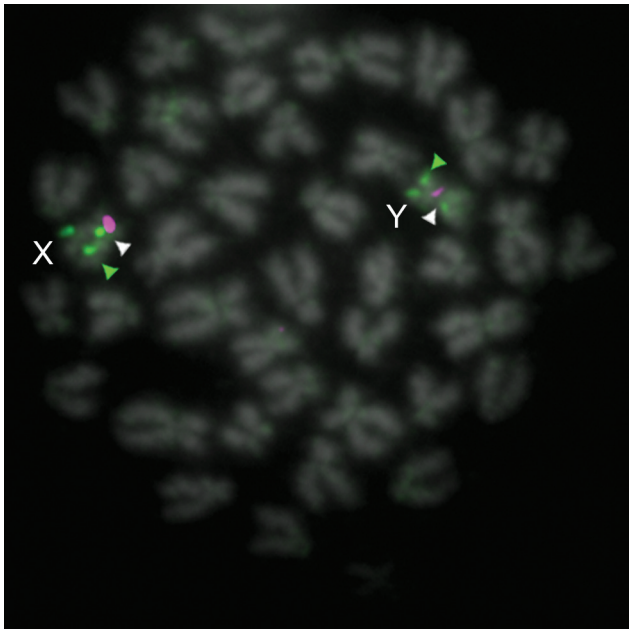


Figure 19. Pericentric inversion on the threespine Y.
Idh probe (green), *Stn187* probe (green), and *Wt1a* probe (purple) hybridized to a male (XY) threespine stickleback metaphase spread (2n=42). While the X marker order is *Wt1a* (purple), centromere (white arrowhead), *Stn187* (green), *Idh* (green; green arrowhead), the order is changed on the Y: *Stn187* (green), centromere (white arrowhead), *Wt1a* (purple), *Idh* (green; green arrowhead).

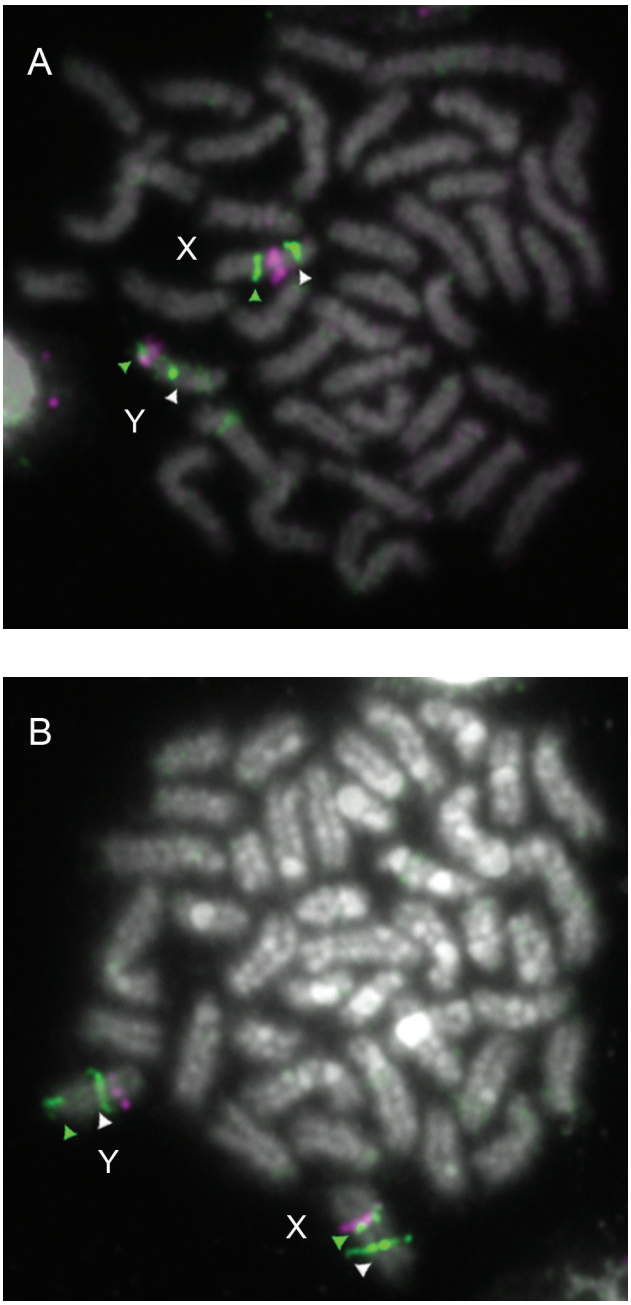


Figure 20. Refining probe orders on the threespine X and Y.
(A) The *Idh* probe (green), *Stn235* probe (purple) and *Wt1a* probe (green) are hybridized to a male (XY) threespine stickleback metaphase spread. White arrowheads denote the positions of centromeres. Green arrowheads denote the green signal corresponding to the *Idh* probe, which is distinguished on the basis of relative position on the chromosomes as seen in Figure 15A. The order of probes on the X is *Wt1a*-CEN-*Stn235*-*Idh*, and the order on the Y is CEN-*Wt1a*-*Stn235*-*Idh*. (B) The *Idh* probe (green), *Stn194* probe (purple) and *Stn187* probe (green) are hybridized to a male metaphase spread. The order of probes on the X is CEN-*Stn187*-*Idh*-*Stn194*; on the Y, the order is *Stn194*-*Stn187*-CEN-*Idh*.

terval 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* (Figure 20A) and *Stn194*-*Stn187*-CEN-*Idh* (Figure 20B). 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 21.

Discussion

In this chapter, I used FISH to compare the physical structures of the threespine stickleback X and Y chromosomes and found that the Y chromosome of the threespine stickleback is heteromorphic, despite prior reports (Chen and Reisman 1970; Klinkhardt and Buuk 1990; Cuñado *et al.* 2002), implying that the chronologically young threespine Y is evolutionarily older (more degenerate) than previously expected. Although there is

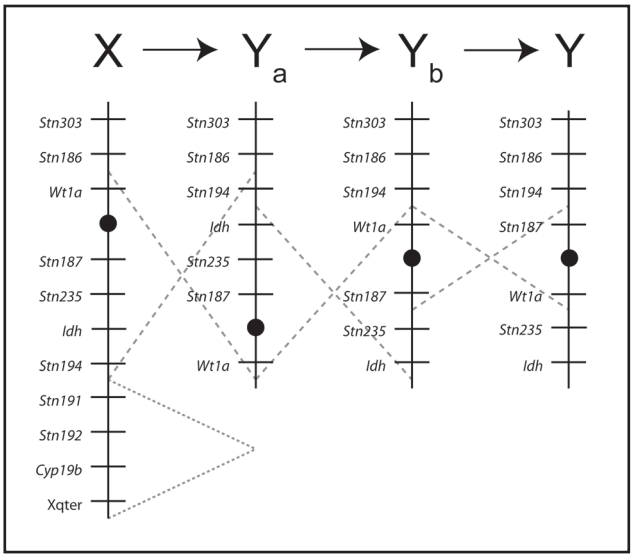


Figure 21. Parsimony model for the evolution of the threespine Y.
This model is the most parsimonious way to use inversions and deletions to arrive at the physical order of markers on the Y (right) having started with the order on the X (left). I hypothesize that three inversions (crossing dashed lines) containing the centromere (black 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.

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)).

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, the possibility cannot be ruled out 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 I 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. Detailed molecular cytogenetic analyses, such as those performed here, will likely 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, my sequence comparison of the X and the Y chromosome 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). It should be noted, though, that coarse

size measurements of the X and Y at metaphase might not correlate with their sequence content.

My model for the evolution of the threespine stickleback Y chromosome (Figure 21) explains the change in position of the centromere from the submetacentric X chromosome to the metacentric Y due to pericentric inversions. My 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. The centromere is likely 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.

My model predicts that multiple inversions have occurred on the stickleback Y chromosome. 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* might be larger than previously determined (Peichel *et al.* 2004). Based on the current genetic and cytogenetic maps, I 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* might 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 non-recombining region. I also conclude that the physical size of the X-Y homologous region, in which all recombination events between the X and Y occur, is less than 3.2 Mbp, extending from pTEL to an inversion breakpoint between it and *Wt1a* (Figures 14 and 21).

Evolutionary strata produced by the succession of inversions might be found on the Y (Lahn and Page 1999; Bergero *et al.* 2007). Older Y chromosome inversions causing loss of recombination should contain regions of greater X-Y divergence than younger

inversions, and comparative sequencing of homologous loci on the X and Y might define the relative order of the putative inversions. Theory predicts that selection for reduced recombination is to preserve linkage between *SEX* and genes with sexually-antagonistic effects (Bull 1983a; Rice 1987b). If inversion caused reduced recombination between the stickleback X and Y, then the physical interval contained in the first inversion should define the Y region in which *SEX* is located. This physical interval would certainly be smaller than the current interval containing *SEX*. Future sequencing and assembly of the threespine stickleback Y chromosome will make it possible to examine levels of sequence divergence across the X and Y and to identify the existence of evolutionary strata corresponding to the inversion events suggested by FISH analysis.

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 (*O. 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 *T. rubripes* lies in a region without synteny either to the stickleback or the medaka 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 genus Oreochromis (Lee *et al.* 2003; Lee *et al.* 2004; Cnaani *et al.* 2007), 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 threespine 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 about 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 2004a).

My 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), supporting the conformational hypothesis of reduced recombination. My present study also provides support for the structural hypothesis: I have shown inversions on a young sex chromosome in a region that lacks recombination. This situation is very similar to the *S. latifolia* Y chromosome, which is also 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).

Although the presence of multiple pericentric inversions on the threespine Y (Figure 21) are consistent with Ohno's prediction that a pericentric inversion could be used to establish sex-chromosome heteromorphy (Ohno 1967), the stickleback Y might be too old to distinguish the conformational and structural hypotheses (Jablonka and Lamb 1990). However, additional analysis of the levels of X-Y divergence across the stickleback sex-chromosome pair might 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 testing of the association between these pericentric inversions and the suppression of recombination.

Sex-chromosome heteromorphy has been reported in the black-spotted stickleback (XY) and in the fourspine stickleback (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 still 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 I used in this study to provide the first report of FISH in sticklebacks, comparative analyses of sex-chromosome evolution in stickleback fishes might help improve our understanding of the process of transition between XY and ZW systems of genetic sex determination in closely related species, which I discuss in Chapter Five.

Chapter 5. Evolution of Sex Determination and Sex Chromosomes in Sticklebacks

Introduction

GSD is prevalent in vertebrates and is generally accompanied by the presence of a heteromorphic chromosome pair in one sex. In birds and snakes, the W sex chromosome is female-limited and carries a female-determining locus; however, neither the bird nor the snake sex determination locus has been identified (reviewed in (Ezaz *et al.* 2006)). Most mammals have an XY heteromorphic pair (Graves 2006), and the male-limited Y sex chromosome bears *SRY*, a male-determining gene (Gubbay *et al.* 1990a; Sinclair *et al.* 1990; Koopman *et al.* 1991) that is found in all but a handful of mammals (Fredga *et al.* 1976; Hoekstra and Hoekstra 2001; Just *et al.* 2002; Wallis *et al.* 2007; Waters *et al.* 2007).

However, this broad conservation of sex chromosome systems across large taxonomic groups is not universal in vertebrates. Both simple (XY and ZW) and complex (polyfactorial) forms of GSD, as well as ESD, are seen in fish, lizards, turtles and amphibians (Ezaz *et al.* 2006). Even closely related species within a genus may have different sex determination systems; for example, the only other known vertebrate sex-determining gene, *DMY* in the medaka fish (Matsuda *et al.* 2002; Nanda *et al.* 2002), is not found in closely related Oryzias species (Kondo *et al.* 2003; Kondo *et al.* 2004).

Sex-chromosome heteromorphy arises because of loss of recombination around sex-determining loci (generically, *SEX*). Once recombination around *SEX* is suppressed, intrachromosomal inversions and deletions and mobile sequence elements tend to accumulate in the nonrecombining region (Rice 1987a; Jablonka and Lamb 1990; Charles-

worth and Charlesworth 2000; Charlesworth *et al.* 2005; Steinemann and Steinemann 2005; Graves 2006). These physical changes to the sex chromosome result in heteromorphy seen in metaphase chromosome spreads, although it is not possible to state *a priori* that the sex chromosome will be either the larger or smaller chromosome of a heteromorphic pair (Graves 1995).

Theoretical studies have suggested that reduced recombination on a sex chromosome is favored when *SEX* arises in proximity to genes with alleles of sexually-antagonistic effect (Charlesworth and Charlesworth 1978; Rice 1987b). For example, an allele that increases male fitness and reduces female fitness in an XY system benefits from absolute linkage with the male-determining *SEX* locus. Selection for linkage of sexually-antagonistic genes to *SEX* may also explain the rapid turnover of sex determination loci and sex chromosomes between closely related species (van Doorn and Kirkpatrick 2007), as seen in medaka (Tanaka *et al.* 2007) and salmonids (Woram *et al.* 2003), respectively. Selection for linkage to sexually-antagonistic genes has also been proposed as an explanation for translocations of parts of sex chromosomes, or fusions of entire sex chromosomes, to autosomes (Charlesworth and Charlesworth 1980). This reasoning can be extended to suggest that an autosome carrying an abundance of genes with sexually-antagonistic alleles could independently evolve into a sex chromosome in closely related lineages. Therefore, it is crucial to investigate which chromosomes and what genes are linked to *SEX*.

Fishes are a particularly useful group to explore the turnover of sex chromosome systems because both XY and ZW systems exist in closely related species (Devlin and Nagahama 2002; Mank *et al.* 2006). For example, both XY and ZW GSD systems have been identified in species of the genus Oryzias (Takehana *et al.* 2007a; Takehana *et al.* 2007b; Tanaka *et al.* 2007), the genus Xiphophorus (Volff and Schartl 2001), and tilapiine cichlid species of the genus Oreochromis (Lee *et al.* 2003; Lee *et al.* 2004; Cnaani *et al.*

2008). Thus, fishes may provide the opportunity to ask whether distinct forms of GSD found in closely-related species have interconverted or evolved independently (Ohno 1967; Traut and Winking 2001; Ezaz *et al.* 2006; Mank *et al.* 2006). Several mechanisms have been proposed for how sex chromosomes arise, including appearance of a novel *SEX* locus on an autosome (Ohno 1967), transposition of a *SEX* locus between chromosomes in different lineages (Woram *et al.* 2003), and fusions between sex chromosomes and autosomes (Charlesworth and Charlesworth 1980). Attempts to address these possibilities will benefit from detailed molecular, genetic, cytogenetic and phylogenetic analyses of sex determination systems that differ between closely related species.

Among fishes, the sticklebacks (family Gasterosteidae) provide a particularly interesting system in which to investigate the evolution of sex determination and sex chromosomes. The first cytogenetic survey in this family reported the presence of a heteromorphic XY pair in the black-spotted stickleback and a heteromorphic ZW pair in the fourspine stickleback (Chen and Reisman 1970). In the same study, evidence of a heteromorphic pair was not seen in the threespine stickleback. The findings of Chen and Reisman's 1970 study, along with reported phylogenetic relationships between the stickleback species, are summarized in Figure 3.

Although later studies (Klinkhardt and Buuk 1990; Cuñado *et al.* 2002) also did not find evidence of a heteromorphic sex chromosome pair in the threespine stickleback, genetic mapping subsequently identified the presence of XY genetic sex determination on LG19 in this species (Peichel *et al.* 2004). Using FISH, I demonstrated that there is a heteromorphic XY pair in threespine sticklebacks (Ross and Peichel in press). Genetic mapping by Michael Shapiro has now demonstrated that the sex determination locus in the ninespine stickleback maps to LG12, which is distinct from the threespine sex chromosome LG19 (Shapiro *et al.* in preparation). In contrast, no heteromorphic sex-chromosome pairs have been reported in the outgroup family Syngnathidae (Vitturi *et al.* 1998;

Libertini *et al.* 2006). Taken together, these data suggest that different sex determination systems and sex chromosomes have evolved within the stickleback family. Combined with the recent development of genetic and genomic resources for both threespine and ninespine sticklebacks (Peichel *et al.* 2001; Kingsley *et al.* 2004; Kingsley and Peichel 2007; Shapiro *et al.* in preparation), these small teleost fish are an excellent system in which to study the evolution of sex chromosomes and GSD.

To systematically characterize the relationships between the sex determination mechanisms and sex chromosome systems in the stickleback family, sex determination loci were genetically mapped and I searched for heteromorphic sex chromosome pairs using FISH in the rest of the North American stickleback species: the black-spotted, brook, and fourspine sticklebacks. In ninespine sticklebacks, I identified a heteromorphic XY pair corresponding to LG12, where the sex determination locus has been mapped in this species (Shapiro *et al.* in preparation). I confirmed the presence of a heteromorphic pair in black-spotted sticklebacks, although I found that males of this species have 41 chromosomes, not 42 as previously reported (Chen and Reisman 1970). Genetic mapping and my molecular cytogenetics demonstrate that the black-spotted Y chromosome consists of a fusion between LG12 and LG19, defining their sex chromosome system as of the X_1X_2Y type and suggesting an evolutionary link between the distinct Y chromosomes of threespine and ninespine sticklebacks.

However, neither LG12 nor LG19 is associated with a sex determination locus or a heteromorphic sex chromosome pair in brook or fourspine sticklebacks. Consistent with previous work (Chen and Reisman 1970), I do find that fourspine sticklebacks have a heteromorphic ZW pair, while brook sticklebacks have no heteromorphic sex chromosome pair. Remarkably, I have confirmed the presence of an independent X_1X_2Y system, first identified by Jun Kitano by genetic mapping, within the Japan Sea population of threespine sticklebacks. The results reported in this chapter, summarized in Figure 1 and

destined for publication (Ross *et al.* in preparation), exhibit the remarkable diversity of genetic mechanisms and chromosomal systems of sex determination that can be present within a single family of fish that have diverged within the past twenty-five million years (Bell 1994; Orti Personal communication).

Materials and Methods

Genetic crosses: Three black-spotted stickleback crosses were generated using males and females collected from Wells, ME in May 2003. Sperm from a single black-spotted male was used to fertilize the eggs of a single black-spotted female (cross 1); the sperm of a second male was used to fertilize the eggs of two different females (crosses 2 and 3). The progeny of each of the three crosses were grown in separate tanks. A single brook stickleback cross was generated using a female collected from Fox Holes Lake (Northwest Territories, Canada) and a male collected from Pine Lake (Wood Buffalo National Park, Alberta, Canada) in June 2005. A single fourspine cross was generated using a single female and a single male collected from Pilgrim Lake (Cape Cod National Seashore, MA) in May 2004. For all crosses, the sex of the progeny was determined by visual inspection of the gonads. DNA was prepared from the caudal fin of each individual by phenol-chloroform extraction, followed by ethanol precipitation.

Microsatellite genotyping: PCR genotyping with threespine microsatellite markers and ninespine microsatellite markers was performed as previously described (Peichel *et al.* 2001; Shapiro *et al.* in preparation), except that the reactions were run on an ABI 3100 and the genotypes were analyzed using ABI GeneMapper 3.7 (Applied Biosystems).

Amplified fragment length polymorphism (AFLP) genotyping: AFLP screens (Vos *et al.* 1995) were performed by James Urton on parents and individuals from the brook and fourspine stickleback crosses. The AFLP Plant Mapping Protocol (Applied Biosystems) was used, with the following alterations. Genomic DNA (1-2µg) was cut

with 50 units of *Eco*RI (New England Biolabs, NEB) and 25 units of *Mse*I (NEB) with 10µg of BSA for 4 hours at 37ºC. Reactions were then heated to 65ºC for 20 minutes. Preselective amplifications were run on a Peltier Thermal Cycler-100 (PTC-100, MJ Research). For the AFLP selective amplifications, each of 8 *Eco*RI primer pairs (E-AAC, E-AAG, E-ACA, E-ACC, E-ACG, E-ACT, E-AGC, E-AGG) were paired with each of 6 *Mse*I primer pairs (M-CAA, M-CAC, M-CAG, M-CAT, M-CTA, M-CA), for a total of 48 primer pairs. *Eco*RI primers were labeled with 6-FAM. Selective amplifications were run on a PTC-100 thermal cycler (MJ Research). Reactions were run on an ABI 3100 and genotypes were analyzed with the "AFLP Default" method on ABI GeneMapper 3.7 (Applied Biosystems).

Linkage analysis: Genetic linkage maps were created in JoinMap3.0 (Van Ooijen and Voorips 2001) using default parameters. Both Kruskal-Wallis tests for significant associations between genotype and sex phenotype and interval mapping were performed in MapQTL4.0 (Van Ooijen *et al.* 2002).

Cytogenetic analysis: Metaphase spreads were prepared as described (Ross and Peichel in press) using black-spotted males collected from Baie de L'Isle-Verte National Wildlife Area (Québec, Canada) in May 2003, black-spotted males and females collected from Demarest Lloyd State Park (Dartmouth, MA) in May 2005 and May 2007, ninespine and brook stickleback males and females collected from Pine Lake (Wood Buffalo National Park, Alberta, Canada) in June 2007, fourspine males collected from Pilgrim Lake (Cape Cod National Seashore, MA) in May 2005, and fourspine males and females collected from Demarest Lloyd State Park (Dartmouth, MA) in May 2005 and 2007.

Animal work was approved by the Fred Hutchinson Cancer Research Center IACUC (protocol #1575). Fish collections were performed with the permission of the Commonwealth of Massachusetts, Division of Marine Fisheries (permit numbers 8002 in 2003, 2004, 2005; 152769 in 2007), the Cape Cod National Seashore (permit num-

ber CACO-2005-SCI-0014 in 2005), the Department of Fisheries and Oceans, Canada (permit numbers SLE-04/05-215 and S-07/08-2005-HR), the Aurora Research Institute (permit numbers 13810R and14163R), and Wood Buffalo National Park (permit numbers WB05-1010 and WB-2007-1007).

Fluorescence *in situ* hybridization (FISH) experiments were performed on metaphase spreads as described (Ross and Peichel in press). Bacterial artificial chromosomes (BACs) from the threespine CHORI-213 library (Kingsley *et al.* 2004) were used as FISH probes. Linkage group (LG) 19 probes (*Stn303*: CH213-035N15; *Idh*: CH213-101E08; and *Wt1a*: CH213-180J08) were identified previously (Ross and Peichel in press). The LG12 probe (CH213-140B10), spanning 0.35 to 0.56 Mbp on the threespine public genome assembly of scaffold 6 (part of the chromosome 12 assembly), was identified as containing genetic marker *Stn144* (Peichel *et al.* 2001) at 0.51 Mbp using a computational approach (Ross and Peichel in press). A BAC clone in the proximity of the LG9 genetic marker *Stn102* (called 9D3b5 in Figure 27) was identified using the same approach. *Stn102* is located at 4.17 Mbp on the public assembly of scaffold 8 (part of the chromosome 9 assembly) and is nonrecombinant with some LG19 markers in males (Figure 27). The LG9 BAC probe used, CH213-031B20, contains the portion of scaffold 8 from 4.20 to 4.41 Mbp.

Results

***SEX* is linked to both LG12 and LG19 in black-spotted sticklebacks**: Master sex determination loci (*SEX*) map to independent Y chromosomes in threespine stickleback (LG19) and ninespine stickleback (LG12) (Peichel *et al.* 2004; Shapiro *et al.* in preparation). To determine whether markers from either chromosome are linked to *SEX* in black-spotted sticklebacks, Catherine Peichel genotyped the 80 progeny (41 females and 39 males) of three black-spotted crosses with threespine and ninespine markers from

Table 5. Sex-linked threespine and ninespine markers used for genotyping.
For each microsatellite marker, the threespine linkage group (LG) designation and position in the threespine genome assembly (http://www.ensembl.org/Gasterosteus_aculeatus/index.html) identified by BLAT are indicated. If a marker was also mapped in black-spotted or ninespine sticklebacks, the LG designation in that species is also indicated. FP (failed PCR) indicates that PCR product was not obtained for a marker in a given species. NP (not polymorphic) indicates that a PCR product was obtained in the species, but was not polymorphic in a cross. NT (not tested) refers to markers for which sex linkage in a species could not be tested. Data provided by Catherine Peichel, Michael Shapiro, James Urton and Jessica Boland.

	G. aculeatus			*G. wheatlandi*		*P. pungitius*		*C, inconstans*		*A. quadracus*	
Marker	**LG**	**Position (mbp)**	**Sex-linked**	**LG**	**Sex-linked**	**LG**	**Sex-linked**	**PCR product**	**Sex-linked**	**PCR product**	**Sex-linked**
Pun99	12	5.576	NT	12/19	yes	12	yes	FP	NT	yes	no
Stn327	12	5.800	no	12/19	yes	FP	NT	NP	NT	NP	NT
Pun7	12	8.475	no	NP	NT	12	yes	NP	NT	yes	no
Stn287	12	9.516	no	12/19	NT	12	yes	NP	NT	yes	no
Stn276	12	9.516	no	12/19	NT	12	yes	NP	NT	yes	no
Stn144	12	11.037	no	FP	NT	12	yes	NP	NT	yes	no
Stn142	12	12.635	no	12/19	yes	NP	NT	FP	NT	yes	no
Pun2	12	12.276	no	12/19	yes	12	yes	NP	NT	NP	NT
Pun234	12	15.613	NT	FP	NT	12	yes	yes	no	yes	no
Stn186	19	1.942	yes	NP	NT	19	no	yes	no	FP	NT
Pun117	19	6.325	yes	12/19	yes	19	no	FP	NT	yes	no
Stn235	19	7.396	yes	12/19	yes	NP	NT	FP	NT	FP	NT
Stn194	19	11.787	yes	12/19	yes	19	no	NP	NT	yes	no
Pun268	19	13.170	FP	FP	NT	19	no	yes	no	FP	NT
Stn284	19	13.658	yes	12/19	yes	NP	NT	NP	NT	FP	NT
Stn168	19	13.736	NP	NP	NT	19	no	yes	no	FP	NT
Cyp19b	19	16.671	yes	12/19	yes	19	no	yes	no	FP	NT

Table 6. Marker genotype-sex phenotype associations in black-spotted sticklebacks.
For each marker from threespine LG12 or LG19, the Kruskal-Wallis test statistic was used to determine whether there were significant differences in phenotype means between the four possible segregating genotypes "ac", "ad", "bc" and "bd". For each marker, the mother was assigned genotype "ab" and the father was assigned genotype "cd". The female sex phenotype was assigned a score of "0" and the male sex phenotype was assigned a score of "1". The total number of individuals with a given marker genotype are indicated (*n*). Data provided by Catherine Peichel.

				Phenotype means			
Marker	**LG**	**Kruskal-Wallis**	**p**	ac (n)	bc (n)	ad (n)	bd (n)
Pun99	12	7.00	<0.01	0.00 (4)	-	1.00 (4)	-
Stn327	12	76.00	< 0.0001	0.00 (24)	0.00 (16)	1.00 (20)	1.00 (17)
Stn142	12	77.00	< 0.0001	0.00 (21)	0.00 (19)	1.00 (24)	1.00 (14)
Pun2	12	43.00	< 0.0001	0.00 (11)	0.00 (14)	1.00 (10)	1.00 (9)
Pun117	19	75.00	< 0.0001	0.00 (23)	0.00 (17)	1.00 (21)	1.00 (15)
Stn235	19	59.00	< 0.0001	0.00 (16)	0.00 (16)	1.00 (16)	1.00 (12)
Stn194	19	61.00	< 0.0001	0.00 (13)	0.00 (17)	1.00 (21)	1.00 (11)
Stn284	19	79.00	< 0.0001	0.00 (18)	0.00 (23)	1.00 (24)	1.00 (15)
Cyp19b	19	65.00	< 0.0001	0.00 (13)	0.00 (20)	1.00 (20)	1.00 (13)

both LG12 and LG19 (Table 5). Five markers from LG19 and six markers from LG12 are polymorphic in at least one of the parents of the three crosses (Table 5). For the nine markers that were polymorphic in a male parent, there is perfect concordance between the marker genotype inherited from the father and the sex phenotype of the progeny (Table 6), demonstrating that black-spotted males are the heterogametic (XY) sex and that markers from both LG12 and LG19 are *SEX*-linked in black-spotted sticklebacks. For all five LG19 markers, the Y-linked allele is a null allele (i.e. no product is amplified), while none of the Y-linked alleles of LG12 markers are null.

To further explore the relationship between LG12 and LG19 markers in these black-spotted crosses, Peichel used the complete genotypes of the ten markers informative in all three crosses to create a linkage map. Even using a stringent LOD score of 10.0, all ten markers are found in a single linkage group (Figure 22A). However, when only the female meiotic data is used to create a linkage map, two independent linkage

groups representing LG12 and LG19 are found (Figure 22B). In contrast, when only the male meiotic data is used to create a linkage map, all markers are completely linked to each other and to *SEX* (Figure 22C).

Cytogenetic evidence of a fusion between LG12 and LG19 in black-spotted males: These genetic mapping data suggested that one chromosome 12 and one chromo-

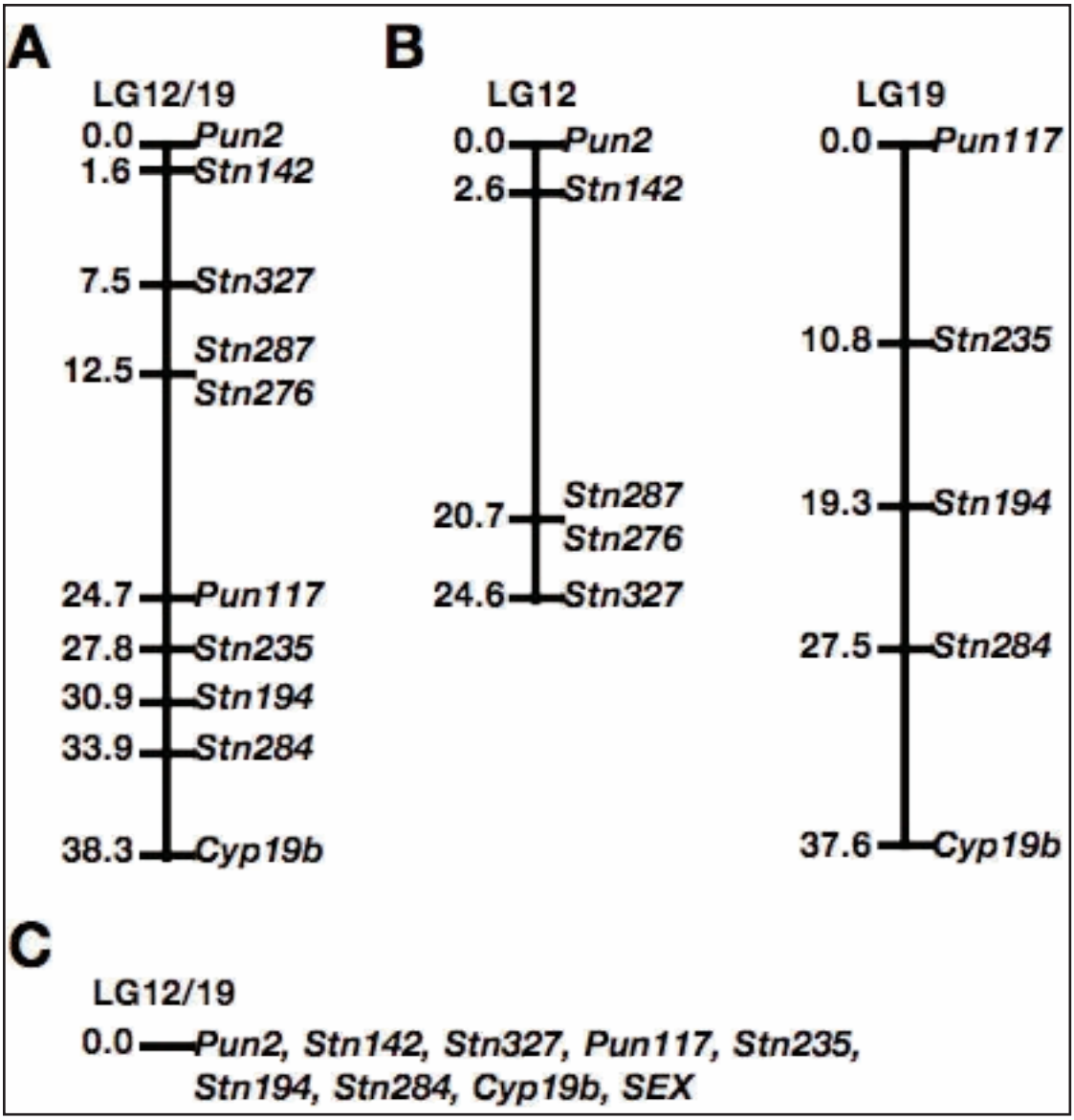


Figure 22. Linkage maps of the black-spotted sex chromosome.
(A) Combined male and female meiotic data. (B) Female meiotic data only. (C) Male meiotic data only. Data provided by Catherine Peichel.

some 19 might be fused in male, but not female, black-spotted sticklebacks. Consistent with these results, a karyogram I made from a male black-spotted metaphase spread (Figure 23A) contains 41 chromosomes (19 pairs and three unpaired). The heteromorphic triad, composed of a large submetacentric, a medium submetacentric, and a medium acrocentric chromosome, is not present in the female karyogram (Figure 23B), which

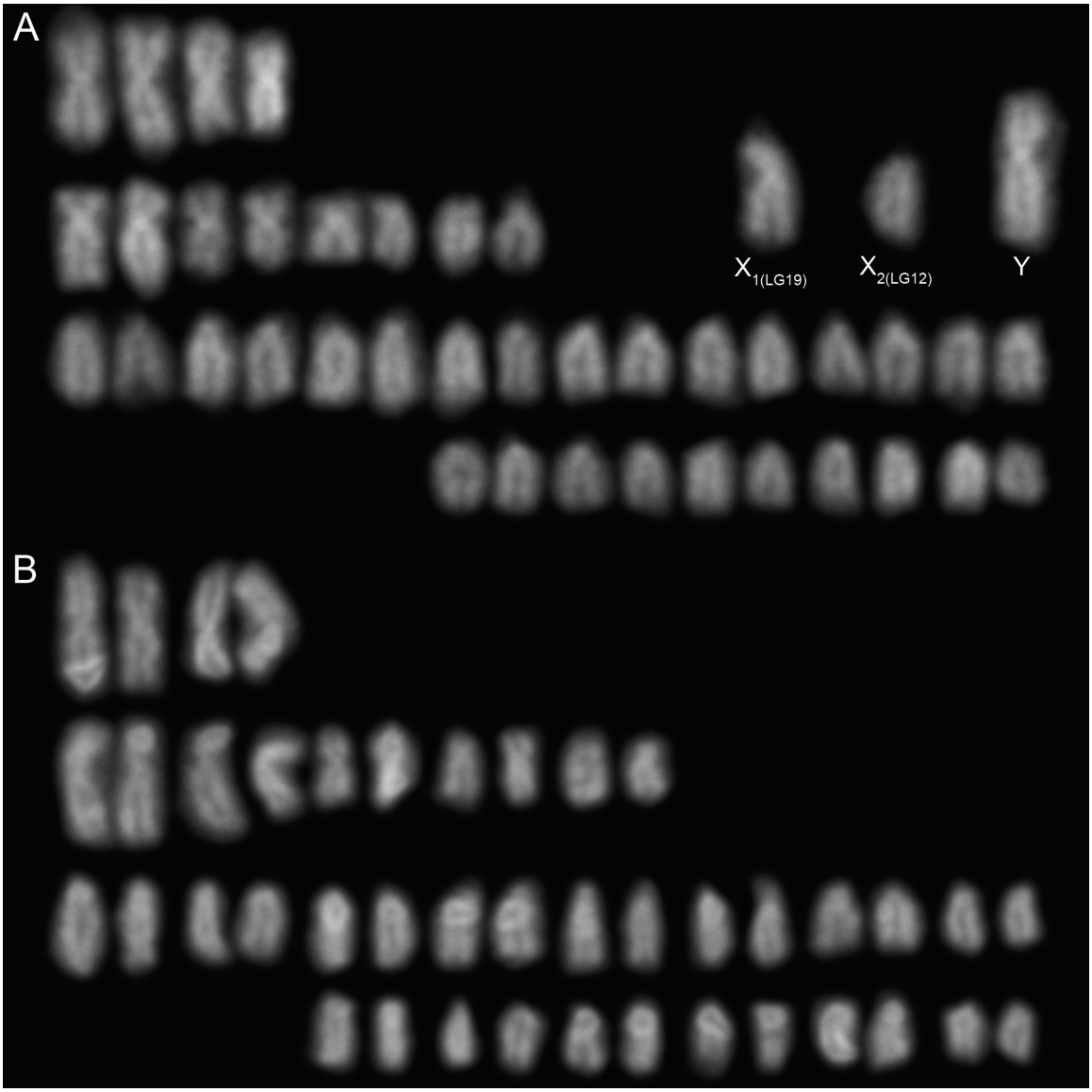


Figure 23. Karyograms of black-spotted sticklebacks.
(A) Male. The two presumed X chromosomes, one from LG12 and one from LG19, and the Y chromosome, are indicated. (B) Female.

Table 7. Karyotype data for stickleback fishes.
For both sexes of each species, column 3 gives the number of individuals from whom diploid chromosome number counts were obtained; the total number of metaphase spreads analyzed is given in column 4. The mode diploid chromosome number, which I present as the true diploid chromosome number, is in column 5. The percentage of total metaphases analyzed having the mode chromosome number is shown in column 6, and the range of chromosome counts for all metaphase spreads is given in column 7. The identification of fewer chromosomes than the mode in a metaphase spread may be due to chromosomes overlapping in a metaphase spread and being counted as one or to nearby cellular debris obscuring chromosomes. The identification of more chromosomes than the mode in a metaphase spread could be due to chromosomes having tortuous morphology or uneven DAPI staining and being counted as two or more chromosomes, or to the presence of chromosomes from neighboring nuclei near the metaphase spread.

Species	Sex	# individuals	# metaphases analyzed	Mode 2n	% metaphases with mode 2n	2n range
black-spotted	Male	4	56	41	87%	36-41
	Female	3	20	42	85%	40-42
ninespine	Male	1	16	42	69%	39-42
	Female	5	33	42	85%	39-43
brook	Male	3	40	46	78%	41-47
	Female	4	40	46	65%	40-47
fourspine	Male	10	16	46	56%	41-47
	Female	11	49	46	85%	35-47

comprises 21 chromosome pairs. Absence of the largest chromosome in the male heteromorphic triad from the female karyogram defines it as the Y (Figure 23A). The presence of 41 chromosomes in male somatic tissue was seen in multiple metaphase spreads from multiple individuals (Table 7).

To examine the relationship between LG12 and LG19 in black-spotted, threespine and ninespine sticklebacks, I hybridized LG12 and LG19 FISH probes to female and male metaphase spreads of all three species. In females of all three species, the LG12 and LG19 pairs appear homomorphic (Figure 24). I had previously demonstrated that the threespine LG19 is heteromorphic in males (Ross and Peichel in press); here I demonstrate that LG12 is homomorphic in males (Figure 24). A heteromorphic pair in the male ninespine karyogram (Figure 25A) is absent from the female karyogram (Figure 25B). However, I find that LG12, and not LG19, is the heteromorphic pair in male ninespine

sticklebacks (Figure 24). Because both copies of chromosome 12 (the X) in female ninespines are metacentric, the metacentric chromosome 12 in ninespine males is the X, and the submetacentric chromosome 12, which appears larger than the X, is therefore the Y chromosome (Figure 24 and Figure 25A).

In male black-spotted metaphase spreads, both the LG12 probe and the LG19 probe for the *Stn303* locus (Ross and Peichel in press) hybridize to the q and p arms, respectively, of one submetacentric chromosome, which is the largest single chromo-

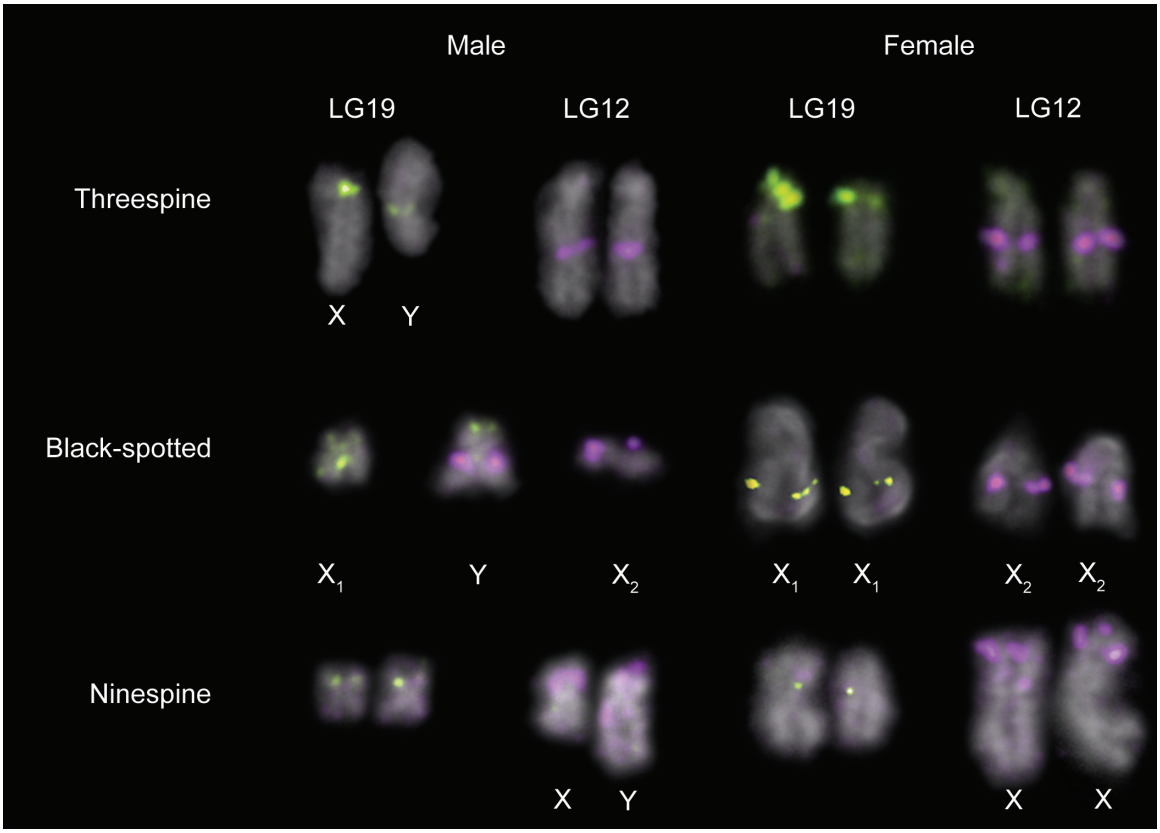


Figure 24. FISH analyses of LG12 and LG19.
The LG19 probe (CH213-180J08 (*Wt1a*), except for CH213-035N15 (*Stn303*), which was hybridized to a black-spotted male metaphase spread) is green; the LG12 probe (CH213-140B10) is purple. For each sex of each species, only chromosomes in a single metaphase spread are shown. The threespine LG19 sex chromosome pair is heteromorphic in males, while LG12 is not. The ninespine male LG12 pair is heteromorphic, while LG19 is not. In black-spotted males, one distinct copy each of LG12 and LG19 is present, while probes to both LGs hybridize to the two arms of a submetacentric, male-specific chromosome, the Y. LG12 and LG19 are homomorphic in females of all three species. The sizes of chromosomes cannot be compared between sexes or species because they were obtained from different metaphase spreads.

some in the karyogram. These probes also identify the other copies of chromosomes 12 (acrocentric) and 19 (submetacentric) by hybridization (Figure 24). In female black-spotted metaphase spreads, the LG12 and LG19 probes hybridize to two independent chromosome pairs that appear the same as the unpaired acrocentric and submetacentric chromosome in the male karyotype (Figure 24). Based on a comparison of chromosome morphologies across species, I arbitrarily define the submetacentric chromosome 19 to be

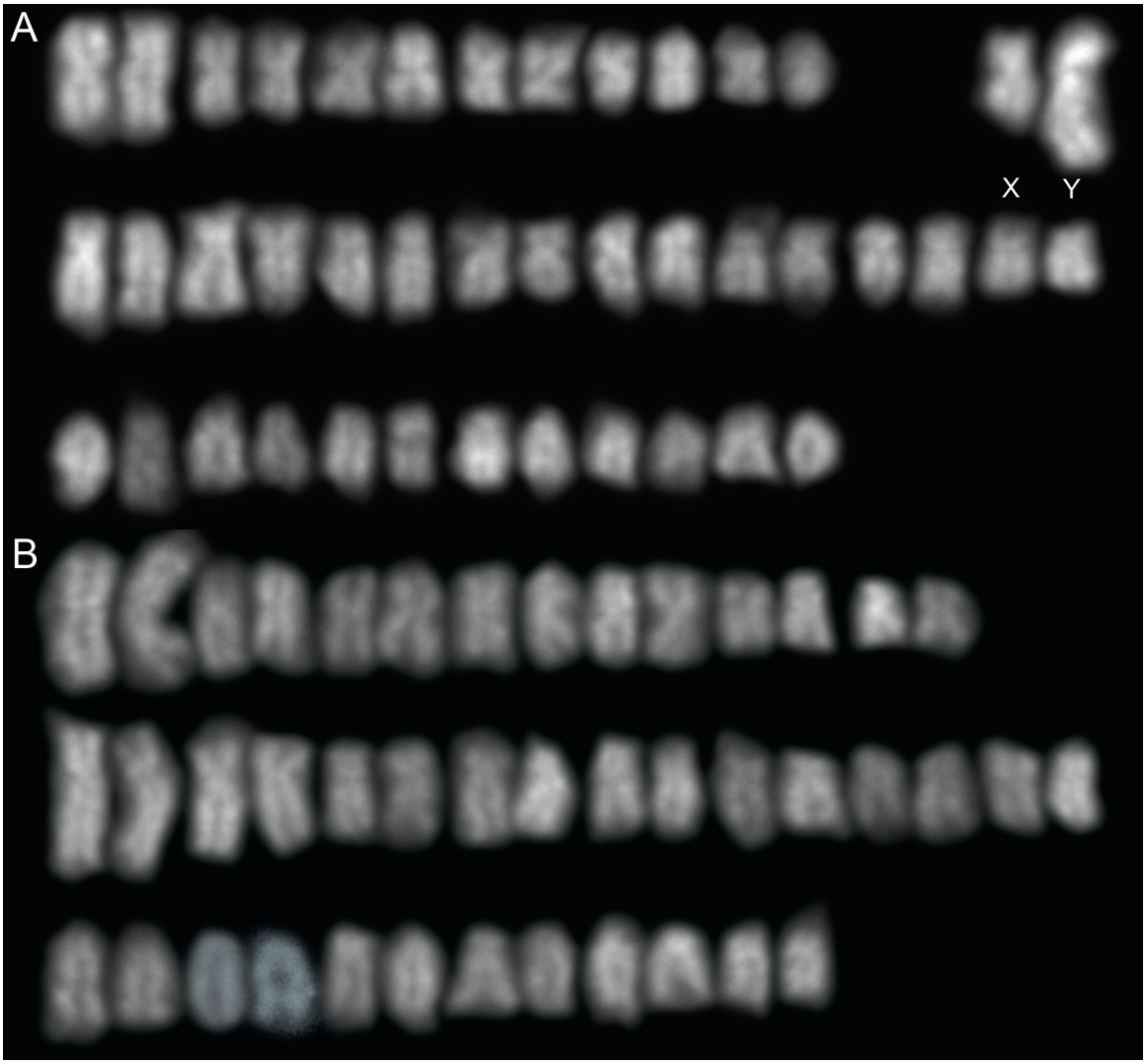


Figure 25. Ninespine stickleback karyograms.
(A) Male and (B) female. The presumed X and Y chromosomes are indicated.

X_1 and the acrocentric chromosome 12 to be X_2 for black-spotted males (Figure 23A and Figure 24).

To further study the black-spotted Y chromosome, I used two additional LG19 FISH probes, one from the *Idh* locus and one from the *Wt1a* locus (Ross and Peichel in press). Although both probes hybridized to two chromosomes in female black-spotted sticklebacks and to one chromosome in male black-spotted sticklebacks, neither of these probes hybridized to the Y in males (Figure 26), indicating that these loci on the threespine Y may be deleted from the black-spotted Y.

Independent sex-chromosome-autosome fusion in the Japan Sea population of threespine sticklebacks: Genetic evidence for an independent Y-autosome fusion in the Japan Sea population of threespine sticklebacks was found by Jun Kitano. The Japan Sea population diverged from the Pacific Ocean sticklebacks when the Japan Sea was landlocked about 1.5–2 MYA (Higuchi and Goto 1996; Kitano *et al.* 2007), but later came into contact with the Pacific Ocean marine threespine stickleback population when ocean levels rose again after the last glacial maximum. Kitano has characterized genetic, morphological and behavioral differences between these two populations (Kitano *et al.* 2007). In regions of overlap between these populations, there are few hybrids (0.6-1%), suggesting that they are reproductively isolated in nature (Higuchi and Goto 1996; Kitano *et al.* 2007). Kitano has shown that divergence in male mating behavior and female preferences for these behaviors, as well as hybrid male sterility, contribute to reproductive isolation between the populations (Kitano *et al.* 2007).

While generating a genetic map of the Japan Sea population, Kitano noticed that Y alleles of LG19 markers were linked to LG9 markers in males; LG9 and LG19 still appeared to be distinct linkage groups in females (Figure 27). This finding is reminiscent of the genetic evidence for the Y-autosome fusion in black-spotted sticklebacks. I therefore performed FISH with probes from LG9 and LG19 and confirmed that the Y is fused to

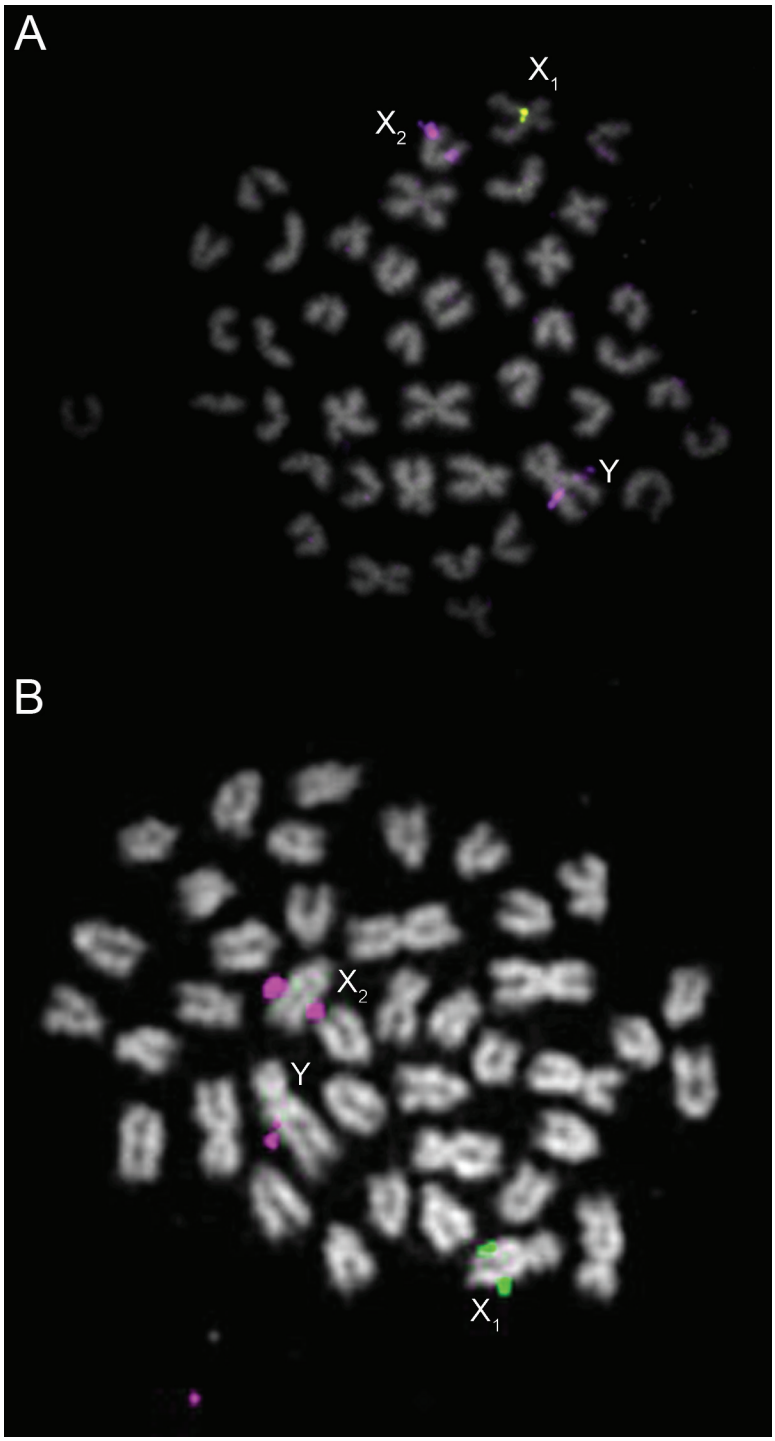


Figure 26. Certain LG19 FISH probes do not hybridize to the black-spotted Y.
The LG19 probes, green, in (A) CH213-180J08 (*Wt1a*) and (B) CH213-101E08 (*Idh*), do not hybridize to the Y, the single largest chromosome, which is hybridized by the LG12 probe. Both LG19 probes hybridize to the X_1 (LG19).

one copy of LG9 in males, but not females, from the Japan Sea (Figure 28). Additionally, Japan Sea males have an odd number of chromosomes, 2n=41, while females are 2n=42 (Figure 28). The fusion of chromosome 9 and the Y and the diploid chromosome number of 41 was present in five Japan Sea male individuals studied (data not shown). The fusion of chromosome 9 and the Y in the Japan Sea threespine sticklebacks defines an X_1X_2Y

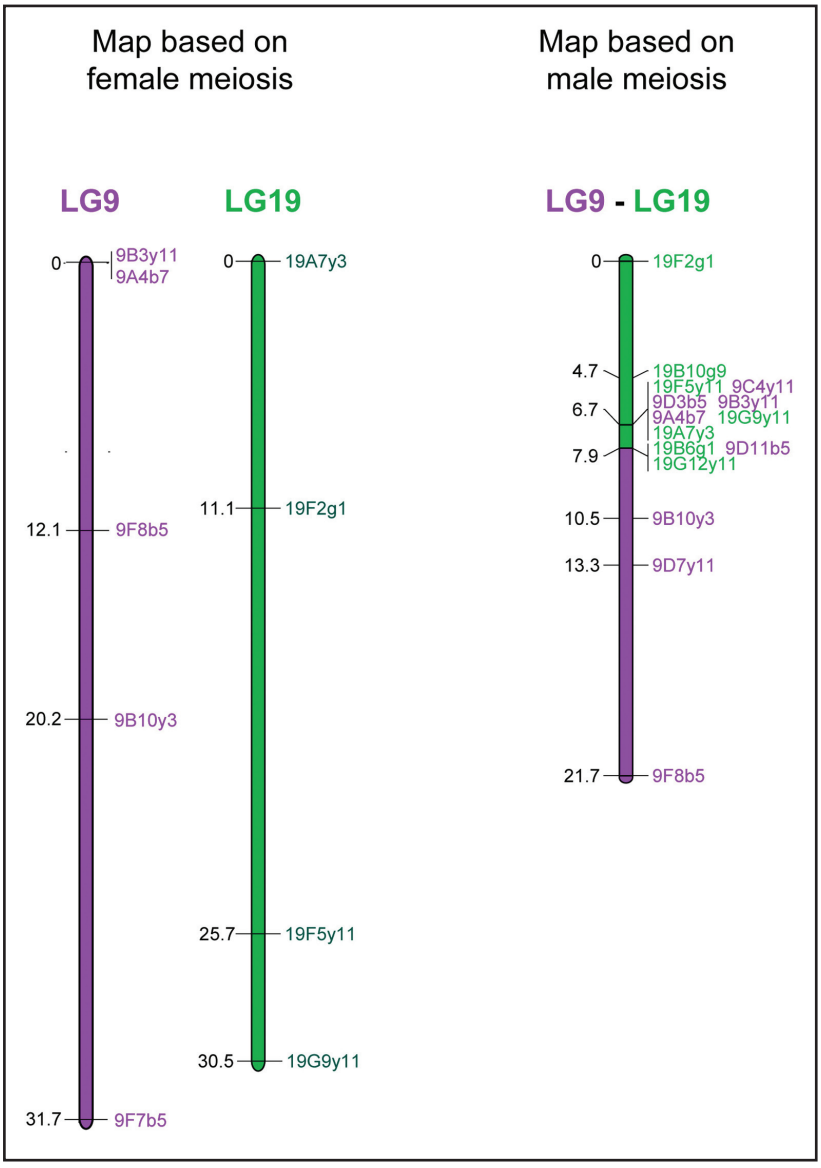


Figure 27. Genetic maps of Japan Sea LG9 and LG19.
Based on female meioses in a Japan Sea x Japan Sea cross, LG9 and LG19 are distinct. Fusion of the two linkage groups in males is evident by analyzing male meioses. Data provided by Jun Kitano.

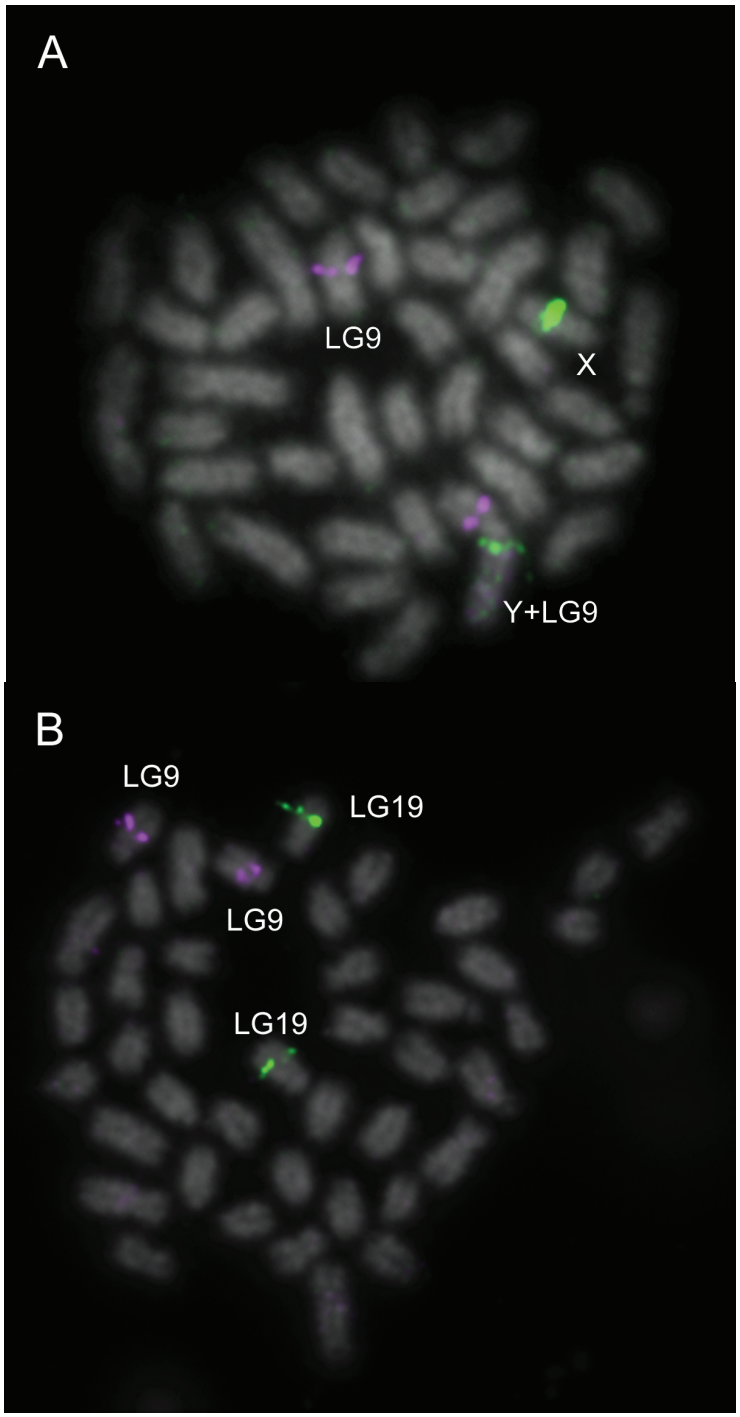


Figure 28. FISH demonstration of LG9-19 fusion in Japan Sea males.
(A) Hybridization of a LG19 probe, CH213-101E08 (green), and a LG9 probe, CH213-031B20 (purple), to a Japan Sea male metaphase spread reveals one distinct LG9 and one LG19 (X). Both probes hybridize to opposite ends of the longest chromosome in the karyogram, the fused Y (Y+LG9). (B) Hybridization of the LG19 and LG9 probes to a Japan Sea female metaphase spread. No fusion is evident: two chromosomes 9 and 19 are indicated by hybridization.

sex-chromosome system in sticklebacks independent of the fusion of the threespine Y to LG12 seen in black-spotted stickleback males.

Interestingly, in Figure 17, in which the *Idh* BAC probe (on LG19) was hybridized to a Japan Pacific Ocean threespine male metaphase spread, a faint *Idh* signal was seen on a third chromosome (arrowhead); this third signal has not been seen in any Japan Sea males or females, Japan Pacific Ocean females, or North American threespine males or females studied. Given the presence of LG12 and LG9 neo-Y chromosomes in stickleback species, I tested the hypothesis that a male-limited region on one of these two linkage groups is present the Japan Pacific Ocean population. Figure 29A shows a male Japan Pacific Ocean metaphase spread in which the two copies of chromosome 19 (the X and Y) are identified by *Idh* hybridization and the autosome exhibiting the third *Idh* signal corresponds to chromosome 9 (LG9 with white arrowhead); the other copy of chromosome 9 (LG9) shows no *Idh* hybridization. The same results were seen in four other Japan Pacific Ocean male individuals studied (data not shown). Figure 29B shows a female Japan Pacific Ocean metaphase spread hybridized with the same probes, in which no cross-hybridization of LG9 and LG19 probes is detected.

To rule out the possibility of introgression of part of the Japan Sea Y^{LG9} into the Pacific Ocean male individual studied, Kitano genotyped the male with a diagnostic panel of markers that can distinguish fish from these two populations and determined that at all loci tested, the individual has Pacific Ocean alleles. Thus, it appears that physical and perhaps genetic relationships exist between LG19 and LG9 exists in the Japan Pacific Ocean population; this relationship might be predictive of the LG9-Y fusion in Japan Sea males.

***SEX* is not linked to LG12 or LG19 in brook or fourspine sticklebacks**: James Urton, Jessica Boland, and Catherine Peichel next worked together to ask whether *SEX*-linked markers from LG12 or LG19 are associated with a single locus controlling male or female sexual development in brook or fourspine stickleback crosses and found no such

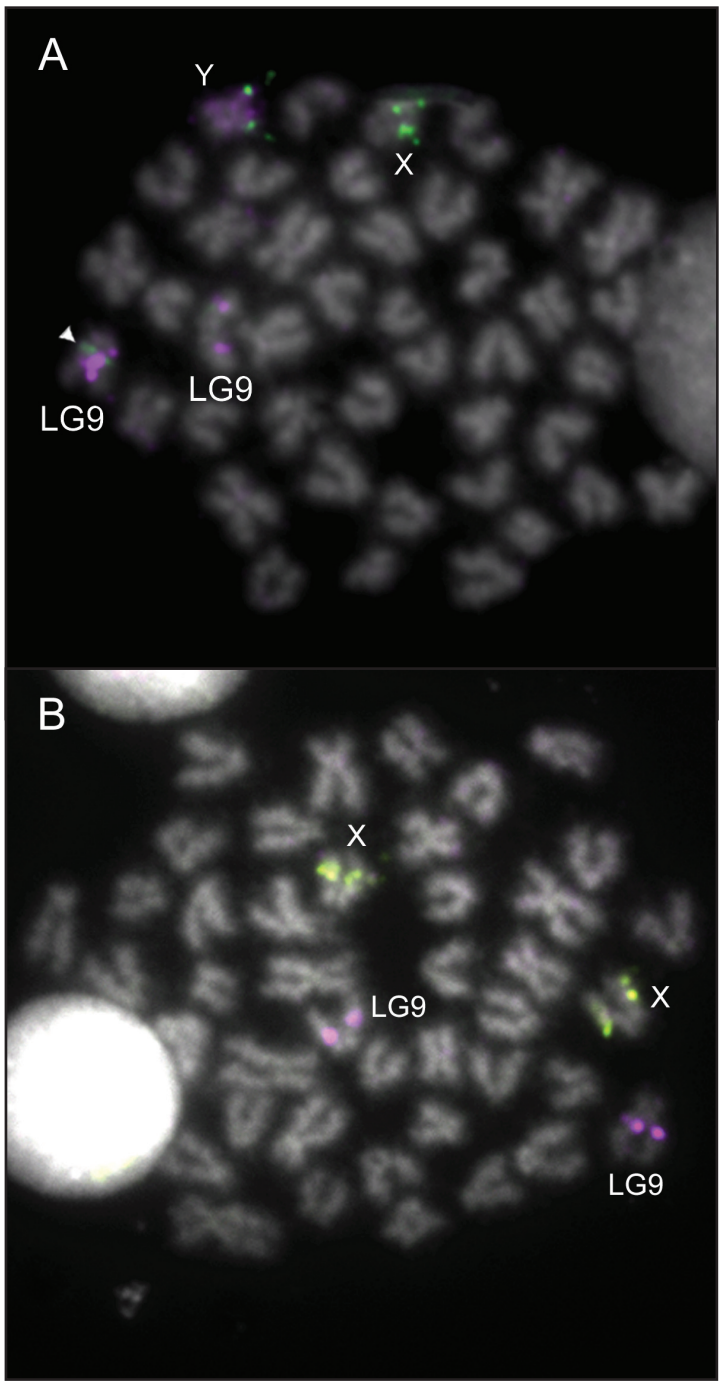


Figure 29. Relationship between LG9 and LG19 in Japan Pacific Ocean males.
(A) *Idh* probe (green) and LG9 probe (purple) hybridized to a Japan Pacific Ocean male metaphase spread. One X and one chromosome 9 are identified by *Idh* and LG9 FISH probe signals. The Y (LG19) has both *Idh* signal as well as diffuse LG9 signal; the other chromosome 9 exhibits an ectopic *Idh* (LG19) signal (white arrowhead). This relationship is not seen in Japan Pacific Ocean females, shown in (B), in which no cross-hybridization of probes is apparent.

associations (Table 5). Seven LG12 markers (4 from threespine sticklebacks and 3 from ninespine sticklebacks) were polymorphic in the fourspine stickleback cross, yet none were sex-linked (Table 5). Similarly, two LG19 markers, *Stn194* and *Pun117,* are linked to *SEX* in threespine but not in fourspine sticklebacks (Table 5). Although very few LG12 and LG19 markers were informative in the brook stickleback cross, neither the LG12 marker *Pun234* nor the four LG19 markers, *Stn186, Cyp19b, Pun168,* and *Pun268,* were linked to *SEX* in brook sticklebacks (Table 5).

To determine whether any of the genome-wide threespine or ninespine genetic markers are associated with a sex-determination locus in these species, Urton genotyped the brook stickleback cross and the fourspine stickleback cross with all available threespine and ninespine markers. There was no evidence for an association between a single marker genotype and sex phenotype in either species. However, many threespine and ninespine markers either failed to work in the other species or were not polymorphic (Table 8). Urton tested markers from 17 (of 21) threespine and 19 (of 30) ninespine linkage groups in the brook stickleback cross, and markers from 15 threespine and 15 ninespine linkage groups in the fourspine cross. Therefore, Urton also used an amplified fragment length polymorphism (AFLP) approach to identify *SEX*-linked sequence polymorphisms in brook and fourspine sticklebacks. Using 48 AFLP primer combinations, 86 polymorphic bands in fourspine sticklebacks and 35 polymorphic bands were identified in brook sticklebacks, but none of these polymorphisms were sex-linked in either species.

Fourspine sticklebacks have a ZW sex-chromosome system: Consistent with the genetic mapping data in both fourspine and brook sticklebacks, I found no evidence for heteromorphy of the chromosomes identified by hybridization with LG12 or LG19 probes in either sex of either species (Figure 30). Furthermore, there was no evidence for obvious heteromorphy of any chromosome pair in brook stickleback males or females (Figure 31). By contrast, a heteromorphic chromosome pair is apparent in the female

Table 8. Markers used for genotyping brook and fourspine stickleback crosses.
The number of microsatellite markers markers developed in threespine and ninespine sticklebacks are shown in column two. Then, for each species, the number of markers that were able to generate a PCR product, were polymorphic, and were sex-linked are listed. Data provided by James Urton, Jessica Boland and Catherine Peichel.

		C. inconstans			*A. quadracus*		
Markers	**n**	**PCR product**	**Polymorphic**	**Sex-linked**	**PCR product**	**Polymorphic**	**Sex-linked**
G. aculeatus	576	225 (39.1%)	66 (11.5%)	0 (0%)	135 (23.4%)	47 (8.2%)	0 (0%)
P. pungitius	162	86 (53.1%)	43 (26.5%)	0 (0%)	51 (31.5%)	26 (16.1%)	0 (0%)

fourspine karyogram (Figure 32), confirming the prior cytogenetic identification of a ZW pair in this species (Chen and Reisman 1970); the W chromosome is larger than the Z in fourspine females.

Discussion

Identification of sex-chromosome heteromorphies in sticklebacks: My cytogenetic survey of the family Gasterosteidae has uncovered a diversity of sex chromosome systems within the stickleback family not identified by others (Chen and Reisman 1970). Although my studies confirm the previous report of a ZW sex chromosome in fourspine sticklebacks and lack of heteromorphic sex chromosomes in brook sticklebacks (Chen and Reisman 1970), my recent study of the threespine Y chromosome (Ross and Peichel in press) and the results of this chapter provide evidence of additional heteromorphic sex chromosome systems in sticklebacks. For example, initial cytogenetic surveys of stickleback fish did not identify a heteromorphic XY pair in ninespine sticklebacks (Chen and

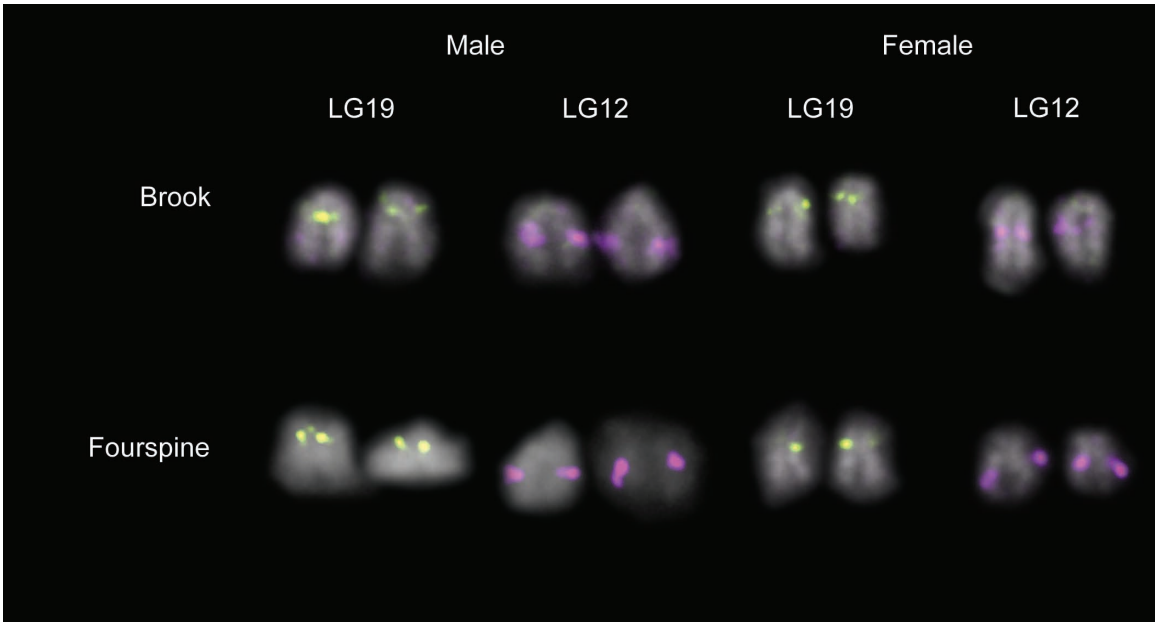


Figure 30. FISH analyses of LG12 and LG19 in brook and fourspine sticklebacks.
The LG19 probe (CH213-180J08) is green; the LG12 probe (CH213-140B10) is purple. For each sex of each species, only chromosomes hybridized by probe in a single metaphase spread are shown. LG12 and LG19 appear homomorphic in both sexes of both species.

Reisman 1970; Klinkhardt and Buuk 1990); however, genetic mapping has shown that ninespine males have an XY pair corresponding to LG12 (Shapiro *et al.* in preparation). Here, I used FISH to demonstrate that chromosome 12 is a heteromorphic pair in ninespine males (Figure 24).

While my black-spotted male karyogram supports the prior report of a heteromorphic XY pair, the same study also reported a male diploid chromosome number of 42 (Chen and Reisman 1970). My karyogram shows that male black-spotted sticklebacks have a 2n=41 karyotype, while females are 2n=42. It is unlikely that presence of an

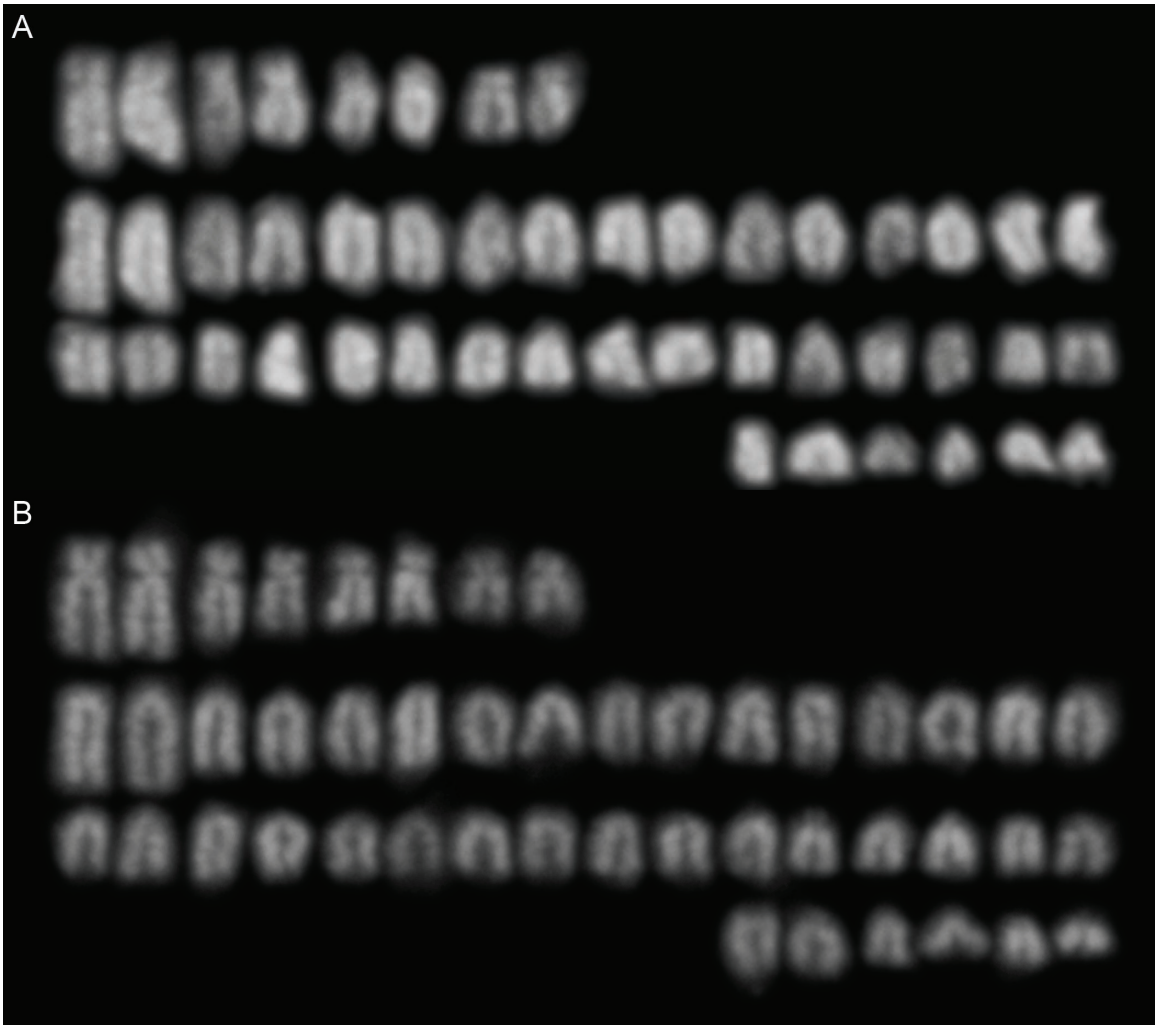


Figure 31. Brook stickleback karyograms.
(A) Male and (B) female.

odd diploid chromosome number in males is due to experimental artifact, as this result was obtained in multiple metaphase spreads from multiple individuals from two natural populations, and the genetic mapping data supporting the relationship between LG12 and LG19 was obtained from a third black-spotted population. I also use primary tissue from wild-caught fish for isolating metaphase nuclei, so chromosomal rearrangement arising spontaneously and fixed by inbreeding or passage in tissue culture can be dismissed.

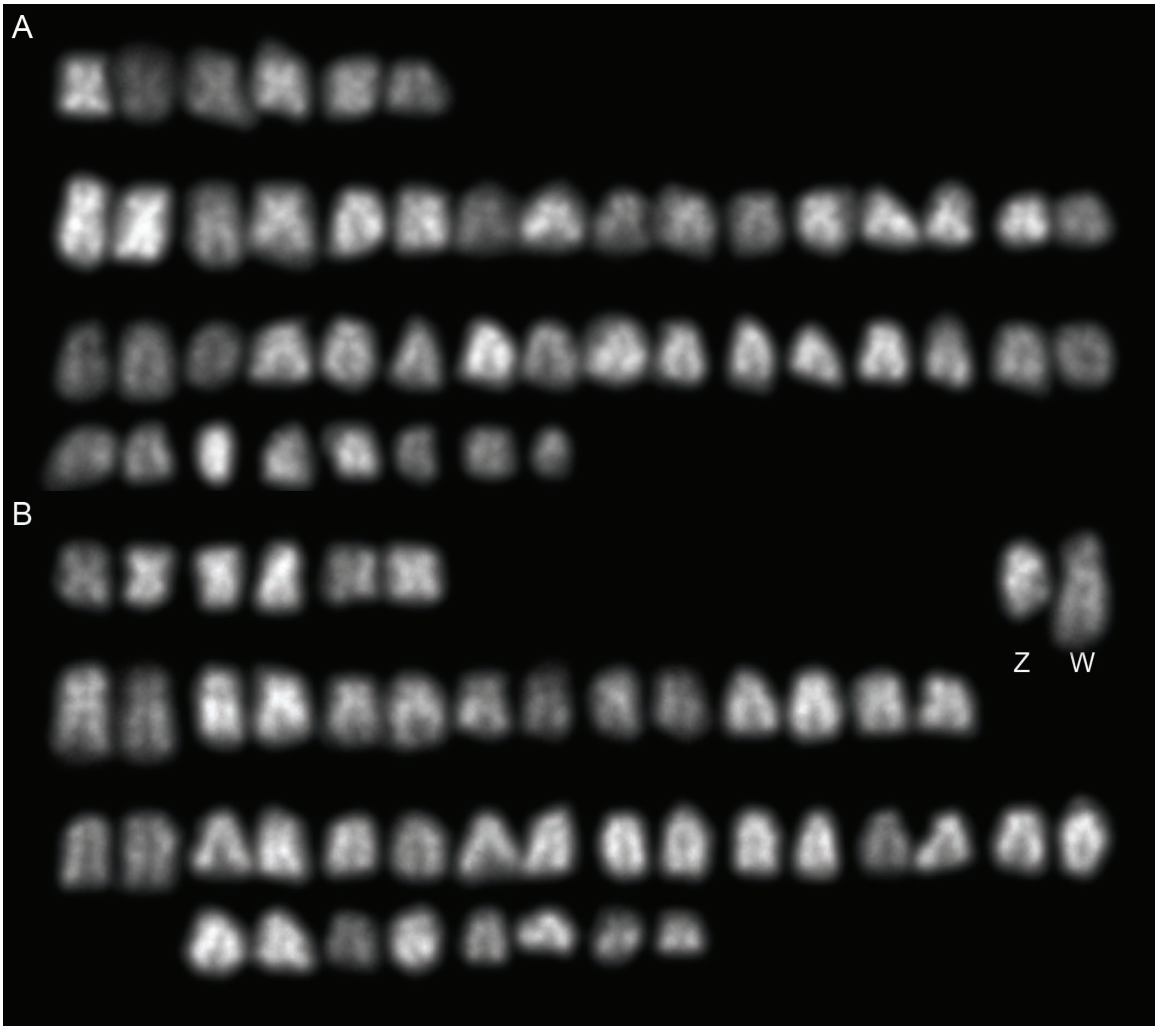


Figure 32. Fourspine stickleback karyograms.
(A) Male and (B) female, with the W and presumed Z chromosomes labeled. In the female, the single largest chromosome, not seen in males, is a W chromosome.

Another potential explanation for the discrepancies between my black-spotted and ninespine male karyotypes and previous karyotypes is that we studied different populations. In the prior study, both of these species had been collected from Reid State Park, Maine, U.S.A (Chen and Reisman 1970). My black-spotted stickleback specimens were also collected from coastal locations in New England, including one site in Maine (Materials and Methods), so it is unlikely that population differences in karyotype explain the differences reported here, although the possibility of intra-species polymorphism cannot yet be ruled out. Finally, my molecular cytogenetic studies of the threespine Y chromosome (Ross and Peichel in press) also revealed heteromorphy that was not previously identified (Chen and Reisman 1970; Klinkhardt and Buuk 1990; Cuñado *et al.* 2002), suggesting that my molecular cytogenetic techniques have provided higher resolution analyses of the karyotypes of these species. It is not uncommon for disagreement of chromosome number counts to persist in the literature. After all, over fifty years passed following the publication of the first of many incorrect determinations of the human diploid chromosome number before Tjio and Levan convincingly established in 1956 that humans have 2n=46 chromosomes (Gartler 2006).

Evolutionary relationships of the stickleback fishes: Knowledge of the evolutionary relationships between the stickleback species are key to the interpretation of the evolution of the different sex chromosome systems that I have identified. Although a number of Gasterosteidae phylogenies have been created (Figure 3), they differ in topology, depending upon which morphological, behavioral and molecular characters are used to create the phylogeny (reviewed in (Mattern 2007)). Most phylogenies agree that threespine and black-spotted sticklebacks are sister taxa and that ninespine and brook sticklebacks are sister taxa (Figure 3). However, the relationship of fourspine sticklebacks relative to these other taxa has not been resolved (Figure 3). Although the Gasterosteus and Pungitius genera both have XY sex determination, they do not form a monophyletic

group, suggesting that XY sex determination might have arisen independently in these lineages.

Chromosome number also appears to have rapidly evolved within this family. Both brook and fourspine sticklebacks have 2n=46 chromosomes (Figure 3); I also found that an outgroup species for the Gasterosteidae, the tubesnout *Aulorhynchus flavidus* (Kawahara *et al.* 2008), also has 2n=46 chromosomes (data not shown). This suggests that a diploid number of 46 chromosomes might be ancestral for the Gasterosteidae and that 2n=42 might have evolved once in Gasterosteus and once in Pungitius.

X_1X_2Y sex-chromosome systems in sticklebacks*:* Genetic and cytogenetic evidence support the conclusion that a fusion between LG12 and LG19 in black-spotted males formed a single Y chromosome. The fusion of two chromosomes, creating a single Y, defines the sex determination mechanism in black-spotted sticklebacks as of the X_1X_2Y type, where one copy each of chromosome 12 and chromosome 19 segregate opposite a Y chromosome comprising the other copy of both chromosomes. This chromosomal rearrangement explains the odd diploid chromosome number of 41 in black-spotted males. Both stickleback phylogenies (Figure 3) suggest that the X_1X_2Y system arose after threespine and black-spotted sticklebacks diverged about 10 MYA (Bell 1994; Mattern 2004; Orti Personal communication).

I have confirmed the presence of a second X_1X_2Y sex-chromosome system within the threespine stickleback species using FISH: males of the Japan Sea population have a fusion between one copy of chromosome 9 and the Y chromosome and 2n=41 chromosomes. The X_1X_2Y system in the Japan Sea is likely to be a derived character in this population as well. Phylogenetic clustering of allele sequences from the Japan Sea Y^{LG19} with the Pacific Ocean Y and from the Japan Sea X_1 with the Pacific Ocean X suggests that the threespine Y^{LG19} predates the divergence of the Pacific Ocean and Japan Sea populations (Peichel *et al.* 2004). Furthermore, neither Japanese Pacific Ocean threespine sticklebacks

(Figure 29) nor North American Pacific Ocean populations (Figure 16) exhibit a Y-LG9 fusion. Given the involvement of the Pacific Ocean Y chromosome in sex determination in Japan Sea threespine sticklebacks, it is likely that they both use the same *SEX* locus.

X_1X_2Y systems have been identified in many species of insects, fish, and mammals, and three mechanisms for the creation of X_1X_2Y systems have been described (White 1973). First, fission of the ancestral X into two chromosomes can create an X_1X_2Y system. However, no evidence for linkage of LG12 and LG19 or LG9 and LG19 exists in any of the stickleback species analyzed aside from black-spotted and Japan Sea males (Figures 24 and 30), suggesting that a fusion of these chromosomes in black-spotted males and Japan Sea males, respectively, is not likely to represent ancestral states. Furthermore, if the black-spotted or Japan Sea X_1X_2Y male karyotypes were created by fission of the X, I would have expected a diploid chromosome number of 2n=43 in males, rather than the observed 2n=41 (Figure 23A and Figure 28A). Second, for species with XX/XO GSD, a fusion between a metacentric X and a metacentric autosome may create an X_1X_2Y system, but the presence of XX/XY GSD in the Pacific Ocean population of threespine stickleback, the same species as the Japan Sea population and sister species to black-spotted sticklebacks (Figure 3), instead suggests that XX/XY GSD was the ancestral sex determination mechanism of black-spotted and Japan Sea sticklebacks.

Instead, I favor the third proposed mechanism of X_1X_2Y system formation as one that occurred in an ancestor of black-spotted and Japan Sea males: fusion of a Y chromosome with an autosome, causing the attached copy of the autosome to segregate with the Y and the unfused copy of the autosome to segregate like an X chromosome. The strongest support for the fusion argument is that black-spotted and Japan Sea males have one less chromosome than both black-spotted and Japan Sea females and than males and females in the sister taxon, Pacific Ocean threespine sticklebacks. This is consistent with other studies in which fish species with X_1X_2Y systems often have one less chromosome

than sister taxa (Uyeno and Miller 1971; de Almeida-Toledo *et al.* 2000), suggesting that derived chromosomal fusions may be the predominant source of X_1X_2Y sex chromosome systems in fishes.

Based on the hypothesis that a fusion between an existing Y chromosome and an autosome is the likely cause of the black-spotted and Japan Sea X_1X_2Y sex chromosome systems, I propose that the black-spotted Y chromosome is derived from a fusion between the Y_{LG19} chromosome and the LG12 autosome found in threespine sticklebacks, rather than a fusion between the Y_{LG12} chromosome and LG19 autosome found in ninespine sticklebacks. All available phylogenies support a closer relationship between black-spotted and threespine sticklebacks than between black-spotted and ninespine sticklebacks (Figure 3).

My cytogenetic and FISH data also support a closer relationship between the threespine and black-spotted karyotypes than between the black-spotted and ninespine karyotypes. First, LG12 is acrocentric in both threespine and black-spotted females, but metacentric in ninespine females (Figure 23 and Figure 25). Second, the LG12 FISH probe hybridizes to an internal position on LG12 in both threespine and black-spotted sticklebacks, and a terminal position on LG12 in ninespine sticklebacks, suggesting that rearrangement(s) of LG12 have occurred between these genera (Figure 24). Third, LG19 is a submetacentric X chromosome in threespine and black-spotted females, but metacentric in ninespine sticklebacks (Figure 24).

The male FISH data also provide evidence for a fusion between an ancestral Y_{LG19} chromosome and the acrocentric LG12 autosome, with a loss of the q arm of Y_{LG19} in black-spotted males. The two threespine LG19 FISH probes that did not hybridize to the black-spotted Y (Figure 26) are located on the q arm of the threespine Y (Ross and Peichel in press), while the LG19 probe that did hybridize to the black-spotted Y (Figure 24) is on the p arm of the threespine Y (Ross and Peichel in press). Furthermore, all four

LG19 markers from the threespine Y chromosome q arm were Y null in black-spotted sticklebacks, supporting loss of that arm, although the fifth LG19 marker, from the p arm of the threespine Y, was also null. However, in threespine sticklebacks, there are also Y-null alleles that result from sequence divergence between the X and the Y and not from deletion (Ross and Peichel in press). In further support of the hypothesis that the Y_{LG19} is ancestral to the black-spotted Y chromosome, none of the LG12 markers had null alleles, suggesting that extensive degeneration has not yet occurred on the LG12 derived region of the black-spotted Y.

To more precisely map the rearrangements that have occurred between the two X chromosomes and the Y chromosome in black-spotted sticklebacks, a more extensive cytogenetic analysis should be performed, as I accomplished for the threespine X and Y (Ross and Peichel in press). However, current data suggest that additional deletions of Y chromosome material have occurred on the black-spotted Y relative to the threespine Y. Both *Wt1a* and *Idh* are deleted from the black-spotted Y chromosome (Figure 26) but present on the threespine Y chromosome (Ross and Peichel in press). In particular, the deletion of the region around the *Idh* FISH probe (Figure 26B) explains why a male-specific allele of this locus was not identified in a previous study (Peichel *et al.* 2004), leading to the erroneous conclusion that the Y-chromosomes of these two species were unrelated.

Taken together, my data suggest that an unbalanced Robertsonian translocation between one copy of chromosomes 12 and 19, causing loss of the q arm of chromosome 19, formed the black-spotted $Y_{LG12\text{-}LG19}$. Robertsonian translocations occur by the fusion of two chromosomes at their centromeres, thereby circumventing issues associated with a single fused chromosome having two centromeres during cell division. Because Robertsonian translocations are common in fish and mammals (White 1973), they may play a regular role in the formation of X_1X_2Y sex chromosome systems. In particular, balanced Robertsonian translocations occur between two acrocentric chromosomes and result in

loss only of the satellite sequences comprising the short arms of the chromosomes and pericentromeric alpha satellite sequence. Thus, a balanced Robertsonian translocation between a Y chromosome and an autosome might not incur fitness costs; such centric fusions are thought to be particularly favored when they bring genes with sexually antagonistic fitness effects into linkage with a sex determination locus (Charlesworth and Charlesworth 1980). However, my existing data suggest that the black-spotted Y chromosome may have resulted from an unbalanced Robertsonian translocation. The loss of the q arm of the Y chromosome could have been tolerated without fitness effects if dosage compensation had evolved in sticklebacks. Although it is not currently known whether sticklebacks have a mechanism for dosage compensation, this possibility should be examined in the future.

The evolutionary history of XY GSD in sticklebacks: The mapping of XY sex determination to LG19 in threespine sticklebacks (Peichel *et al.* 2004) and to LG12 in ninespine sticklebacks (Shapiro *et al.* in preparation) originally suggested that these two genetic sex determination systems might have arisen independently. However, the genetic and cytogenetic evidence for linkage of both LG12 and LG19 to the black-spotted Y chromosome suggested that there might be a relationship between these XY sex chromosome systems. Because these three species are not monophyletic, it is difficult to assess whether the Gasterosteus and Pungitius XY sex determination systems are related or have arisen independently.

One possibility is that threespine, black-spotted, and ninespine sticklebacks all share a common sex-determining locus, but that *SEX* has transposed between LG12 and LG19 in the two lineages; a similar transposition of the *SEX* locus to four different chromosomes has been seen in salmonids (Woram *et al.* 2003). It is also possible that, despite the interrelationship between the sex chromosomes of threespine, black-spotted, and ninespine sticklebacks, sex determination arose independently in ninespine sticklebacks.

Although I have argued that the black-spotted Y was derived from the threespine Y, it is also possible that sex determination may have arisen independently in this species, or is derived from the ninespine sex determination system.

As in sticklebacks, the *SEX* locus in salmonids has also been reported to move between linkage groups. While a set of markers near *SEX* is conserved, *SEX* also appears linked to different autosomes in different species, and the same possibilities for the evolutionary history of *SEX* were raised: that Robertsonian fusions of the Y chromosome, translocations containing *SEX*, or independent evolution of *SEX* could explain the findings (Woram *et al.* 2003). In the fish of the genus Oryzias, independent evolution of *SEX* likely explains how both XY and ZW species have *SEX* loci on different linkage groups (Takehana *et al.* 2007b). As the sex-determining gene *DMY* in *O. latipes* was found in some, but not other, closely-related species (Kondo *et al.* 2003; Matsuda *et al.* 2003; Veith *et al.* 2003; Volff *et al.* 2003; Kondo *et al.* 2004; Matsuda 2005). Thus, translocation was ruled out as a cause for the appearance of *SEX* on different linkage groups. XY Oryzias species lacking *DMY* must use different sex determination triggers.

In order to distinguish the degree of conservation of *SEX* in threespine, black-spotted and ninespine sticklebacks, the identity of *SEX* in these three species must be determined. If all three species share a common sex-determining factor, then transposition of *SEX* between linkage groups has likely occurred; the identification of different sex-determining factors in the three species would provide evidence of the independent evolution of XY sex determination in sticklebacks.

Forces causing an autosome to become a sex chromosome: In these scenarios, LG12 appears to have been selected for *SEX* linkage at least two independent times: by fusion to the Y_{LG19} chromosome in black-spotted sticklebacks, and either by transposition of an existing *SEX* locus or independent evolution of a new *SEX* locus in ninespine sticklebacks. In all of these cases, sexually antagonistic selection is believed to be the underly-

ing evolutionary force (Charlesworth and Charlesworth 1980; van Doorn and Kirkpatrick 2007), suggesting that LG12 in sticklebacks might have an abundance of genes with differential fitness effects in males and females and thus be predisposed to becoming a sex chromosome.

Independent evolution of the same autosome into a sex chromosome has occurred in the Japanese frog *R. rugosa*, in which chromosome 7 has become XY pair and a ZW pair in independent populations (Ogata *et al.* 2008). Three genes involved in sex differentiation in vertebrates (*AR, SF1* and *SOX3*) map to these XY and ZW pairs (Uno *et al.* 2008), suggesting the possibility that such genes might have driven the selection of chromosome 7 as a sex chromosome. Comparative genomic analysis of the autosomal and sex-linked forms of LG12 in the different stickleback species may yield insight into the types of genes present on these chromosomes that might have sexually antagonistic alleles and thus play an important role in the evolution of sex chromosomes in sticklebacks.

Similar evolutionary forces might explain the Japan Sea Y-LG9 fusion that Jun Kitano first identified. He has since found that a locus controlling hybrid male sterility maps to the ancestral X chromosome (LG19) and that dorsal pricking, a trait involved in the Japan Sea male mating display, maps to the neo-X chromosome (LG9). Thus, loci controlling the Japan Sea-specific mating behaviors that contribute to reproductive isolation of the Japan Sea and Pacific Ocean sticklebacks are found on the neo-X chromosome, supporting a prediction that many speciation genes will be linked to X chromosomes (Lemmon and Kirkpatrick 2006).

The identification of a sexually-dimorphic gene on a neo-X chromosome supports an existing theoretical model for the evolution of fusions between sex chromosomes and autosomes (Charlesworth and Charlesworth 1980). The mapping of traits that contribute to reproductive isolation to the old and new X chromosomes in these populations also agrees with findings in other species, particularly in Drosophila, that X chromosomes

tend to be enriched for speciation genes, the so-called "large X effect" (Coyne and Orr 2004). In backcross hybrids in Drosophila, the X chromosome often contains genes involved in hybrid sterility or inviability (reviewed in (Presgraves 2008)); up to four times more hybrid male sterility genes can be found on the Drosophila X relative to a comparably-sized autosome (Masly and Presgraves 2007).

Relationship between LG9 and LG19 in Japan Pacific Ocean threespine sticklebacks: Presence of an *Idh* signal on three chromosomes in a male (Figure 29A) but on two chromosomes in a female (Figure 29B) suggests that the third signal, on an autosome, is male-limited like a Y chromosome. I used FISH to show that this autosome is LG9 (Figure 29A). The cross-hybridization of a LG19 probe to one copy of chromosome 9, and of a LG9 probe to the Y chromosome, was seen in multiple males. Thus, it appears that one copy of LG9 is inherited in a male-limited fashion in the Japan Pacific Ocean population.

The physical basis for the hybridization of *Idh* to LG9 is not known. It is possible that the *Idh* locus has duplicated onto one copy of chromosome 9; it is also possible, though unlikely, that repetitive elements present on the *Idh* probe (which contains the X chromosome allele of *Idh*) mediate cross-hybridization to a mobile element-rich male-specific region of LG9. Future efforts will employ additional LG9 FISH probes to determine whether the same FISH results are obtained using probes from multiple loci.

Ultimately, the segregation of a male-limited region of one chromosome in a population in physical proximity to one in which the same chromosome fused to the Y supports the prediction that certain autosomes are more likely to evolve into sex chromosomes because of preexisting features such as a local abundance of mobile elements or presence of SA genes. Indeed, Kitano has mapped candidate SA loci on LG9; obtaining sequence of LG9 from Japan Sea and Pacific Ocean males will be useful in understanding the molecular basis of the LG9 male-limited region, whether it contains a duplication of

LG19 sequence, perhaps containing a SA gene, or an abundance of mobile sequence elements. The production of genetic maps of LG9 and LG19 in a Japan Pacific Ocean cross might also provide genetic evidence of linkage of part of LG9 with the Y chromosome.

Evolution of XY and ZW systems: Both cytogenetic and genetic data suggest that the ZW system of fourspine sticklebacks is not related to the XY systems of threespine, black-spotted, or ninespine sticklebacks, raising the possibility that the ZW system arose independently. However, the stickleback phylogenies do not agree on the position of the fourspine stickleback (Figure 3), so an accurate parsimony-based reconstruction of the evolution of XY and ZW GSD in sticklebacks is not currently possible. It will be informative to karyotype the European fifteenspine stickleback, (*Spinachia spinachia*), a close relative of the fourspine stickleback, to determine whether it has 46 chromosomes and a heteromorphic ZW pair. Furthermore, we are using unbiased methods to identify *SEX*-linked sequences in fourspine sticklebacks and using FISH with threespine BAC probes to identify the linkage group comprising the ZW pair in fourspine sticklebacks. These studies will allow the determination of which autosome(s) gave rise to the sex chromosome in fourspine sticklebacks.

Efforts will also focus on identifying the sex determination mechanism of brook sticklebacks. Although sex-linked markers have not been identified in either brook or fourspine sticklebacks, these studies have been limited by the availability of polymorphic markers. It is still possible that there is a simple genetic sex determination mechanism in brook sticklebacks, although it is also possible that brook sticklebacks use environmental sex determination or complex genetic sex determination. Identifying the sex determination mechanism in brook sticklebacks may shed light on the transition between XY and ZW systems in this family of fish.

Transitions between XY and ZW GSD may occur via indirect or direct mechanisms. For example, an interim period of ESD may facilitate an indirect transition be-

tween two forms of GSD (Ezaz *et al.* 2006). There may also be a more direct transition between XY and ZW forms, as exemplified by recent work in several vertebrate taxa. In tilapiine cichlid fishes, two species (*O. aureus* and *O. mossambicus*) have complex genetic sex determination in which LG1 and LG3 are both associated with sex determination loci (Lee *et al.* 2004; Cnaani *et al.* 2008). The phylogenetic positions of these species provide a direct link between two related species in which LG1 is associated with a simple XX/XY system, and two other species in which LG3 is associated with a simple ZZ/ZW system (Cnaani *et al.* 2008). A similar link exists in the platyfish, where some populations have W, X, Y and Z chromosomes, while closely-related species have either XY or ZW GSD (Volff and Schartl 2001).

In the Japanese frog (*R. rugosa*), there is evidence that an existing XY sex chromosome became a ZW sex chromosome in a derived population (Ogata *et al.* 2008). Comparative mapping of the platypus sex chromosome chain suggests that the monotreme XY GSD system is related to the ZW system in birds, while the therian XY system is now hypothesized to have evolved independently (Grutzner *et al.* 2004b; Ezaz *et al.* 2006; Wallis *et al.* 2007; Veyrunes *et al.* 2008). Additional genetic and genomic analyses of all the stickleback species may elucidate whether the ZW and XY systems directly interconverted or were independently derived in sticklebacks.

At least two models for how XY and ZW systems interconvert have been suggested. Transition between these two forms of simple GSD may be brought about by selection for a stable sex ratio in a species experiencing a sex ratio bias (Ogata *et al.* 2003). In such a case, it has been suggested that a male bias selects for establishment of a ZW system and a female bias for an XY system. To explain the transition from an XY to a ZW system in *R. rugosa*, it was suggested that a male sex-ratio bias drove the recruitment of a dominant female-determining gene on the X, establishing a ZW system (Ogata *et al.* 2008). This model explains how the same chromosome pair evolved to be XY and ZW in

different populations, although theoretical work suggests that transition of an X to a W is no more beneficial than when an autosome becomes a W (Vuilleumier *et al.* 2007). XY-ZW transition may also occur if a new *SEX* locus arises in linkage with a gene of adaptive value that can drive *SEX* to fixation by hitchhiking (Bull and Charnov 1977). However, a recent study has concluded that drift and sex ratio selection alone could cause fixation of a new *SEX* locus (Vuilleumier *et al.* 2007).

In a second model, an autosome pair (A, A) fuses to the Z chromosomes of a ZZ male (AZ, AZ) and then a male-determining locus evolves on one of the autosomes, making it a Y (AZ, YZ) (Ezaz *et al.* 2006). This chromosomal constitution is the equivalent of an XY system. As seen in *R. rugosa* and *X. maculatus*, it is likely that intra-species polymorphism in sex determination loci or in sex chromosomes will exist prior to a transition between forms of GSD; it has also been suggested that polymorphism for a chromosomal rearrangement may precede chromosomal speciation (McAllister *et al.* 2008). Because earlier cytogenetic studies had failed to identify some of the heteromorphic sex chromosome systems I have found in this study, it is possible that sex chromosome polymorphisms exist within the stickleback species and have facilitated the transitions inferred here. My identification of male black-spotted sticklebacks with 2n=41, X_1X_2Y chromosomes, when 2n=42, XY had been reported previously in a different population, could indicate that the sex chromosome system in black-spotted sticklebacks may be polymorphic; surveys of additional populations should be accomplished to address this possibility.

Dynamics of sex-chromosome heteromorphy: Although the threespine Y chromosome has experienced inversions and deletion (Ross and Peichel in press), the Y is of similar size to the X at metaphase. Contrary to the common notion, based primarily on studies of the broadly-conserved, and therefore old, mammalian Y and snake W chromosomes, that sex chromosomes are smaller than their homologs (Ming and Moore 2007), here I report that the threespine Japan Sea, black-spotted, and ninespine Y chromosomes

are larger than the X chromosomes and that the fourspine W is larger than the Z. The presence of Y or W chromosomes larger than their homologs is not uncommon in fish or plants. In *O. latipes*, the size of the Y chromosome was increased by addition of a duplicated sequence block that arose from an autosome (Kondo *et al.* 2006); such additions of sequence to the mammalian Y chromosome are also thought to have taken place repeatedly during its evolution (Graves 1995). The W is larger than the Z in *O. hubbsi* (Takehana *et al.* 2007b); in the plant *S. latifolia*, the Y chromosome has increased in size almost 1.5 fold since its inception about 10 MYA (Nicolas *et al.* 2005).

What forces might explain the trend that young sex chromosomes are larger than their homologs while older sex chromosomes tend to be smaller and more degenerate? Certainly, the addition of mobile sequence elements and transposed blocks of sequence to the nonrecombining region of a sex chromosome would increase its size, but at some point addition must give way to attrition to yield what is thought of as a typical degenerated sex chromosome like the mammalian Y. The first stages of sex chromosome evolution may involve an addition of mobile elements until a critical mass of such elements is reached, at which point they mediate intrachromosomal rearrangements leading to deletions, causing a reduction in sex chromosome size (Graves 1995). As these deletion events could occur with stochastic timing, the *caveat* is that the degree of heteromorphy of any sex chromosome, regardless of whether it is larger or smaller than its homolog, need not be linearly correlated with its chronological age.

The Y chromosomes in male black-spotted and Japan Sea threespine sticklebacks are clearly larger than either X due to the fusion of the ancestral Y with an autosome. Although I argue that one arm of the ancestral Y^{LG19} was lost in black-spotted males, the Y is still the single largest chromosome in the karyogram, suggesting that it may have experienced an increase in size as well.

Another mechanism of increase in size of a chromosome relative to its homolog could be meiotic drive. I have argued that the Y chromosomes in black-spotted and Japan Sea males fused to autosomes by Robertsonian translocations, which occur at the centromeres of the two participating chromosomes. Centromeres are selfish genetic elements (Henikoff and Malik 2002; Malik and Henikoff 2002) composed of repetitive DNA and may gain a transmission advantage by evolving to capture more microtubules at meiosis by increasing in size (Malik and Bayes 2006). By joining two chromosomes at their centromeres, Robertsonian translocations provide a way to create a larger centromere.

Although meiotic drive may also cause an expansion of centromere repeats, thus increasing the size of a chromosome relative to its homolog, sex chromosomes are not good candidates for centromere drive because the preferential transmission of a sex chromosome would lead to a skewing of the sex ratio, which then leads to selection for autosomal loci that suppress the drive (Burt and Trivers 2006). Although the effects of drive have not yet been sought in black-spotted sticklebacks, Jun Kitano found no evidence for transmission bias of the fused Y chromosome in the threespine Japan Sea population, suggesting either that centromere drive did not cause the fusion of LG19 and LG9 or that a suppressor of drive has already evolved.

Because the ninespine Y is larger than the X, it may still be in a phase of addition prior to sex chromosome attrition (Graves 1995) and thus evolutionarily younger than the threespine Y. It is also possible that the degree of physical degeneration of the threespine and ninespine Y chromosomes differs because they have experienced different repertoires of rearrangements. If *SEX* transposed from LG19 to LG12 in the *Pungitius* lineage, then the addition of a duplicated sequence block to the ninespine Y^{LG12} might explain the increase in size relative to the X. It would be useful to perform a physical characterization of the ninespine XY pair to identify whether any sex chromosome rearrangements are

shared in common with the threespine Y and to seek the presence of LG19 material on the ninespine Y chromosome that might suggest translocation of *SEX*.

Another possibility to consider is whether any sex chromosome in sticklebacks is a B chromosome, as has been suggested to be the origin of the Y chromosome in Drosophila (Hackstein *et al.* 1996; Carvalho 2002). Selfish B chromosomes are thought to have evolved from supernumerary "dot" chromosomes and persist by their ability to pair with other unpaired chromosomes, particularly the lone X in XO individuals, which may dispose B chromosomes to appear to be Y chromosomes. In such a case, although not being involved in sex determination, B chromosomes can explain the presence of a heteromorphic chromosome pair and could reasonably be larger than the X, as was found in the fish *Alburnus alburnus*, in which a B chromosome was larger than all of the "A" chromosomes (any of the required complement of chromosomes)(Ziegler *et al.* 2003).

The stickleback Y chromosomes are unlikely to be derived from B chromosomes because of the presence of X and Y alleles of genetic markers spanning the X-Y pairs in these species and because FISH probes hybridize to both the X and Y in threespine, black-spotted, and ninespine sticklebacks, demonstrating homology of the X and Y chromosomes, something not expected of an X and a B chromosome. Additionally, no XO GSD system has been described in sticklebacks. However, because no genetic, cytogenetic, or sequence data bear on the homology of the Z and W in fourspine sticklebacks, the possibility of a B chromosome in this species cannot be ruled out at present.

Conclusion: Fish are useful organisms in which to study the evolution of sex determination and sex chromosomes because of the lability of these features, and several fish species have been identified as having X_1X_2Y sex chromosome systems (Uyeno and Miller 1971; de Almeida Toledo *et al.* 1984; Saitoh 1989; Almeida-Toledo *et al.* 2000; de Almeida-Toledo *et al.* 2000; de Almeida Toledo and Foresti 2001; Bertollo *et al.* 2004; Centofante *et al.* 2006). While a benefit of fusion of sex chromosomes to autosomes has

been shown (Charlesworth and Charlesworth 1980), this is the first report to my knowledge of the evolution of an X_1X_2Y system in which the Y comprises two distinct chromosomes that are Y-chromosomes in closely-related species.

Although X_1X_2Y sex chromosome systems are relatively easy to identify because of the obviously larger fused Y chromosome, these systems are not thought to be present in as many species as XY or ZW systems, which could mean that they exist predominantly as transitionary states (Charlesworth and Charlesworth 2005). For example, following the fusion of a Y to an autosome, the X_1 and X_2 chromosomes might fuse to restore diploidy; at the same time, a more complicated sex chromosome system, such as the $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ sex chromosome chain found in the platypus, might subsequently evolve (Rens *et al.* 2004). The discovery of an X_1X_2Y system in black-spotted sticklebacks, sister species to threespine sticklebacks, for which many molecular, genetic and genomic tools have been developed (Peichel *et al.* 2001; Kingsley *et al.* 2004; Kingsley and Peichel 2007), will facilitate further characterization of the mechanisms and evolutionary forces underlying the transition between simple genetic XY sex determination and the X_1X_2Y type. In particular, the identification of two GSD systems in distinct populations of threespine stickleback, XY in the Pacific Ocean and an X_1X_2Y system in the Japan Sea, provides a connection between sex chromosomes and speciation genes and will permit the genetic dissection of the process of incipient speciation in a vertebrate system.

Chapter 6. Summary and Future Directions

As a result of my discoveries, the potential of stickleback fishes as model systems for studying the evolution of GSD and sex chromosomes has been strengthened. I developed the technique of fluorescence *in situ* hybridization (FISH) for sticklebacks as well as methodologies for cloning a sex chromosome, including the FISH identification of BAC clones being from the X or Y. Through these efforts, I have produced minimum tiling paths of Y BAC clones to be sequenced and also helped verify the identity of additional Y clones to sequence in an effort to seek the identity of the *SEX* locus in threespine sticklebacks. The sequence of the male-specific region of the Y chromosome will also be useful in expanding the sequence characterization of the X and Y I have already performed, in which I showed that the Y chromosome has expanded relative to the X at the *Idh* locus and that it exhibits sequence characteristics of evolved sex chromosomes: a decrease in X-Y homology and an increase in mobile sequence elements (Peichel *et al.* 2004).

I then used FISH to show that the threespine stickleback, despite previous reports, does have a cytogenetically visible sex chromosome that has already experienced intrachromosomal rearrangements (Ross and Peichel in press). My model of the evolution of the threespine Y, which represents the most exhaustive attempt yet to enumerate the rearrangements experienced by a young vertebrate sex chromosome, proposes that a series of pericentric inversions occurred on the Y chromosome. Along with at least one large deletion on the Y, these rearrangements are responsible for the heteromorphy of the XY pair: a change in centromere position from the X to the Y. That the X and Y appear similar in size at metaphase, despite an increase in size of the Y at *Idh* and a large deletion from

the Y containing *Stn191, Stn192* and *Cyp19b,* indicates that a chronologically young sex chromosome might increase in size relative to its homolog.

By investigating the presence of heteromorphic sex-chromosome pairs in other stickleback species, I have verified the presence of a ZW pair in fourspine sticklebacks and identified a heteromorphic XY pair in ninespine sticklebacks. The fourspine W and ninespine Y are both larger than their homologous chromosomes. Although I cannot yet conclude whether these sex chromosome systems are evolutionary young, the phylogenetic independence of the fourspine ZW system and the ninespine Y^{LG12} system from the Y^{LG19} system in threespine sticklebacks suggests that they have recently evolved. The sex chromosome systems in these species thus provide evidence to validate the addition/attrition hypothesis that sex chromosomes first increase in size before degenerating.

The Y chromosomes in black-spotted and threespine Japan Sea sticklebacks are also the largest chromosomes in their respective karyograms. However, the mechanism of heteromorphy in these clades are different than those above. I demonstrated the physical linkage of LG12 and Y^{LG19} in black-spotted males, which had been predicted by genetic mapping by Catherine Peichel. A similar fusion of LG9 and Y^{LG19} was predicted by Jun Kitano from mapping data in the Japan Sea threespine stickleback population, and I verified this fusion using FISH.

Altogether, I have obtained cytogenetic evidence of at least four independent sex-chromosome systems in the stickleback family: two independent X_1X_2Y systems in the threespine Japan Sea and in black-spotted sticklebacks, XY systems in threespine and ninespine sticklebacks, and ZW GSD in fourspine sticklebacks. The Y-autosome fusion in black-spotted males has provided critical evidence to support theoretical work suggesting that certain chromosomes will become sex chromosomes because of preexisting features of those chromosomes. In the case of sticklebacks, it appears that LG12 became associ-

ated with a Y chromosome twice. Once as the Y chromosome in ninespine sticklebacks, and again with the fusion of one copy of LG12 to the Y^{LG19} in black-spotted sticklebacks.

My research has provided new insights into the details of the early stages of sex-chromosome evolution in vertebrates and stimulated a number of additional questions to be asked. As with any productive course of research, as one conclusion is reached, more lines of experimentation arise. I will elaborate on some of the next questions to be addressed in the following sections.

Materials and Methods

BAC fingerprint mapping: 160 ng of BAC DNA was digested with the restriction endonuclease *Eco*RI (Roche) according to the manufacturer's instructions. The digest was then applied to a 0.8% agarose gel made with 2x GGB (80 mM TRIS base, 40 mM sodium acetate, 4 mM EDTA, 52 mM glacial acetic acid, pH 8.0) (Olson *et al.* 1986) with 6x GGB loading dye (15% glycerol, 1.7x GGB and 3.7 mM bromophenol blue). A custom DNA ladder was applied to the gel, consisting of 8 µg High Molecular Weight marker (Invitrogen) and 15 µg 1 kb Ladder (Invitrogen) in 0.63x TE pH 8.0 and 1.07x GGB loading dye. The ladder was denatured at 65°C for 10 min prior to loading on the gel. Gel electrophoresis was performed in a mapping gel box with recirculated, cooled 2x GGB buffer at 200 V for eighteen hours. The gel was then stained in a 1:2000 dilution of Vistra Green (Applied Biosystems) in water and visualized using a Typhoon imaging system (GE Life Sciences).

Identification of *SEX* in Sticklebacks

Although I initially used a positional cloning strategy in an attempt to identify *SEX*, this approach became impractical given the large physical size (about 16 Mbp) of the nonrecombining region on the Y (Ross and Peichel in press). Many additional ap-

proaches can be used to identify the sex-determining factor. Because the main barrier to positional cloning on a sex chromosome is lack of recombination in the region of interest, producing YY fish would be beneficial, as the two Y chromosomes would be homologous over their length and might not exhibit reduced recombination around *SEX*.

Fish are generally amenable to sex-reversal by application of exogenous steroids (Devlin and Nagahama 2002). Treating stickleback fry with estrogen in an attempt to feminize genotypic males might lead to the creation of XY female fish. By then crossing an XY female with an XY male, YY males might be produced and can be identified by *Idh* genotype. Using such a male as a parent in a mapping cross might allow creation of a genetic map of the Y in which the genetic order of markers on the Y, and the position of *SEX*, are more refined. This approach might also provide the additional benefit of demonstrating whether significant functional divergence of the Y from the X has occurred. If YY males are viable, it would suggest that coding regions on the Y have not yet degenerated and that dosage compensation has not yet needed to evolve.

It is also important to determine whether *SEX* is Y-dominant or X-recessive in order to focus efforts to identify *SEX* by looking for a Y-present gene or an X-absent gene. In mammals, rare sex-chromosome aneuploids exist; their presence was instrumental in determining whether the mammalian sex-determining factor acted in a dominant or recessive manner. Most male humans have 46 chromosomes, including an XY pair (46, XY) and females are (46, XX), but some phenotypic females have only one X chromosome (45, XO), a condition known as Turner's Syndrome (Ford *et al.* 1959), and a small percent of phenotypically male humans have an extra copy of the X (47, XXY), called Klinefelter's Syndrome (Jacobs and Strong 1959). Together, the phenotypes of individuals with these conditions defined the human sex-determination locus as a Y-dominant one. In Klinefelter's patients, a Y, despite the presence of two X chromosomes, causes

maleness. In Turner patients, a single X chromosome is not sufficient to produce a male phenotype, which it would if X chromosome dosage played a sex-determining role.

Similar sex-chromosome aneuploids would be useful to help define the dominance of the stickleback *SEX* locus. A method for producing triploid threespine sticklebacks has been published (Swarup 1959). By fertilizing eggs and then subjecting them to cold treatment, meiosis II can be prevented, generating triploid offspring with sex-chromosome constitutions of XXX or XXY. The presence of one triploid male (XXY) produced in this manner has been reported (Swarup 1959), suggesting that the Y carries a dominant sex-determining factor. This work should be replicated on a larger scale, and the sex-chromosome constitution of triploid males should be identified using FISH, to confirm the dominance of the threespine stickleback *SEX* locus. Undertaking this study will be crucial for identifying *SEX*, regardless of which approach outlined below is ultimately taken.

Although the non-recombining *SEX* interval is large, the plan to sequence the Y chromosome still has merit, both to identify *SEX* and also to more extensively characterize a young vertebrate sex chromosome. Considering the difficulties I encountered during the process of cloning a sex chromosome, new strategies are needed to reach this goal.

Cloning the threespine Y: The method I employed during chromosome walking to determine whether clones originated from the X or Y involved identification of sex-specific alleles. In some cases, obtaining sufficient sequence to locate an easily scorable sequence difference might not be feasible. Moreover, some markers had null Y alleles, preventing the positive assignment of clones to the Y. While some of the null alleles are explained by deletions from the Y (Figures 18 and 21), it is possible that some null alleles are due to sequence divergence at the marker, not deletion. In this case, different primers are needed to identify Y chromosome clones.

Additionally, although I was able to determine the chromosomal origin of BACs containing genetic markers that were not Y-null, such as *Idh* and *Stn194*, I rarely identified useful (i.e., polymorphic and chromosome-specific) microsatellite or insertion-deletion markers during the process of BAC end sequencing. Identification of X-Y single nucleotide polymorphisms (SNPs) might be a good solution to the problem of identifying new X-Y diagnostic markers that can assign genomic library clones to their chromosomes of origin.

Fingerprint mapping to identify Y clones: X and Y clones might also be distinguished by their distribution of restriction sites. This idea prompted me to use available BAC fingerprint contigs (FPCs) of the CH213 library (Kingsley *et al.* 2004) to aid my chromosome walking (Chapter 3). Fingerprint mapping is the process of digesting BAC clones with a restriction endonuclease and then examining the digests with agarose gel electrophoresis. I produced fingerprint maps to validate BAC contigs defined by STS content. For example, all BACs in the *Idh* and *Stn194* CH215 BAC contigs were identified as members of their respective contig by library screen using *Idh* and *Stn194* probes, fingerprint maps of the BACs from the contigs reveal shared differences between two classes of BAC: those known to be from the Y and the X based on *Idh* genotype (Figure 33). BAC fingerprinting was also useful in confirming that certain clones, whose STSs were present on more clones in their contig than expected, also had aberrant restriction maps compared to the bulk of the clones. These might be Y clones. Although an aberrant BAC fingerprint cannot provide conclusive evidence of the chromosome of origin of a clone, it might be sufficient to tip the balance of evidence in favor of the X over the Y, or *vice versa*. By itself, a different restriction fragment pattern might simply indicate that the clone is from elsewhere in the genome.

Fingerprint maps were also useful in validating and extending contigs I assembled by STS content mapping. When I completed an STS content analysis of a contig, I then

determined whether all members of the contig were members of the same FPC. X and Y clones were rarely identified as members of the same FPC, suggesting that chromosome walking could be aided by identifying one Y clone and then studying other clones from the same FPC. My current method for cloning the Y by chromosome walking is to extend a Y BAC contig *in silico* with an MTP of clones from FPCs, which I then validate as be-

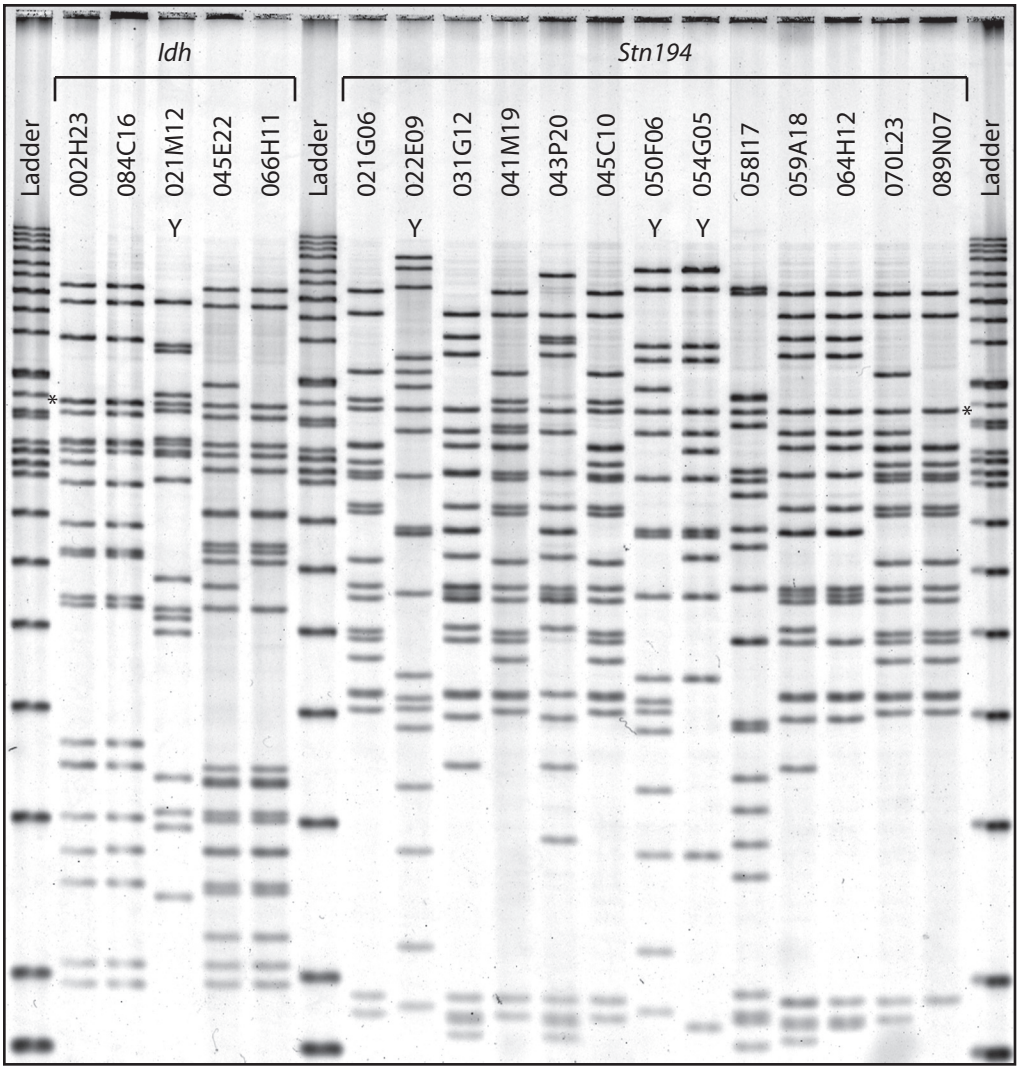


Figure 33. BAC fingerprint mapping of CH215 contigs.
Clones positive for the *Idh* and *Stn194* markers are indicated with brackets. The clones containing the Y chromosome alleles are indicated (Y). Within the *Idh* contig of five BACs, the Y clone has a visibly different restriction fingerprint. Similarly, the three *Stn194* Y clones, while significantly overlapping all of the X clones, share some diagnostic restriction products not present in X clones. One band present in every clone (marked with * on each side of the gel) represents the BAC vector.

ing members of the contig by STS content mapping. This combination of physical mapping techniques (STS content analysis and restriction-based BAC fingerprint mapping) has enhanced the accuracy and efficiency of the X and Y chromosome cloning process by providing two independent methods of assessing the physical relationships among clones in a contig.

However, this modified process, while more efficient than the original, fails at the same point: when the boundaries of an FPC contig are reached, a new Y-specific probe for library screening must still be found. At present, members of the Centers for Excellence in Genome Sciences (CEGS) at Stanford University are assisting in the cloning of the Y chromosome by integrating the FPC data with BAC end sequence and the female genome sequence data to identify putative Y-specific FPCs.

FISH identification of Y clones: The key improvement in the Y cloning process has been the use of FISH in identifying the chromosome of origin of BACs. I first discovered that, although an *Idh* X clone (CH213-101E08) hybridizes both to the X and Y (Figure 34A), an *Idh* Y clone (CH213-119K16) hybridizes only to the Y (Figure 34A). In a different clone library, a *Stn188* Y clone (CH215-029H19) hybridizes only to the Y (Figure 34B) and a *Stn188* X clone (CH215-007C15) hybridizes only to the X (Figure 34C). Specificity of clone hybridization to the chromosome of origin might be due to the increased repetitive element content on the Y, because although an entire X clone might have homologous sequence on the Y, a significant percentage of a Y clone might have no homology to the X, perhaps reducing its hybridization below the level of detection.

Y clone sequencing: At present, the recommended protocol for continuing to clone the stickleback Y is for the Stanford CEGS group to identify putative Y clone contigs, and then to FISH one clone from each contig and ask whether it hybridizes only to the Y or to the X and Y. The CEGS group then sequences the clones that hybridize only to the Y and continues to study the remaining clones in fingerprint contigs with

FISH-verified Y clones. At present, in addition to the two CH213 Y BACs containing *Idh* analyzed in Chapter 2, twelve CH215 clones confirmed to be Y-specific by FISH are in various stages of sequencing and currently represent 1.9 Mbp of Y sequence; another seven clones were recently added to the sequencing queue. This process of computational

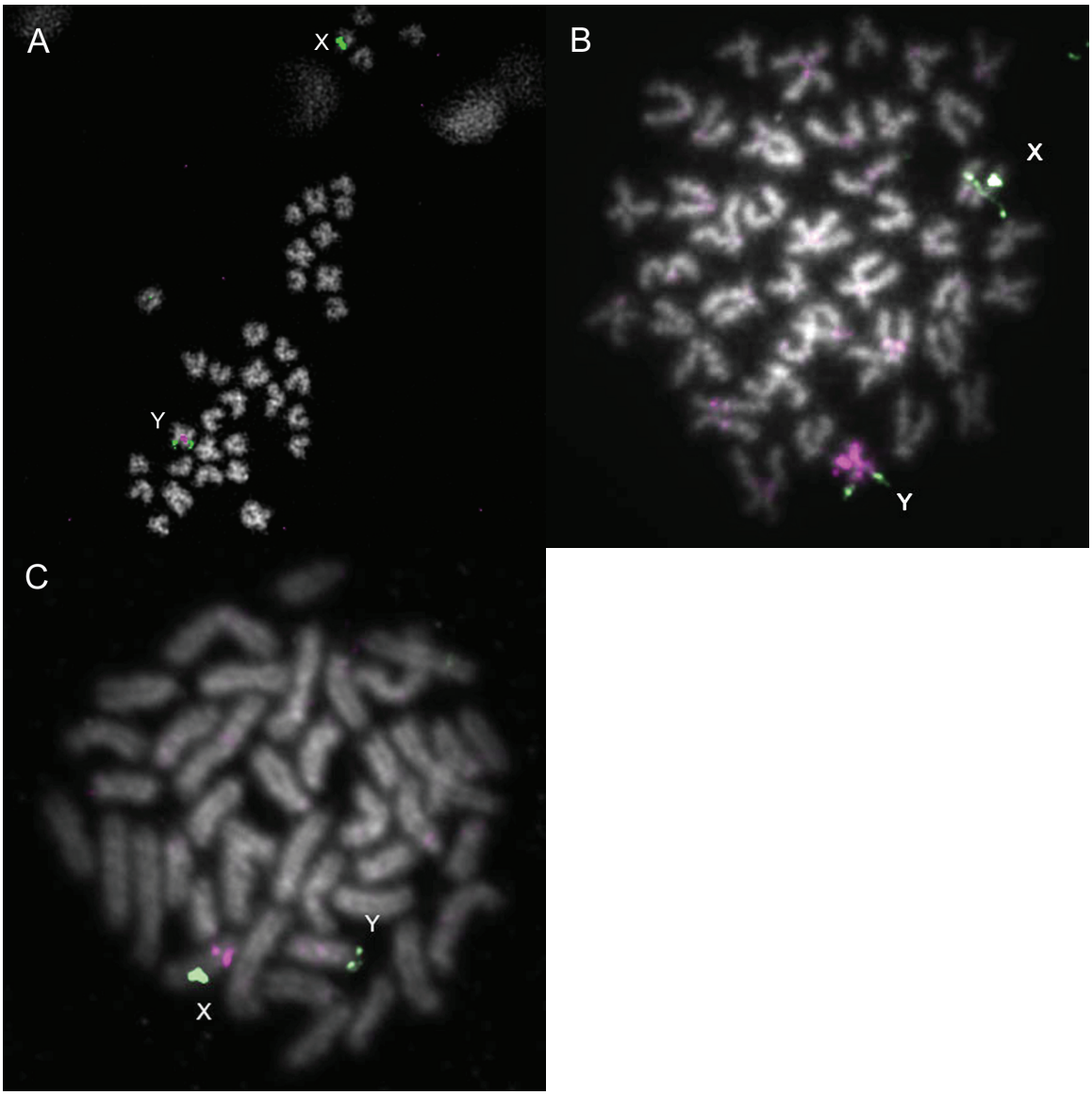


Figure 34. Use of FISH to assign chromosomal origin of probes.
The clone CH213-101E08 is green in each panel. (A) Threespine male metaphase spread hybridized with CH213-119K16 (purple) and CH213-101E08 (green). While the *Idh*-X probe 101E08 hybridizes to the X and Y, the *Idh*-Y clone 119K16 hybridizes only to the Y. (B) The *Stn188* Y clone CH215-029H19 (purple) hybridizes only to the Y but not to the X. (C) The *Stn188* X clone CH215-007C15 (purple) hybridizes to the X but not to the Y.

chromosome walking with FISH validation should allow the efficient cloning of much of the approximately 10 Mbp of the male-specific region of the threespine stickleback Y chromosome (Ross and Peichel in press).

Comparative approaches: Efforts are currently underway to identify *SEX* in the threespine stickleback by cloning and sequencing the nonrecombining Y, and the MTP of clones from the Y can also be used in a transgenics screen to search for a Y clone that causes sex-reversal of sticklebacks (Hosemann *et al.* 2004). Another approach to consider for sequencing the Y is microdissection of the threespine Y, followed by random amplification and sequencing. However, given my findings in threespine Japan Sea and black-spotted males, it is worth asking whether identification of a *SEX* locus in sticklebacks might be better accomplished in one of these two taxa.

The fused Y chromosomes in the Japan Sea threespine sticklebacks and in black-spotted sticklebacks, both of which may use the same *SEX* locus as the North American populations of threespine stickleback, are clearly distinguishable in metaphase spreads by size alone for microdissection. Using a massively parallel sequencing approach on the microdissected threespine Japan Sea or black-spotted Y chromosomes would yield some sequence homologous to the threespine Y and also result in the generation of neo-Y chromosome sequence. Deletions of Y sequence that may have occurred during the black-spotted Y-autosome fusion (Chapter 5) suggest that the physical region containing *SEX* may be the smallest in this species, requiring less sequencing. Although a microdissection-based sequencing effort would by necessity also generate sequence from the autosome that fused to the Y, this would provide an excellent resource for studies of the evolution of neo-sex chromosomes.

Candidate gene approaches: Aside from chromosome-wide sequencing approaches, I had also considered cDNA subtraction methods to seek transcripts present only in males, particularly in gonadal and surrounding tissues during the period of sex

determination. Amanda Bruner has performed a developmental analysis of threespine sticklebacks to identify the earliest point at which sex-specific changes are visible histologically, defining the developmental timepoint prior to which sex determination must have occurred as 12 days post-fertilization (around 5–7 mm total length), in agreement with a recent report (Lewis *et al.* 2007). James Urton is currently working to identify the sex-determination gene in threespine stickleback using transcripts present at and around this critical timepoint to perform cDNA subtraction.

Demonstration of role of a candidate gene in affecting male sex determination can involve many lines of evidence. Studies concluding that *SRY* and *DMY* are the sex-determining genes in mammals and the medaka fish, respectively, showed that absence of the gene prevented male development (Berta *et al.* 1990; Gubbay *et al.* 1990a; Gubbay *et al.* 1990b; Jager *et al.* 1990; Otake *et al.* 2006) and that presence of the gene caused male development (Sinclair *et al.* 1990; Koopman *et al.* 1991; Matsuda *et al.* 2007). These lines of evidence have come from knockouts, naturally-occurring mutants and lines carrying candidate transgenes. As techniques for making deletion lines and knockouts in sticklebacks have not yet been developed, screening for naturally-occurring sex revertants (XY phenotypic females) might be important to show that mutation or deletion of a candidate gene correlates with lack of male development in an XY individual.

BAC transgenic techniques have been developed for the threespine stickleback, making it possible to achieve germline transmission of BAC clones (Hosemann *et al.* 2004). If *SEX* in threespine sticklebacks acts in a dominant fashion, insertion of BACs carrying candidate sex-determination genes into the genomes of stickleback embryos and later assessing the presence of XX males can provide critical evidence of the role of such a candidate gene in causing male sex determination.

In the meantime, I identified BACs containing two candidate genes, *Cyp19b* and *Wt1a*, as being deleted from the Y and present on the Y, respectively (Chapter 4); both are

in the nonrecombining region of the threespine Y, in which *SEX* is located. It is unknown whether sex in threespine sticklebacks is determined by the presence of a Y-specific locus or the absence of a dosage-sensitive locus on the Y, although a single report of the production of a triploid XXY male suggests that *SEX* might act in a dominant fashion (Swarup 1959). Thus, candidate *SEX* genes may be in the region of the Y chromosome that does not recombine with the X (10 Mbp) or in the region of the X deleted from the Y (6 Mbp).

Wt1a was involved in all three pericentric inversions in my model of the threespine Y chromosome evolution, and *Cyp19b* was in the Y deletion (Figure 21). *Wt1a* is a transcription factor involved in gonad development in mammals (Wagner *et al.* 2003), and *Cyp19b* is an aromatase responsible for converting testosterone into estrogen (Conley and Hinshelwood 2001). Paralogs of these genes were considered as candidate *SEX* genes in the Nile tilapia (*O. niloticus*), where *Cyp19a1* and *Wt1b* map to the sex chromosome. However, both genes were eventually excluded as tilapia sex-determination genes by detailed genetic mapping (Lee and Kocher 2007). Future studies in sticklebacks should seek to determine whether either of these genes is expressed in a sex-specific and tissue-specific manner during early development by *in situ* hybridization. For a candidate gene found to be expressed only in males prior to sex determination, a BAC carrying the gene should be used to generate transgenic sticklebacks to determine whether expression of the gene causes development of XX males.

Comparative Studies

Comparison of sex-chromosome characteristics: The presence of XY and ZW GSD in stickleback species will motivate comparative analyses of these sex-chromosome systems. The first step will be to genetically and cytogenetically identify the heteromorphic ZW pair in fourspine sticklebacks. The identification of *SEX*-linked markers and of

the linkage group corresponding to the heteromorphic pair will be important in characterizing the Z and W chromosomes at the genetic and eventually sequence levels. However, it should be noted that an increase in chromosome number from threespine (2n=42) to fourspine sticklebacks (2n=46) suggests that the linkage groups of one species will not correspond exactly to those in the other. In fourspine sticklebacks, the presence of two more chromosome pairs suggests that two threespine LGs might be split into four in fourspine sticklebacks.

My FISH characterization of fourspine sticklebacks to this point has not been comprehensive enough to rule out the possibility that the sex chromosome in threespine or ninespine sticklebacks was rearranged as part of the change in karyotype between the 2n=42 and 2n=46 species. Hence, a more exhaustive cytogenetic study of stickleback species to catalog the rearrangements involved in the evolution of their karyotypes would be an excellent resource for future studies. An initial approach would be to identify two threespine BAC clones from each chromosome, one from each end, and co-hybridize them as FISH probes to metaphase spreads from both sexes of the other stickleback species. Identification of two probes from a single threespine chromosome hybridized to two chromosomes in other species would provide evidence of a chromosomal rearrangement.

***SEX* identification in other stickleback species**: Once a sex-determining gene is identified for a stickleback species, it then becomes easy to determine whether the same gene exists and plays the same role in sex determination in closely related species. Such a survey was performed in relatives of the medaka fish (Matsuda *et al.* 2003; Veith *et al.* 2003; Volff *et al.* 2003; Takehana *et al.* 2007a; Takehana *et al.* 2007b); this work showed that *DMY* had arisen recently enough that other fish in the same genus did not have it. Given the relationships between sex chromosomes of threespine, black-spotted, and ninespine sticklebacks, the first prediction to test will be that they all use the same *SEX* locus. I would not expect to find that fourspine sticklebacks share *SEX* with these three

species, as fourspines have ZW GSD and do not exhibit linkage of sex determination with LG12 or LG19 markers. Similarly, given lack of genetic or cytogenetic evidence of GSD in brook sticklebacks, I would not expect them to share *SEX* with the male heterogametic species.

Sex-chromosome degeneration: The variety of sex-chromosome systems in closely related species makes the stickleback family an excellent system in which to perform comparative analyses of sex determination and sex-chromosome evolution. Apart from identifying *SEX* loci and assessing their degree of conservation across taxa, many cytogenetic studies might be pursued to assess the degree of sex-chromosome degeneration in the family Gasterosteidae.

One avenue of study made possible by the availability of the female threespine stickleback genome sequence is to seek BAC clones whose end sequences appear inverted relative to the X, or are much closer together or farther apart than predicted based on the clone insert size. These might represent Y clones containing inversion, insertion, or deletion breakpoints. Identifying such clones enables future study of the mechanisms of chromosomal inversions by providing the reagents necessary for cloning and characterizing the breakpoints.

It would also be interesting to perform a comparative analysis of sex-chromosome degeneration in sticklebacks. By creating cytogenetic maps of the heteromorphic pairs in ninespine and fourspine sticklebacks to identify deletions and inversions as I did for threespine sticklebacks (Ross and Peichel in press), trends in the degeneration of sex chromosomes, particularly between XY and ZW systems, might be identified. Similarly, the evolution of two independent X_1X_2Y systems provides an unparalleled opportunity to characterize and compare the degeneration of neo-sex chromosomes in vertebrates. Similar studies of a neo-Y chromosome in Drosophila suggest that neo-Y chromosomes begin to degenerate once they fuse with an ancestral Y chromosome (Steinemann and

Steinemann 2000). It would be particularly interesting to compare sequences from the X_1, X_2 and the Y and neo-Y portions of the sex chromosome in threespine Japan Sea and in black-spotted males.

The Future of Sex Chromosomes

The ultimate outcome of sex-chromosome degeneration is uncertain; many predictions have been made. First, translocation of a degenerating sex chromosome to an autosome, creating an X_1X_2Y/X_1X_2 sex-chromosome system, or transposition of *SEX* to an autosome might rescue a sex chromosome from completely eroding while putting another autosome at risk of degeneration. Perhaps the best example to date of recycling of the Y by autosomal fusion is in Drosophila (Carvalho and Clark 2005). Although Y-autosome fusion seems ultimately detrimental, the presence of such systems suggests that certain autosomal destinations might be beneficial if they contain sexually antagonistic genes. This hypothesis is currently being tested in the Peichel lab using sticklebacks.

Other possibilities have been put forth. The loss of an entire sex chromosome, including *SEX*, could favor the evolution either of a more-stable XO system, in which X chromosome dosage might assume the role of sex determination, or ESD might arise. Some have predicted that the ultimate fate of the human Y is complete loss in about 15 MY based on estimates of the rate of gene loss from the mammalian Y (Aitken and Graves 2002). Before that point, I hope that research stimulated by the findings I report here will provide clearer ideas of how sex chromosomes and genetic sex determination mechanisms transition from one system to another.

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Curriculum Vitae

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Education and Research

2002—2008	University of Washington and Fred Hutchinson Cancer Research Center Advisor: Catherine L. Peichel, Ph.D. Ph.D. in Molecular and Cellular Biology
2001—2002	Fred Hutchinson Cancer Research Center Research Technician, Barbara J. Trask Lab "Mouse olfactory epithelium cDNA library screen."
1999—2000	University of Oregon Institute of Molecular Biology Advisor: Diane K. Hawley, Ph.D. "DNA-TATA Binding Protein interactions during transcription initiation."
1996—2000	University of Oregon B.A. Biochemistry, *magna cum laude*, Departmental Honors

Awards and Funding

2006	Fred Hutchinson Cancer Research Center Student/ Postdoc Advisory Committee Conference Travel Award, $1,500
2004—2007	Cell and Molecular Biology Training Grant Public Health Service, National Research Service Award T32 GM07270, from the National Institute of General Medical Sciences
2004	NSF Graduate Research Fellowship Honorable Mention
1999	HHMI Summer Undergraduate Research Fellow
1996—2000	Presidential Scholarship, University of Oregon
1996	University of Oregon Alumni Association Scholarship

Peer-reviewed Publications

Ross JA and Peichel CL. "Molecular cytogenetic evidence of rearrangements on the Y chromosome of the threespine stickleback fish." *Genetics* (2008) accepted.

Peichel CL, **Ross JA**, Matson CK, Dickson M, Grimwood J, Schmutz J, Myers RM, Mori S, Schluter D and Kingsley DM. "The master sex-determination locus in threespine sticklebacks is on a nascent Y chromosome." *Current Biology* (2004) **14**:1416-1424.

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Other Publications

Ross JA. Correspondence. *Nature* (2007) **445**:593.

Selected Presentations

"Evolution of sex chromosomes in stickleback fishes." Fourth International Symposium on Vertebrate Sex Determination. Kona, HI. 13 April 2006.

"Sex chromosome evolution in the threespine stickleback *Gasterosteus aculeatus*." Basic Sciences and Human Biology Divisions Seminar Series, Fred Hutchinson Cancer Research Center. 28 January 2005.

"Sex determination and sex chromosome evolution in stickleback fish." Genetics and Genomics Group, Fred Hutchinson Cancer Research Center. 10 December 2003.

"Transcription initiation: DNA-TATA binding protein (TBP) interactions." Howard Hughes Medical Institute Undergraduate Summer Research Symposium. University of Oregon. 1999.

Selected Posters (presenter)

Bruner A, Ross J and Peichel C. "Evolution of sex determination in stickleback fishes" Society of Integrative and Comparative Biology. Orlando, Florida. January 2006.

Ross JA, Blahm AM and Peichel CL. "Evolution of genetic sex determination in sticklebacks (Gasterosteus spp.)" Northwest Reproductive Sciences Symposium. Seattle, Washington. 22-23 April 2005.

Ross JA and Peichel CL. "Evolution of sex determination mechanisms and sex chromosomes in stickleback fish." EVO-WIBO (Evolutionary Biology Conference of Washington, Idaho, British Columbia and Oregon). Port Townsend, Washington. 17 April 2004.

* Student Presentation Award *

Ross JA and Peichel CL. "Evolution of sex determination mechanisms and sex chromosomes in stickleback fish." American Indian Science and Engineering Society National Conference. Albuquerque, New Mexico. 22 November 2003.

* Best Graduate Student Poster Award *

Young J, Shykind B, Lane R, Tonnes-Priddy L, Ross J, Walker M, Williams E, Axel R and Trask B. "Odorant receptor ESTs demonstrate olfactory expression of over 400 genes, extensive alternate splicing, and unequal expression levels." Cold Spring Harbor 68th Symposium: The Genome of Homo Sapiens. May 2003.

Young JM, Friedman C, Tonnes-Priddy L, Ross J, Lane RP and Trask BJ. "The human and mouse olfactory receptor gene families are shaped by different evolutionary processes." Cold Spring Harbor Symposium: Genome Sequencing and Biology. May 2001.