Purifying Selection Maintains Dosage-Sensitive Genes during Degeneration of the Threespine Stickleback Y Chromosome

Michael A. White,*1 Jun Kitano,2 and Catherine L. Peichel1

1Division of Human Biology and Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA
2Ecological Genetics Laboratory, National Institute of Genetics, Shizuoka, Japan
*Corresponding author: E-mail: mawhite@fhcrc.org

Abstract

Sex chromosomes are subject to unique evolutionary forces that cause suppression of recombination, leading to sequence degeneration and the formation of heteromorphic chromosome pairs (i.e., XY or ZW). Although progress has been made in characterizing the outcomes of these evolutionary processes on vertebrate sex chromosomes, it is still unclear how recombination suppression and sequence divergence typically occur and how gene dosage imbalances are resolved in the heterogametic sex. The threespine stickleback fish (Gasterosteus aculeatus) is a powerful model system to explore vertebrate sex chromosome evolution, as it possesses an XY sex chromosome pair at relatively early stages of differentiation. Using a combination of whole-genome and transcriptome sequencing, we characterized sequence evolution and gene expression across the sex chromosomes. We uncovered two distinct evolutionary strata that correspond with known structural rearrangements on the Y chromosome. In the oldest stratum, only a handful of genes remain, and these genes are under strong purifying selection. By comparing sex-linked gene expression with expression of autosomal orthologs in an outgroup, we show that dosage compensation has not evolved in threespine sticklebacks through upregulation of the X chromosome in males. Instead, in the oldest stratum, the genes that still possess a Y chromosome allele are enriched for genes predicted to be dosage sensitive in mammals and yeast. Our results suggest that dosage imbalances may have been avoided at haploinsufficient genes by retaining function of the Y chromosome allele through strong purifying selection.

Key words: threespine stickleback, sex chromosome evolution, dosage compensation.

Introduction

Heteromorphic sex chromosomes (i.e., XY or ZW) have repeatedly evolved from autosomal ancestors across a diverse array of taxa and exhibit evolutionary patterns distinct from the remainder of the genome. Theoretical work as well as empirical studies in vertebrates, invertebrates, plants, and fungi (Ellegren 2011; Bachtrog 2013) suggest that the independent evolution of heteromorphic sex chromosomes in diverse taxa follows a similar trajectory. First, suppression of recombination is a hallmark of heteromorphic sex chromosomes, and is thought to occur as a result of selection for linkage between a sex-determination locus and genes with sexually antagonistic alleles (Bull 1983; Rice 1987a; Charlesworth 1991, 1996). Suppression can either occur through inversions or changes in genetic modifiers controlling recombination rate (Charlesworth et al. 2005). Once recombination is suppressed, the efficacy of natural selection is reduced, and deleterious mutations can quickly accumulate on the Y or W chromosome (Charlesworth 1978; Rice 1987b; Bachtrog 2013).

These evolutionary milestones have been studied in a variety of sex chromosome systems. In humans, the Y chromosome evolved over the last 180 My through at least four independent steps of recombination suppression, creating regions of different ages (termed “evolutionary strata”) (Lahn and Page 1999; Skaletsky et al. 2003; Hughes et al. 2012; Bellott et al. 2014; Cortez et al. 2014). At least in the younger strata, inversions seem to have suppressed recombination between the X and Y chromosomes (Lemaitre et al. 2009). Inversions have also been correlated with evolutionary strata on the papaya sex chromosomes (~7 My old) (Wang et al. 2012). However, the relationship between evolutionary strata and inversions is less clear in other systems that span a variety of ages from the young sex chromosomes of the plant Silene latifolia (~5–10 My old) (Filatov and Charlesworth 2002; Filatov 2005; Bergero et al. 2008, 2013; Rautenberg et al. 2010; Chibalina and Filatov 2011) to the old sex chromosomes of birds (140 My old) (Cortez et al. 2014; Wright et al. 2014). Furthermore, suppression of recombination does not occur in many sex chromosome systems, and even ancient sex chromosomes can remain homomorphic (Bachtrog et al. 2014). Thus, the evolutionary forces and molecular mechanisms that lead to the suppression of recombination on sex chromosomes are not completely understood.

As a result of suppressed recombination, many genes are eventually lost from the Y chromosome. This is exemplified by mammalian Y chromosomes, in which only a small fraction of genes remain on the Y chromosome, compared with their X-linked gametologs (Hughes et al. 2012; Bellott et al. 2014; Cortez et al. 2014). In response to sequence degeneration and gene loss, different mechanisms have evolved across taxa to restore gene dosage balance in the heterogametic sex. In some old systems, like Drosophila melanogaster...
Therefore, we explored whether local dosage compensation has evolved at individual genes by comparing gene expression on the three-spined stickleback sex chromosomes with their autosomal orthologs in a closely related outgroup species, the ninespine stickleback (*Pungitius pungitius*). This species does not possess the threespine stickleback XY sex chromosome system and diverged approximately 13–16 Ma from the threespine stickleback (*Bell et al. 2009; Kawahara et al. 2009; Ross et al. 2009; Aldenhoven et al. 2010*). Our results highlight the power in merging patterns of molecular evolution with allele-specific gene expression in males to understand the evolution of heteromorphic sex chromosome pairs.

**Results and Discussion**

**Protein-Coding Divergence between the X and Y Chromosomes Reveals Two Evolutionary Strata**

Divergence between the X and Y chromosomes was significantly higher among genes in the nonrecombinating region than among genes within the recombinating pseudoautosomal region (PAR). This pattern was evident for both synonymous site divergence ($d_{s}$) (median XY nonrecombinating: 0.0139, *N* = 657 genes; median XY recombinating PAR: 0.0000, *N* = 87 genes; Mann–Whitney *U* test, *P* < 0.001) (fig. 1) and nonsynonymous site divergence ($d_{N}$) (median XY nonrecombinating: 0.0056, *N* = 657 genes; median XY recombinating PAR: 0.0000, *N* = 87 genes; Mann–Whitney *U* test, *P* < 0.001) (fig. 1). In addition, divergence between the X and Y chromosomes showed evidence of significantly less purifying selection (i.e., higher $d_{N}/d_{S}$) in genes in the nonrecombinating region than in genes in the recombinating PAR of the sex chromosomes (median $d_{N}/d_{S}$ XY nonrecombinating: 0.2832, *N* = 562 genes; median $d_{N}/d_{S}$ XY recombinating PAR: 0.0000, *N* = 84 genes; Mann–Whitney *U* test, *P* < 0.001).

We investigated whether there were differences in sequence divergence among the three major cytogenetically characterized pericentric inversions (here referred to as A, B, and C) of the *G. aculeatus* Y chromosome (*Ross and Peichel 2008*). This difference in $d_{S}$ would support a model where inversions suppressed recombination in a stepwise fashion across the *G. aculeatus* Y chromosome. Despite the presence of pericentric inversions, we detected no significant differences in median nonsynonymous and synonymous site divergence ($d_{N}$ and $d_{S}$) (fig. 1) or in median $d_{N}/d_{S}$ ratio between the X and Y among the three pericentric inversions (median $d_{N}/d_{S}$: A: 0.0132, *N* = 180 genes; B: 0.0140, *N* = 378 genes; C: 0.0129, *N* = 24 genes; median $d_{N}/d_{S}$: A: 0.0052, *N* = 180 genes; B: 0.0059, *N* = 378 genes; C: 0.0049, *N* = 24 genes; Mann–Whitney *U* test, *P* > 0.05 in all comparisons using Mann–Whitney *U* test). Similar values of $d_{N}$ suggest that recombination ceased at a comparable time across the three regions. These results are consistent with several scenarios. In one scenario, a single large inversion could have suppressed recombination simultaneously across the region. If two nested inversions then formed within the prior inversion (*Ross and Peichel 2008*), delineating regions A, B, and C, the nested inversions would have no differential effect on $d_{S}$ because
recombination was already suppressed across the region. A second plausible scenario is that inversions A, B, and C were not nested within an initial large inversion and instead occurred sequentially, shutting down recombination in a stepwise manner across the Y chromosome. If these inversions occurred close enough together temporally, $d_S$ may not be discernable between the strata. Indeed, simulations indicate that the ages must be considerably different to generate detectable strata in the values of $d_S$ (Chibalina and Filatov 2011).

Third, it is possible that other genetic modifiers evolved to suppress recombination (Charlesworth et al. 2005) and that the pericentric inversions did not play a role. Finally, intrachromosomal rearrangements have been documented on Y chromosomes between closely related species (Hughes et al. 2010) and even within species (Knebel et al. 2011; Lange et al. 2013). Thus, it is possible that the lack of differentiation between the inversions is because the inversions are polymorphic within the Japanese Pacific Ocean *G. aculeatus* population used in both this study and the previous cytogenetic study (Ross and Peichel 2008). Regardless of the reason, because we found equal $d_S$ across A, B, and C, we combined these regions into a single region for subsequent analyses.

In addition to the pericentric inversions, previous cytogenetic evidence indicated that the distal approximately 6 Mb of the X chromosome is largely missing from the Y chromosome (Ross and Peichel 2008). Next-generation sequencing and microarrays from multiple worldwide freshwater and marine populations have also revealed a similar pattern, with read coverage or hybridization in this region nearly half of that in females (Leder et al. 2010; Roesti et al. 2013; Yoshida et al. 2014; Schultheiss et al. 2015). This indicates that the region is largely degenerated and/or deleted, and that these changes are likely fixed within the species. Surprisingly, we found a number of Y-linked transcripts that aligned to this region of the X, revealing that some genes from this region still exist on the Y chromosome (fig. 1). Together, these results raise the intriguing possibility that this region is an older evolutionary stratum. To test this possibility, we estimated divergence in the remaining genes of this distal region (D). Median $d_S$ between X and Y in region D was significantly
higher than in region ABC (D: 0.0179, N = 75 genes; ABC: 0.0135, N = 582 genes; Mann–Whitney U test, P = 0.021) (fig. 1), suggesting that recombination first ceased between the X and Y chromosomes in region D. Interestingly, a previous study did not report a significant difference in \( d_{NS} \) between these regions (Schultheiss et al. 2015). However, the previous study was likely underpowered to detect a difference in divergence as \( d_{NS} \) was estimated among a smaller pool of genes in the oldest stratum \( (N = 38 \) in Schultheiss et al. 2015; \( N = 75 \) in this study).

Median \( d_{NS} \) in region D (0.0044, \( N = 75 \) genes) was significantly lower than in region ABC (0.0057, \( N = 582 \) genes) (Mann–Whitney U test, \( P = 0.010 \)) (fig. 1). Consequently, genes within region D have been under stronger purifying selection (i.e., lower \( d_{NS}/d_{AS} \)) than genes in the rest of the non-recombining region since the divergence of the X and Y chromosomes from their autosomal ancestor (D: 0.1500, \( N = 74 \) genes; ABC: 0.3085, \( N = 488 \) genes; Mann–Whitney U test, \( P = 0.019 \)). Similar patterns were observed in the oldest regions of the S. latifolia sex chromosomes, where high \( d_{NS} \) was correlated with low \( d_{NS}/d_{AS} \) ratios (Chibalina and Filatov 2011). After large-scale loss of coding and intergenic regions, the only genes that will remain in old strata are those that are critically important for biological functions. This has been observed in mammals, where a highly conserved set of genes involved in transcription and translation independently survived deletion across Y chromosome lineages (Bellott et al. 2014; Cortez et al. 2014). As in threespine sticklebacks, this conserved set of genes had significantly lower \( d_{NS}/d_{AS} \) ratios than the remainder of genes on the X chromosome (Bellott et al. 2014). Hereafter, we refer to the older evolutionary stratum (D) as stratum 1 and the younger region (ABC) as stratum 2, to be consistent with previous literature on sex chromosome evolution (fig. 1) (Lahn and Page 1999; Ross et al. 2005; Bellott et al. 2014; Cortez et al. 2014). Note that this nomenclature differs from previous studies of the threespine stickleback sex chromosomes (Roesti et al. 2013; Schultheiss et al. 2015).

Stratum 1 may have evolved structurally under a variety of scenarios. One possibility is that the distal end of the Y chromosome was not subject to a single large deletion. Instead, most of the genes and intergenic regions could have degraded over a long period of time, leaving little homologous sequence for aligning next-generation sequencing reads (Roesti et al. 2013; Yoshida et al. 2014; Schultheiss et al. 2015) or for hybridizing cytogenetic probes (Ross and Peichel 2008). Under this model, if most of the genes and intergenic regions have simply degraded over time, the remaining genes under purifying selection would be clustered together. Alternatively, biologically important genes from the old stratum could have duplicated and translocated elsewhere on the Y chromosome over time. Following these gene duplication events, the ancestral copies of stratum 1 would be deleted from the Y chromosome in a single event. This scenario is less parsimonious as it requires multiple translocation steps; however, extensive Y chromosome rearrangements have been documented in mammals (Hughes et al. 2010). Under this model, the remaining genes of stratum 1 would be scattered in different regions of the Y chromosome and would not be syntenic with the X chromosome. Ultimately, the complete sequencing of the Y chromosome will reveal the order and location of all transcripts from stratum 1, allowing us to distinguish these models.

### Lineage-Specific Sequence Divergence of the X and Y Chromosomes

Substitutions occur at different rates along the X and Y chromosome lineages for several reasons, including higher mutation rates in males (Shimmin et al. 1993; Li et al. 2002; Ellegren 2007; Wilson Sayres and Makova 2011), Y-specific sequence degeneration (Charlesworth and Charlesworth 2000; Bachtrog 2013), and the faster-X effect (X-linked divergence occurs at a higher rate than divergence on the autosomes) (Charlesworth et al. 1987; Vico and Charlesworth 2006; Mank et al. 2010; Meisel and Connallon 2013). To separate lineage-specific evolution of the X and Y chromosome of G. aculeatus, we used the sequence of a P. pungitius female as an outgroup. This species has independently evolved an XY sex chromosome system from chromosome 12 (the G. aculeatus XY sex chromosome system evolved from chromosome 19) and so the sex-linked transcripts of G. aculeatus are autosomal in P. pungitius (Ross et al. 2009; Shapiro et al. 2009). There were no significant differences in \( d_{NS} \) among the X and Y lineages in any regions of the sex chromosomes (fig. 2A) (Kruskal–Wallis test, \( P = 0.227 \)), indicating an absence of a biased mutation rate in male threespine sticklebacks. However, we found different levels of \( d_{NS} \) among regions of the sex chromosomes (fig. 2B) (Kruskal–Wallis test, \( P < 0.001 \)). \( d_{NS} \) was higher in stratum 2 of the Y chromosome as compared with the autosomes, although this difference was not significant when corrected for multiple comparisons (autosomes, \( N = 27,858 \) genes; stratum 2, \( N = 490 \) genes; post hoc Mann–Whitney U test, \( P = 0.016 \); corrected for multiple comparisons using Holm’s method, \( P = 0.097 \); Holm 1979). \( d_{NS} \) was significantly higher in nonfunctional stratum 2 genes that contain frameshifts or nonsense mutations on the Y chromosome as compared with the autosomes (autosomes, \( N = 27,858 \) genes; stratum 2, \( N = 92 \) genes; post hoc Mann–Whitney U test, \( P < 0.001 \)) (fig. 2B). Similarly, the \( d_{NS}/d_{AS} \) ratio was significantly higher in nonfunctional Y chromosome genes of stratum 2 than autosomes (autosomes, \( N = 27,328 \) genes; stratum 2, \( N = 91 \) genes; Kruskal–Wallis test, \( P < 0.001 \); post hoc Mann–Whitney U test, \( P = 0.006 \)) (supplementary fig. S1, Supplementary Material online), consistent with a loss of selective constraint on Y chromosome genes as they degenerate.

Synonymous divergence between the sex chromosomes should be similar to \( d_{AS} \) between P. pungitius and G. aculeatus autosomal orthologs if the sex chromosomes evolved immediately after the species split (~13–16 Ma) (Bell et al. 2009; Aldenhoven et al. 2010). Instead, we found that XY \( d_{AS} \) was significantly lower than autosomal \( d_{AS} \) between the species in both strata (autosomes: 0.1417, \( N = 27,858 \) genes; stratum 1: 0.0179, \( N = 75 \) genes; stratum 2: 0.0135, \( N = 582 \) genes; \( P < 0.001 \) in both comparisons using Mann–Whitney U test), indicating that both strata are considerably younger.
than the split between *G. aculeatus* and *P. pungitius*. It is possible that we were unable to recover all evolutionary strata with next-generation sequencing. Smaller, ancient strata on the Y chromosome may lack adequate sequence coverage because of difficulty mapping divergent reads to the X chromosome. A Sanger sequenced assembly of the *G. aculeatus* Y chromosome will be necessary to reveal the complete set of evolutionary strata and to accurately estimate the age of the sex chromosomes.

Recessive male beneficial mutations are predicted to accumulate more rapidly in regions of the X chromosome that are hemizygous in males due to the faster-X effect (Charlesworth et al. 1987; Vicoso and Charlesworth 2006; Mank et al. 2010; Meisel and Connallon 2013). To explore whether genes on the X chromosome also evolve faster in the threespine stickleback, we examined whether $d_{NS}/d_S$ ratios (between the *G. aculeatus* X chromosome and *P. pungitius* orthologs) were higher in hemizygous X chromosome genes from stratum 2 and stratum 2, and in genes that had nonfunctional alleles on the Y chromosome (i.e., functionally hemizygous) from stratum 2. When compared with autosomes (median $d_{NS}/d_S$: 0.1798, $N = 27,328$ genes), the median $d_{NS}/d_S$ ratio was higher for X chromosome genes of stratum 2 that were hemizygous (median $d_{NS}/d_S$: 0.2530, $N = 148$ genes; Mann–Whitney one-tailed $U$ test, $P = 0.006$) or that had nonfunctional alleles on the Y chromosome (median $d_{NS}/d_S$: 0.2712, $N = 91$ genes; Mann–Whitney one-tailed $U$ test, $P = 0.002$), and for hemizygous genes in stratum 1 (median $d_{NS}/d_S$: 0.1981, $N = 591$ genes; Mann–Whitney one-tailed $U$ test, $P = 0.048$). Our results complement previous studies on relatively old sex chromosome systems that have demonstrated the faster-X effect (Baines and Harr 2007; Mank et al. 2007; Baines et al. 2008; Meisel and Connallon 2013). Previous work on the threespine stickleback did not detect elevated $d_{NS}/d_S$ ratios on the X chromosome (Yoshida et al. 2014). However, the study was underpowered to detect elevated rates of evolution on the X chromosome. First, the authors did not focus on hemizygous regions of the sex chromosomes, where the effect should be

![Fig. 2](http://mbe.oxfordjournals.org/)
strongest. In addition, the analysis did not use an autosomal outgroup for the sex chromosomes, effectively analyzing $d_{H}/d_{S}$ over a shorter time period. Here, we use an autosomal outgroup and show an elevated rate of evolution in the hemizygous regions of two differently aged strata, indicating that selection can act rapidly to increase beneficial mutations on the X chromosome.

We used the outgroup to show that insertions, deletions, and nonsense mutations were also distributed differently across the X and Y chromosomes. Within stratum 2, there were significantly more indels on the Y chromosome than the X chromosome ($\chi^2 = 75.06, df = 1, P < 0.001$). Across the genome, deletions are primarily driven by replication errors, rather than recombination (Kvikstad et al. 2007, 2009). Therefore, in nonrecombining regions of the genome, such as the Y chromosome, deletions should be favored because mutations would primarily be driven by replication errors (Wilson Sayres and Makova 2011). Consistent with this prediction, we detected a higher frequency of deletions on the G. aculeatus Y chromosome than insertions ($\chi^2 = 9, df = 1, P = 0.003$) (table 1). Frameshift indels were nonexistent on the X chromosome; however, both frameshifs and nonframeshift indels occurred at similar frequencies on the Y chromosome ($\chi^2 = 0.1011, df = 1, P = 0.751$), indicating a lack of selection to purge deleterious frameshift indels from the Y chromosome. Within stratum 1, indels only occurred on the Y chromosome and were at a significantly lower frequency than stratum 2 (stratum 1, $N = 75$ genes; stratum 2, $N = 582$ genes; permutation test with 10,000 random samples, $P = 0.010$), indicating that purifying selection is also acting to purge deleterious insertions and deletions from the remaining genes of stratum 1.

Accumulation of Pseudogenes on the Threespine Stickleback Y Chromosome

Pseudogenes have been quantified as a measure of degeneration on Y and W chromosomes in other systems. We defined pseudogenes on the G. aculeatus Y chromosome as the genes that contain frameshift or nonsense mutations along with the genes that are hemizygous based on the DNA read depth threshold (see Methods). This is likely an overestimate, as hemizygous genes in our analysis combine two types of gene loss. Lower read depth can result from poor sequencing read alignments to highly divergent pseudogenes, or lower read depth can reflect true deletions. Still, these results allow for comparison of the threespine stickleback Y chromosome to other sex chromosome systems. By our estimates, 8.9% of genes were pseudogenes, whereas 53.5% of genes were hemizygous in males. Thus, 62.4% of genes are predicted to be nonfunctional pseudogenes on the G. aculeatus Y chromosome.

Lack of Global Dosage Compensation in Threespine Sticklebacks

In light of this extensive Y chromosome degeneration, we wanted to test whether dosage compensation has evolved in threespine sticklebacks. Thus, we compared gene expression of the X chromosome with orthologous genes in the ninespine stickleback, P. pungitius. In these analyses, we assume that the expression levels in the outgroup reflect the ancestral levels of expression on the proto-X chromosome (Julien et al. 2012; Lin et al. 2012; Mank 2013; Vicoso, Emerson, et al. 2013). Using RNA-seq data from brain tissues of males and females, we first compared the expression of autosomal genes in threespine stickleback relative to their expression in ninespine stickleback. As expected, autosomal gene expression in both males and females was highly correlated with the expression of orthologous genes in the outgroup (Spearman’s rank correlation; male rho = 0.888, $N = 24,308$ genes, $P < 0.001$; female rho = 0.876, $N = 24,308$ genes, $P < 0.001$) (fig. 3A). Median autosomal expression ratios were also nearly identical between males and females (Mann–Whitney $U$ test, $N = 24,308$ genes, $P = 0.926$) (fig. 3B). Across the sex chromosomes, gene expression significantly deviated from ancestral levels, with both region-specific and sex-specific effects. In the youngest regions, the PAR and stratum 2, females had expression ratios that were significantly greater than ancestral expression (one-sample Mann–Whitney $U$ test, $mu = 0$; PAR: $N = 64$ genes, $P = 0.039$; stratum 2: $N = 496$ genes, $P = 0.009$), whereas expression levels in males were indistinguishable from ancestral levels (one-sample Mann–Whitney $U$ test, $mu = 0$; PAR: $N = 64$ genes, $P = 0.075$; stratum 2: $N = 496$ genes, $P = 0.291$) (fig. 3B).

Different patterns were observed in the older stratum 1. Males had gene expression levels significantly lower than the ancestor (one-sample Mann–Whitney $U$ test, $mu = 0$; stratum 1 X and Y alleles: $N = 65$ genes, $P < 0.001$; stratum 1 X hemizygous: $N = 522$ genes, $P < 0.001$), and females had expression levels that matched ancestral levels (one-sample Mann–Whitney $U$ test, $mu = 0$; stratum 1 X and Y alleles: $N = 65$ genes, $P > 0.926$).

---

**Table 1.** Insertions and deletions across the sex chromosomes.

<table>
<thead>
<tr>
<th>Stratum 2</th>
<th>X Chromosome Deletion</th>
<th>X Chromosome Insertion</th>
<th>Y Chromosome Deletion</th>
<th>Y Chromosome Insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonframeshift</td>
<td>0</td>
<td>5</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>Frameshift</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>14</td>
</tr>
<tr>
<td>Stratum 1, X and Y alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonframeshift</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Frameshift</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
\[ N = 65 \text{ genes}, P = 0.092; \text{stratum 1 X hemizygous: } N = 522 \text{ genes, } P = 0.520 \] (fig. 3B).

Stratum 2 and the PAR exhibited strong patterns of feminization. Feminization of the X chromosome has been observed in other XY systems (Bachtrog 2006; Vicoso and Charlesworth 2006; Dean and Mank 2014) and can result from two mechanisms. Because the X chromosome is transmitted more frequently through the female germline, female-beneficial traits are selected for more efficiently than male-beneficial traits (Rice 1984; Bachtrog 2006; Dean and Mank 2014). In addition, genes can evolve sex-biased expression in recombining regions of sex chromosomes to resolve conflict from sexually antagonistic mutations in the absence of recombination suppression (Rice 1984; Scotti and Delph 2006; Otto et al. 2011; Vicoso, Kaiser, et al. 2013). In this case, genes would be upregulated in the sex they benefit. Consistent with these patterns, we observed significant upregulation of genes only in females in the youngest regions of the sex chromosomes. Within the PAR, feminization could be a mechanism to resolve sexual conflict. In stratum 2, female gene expression was still higher than ancestral expression levels, but lower than female expression levels in the PAR (Mann–Whitney U test, \( N = 65 \text{ genes}, P = 0.047 \)) (fig. 3B). In this stratum, feminization could reflect its recent history as a recombining region where sexually antagonistic genes once needed to resolve sexual conflict by evolving sex-biased expression. Alternatively, female-beneficial genes may have accumulated after recombination ceased. Feminization in stratum 2 may also reflect a combination of these two mechanisms.

**Fig. 3.** Threespine stickleback gene expression compared with outgroup gene expression levels. (A) Gene expression of autosomal genes in the threespine stickleback (Gasterosteus aculeatus) is highly correlated with gene expression of orthologous genes in the ninespine stickleback (Pungitius pungitius) (males: \( N = 24,308 \text{ genes}; \) females: \( N = 24,308 \text{ genes}; \) CPM: counts per million). Linear regression lines are shown. (B) Gene expression levels in male and female threespine stickleback and male and female ninespine stickleback were normalized to their respective median autosomal gene expression. Median threespine stickleback:ninespine stickleback expression ratios were measured across the autosomes and the threespine stickleback sex chromosomes. Medians that are significantly greater than ancestral levels are indicated in green, medians that are significantly less than ancestral levels are indicated in red, and medians that do not differ from ancestral levels are indicated in black (Mann–Whitney U test, \( \mu = 0 \), autosomes and the sex chromosomes were corrected for multiple comparisons separately). Within each region (autosomes: \( N = 24,308 \text{ genes}; \) PAR: \( N = 64 \text{ genes}; \) stratum 2: \( N = 496 \text{ genes}; \) stratum 1 X and Y alleles: \( N = 65 \text{ genes}; \) stratum 1 X hemizygous: \( N = 522 \text{ genes} \)), significant differences between males and females are marked with asterisks (pairwise Mann–Whitney U test, corrected for multiple comparisons, **\( P < 0.05 \).** "X & Y alleles" refer to the genes within stratum 1 that retain coding sequence on the Y chromosome. Whiskers are \( 1.5 \times \) the interquartile range. Outliers are not shown.
Reduced gene expression in males within older stratum 1 might reflect a lack of global dosage compensation. To more closely assess whether dosage compensation may be operating in males, we compared male and female gene expression ratios in each of the regions across the sex chromosomes. In the PAR and stratum 2, the expression in males and females did not differ significantly (Mann–Whitney U test; PAR: \(N = 65\) genes, \(P = 0.569\); stratum 2: \(N = 496\) genes, \(P = 0.168\)) (fig. 3B). In stratum 1, there were two different patterns. Among the genes of stratum 1 that retained a Y chromosome allele, male gene expression levels were lower than females, but this reduction was not statistically significant (Mann–Whitney U test, \(N = 65\) genes, \(P = 0.183\)), suggesting some maintenance of gene expression in males. In the hemizygous genes, males had a significantly lower median gene expression than females (Mann–Whitney U test, \(N = 522\) genes, \(P < 0.001\)), matching female-biased expression patterns previously observed in this region (Leder et al. 2010; Schultheiss et al. 2015). Lower gene expression in hemizygous genes in males argues against a global mechanism for dosage compensation. Our results contrast previous work which postulated that dosage compensation was evolving in stratum 1 on the basis of sex chromosome to autosome expression ratios of hemizygous genes with no comparison with ancestral expression levels (Schultheiss et al. 2015). By comparing the expression of sex-linked genes with their autosomal orthologs in the outgroup, we find no evidence for upregulation of hemizygous genes in either males or females, indicating a general lack of dosage compensation across stratum 1.

**X-Biased Gene Expression in Males**

Males had similar median gene expression ratios to females in regions of the sex chromosomes that still possessed a Y chromosome allele (stratum 2 and stratum 1 X and Y alleles). However, simply assessing relative expression levels in males and females alone does not reveal whether similar expression ratios in these regions are the result of increased expression from the X chromosome and/or maintenance of transcript expression from the Y chromosome. To distinguish these possibilities, we examined allele-specific expression to quantify whether Y chromosome alleles matched the expression of X chromosome alleles. In both stratum 2 and stratum 1, X chromosome alleles were expressed at significantly higher levels than Y chromosome alleles (one-sample Mann–Whitney U test, \(mu = 0\), \(N = 52\) genes, \(P = 0.003\); stratum 2: \(N = 414\) genes, \(P < 0.001\)), indicating either some loss of Y chromosome expression or an upregulation of the X chromosome (i.e., dosage compensation) (fig. 4). To distinguish the two possibilities, we examined whether male or female gene expression (normalized to ancestral proto-X expression) changed in response to decreasing expression of the Y chromosome allele. If X-biased gene expression is caused by dosage compensation, overall male gene expression should remain equal to female expression as transcription from the Y chromosome decreases. On the other hand, if X-biased gene expression is mainly due to downregulation of Y chromosome genes without dosage compensation, male gene expression should decrease as gene expression is lost from the Y chromosome. In both strata, among genes where X chromosome expression more closely matched Y chromosome expression, male and female median gene expression was more similar (Mann–Whitney U test; stratum 1: \(N = 8\) genes, \(P = 0.557\); stratum 2, \(N = 65\) genes, \(P = 0.557\)) (fig. 5). In stratum 2, when X-biased expression increases, male gene expression decreases compared with females (Mann–Whitney U test, \(N = 67\) genes, \(P < 0.001\)), suggesting no dosage compensation (fig. 5A). In stratum 1, male gene expression was reduced to a similar magnitude among the most X-biased genes, but this result was not significant (Mann–Whitney U test, \(N = 8\) genes, \(P = 0.482\)) (fig. 5B). The lack of significance was likely due to the small sample size of stratum 1 (upper quartile of X-biased genes in stratum 1, \(N = 8\); upper quartile of X-biased genes in stratum 2, \(N = 67\)). Unlike in *S. latifolia* where local dosage compensation may be evolving (Muyler et al. 2012), our results suggest that X-biased gene expression in *G. aculeatus* is primarily driven by a decay of transcription from the Y chromosome. This argues that most genes that have degenerated across the sex chromosomes do
not have strong haploinsufficiency phenotypes that would require a fine-tuning of dosage in the heterogametic sex.

**Retained Genes Are Enriched for Dosage-Sensitive Functions**

However, some genes were retained under purifying selection in stratum 1, so we explored whether they were enriched for certain classes of genes. Using the X chromosome hemizygous genes in stratum 1 as the reference pool, there was a significant overenrichment of genes predicted to have a role in intracellular protein transport among the genes that retained a Y chromosome allele (stratum 1 X and Y alleles, \( N = 49 \) total genes, 8 intracellular protein transport genes; stratum 1 X hemizygous genes, \( N = 398 \) total genes, 7 intracellular protein transport genes; Fisher’s exact test, \( P = 0.026 \), false discovery rate [FDR] corrected) (fig. 6). We conducted permutation tests to explore whether stratum 1 had an unusually high complement of intracellular protein transport genes by randomly drawing stratum 1-sized regions across the autosomes. We did not detect a significantly higher density of these genes in stratum 1 than on the autosomes (8 Mb regions, 10,000 random permutations, \( P = 0.070 \)). Therefore, our results do not reflect ancestral clustering of genes with a function in intracellular transport and instead likely reflect the outcome of strong purifying selection to maintain protein-coding function of important housekeeping genes on the Y chromosome. A core set of housekeeping genes are also conserved across a wide range of highly degenerate mammalian Y chromosomes (Bellott et al. 2014; Cortez et al. 2014) preserved under purifying selection because of dosage constraints. Interestingly, known dosage-sensitive genes in the yeast genome are enriched for intracellular transport (Makanae et al. 2013), indicating that this function is particularly sensitive to dosage imbalances.

Genes involved in protein complexes should also be sensitive to dosage imbalances, because altering the dosage of a single member will imbalance the stoichiometry of the entire protein complex (Papp et al. 2003; Pessia et al. 2012; Makanae et al. 2013). We searched for orthologs of mammalian protein complex genes (identified using the Comprehensive Resource of Mammalian Protein Complexes [CORUM] database) within the remaining genes of stratum 1. We found a highly significant enrichment of genes involved in protein complexes in the remaining genes of stratum 1, compared with the hemizygous genes of stratum 1 (stratum 1 X and Y alleles, \( N = 65 \) total genes, 21 protein complex genes; stratum 1 X hemizygous genes, \( N = 522 \) total genes, 58 protein complex genes; Fisher’s exact test, \( P < 0.001 \)). It is possible that this enrichment could be an artifact resulting from ancestral clustering of protein complex genes within stratum 1. To investigate this, we conducted the same permutation tests as above to determine whether stratum 1 had a significantly higher density of protein complex genes than the autosomes. Unlike the intracellular protein transport genes, stratum 1 did contain a higher number of protein complex genes than most of the autosomal regions (8 Mb regions, 10,000 random permutations, \( P = 0.050 \), indicating some ancestral clustering. However, ancestral clustering would not necessarily result in enrichment of protein complex genes preferentially within regions that retained Y chromosome alleles. If gene loss within stratum 1 was a stochastic process, there should not be a difference in the number of protein complex genes between the hemizygous genes and the genes that still possess a Y chromosome allele. Instead, our results suggest that selection differentially favored the retention of Y chromosome alleles for many of the protein complex genes. Consistent with this, protein complex genes that retained a Y chromosome allele were significantly overenriched for functions related to intracellular protein transport.
chromosome allele interacted with a higher number of proteins than protein complex genes that are hemizygous (median number of interacting proteins of remaining genes in stratum 1: 25, N = 21; median number of interacting proteins of hemizygous genes in stratum 1: 9, N = 58; Mann–Whitney U test, P = 0.001). Proteins with the highest number of interactions in complexes were found to be the most dosage sensitive in mammals (Pessia et al. 2012). Thus, our data suggest that the genetic architecture of the threespine stickleback Y chromosome has been shaped by dosage sensitivity.

Conclusions

Our results provide a detailed characterization of Y chromosome evolution in the threespine stickleback. Despite extensive sequence divergence and gene loss, we did not find clear evidence of local dosage compensation across a majority of genes. Instead, X-biased gene expression in males was largely due to reduced expression of the Y chromosome allele. Dosage balance was likely maintained at some haploinsufficient genes by preserving function of the Y chromosome allele. Evidence of local dosage compensation across a majority of genes on this chromosome are already diverging from the neo-X (Yoshida et al. 2014). Furthermore, the fourspine stickleback (Apeltes quadracus) will be useful to compare male and female heterogametic systems. The fourspine stickleback ZZ/ZW system (Chen and Reisman 1970; Ross et al. 2009; Urton et al. 2011) is approximately of the same age as the threespine stickleback XX/XY system, enabling a direct comparison of whether the Y or W chromosomes degrade faster (Naurin et al. 2010). Thus, the stickleback family (Gasterosteidae) offers a rich resource to explore sequence divergence and dosage compensation across the early stages of sex chromosome evolution in vertebrates.

Materials and Methods

DNA and RNA Sequencing

All research on live animals was approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol 1575) and the National Institute of Genetics, Japan (protocol 25-15). Genomic DNA was isolated from four male and four female adults, which were the laboratory-reared progeny of wild-caught fish collected in the Bekanbeushi River, Akkeshi, Japan, from the Japanese Pacific Ocean population of G. aculeatus (Kitano et al. 2007, 2009), and from a single female P. pungitius collected from a tidepool in Biwase, Japan (Ishikawa et al. 2013). Whole caudal fin clips were digested overnight in 0.33 μg/ml proteinase-K at 55°C. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), following manufacturer recommended protocols. For G. aculeatus, paired-end reads were sequenced for 50 cycles using the Illumina Genome Analyzer Ix (GAIIx) system by the Genomics Shared Resource at The Fred Hutchinson Cancer Research Center. For P. pungitius, paired-end reads were sequenced for 100 cycles on a HiSeq2000 by the Takara Bio Dragon Genomics Center (Mie, Japan). Each individual was sequenced to at least 10× coverage on average, with one Pacific Ocean male sequenced to 40× coverage.

Total RNA was isolated from the whole brain tissue of nine male and nine female G. aculeatus individuals, which were siblings of the Japanese Pacific Ocean fish used for DNA sequencing. Total RNA was also isolated from the whole brain tissue of three male and three female P. pungitius individuals, which were laboratory-reared fish raised from a single male and female collected from the tidepool in Biwase, Japan. Brain tissue was homogenized in TRIzol reagent (Invitrogen) and RNA was extracted following manufacturer recommended protocols. For G. aculeatus, each RNA sample replicate was composed of a pool of three different individuals of each sex. There were a total of three male replicates and three female replicates. Single-end reads were sequenced for 50 cycles using the Illumina Genome Analyzer Ix (GAIIx) system by the Genomics Shared Resource at The Fred Hutchinson Cancer Research Center. For P. pungitius, RNA was sequenced from individual brains of each male and female, rather than pooled samples, also resulting in three male replicates and three female replicates. For P. pungitius, RNA libraries were constructed with the TruSeq RNA Sample Preparation Kit (Illumina). Paired-end reads were sequenced for 150 cycles on the rapid run mode of HiSeq2500 by the Riken Genesis (Yokohama, Japan). For both the RNA and DNA sequencing, the reads were cleaned of adapters and barcodes, and only reads that passed the default Illumina
CASAVA chastity threshold (≥0.6) were used in downstream analyses.

Sequence Assembly and Transcriptome Annotation
Paired-end DNA sequencing reads were aligned using Bowtie 2 (v. 2.0.2) (Langmead and Salzberg 2012) to the G. aculeatus reference genome, which was generated from a single individual female from a lake population in Alaska (BROADS1) (Jones et al. 2012). Physical positions of the X chromosome (i.e., chromosome 19) were based on a revised version of the X chromosome assembly (Ross and Peichl 2008). The PAR boundary and putative deleted region (region D) boundary were set at 2.50 and 12.00 Mb, respectively (Roesti et al. 2013). Boundaries for the pericentric inversions (regions A, B, and C) were set as the midpoints between Snt187/Snt 235 (6.25 Mb) and Idh/Snt194 (11.50 Mb) (Ross and Peichl 2008). For the sequencing reads from the Japanese Pacific Ocean G. aculeatus fish, the following parameters were used: -D 15 -R 2 -N 0 -L 22 -i S,1,1,15 –rdg 5,3 –rfg 5,3 –mp 6,2. These default parameters produced an average alignment rate of 90.8% of female reads and 89.5% of male reads. For the sequencing reads from the P. pungitius fish, less stringent alignment parameters were used to compensate for greater sequence divergence between the two species (Bruneaux et al. 2013) (-D 20 -R 3 -N 1 -L 20 -i S,1,0,50 –rdg 3,2 –rfg 3,2 –mp 3). The less stringent parameters resulted in an alignment rate of 46.4% of the female G. aculeatus reads to the G. aculeatus reference genome (only 12.2% of the reads aligned using the default parameters above).

RNA sequencing reads were aligned to the G. aculeatus reference genome and assembled using a combination of TopHat (v. 2.0.6) (Kim et al. 2013) and Cufflinks (v. 2.2.1) (Roberts et al. 2011). For G. aculeatus RNA sequencing reads, the following parameters were used: -b2-D 15 –b2-R 2 –b2-N 0 –b2-L 20 –b2-i S,1,1,15 –rdg 5,3 –rfg 5,3 –b2-mp 6,2 -N 2 –max-insertion-length 3 –max-deletion-length 3 –read-gap-length 2 –read-edit-dist 2. These default parameters produced an average alignment rate of 86.6% of female reads and 83.4% of male reads. Similar to the DNA alignments, less stringent TopHat parameters were used to increase the alignment rate of P. pungitius RNA sequencing reads to the reference genome (–b2-D 20 –b2-R 3 –b2-N 1 –b2-L 20 –b2-i S,1,0,50 –b2-rdg 3,2 –b2-rfg 3,2 –b2-mp 3,1 -N 36 –max-insertion-length 12 –max-deletion-length 12 –read-gap-length 24 –read-edit-dist 50). With these parameters, 70.3% of female reads and 63.6% of male reads aligned to the reference genome (only 2.3% of female reads aligned using the default parameters).

To define the total G. aculeatus transcriptome, only female RNA sequencing reads were used. Male reads were not included in the transcript annotations, as transcripts on the Y chromosome harbor a large number of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels), complicating transcript assembly when included with the X chromosome. All three pools of female RNA reads were combined and aligned to the G. aculeatus reference genome with TopHat and assembled with Cufflinks, both using default parameters. The total set of transcripts used for analyses included all Cufflinks transcripts (1,108 transcripts from 848 genes) as well as all predicted transcripts in the Ensembl (release 69) database (846 transcripts from 661 genes), allowing for overlapping transcripts between the two annotation methods (1,954 combined transcripts from 1,509 combined genes).

Molecular Evolution of the X and Y Chromosomes
SNP and indel variants were called using the Genome Analysis Toolkit (GATK v. 2.2-16) (McKenna et al. 2010; DePristo et al. 2011). First, reads were locally realigned around indels in each male and female G. aculeatus DNA sample (using the RealignerTargetCreator and IndelRealigner tools). Variants were then called simultaneously across the four male samples and simultaneously across the four female samples. To maximize the number of variants detected from the Y chromosome, variants were called less stringently with UnifiedGenotyper (–genotype_likelihoods_model BOTH -stand_call_conf 4 –stand_emit_conf 0 -dcov 200). Variants were called in the single female P. pungitius DNA sample using the same parameters for UnifiedGenotyper (–genotype_likelihoods_model BOTH -stand_call_conf 4 –stand_emit_conf 0 -dcov 200). To reduce the number of false positives in G. aculeatus, variants were only considered if the SNP or indel was heterozygous in all four males and homozygous in all four females. The allele that was homozygous in females was treated as the X chromosome allele, whereas the alternate allele in males was treated as the Y chromosome allele. Custom Perl scripts were used to filter the SNPs and indels and to call the X and Y chromosome alleles (available from Dryad Digital Repository).

Synonymous ($d_{s}$) and nonsynonymous ($d_{n}$) site divergence was quantified in protein-coding regions of the transcripts defined above; the protein-coding regions were defined as the longest open reading frame (ORF) among all six reading frames. Divergence was only estimated in ORFs greater than 200 bp in length. The X chromosome, Y chromosome, and P. pungitius DNA sequences of each ORF were constructed from the GATK variants and G. aculeatus reference genome by substituting in the X chromosome, Y chromosome, and P. pungitius SNP and indel variants from the filtered UnifiedGenotyper output into the G. aculeatus reference genome. Nucleotide positions where the P. pungitius read depth was less than two were considered missing and were coded as “N” in the analysis. Divergence was not calculated where more than 10% of the ORF sequence was missing from P. pungitius. In the G. aculeatus data, coding sequences with no variants can reflect no divergence within the region, but can also be due to a lack of Y chromosome reads aligning to the region. Therefore, average read depth across all four males in the coding region was used to determine if a region was missing Y chromosome sequence, that is, was hemizygous. The minimum read depth was empirically determined from coding regions within the recombining PAR (average read depth: 59.20) and from coding regions in the nonrecombining region where Y chromosome variants were detected.
to more than one location in the genome were not considered in the calculation of gene expression ratios. Genes with a DNA read depth below 45 on the autosomes, within the PAR of the sex chromosomes, and within stratum 2 of the sex chromosomes were not considered (see above). Reads were normalized across the three male G. aculeatus replicates, the three female G. aculeatus replicates, the three male P. pun-gitius replicates, and the three female P. pun-gitius replicates by calculating scaling factors with the trimmed mean of M-values method implemented in the Bioconductor package, edgeR, which minimizes the log-fold changes of gene expression between samples (Robinson et al. 2010). To calculate a single gene expression value for female G. aculeatus, male G. aculeatus, female P. pun-gitius, and male P. pun-gitius, the normalized read counts per million (Robinson et al. 2010) were averaged across the three replicates in each of these groups.

Allele-Specific Transcript Expression in Gasterosteus aculeatus Males

Male RNA reads were combined across the three sample replicates. Ambiguous reads that mapped to more than one location in the genome were not considered in the counts. The X and Y SNP variants discovered by the UnifiedGenotyper module of GATK (see above) were used to identify X and Y RNA SNP variants within each ORF. The number of X and Y RNA alleles present at each SNP within an ORF were summed to derive the total X:Y RNA expression ratio. Because transcripts expressed at low levels have a higher chance of being skewed stochastically toward either allele, only SNPs with a minimum total RNA read depth of four were considered. Short sequencing reads are subject to mapping biases (Dohm et al. 2008; Bullard et al. 2010), which could differentially affect the X chromosome or Y chromosome read counts. To correct for mapping biases, the X:Y RNA expression ratio was normalized to the X:Y DNA ratio. To have an accurate estimate of X and Y chromosome allele counts in the DNA sequences, SNPs were only used if there was a minimum read depth of six in each of the four male DNA sequences. It is important to note that transcripts were only considered if they contained at least one variant between the X and Y chromosomes within the DNA samples; thus, the status of hemizygous genes that no longer have a recognizable homolog on the Y chromosome could not be investigated in this analysis. If a gene had multiple transcripts, the X and Y chromosome read counts were averaged across transcripts to provide single gene estimates. A custom Perl script was written to compute the normalized X:Y RNA expression ratios (available from Dryad Digital Repository).

Functional Annotation of Genes

The total pool of genes were functionally annotated using the Blast2GO software package (v. 2.6.6) (Götz et al. 2008). If there were multiple transcripts per gene, genes consisted of the union of all transcripts. The remaining genes within region
Dosage-Sensitive Genes during Y Degeneration


