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Accumulation of Deleterious Mutations on the Neo-Y Chromosome of Japan Sea Stickleback (*Gasterosteus nipponicus*)

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Abstract

Degeneration of Y chromosomes is a common evolutionary path of XY sex chromosome systems. Recent genomic studies in flies and plants have revealed that even young neo-sex chromosomes with the age of a few million years show signs of Y degeneration, such as the accumulation of nonsense and frameshift mutations. However, it remains unclear whether neo-Y chromosomes also show rapid degeneration in fishes, which often have homomorphic sex chromosomes. Here, we investigated whether a neo-Y chromosome of Japan Sea stickleback (*Gasterosteus nipponicus*), which was formed by a Y-autosome fusion within the last 2 million years, accumulates deleterious mutations. Our previous genomic analyses did not detect excess nonsense and frameshift mutations on the Japan Sea stickleback neo-Y. In the present study, we found that the nonrecombining region of the neo-Y near the fusion end has accumulated nonsynonymous mutations altering amino acids of evolutionarily highly conserved residues. Enrichment of gene ontology terms related to protein phosphorylation and cellular protein modification process was found in the genes with potentially deleterious mutations on the neo-Y. These results suggest that the neo-Y of the Japan Sea stickleback has already accumulated mutations that may impair protein functions.

Subject area: Genomics and gene mapping
Key words: fish, sex chromosome, stickleback

Sex chromosomes have evolved from an ordinary pair of autosomes independently in multiple taxa (Bull 1983; Bachtrog et al. 2014; Beukeboom and Perrin 2014). One of the common features of sex chromosome evolution is the degeneration of Y chromosomes in XY systems and W chromosomes in ZW systems, where genes on the Y or W become functionally impaired and are often completely lost (Bachtrog 2013; Charlesworth and Charlesworth 2000; Charlesworth et al. 2005). Erosion of Y (or W) chromosomes can occur as a consequence of recombination suppression via multiple mechanisms, such as Muller's ratchet, background selection, and hitchhiking (reviewed in Charlesworth and Charlesworth 2000). Well-studied sex chromosomes, such as Y chromosomes in mammals and *Drosophila*, show extensive erosion of Y such that only a handful of functional genes are retained. Furthermore, recent genomic studies have revealed that even young neo-sex chromosomes in *Drosophila miranda* (about 1 million years old), *Rumex hastatulus* (15–16 million years old), and *Silene latifolia* (less than 10 million years old) showed several signs of degeneration, such as accumulation of frameshift and nonsense mutations (Bachtrog et al. 2008; Zhou and Bachtrog 2012; Hough et al. 2014; Papadopulos et al. 2015).

Therefore, the evolution of degenerate and heteromorphic sex chromosomes seems to be an inevitable path for sex chromosomes.

However, some taxa, including fishes and amphibians, often have homomorphic sex chromosomes (Beukeboom and Perrin 2014). The absence of degeneration may be due to the young ages of sex chromosomes because of frequent turnovers of sex chromosomes in these taxa (Blaser et al. 2013). However, old homomorphic sex chromosomes have often been found (Beukeboom and Perrin 2014). It is also possible that rare recombination (Guerrero et al. 2012), such as recombination in YY and WW individuals that originated from sex-reversed individuals (Perrin 2009), contribute to the absence of degeneration. Because the extensively investigated neo-sex chromosomes in *Drosophila* do not recombine in males, which might promote the neo-Y degeneration in these taxa (Bachtrog et al. 2008; Zhou and Bachtrog 2012), it is difficult to disentangle the factors that lead to the evolution of heteromorphic sex chromosomes by studying a few systems. Therefore, studies on sex chromosomes with different ages and different levels of recombination suppression across diverse taxa are essential for a better understanding of the general patterns of sex chromosome evolution.

In the present study, we investigated whether a neo-Y chromosome of a stickleback fish species accumulates deleterious mutations. The stickleback family is a good model system for studying sex chromosome evolution because different species within the stickleback family have different sex chromosomes of different ages (Peichel et al. 2004; Ross et al. 2009; Urton et al. 2011). For example, linkage group (LG) 19 is linked to sex in threespine stickleback (*Gasterosteus aculeatus*) (Peichel et al. 2004; Ross and Peichel 2008; White et al. 2015), while LG9 is additionally linked to sex in a closely related species, the Japan Sea stickleback (*Gasterosteus nipponicus*) (Kitano et al. 2009; Yoshida et al. 2014). In the Japan Sea stickleback, LG9 fused with the Y chromosome within the last 2 million years ago and suppression of recombination has evolved near the fusion with recombination rate decreasing toward the fusion end (Kitano et al. 2009; Natri et al. 2013). Previously, we conducted whole genome sequencing of the Japan Sea stickleback and found that the neo-X and neo-Y have started to diverge at nucleotide sequence levels near the fusion end (Yoshida et al. 2014). However, we did not find any clear signs of neo-Y degeneration, such as deletion of genes or accumulation of frameshift and nonsense mutations. To further investigate whether the Japan Sea neo-Y has accumulated any deleterious mutations, we employed a Protein Variation Effect Analyzer (PROVEAN) (Choi et al. 2012; Choi and Chan 2015) and a SIFT (*s*orts *i*ntolerant *f*rom *t*olerant substitutions) (Kumar et al. 2009), which can detect amino acid-changing mutations that alter phylogenetically conserved residues (for details, see below). Because phylogenetically conserved residues are generally considered to be functionally important, any mutations that occur at highly conserved sites are predicted to disturb protein function (Ng and Henikoff 2006).

After detecting genes with potentially deleterious mutations using PROVEAN and SIFT, we investigated whether the neo-Y genes with and without deleterious mutations differ in any functional characteristics. Studies on the Y chromosomes of mammals and threespine sticklebacks have indicated that the majority of genes retained on the Y are dosage-sensitive genes (Bellott et al. 2014; Cortez et al. 2014; White et al. 2015). In *Drosophila*, male-biased genes tend to be retained on Y chromosomes (Kaiser et al. 2011). Therefore, we tested whether intact genes on the Japan Sea stickleback neo-Y are more likely to be dosage-sensitive and/or show more male-biased expression than genes with deleterious mutations in an ancestral threespine stickleback population that lacks the neo-sex chromosomes (Pacific Ocean population).

Materials and Methods

Screening of Deleterious Mutations

Whole genome sequencing of 5 Japan Sea males, 5 Japan Sea females, and 5 Pacific Ocean threespine stickleback females was conducted with a 100 bp paired-end mode of HiSeq2000 at high coverage (over 40× coverage per fish) and described previously (Yoshida et al. 2014). All sequence data are available from DDBJ (DRA001136). Single nucleotide polymorphism (SNP) calling and identification of putatively neo-X-specific and neo-Y-specific SNPs were also described previously (Yoshida et al. 2014). Briefly, when all 5 females of the Japan Sea stickleback have a homozygous genotype at a particular nucleotide position, all 5 females of the Pacific Ocean stickleback have a different homozygous genotype at the same site, and all 5 males of the Japan Sea stickleback have a heterozygous genotype, this SNP was categorized as a putatively neo-X-specific SNP. When all females of the Japan Sea stickleback ($N = 5$) and the Pacific Ocean stickleback ($N = 5$) have the same homozygous genotype and all 5 males of Japan Sea stickleback have a heterozygous genotype, this SNP was categorized as a putatively neo-Y-specific SNP. Our previous study showed that these SNPs were accumulated at the fusion end of LG9 (Figure 1A), and some of these SNPs were located in the coding regions (Figure 1B) (Yoshida et al. 2014).

To compare these sex chromosome-specific SNPs with autosomal SNPs, we additionally identified Japan Sea-specific autosomal SNPs: when all 5 females of Pacific Ocean stickleback and one female of an outgroup species, *Gasterosteus wheatlandi*, have the same homozygous genotype, but all 5 females of Japan Sea stickleback have a different homozygous SNP, this SNP was judged as a Japan Sea-specific autosomal SNP. Whole genome sequences of *G. wheatlandi* have been reported previously (Yoshida et al. 2014) and are available from DDBJ (DRA001086).

To search for mutations that can cause deleterious amino acid changes, we used PROVEAN (Choi et al. 2012) and SIFT (Kumar et al. 2009). Both methods are based on the idea that phylogenetically conserved amino acids are functionally important and substitutions altering the conserved sites are deleterious (Ng and Henikoff 2006). PROVEAN analysis was conducted as described previously (Yoshida et al. 2016). PROVEAN collects orthologs of other species from the NCBI NR protein database and examines the differences in the alignment scores between a protein query sequence and its variant. We calculated the PROVEAN scores of nonsynonymous mutations (http://provean.jcvi.org) (Choi and Chan 2015), which reflect the impacts of mutations on the alignment scores. The threshold of PROVEAN scores for definition of deleterious mutations was set to −2.5 as recommended by Choi et al. (2012). To confirm our results, we used another method SIFT (Kumar et al. 2009). SIFT also searches for orthologs of other species and conducts multiple alignments to calculate normalized probabilities of substitutions at each position (Kumar et al. 2009). Substitutions at a site with normalized probabilities lower than 0.05 were considered to be deleterious. SIFT is reported to have higher sensitivity, but lower specificity than PROVEAN (Choi et al. 2012), so the use of both methods is important. PROVEAN and SIFT results are available from Dryad (http://dx.doi.org/10.5061/dryad.v8rq0). Sliding window analysis of the density of deleterious SNPs per nucleotides was done as described previously (Yoshida et al. 2014) except that we used the physical positions of the revised genome assembly of threespine stickleback

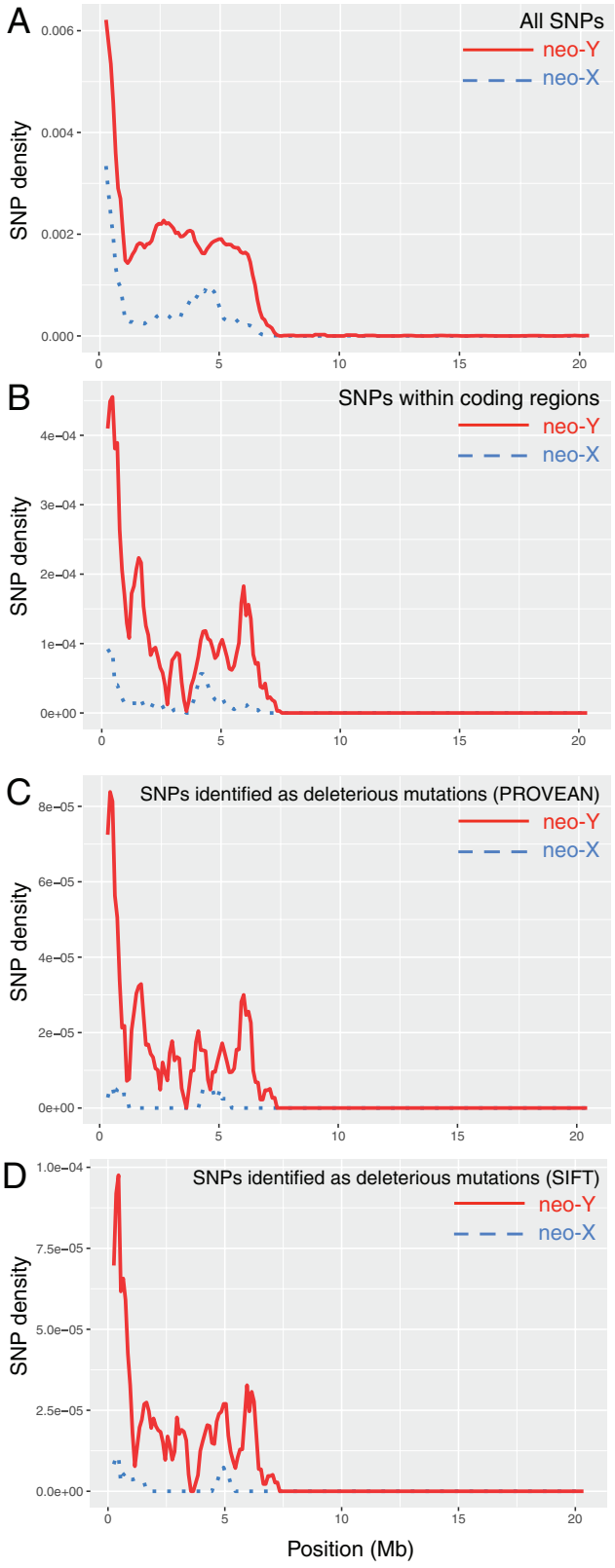

Figure 1. Sliding window analyses of density of putatively neo-X-specific and neo-Y-specific SNPs on LG9 in the Japan Sea stickleback (*Gasterosteus nipponicus*). The *y* axis indicates the density of SNPs among total number of nucleotides within the window: all SNPs (**A**), SNPs within coding regions (**B**), and deleterious SNPs identified with PROVEAN (**C**) and SIFT (**D**). The window size and step size were 500 and 100 kb, respectively. Here, we used the physical positions of the revised genome assembly of threespine stickleback (Glazer et al. 2015).

(Glazer et al. 2015). The window size and step size were 500 and 100 kb, respectively.

Previously, we found that neo-X and neo-Y specific SNPs accumulated at the fusion side of LG9 (left side of LG9 in Figure 1A) (Yoshida et al. 2014). Therefore, only the X-Y diverged region (<7.5 Mb) on LG9 was analysed in the present study (also see the Results section). Within the 7.5 Mb region, we tested whether the presence/absence of deleterious mutations in a gene is associated with its physical position using a generalized linear model with a logit link function. We used the midpoint of each gene as its physical position.

Characterization of Genes with Potentially Deleterious Mutations

For functional enrichment analyses of genes with deleterious mutations, we downloaded the gene ontology database from the Ensembl database (http://asia.ensembl.org). Then, we tested whether any GO terms were enriched or under-represented in the genes with deleterious mutations. We conducted 2-tailed tests with mid *P*-values of a hypergeometric distribution (Rivals et al. 2007) and calculated *q*-values (false discovery rate-corrected *P*-values) (Benjamini and Hochberg 1995). For the neo-Y specific genes, we compared GO terms between genes with neo-Y-specific deleterious mutations and all genes located on the X-Y diverged region of LG9. For testing Japan Sea-specific deleterious mutations, genes with deleterious mutations on the autosomes (all LGs except LG9 and LG19) were compared with all genes on the autosomes. When only a few genes in the region of X-Y divergence have a particular GO term, over- or under-representation of that GO term cannot be tested, so we analyzed only GO terms that were annotated with 5 or more genes in the region.

To investigate whether the magnitude of sex bias in gene expression in ancestral species is correlated with the level of gene degeneration on the neo-Y, we used previously published gonad microarray expression data of 4 males and 4 females from the Pacific Ocean population in which LG9 is an autosome; the microarray contained 48 009 unique oligonucleotide probes representing 23 224 genes (Yoshida et al. 2014). First, the median expression value of each sex was calculated for each probe and then the median $\log_2$-fold difference of the expression value between sexes was calculated for each gene when multiple probes were designed for a single gene. We used a likelihood ratio test to compare the goodness of fit between a model with only the physical position of each gene in the explanatory variable (null model) and a model with the $\log_2$-fold sex difference as an additional explanatory variable (alternative model).

Finally, we tested the hypothesis that dosage-sensitive genes are less likely to accumulate deleterious mutations. Because protein complex members tend to be dosage sensitive (Makino et al. 2009; Makanae et al. 2013), we statistically tested whether there is an under-representation of genes encoding protein complex members in the genes with the deleterious mutations. Information of protein complex members in humans was downloaded from Human Protein Reference Database (HPRD; http://www.hprd.org), as described previously (Makino and McLysaght 2010). Because this protein database was annotated with human gene lists, we identified human orthologs of stickleback genes using the output table of Ensembl Biomart (http://www.ensembl.org/biomart/, Ensembl Gene 83) downloaded from the threespine stickleback dataset with "Human Orthologues" in "Attributes." It should be noted that not all stickleback genes were annotated with human orthologs, so the number of genes used for the analysis of protein complex membership was lower than the number of stickleback genes

with deleterious mutations. We used a likelihood ratio test to compare between a model with only the physical position of each gene in the explanatory variable (null model) and a model including the binary information about whether the gene is a member of protein complexes as an additional explanatory variable (alternative model). Because some stickleback genes have multiple human orthologs, we used an iterative approach (100 iterations) to avoid pseudoreplications: in each iteration, we randomly chose one human ortholog from multiple candidate human orthologs and calculated the median *P*-value.

Results

Figure 1 shows the density of neo-X and neo-Y specific SNPs within 500 kb-sliding windows on LG9. SNPs of these categories accumulated near the fusion end (left side on LG9 in Figure 1A, B) (Yoshida et al. 2014). Accordingly, deleterious neo-X and neo-Y specific SNPs were found only within 7.5 Mb from the fusion end (Figure 1C, D). Even within this 7.5 Mb-region, the side closer to the fusion had more deleterious SNPs (generalized linear model; $P < 0.001$; Supplementary Table S1).

PROVEAN analysis revealed 7 neo-X-specific deleterious mutations in 7 genes, 101 neo-Y-specific deleterious mutations in 65 genes, and 1459 Japan Sea-specific autosomal deleterious mutations in 1208 genes (Figure 1C and Table 1). A significantly higher percentage of mutations in coding regions were deleterious in the neo-Y-specific SNPs (15.2%) compared to the Japan Sea-specific autosomal SNPs (4.1%; Fisher's exact test, $P < 0.001$). In contrast, similar percentages of mutations in coding regions were deleterious between the neo-X-specific SNPs (6.6%) and the Japan Sea-specific autosomal SNPs (Fisher's exact test, $P = 0.212$). Qualitatively similar results were obtained using SIFT for detecting deleterious mutations (Figure 1D and Table 1).

Because the average number of SNPs per gene was lower for neo-Y-specific SNPs than for the Japan Sea-specific autosomal SNPs (Supplementary Table S2), we could exclude the possibility that the higher frequencies of deleterious mutations on the neo-Y were biased by a higher frequency of neo-Y-specific SNPs per gene. The lower frequency of neo-Y-specific SNPs per gene than that of the Japan Sea-specific autosomal SNPs is likely due to the different ages of divergence: divergence between the Japan Sea and the Pacific Ocean autosomes might have occurred earlier than divergence between the Japan Sea neo-X and neo-Y.

GO analysis showed that several GO terms were significantly enriched in the genes with neo-Y-specific deleterious mutations (Table 2). Enriched GO terms were similar between PROVEAN and SIFT analyses: enriched terms detected in both analyses included ion binding, calcium binding, protein binding, cellular protein modification process, and kinase activity. Although ion binding, calcium binding, and protein binding were also enriched in Japan Sea-specific autosomal SNPs (Supplementary Tables S3 and S4), cellular protein modification process and kinase activity were enriched only in the genes with neo-Y specific deleterious SNPs.

Next, we tested whether there is an association between the magnitude of sex bias in gene expression in gonads of the Pacific Ocean stickleback and the accumulation of deleterious mutations on the Japan Sea neo-sex chromosomes. No associations were found: likelihood ratio tests; $P = 0.10$ and $P = 0.58$ for PROVEAN and SIFT analyses, respectively. Finally, we tested whether genes encoding protein complex members tend to remain intact on the neo-Y, but no significant under-representation of genes encoding protein complex members was found in the genes with the deleterious mutations (Supplementary Table S4): likelihood ratio tests; median $P = 0.77$ and median $P = 0.75$ for PROVEAN and SIFT analyses, respectively.

Discussion

Here, we found that the Japan Sea neo-Y chromosome has started to accumulate deleterious mutations, although no clear signs of neo-Y degeneration such as deletion of genes or accumulation of frameshift and nonsense mutations were found in our previous study (Yoshida et al. 2014). Our present data indicate that 65 and 79 genes on the neo-Y have at least one potentially deleterious mutations in the PROVEAN and SIFT analyses, respectively. The accumulation of potentially deleterious mutations at the fusion end on the neo-Y is also consistent with the general idea that recombination suppression leads to the accumulation of deleterious mutations on Y chromosomes, because the region near the fusion end on LG9 does not recombine between the neo-X and neo-Y (Kitano et al. 2009; Natri et al. 2013). Thus, our results indicate that the accumulation of deleterious mutations altering functionally important amino acids may be one of the first steps of Y degeneration.

Our GO analysis showed that the accumulation of deleterious mutations on the neo-Y may not be a simple random process, because genes involved in protein phosphorylation and protein modification were over-represented in the genes with deleterious mutations on the Japan Sea neo-Y, but not on the autosomes. Because sticklebacks lack chromosome-wide mechanisms of dosage compensation (Leder et al. 2010; White et al. 2015), deleterious mutations on the neo-Y should

Table 1. Number of nonsynonymous mutations and deleterious mutations identified with PROVEAN and SIFT

Method	Category	Neo-X-specific SNP[a]	Neo-Y-specific SNP[a]	Japan Sea-specific autosomal SNP
SNP-based count	Mutations within coding regions	106	663	35409
	Nonsynonymous mutations[b]	40 (37.7%)	335 (50.5%)	12491 (35.3%)
	Deleterious mutations (PROVEAN)[c]	7 (6.6%)	101 (15.2%)	1459 (4.1%)
	Deleterious mutations (SIFT)[c]	9 (8.5%)	119 (17.9%)	1958 (5.5%)
Gene-based count	Genes with mutations	63	188	11685
	Genes with nonsynonymous mutations[b]	34 (54.0%)	143 (76.1%)	6582 (56.3%)
	Genes with deleterious mutations (PROVEAN)[c]	7 (11.1%)	65 (34.6%)	1208 (10.3%)
	Genes with deleterious mutations (SIFT)[c]	7 (11.1%)	79 (42.0%)	1615 (13.8%)

[a]Neo-X-specific SNPs and neo-Y specific SNPs were analysed only in the 7.5 Mb X-Y diverged region on LG9, because no mutations in coding regions were found outside this region.

[b]The parentheses show the percentage of mutations in coding regions that are nonsynonymous or deleterious.

[c]The parentheses show the percentage of genes with mutations that have nonsynonymous or deleterious mutations.

Table 2. GO terms enriched in genes with putatively neo-Y-specific deleterious mutations

Method	GO category	GO	Proportion of genes[a]	*P*-value	*q*-Value
PROVEAN	Slim	0043167 // ion binding	25/82	1.4.E-03	0.026
		0006464 // cellular protein modification process	7/13	2.0.E-03	0.026
		0016301 // kinase activity	5/11	0.020	0.173
	Biological process	0006468 // protein phosphorylation	5/9	7.0.E-03	0.070
		0007165 // signal transduction	5/13	0.043	0.216
	Molecular function	0005515 // protein binding	25/71	8.2.E-05	1.1.E-03
		0005524 // ATP binding	10/24	3.1.E-03	0.022
		0004672 // protein kinase activity	5/9	7.0.E-03	0.024
		0004713 // protein tyrosine kinase activity	5/9	7.0.E-03	0.024
		0005509 // calcium ion binding	4/8	0.023	0.065
SIFT	Slim	0043167 // ion binding	29/82	1.8.E-03	0.047
		0006464 // cellular protein modification process	7/13	6.7.E-03	0.088
		0016301 // kinase activity	5/11	0.045	0.293
		0016887 // ATPase activity	3/5	0.042	0.293
	Biological process	0006468 // protein phosphorylation	5/9	0.017	0.168
	Molecular function	0005509 // calcium ion binding	6/8	1.1.E-03	0.015
		0005515 // protein binding	25/71	5.1.E-03	0.035
		0004672 // protein kinase activity	5/9	0.017	0.059
		0004713 // protein tyrosine kinase activity	5/9	0.017	0.059

[a]Ratios of the numbers of genes with neo-Y-specific deleterious mutations per total numbers of genes in the X-Y diverged regions.

cause haplo-insufficiency in males. However, amino acid changes of conserved residues are not always deleterious (Richards et al. 2015). Because protein phosphorylation and ubiquitination are involved in sexual differentiation (Bernard and Harley 2007; Mohapatra et al. 2013; Gao et al. 2014), we cannot exclude the possibility that neo-Y specific mutations may underlie some adaptive sex differences in the Japan Sea stickleback. Further molecular studies will be essential to understand the impacts of these mutations. Another caveat of our analyses is that both PROVEAN and SIFT rely on multiple alignments to seek mutations altering amino acids of conserved residues, so we may overlook deleterious mutations in rapidly evolving proteins (Ng and Henikoff 2006).

Although degeneration seems to be an inevitable fate of Y chromosomes (Charlesworth and Charlesworth 2000; Charlesworth et al. 2005), old homomorphic sex chromosomes have often been found in several taxa (Beukeboom and Perrin 2014). Rare events of recombination between X and Y are suggested to prevent the accumulation of deleterious mutations on Y (Perrin 2009; Guerrero et al. 2012). The absence of deleterious mutations past 7.5 Mb on LG9 is consistent with this idea, because the right side of LG9 can recombine at very low frequencies (Natri et al. 2013). Much more apparent signs of degeneration were found in the neo-Y chromosome of *Drosophila miranda* with a similar age to that of the Japan Sea stickleback (Bachtrog et al. 2008; Zhou and Bachtrog 2012), probably because recombination is completely suppressed in male *Drosophila*. Our results also suggest that detailed genomic studies may be able to determine subtle and initial signs of Y degeneration, such as the excess of mutations altering phylogenetically conserved amino acids, even in seemingly homomorphic Y chromosomes. Further genomic studies on sex chromosomes with varying ages and varying extents of recombination suppression across diverse taxa will lead to a better understanding of the general patterns of sex chromosome evolution. Because independently derived sex chromosomes with different ages can be found in fishes, including sticklebacks (Peichel et al. 2004; Kitano et al. 2009; Ross et al. 2009; Urton et al. 2011), medakas (Takehana 2011; Myosho et al. 2015), and cichlids (Lande et al. 2001; Ser et al. 2010; Yoshida et al. 2011), fishes offer further opportunities to investigate the general patterns of Y degeneration in independently derived young sex chromosomes.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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