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Sex-Specific Mutation Rates in Salmonid Fish

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Abstract. If germline mutations arise because of replication errors, the mutation rate may differ between males and females given that they differ in their number of germ cell divisions. As males of many higher organisms produce more gametes than females, this has led to the idea of "male-driven evolution." The extent of such male bias to the mutation rate is currently debated. For human some recent data suggest a very low bias, at a factor 1.7 only, while other approaches have given values of α_m (the male-to-female mutation rate ratio) of 5, which is more close to what might be expected from male and female germ cell biology. Comparative analyses of sex-specific mutation rates in other organisms may be necessary for understanding the generality of an effect of sex and the number of germline DNA replications on the mutation rate. In this study we estimate for the first time sex-specific mutation rates in fish. Comparing the intronic substitution rates of the autosomal *GH-2* gene and its duplicated Y-linked and male-specific copy *GH-2Y* (447–468 bp of each gene), we estimate α_m to be 5.35–6.60 in salmonid fish of the genus *Oncorhynchus*. To the observations previously made among mammals and birds, this adds evidence from another class of vertebrates showing that a majority of mutations are of paternal origin. This would suggest that replication errors play a major role for the generation of new mutations.

Key words: *Oncorhynchus* — Y chromosome — Germline — Mutation rate — Sex-bias

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Introduction

If germline mutations arise in connection with cell division (i.e., replication error), it may be expected that the mutation rate correlates with the number of germline cell divisions, everything else being equal. This simple assumption has led to the idea of "male-driven evolution" (Miyata et al. 1987), which implies that if males produce many more gametes than females, most mutations should arise in the male germline (and assuming the mutation rate per germline cell division being similar in the two sexes; hereafter the term mutation rate is understood as the rate of mutation per generation or time unit, not per cell division). A significant excess of cell divisions in spermatogenesis compared to oogenesis (the ratio of which is termed α) occurs in many phyla; for instance, in human α corresponds to $\approx$10 at mean age of reproduction (Vogel and Motulsky 1997). The positive relationship between number of germline cell divisions and mutation rate was recognised by Haldane (1947), who suggested that a majority of cases of *de novo* mutations to hemophilia could be traced back to paternal meiosis.

A difference in the mutation rate between sexes should translate into different (neutral) evolutionary rates of sequences on autosomes (A) and sex chromosomes, depending on their relative time spent in the male and female germline, respectively. Specifically, the Y chromosome should evolve solely according to the male mutation rate in organisms with male heterogamety (XY). In contrast, the X chromosome, which spends two-thirds of the time in female germline in such systems, should be affected by male and female mutation rates to the relationship

1:2. Finally, male and female mutation rates should obviously have equal influence on the evolutionary rate of autosomes. Comparisons of substitution rates in neutral sequences on different types of chromosomes can thus be used to estimate sex-specific mutation rates. This was first applied by Miyata et al. (1987), and later extended by Li and colleagues (e.g., Shimmin et al. 1993a; Huang et al. 1997), who demonstrated that the evolutionary rate of neutral sequences on human chromosomes follow the pattern Y > A > X, suggestive of a male-bias to the mutation rate. Quantitative analyses indicated that this mutation bias (denoted α_m to distinguish it from the cell division bias) may be in the order of 3–6.

More recent analyses of sex-specific mutation rates have not necessarily clarified the picture. It has been suggested that estimates of α_m from sex chromosome analyses may be flawed because of a specific reduction in the X chromosome mutation rate, not related to germline biology, for adaptive reasons (McVean and Hurst 1997). However, sequence data from human autosomes and the X chromosome do not support this idea (Nachman and Crowell 2000). Of more serious concern, a recent report by Bohossian et al. (2000) indicates that α_m in human may be as low as 1.7, a figure derived from extensive data on the degree of divergence between sequences recently transposed to the homonoid Y chromosome. On the other hand, the study by Nachman and Crowell (2000) reports α_m to be ≈4 (from A–X comparisons). Direct counts in pedigrees of *de novo* mutations at hypermutable human microsatellite loci similarly give α_m ≈3–5 (Ellegren 2000; Xu et al. 2000).

Comparative analyses of the extent of male-biased mutation in different groups of organisms are rare but necessary for understanding the generality of an effect of sex and the number of germline DNA replications on the mutation rate. In fish, the process of sex determination is complex and far from being fully understood. However, in salmonid fishes it is evident that sex is genetically determined and the karyotype is consistent with male heterogamety (Hartley et al. 1987). The X and Y chromosomes of salmonids show only very limited differentation and no Y-specific gene conserved across all salmonid species has yet been identified. However, in some *Oncorhynchus* species, including the coho salmon (*Oncorhynchus kisutch*) and the chinook salmon (*O. tshawytscha*), the autosomal gene for growth hormone 2 (*GH-2*) has a male-specific pseudogene copy present on the nonrecombining part of the Y chromosome (Du et al. 1993; Forbes et al. 1994; Nakayama et al. 1999; Devlin et al. 2001; which we herein denote *GH-2Y*). In this study we have used intron sequences from these two gene copies for measuring the frequency of nucleotide substitution on an autosome and the Y chromosome of *O. kisutch* and *O. tshawytscha,* thereby being able to estimate sex-specific mutation rates in salmonid fish.

Material and Methods

DNA samples from chinook salmon were kindly provided by D.Jim Shaklee. The autosomal and the Y-linked copy of the third intron of chinook salmon *GH-2* was obtained by PCR amplification from female (*GH-2*) and male (*GH-2Y*) DNA, respectively. PCR reactions contained 5 pmol each of the primers GH-2 Left and GH-2 Right (Forbes et al. 1994), 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* dNTP, 0.5 U of AmpliTaq DNA polymerase (Perkin Elmer), and approximately 100 ng of template DNA, in a total volume of 25 μl. A touchdown-PCR profile was used, consisting of a 2 min denaturation step at 94°C, followed by 10 cycles of 94°C for 30 s, 62°C to 52°C (decreasing 1°C per cycle) for 30 s and 72°C for 30 s. Then 25 cycles of the same profile were run at a constant annealing temperature of 52°C, and a final extension step of 72°C for 10 min was added after the last cycle. The male-specificity of *GH-2Y* was supported by successful amplification in 10 males but not in 10 females.

PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and fragments were ligated into the pGEM-T vector (Promega). The inserts of ten clones from each ligation were screened by PCR using the original primers (according to above) and separated by agarose gel electrophoresis or polyacrylamide gel electrophoresis, in the latter case following standard protocols for single strand conformation polymorphism (SSCP) analysis. Plasmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen) and sequenced with fluorescently labeled M13F and M13R Big Dye primers (Perkin Elmer). Automated sequencing was performed on an ABI 377 (Perkin Elmer) instrument and resulting sequences were analyzed and aligned by Sequencer 3.0 (Gene Codes Corp.), Sequence Navigator, or AutoAssembler (Applied Biosystems). The sequences obtained in this study have been deposited to GenBank under the accession numbers AF281143-4. From GenBank we obtained *GH-2* and *GH-2Y* intron 3 sequences of *O. kisutch* (U04930 and U04931; Forbes et al. 1994), and the *GH-2* sequence of Atlantic salmon (*S. salar*) (M21573; Johansen et al. 1989). For the analysis of intron 4, the *GH-2* sequences of *O. tshawytscha* and masu salmon (*O. masou*) were from GenBank (U28157 and U28361), while the homologous sequence *of GH-2Y* in these two species was from Zhang et al. (2001). Substitution rates and bootstrap standard errors were calculated using Tajima and Nei correction, and by removing gaps in pairwise comparisons. A maximum parsimony analysis of the sequence data was made by uniform weighting and close-neighbor–interchange searches to determine the most parsimonious tree. The robustness of the tree was tested using the bootstrap method. All analyses were done in MEGA (Kumar et al. 2001).

Results

Amplification of the third intron *of GH-2* in *O. tshawytscha,* using flanking exonic primers, consistently gave one PCR product in females (550 bp) and two products distinguishable by SSCP in males. Tentative assignments of these products as the autosomal *GH-2* gene (the fragment obtained in both female and male amplifications) and the homologous Y-linked *GH-2Y* gene (the additional fragment seen in males only) were subsequently verified following sequencing and sequence alignment with *O. kisutch*

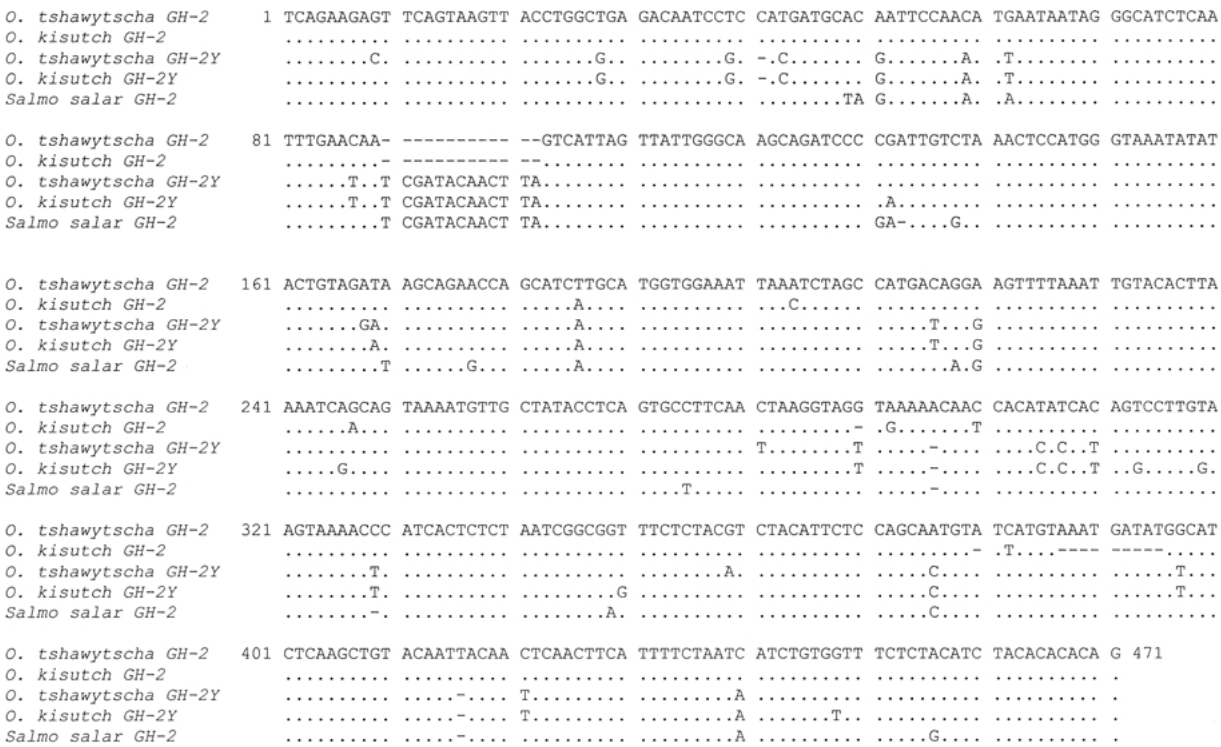

Fig. 1. Alignment of intron 3 sequences of the *O. kisutch* and *O. tshawytscha GH-2* and *GH-2Y* genes. Identical positions are denoted by a dash. The *GH-2* sequence of *S.salar* is also included.

Table 1. Estimated number of nucleotide substitutions per site and their standard errors for salmonid *GH-2* and *GH-2Y* intron 3 sequences[a]

	O. tsawytscha GH-2	*O. kisutch GH-2*	*O. tsawytscha GH-2Y*	*O. kisutch GH-2Y*
O. tsawytscha GH-2	—	0.0138 ± 0.0046	0.0520 ± 0.0113	0.0571 ± 0.0118
O. kisutch GH-2	—	—	0.0620 ± 0.0126	0.0717 ± 0.0131
O. tsawytscha GH-2Y	—	—	—	0.0232 ± 0.0070

[a] Length of intron sequences were as follows: *O. tsawytscha GH-2* 458 bp, *O. kisutch GH-2* 447 bp, *O. tsawytscha GH-2Y* 468 bp, *O. kisutch GH-2Y* 468 bp.

GH-2 and *GH-2Y* sequences (Fig. 1). From these sequences we calculated the frequency of nucleotide substitution for all possible pairwise comparisons (Table 1). Clearly, the degree of divergence between the *GH-2* and *GH-2Y* sequences of each species exceeds the degree of interspecific divergence between the two *GH-2* or the two *GH-2Y* sequences. This is consistent with the hypothesis that the ancestral *GH-2* gene was duplicated into *GH-2Y* prior to the split of the two lineages leading to *O. tshawytscha* and *O. kisutch,* respectively (cf. below).

The frequency of nucleotide substitution between the *GH-2Y* introns was higher than that between the *GH-2* introns, giving a Y/A ratio of 0.0232/0.0138 = 1.685. According to Miyata et al. (1987), this translates to $\alpha_m = 5.36$. Using the formula of Shimmin et al. (1993a) to calculate a 95% confidence interval for this estimate gives a very large distribution of $\alpha_m 0.07 - \infty$. Although with this enormous interval the possibility that there is no difference in the mutation rate between sexes cannot be rejected, it should be noted that calculation of confidence intervals for α_m is not without difficulties. Alternative statistical approaches may therefore be required.

To extend the analysis we sought to estimate the total number of substitutions that have accumulated in the *GH-2* and *GH-2Y* lineages since the *GH-2* gene was duplicated in an ancestor to *O. kisutch* and *O. tshawytscha*. As *GH-2* only exists in one copy in many other salmonid species (Devlin et al. 2001), it is reasonable to assume that *GH-2* was duplicated after the lineage leading to *O. kisutch* and *O. tshawytscha* split from other salmonid species. This is also supported by a parsimony analysis based on *GH-2* and *GH-2Y* sequences from *O. kisutch* and *O. tshawytscha,* and the *GH-2* sequence from Atlantic salmon (*S. salar*) (Fig. 2), as well as by a similar analysis made by Devlin et al. (2001).

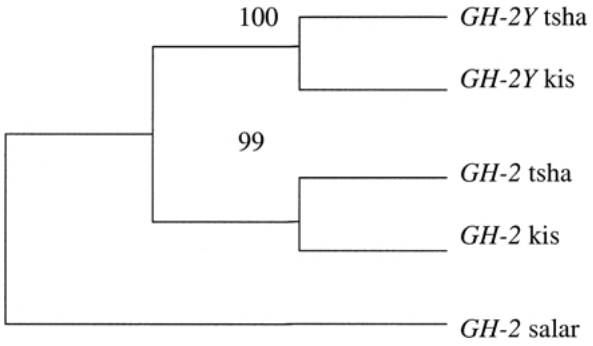

Fig. 2. Phylogenetic relationships between *Oncorhynchus GH-2* and *GH-2Y* intron 3 sequences based on parsimony analysis, with bootstrap support. kis is *O. kisutch,* tsha is *O. tshawytscha*, and salar is *S. salar*.

The respective number of nucleotide substitutions arising in the *GH-2* and *GH-2Y* lineages subsequent to their differentiation can be estimated from an alignment with the *S. salar* outgroup sequence (Fig. 1), based on the parsimony principle for inferring ancestral states. For example, a substitution which distinguishes both copies of *GH-2Y* from both copies *of GH-2* as well as from the outgroup sequence, was taken to have arisen in the Y chromosome lineage subsequent to the duplication of *GH-2* but prior to the split of *O. kisutch* and *O. tshawytscha* (e.g., position 28 in Fig. 1). Twelve such positions were identified for *GH-2Y* and five for *GH-2*. For two additional positions the ancestral state could not be inferred since the outgroup sequence displayed a unique state; these sites were not further regarded. To the substitutions arisen subsequent to the duplication of *GH-2* but prior to the split of *O. kisutch* and *O. tshawytscha* should be added substitutions arisen following the split of the two species. These are equal to the substitutions behind the estimate of α_m made above, and amount to 9 in *GH-2Y* and 6 in *GH-2* (Fig. 1). Thus, in total, we estimate that there have been 21 substitutions in the Y chromosome lineage leading *to GH-2Y* and 11 substitutions in the autosomal lineage leading to *GH-2,* following the duplication of *GH-2*.

It should be noted that the analyses presented above are with the assumption that variation in intronic substitution rates is mainly due to mutation rate variation and is not affected by possible functional constraints. This is an often made assumption in molecular evolutionary studies but recent large-scale analysis of genome sequence data have indicated that non-coding DNA (intronic as well as intergenic) may contain blocks of conserved sequences (Hardison 2000; Frazer et al. 2001). Furthermore, in this particular case it might potentially be argued that introns of *GH-2*, but not *GH-2Y,* could be under selection since *GH-2* is functional while *GH-2Y* is a pseudogene, possibly explaining the faster evolution of *GH-2Y* than *GH-2* (a 'pseudogene effect'). If so, the intronic substitution rate of *GH-2* in *O. kisutch* and *O. tshawytscha* should be expected to be lower than the rate of substitution at silent sites (K_S) of this gene, assuming silent sites being neutral (Eyre-Walker and Hurst 2001). However, this is not the case (Tables 1 and 2). The rate of substitution in 455 bp of intron 3 sequence from *GH-2* is 0.014 ± 0.005 while K_S for 202 bp of coding sequence of this gene is 0.010 ± 0.007. Moreover, the intronic rate is not lower than the mean K_S of the four genes sequenced in the two species (0.013 ± 0.002; see Table 2). It should be acknowledged that this is an indirect test of a possible pseudogene effect as it suggests that substitution rates at silent sites and in introns of the functional gene are similar, rather than testing this for the pseudogene. The indirect test can be motivated by that if any gene copy could possibly be expected to exhibit selective constraints in non-coding regions it would be the functional gene. Unfortunately, there is not sufficient amount of coding sequence data for *GH-2Y* to allow a corresponding test for the pseudogene.

Finally, the sequences analysed above can be augmented with data from a ≈ 270 bp region, including part of exon 4, intron 4 and part of exon 5, of *GH-2Y* from *O. tshawytscha* and masu salmon (*O. masou*). Besides the intron 3 data analyzed above these are the only available *GH-2Y* sequences. As *GH-2Y* is a pseudogene exons and introns can be combined and in total there are 12 substitutions distinguishing *O. tshawytscha* and *O. masou* over 266 bp. Intron 4 of *GH-2* has not been sequenced in *O. tshawytscha* and *O. masou* so we use data from intron 3 where the two species differ at 8 out of 435 sites. For these sequences we thus obtain 12 substitutions in the Y chromosome lineage leading to *GH-2Y* and 8 in the autosomal lineage leading to *GH-2*. Although this means comparing two different introns of different length, the comparison is conservative for the detection of a possible male-bias as a much longer sequence is considered for the autosomal lineage. In total we now have 21 + 12 = 33 substitutions in the Y chromosome lineage and 11 + 8 = 19 in the autosomal lineage of *Oncorhynchus* species. This rejects a null hypothesis of equal mutation rates in the two sexes at $p = 0.05$ (Binomial test, two-tailed). An estimate of $\alpha_m = 6.60$ with a 95% confidence interval of $0.99 - \infty$ is obtained. We conclude that the data strongly indicate the mutation rate of salmonid fish to be male-biased.

Discussion

The observation of $\alpha_m = 5.35 - 6.60$ in salmonid fish was derived from data on two paralogous genes which are of relatively recent origin. Given that there

Table 2. Synonymous substitution rates (K_S) in nuclear genes of *O. kisutch* and *O. tshawytscha*

Gene	$K_S \pm$ SE	Length (bp)	GenBank #
chemokine 1	0.007 ± 0.005	297	AF093803, AF093805
growth hormone 1	0.013 ± 0.005	627	M19999, S50867
growth hormone 2	0.010 ± 0.007	202	U28359, U28157
transferrin	0.022 ± 0.003	2024	AF223786-92, AF114849-53

is only one *GH-2* gene in several other *Oncorhynchus* species, *GH-2* is likely to have differentiated into *GH-2Y* after the split of the lineage leading to *O. kisutch* and *O. tshawytscha* (Devlin et al. 2001). This split is estimated to have taken place approximately five million years ago (Oohara et al. 1997; Oakly and Philips 1999). In theory, *GH-2* could have diverged much earlier and the *GH-2Y* copy then got lost in other *Oncorhynchus* species. However, the observed degree of divergence between *GH-2Y* and *GH-2* (≈6%) is compatible with the two genes having evolved after the split of the *O. kisutch–O. tshawytscha* lineage from other *Oncorhynchus*.

It has been suggested that the use of highly similar sequences is advantageous for obtaining accurate and precise estimates of α_m (Shimmin et al. 1993b). This should reduce the potential effect of nucleotide sequence context differently influencing the substitution rate in the genes under study (Bohossian et al. 2000). It may thus be argued that any such contextual bias should be negligible for the closely related *GH-2Y* and *GH-2* genes. It is noteworthy that X–Y gene pairs often used for estimating α_m in human (e.g., *ZFX*/*ZFY*) represent much larger genetic distances (80 to 130 million years ago; Lahn and Page 1999).

The two salmonid species used for estimating α_m in fish represent rather recent divergences, as indicated above. Using closely related species for estimating α_m may be associated with ancient polymorphism differently affecting the estimation of degree of divergence different chromosomes (Makova and Li 2002). As the X and Y chromosomes have different effective population sizes, and may also differ in polymorphism levels for other reasons (Charlesworth 1996), a population reduction can differently affect divergence of these chromosomes in a short term perspective. Makova and Li (2002) have shown that this results in lower estimates of α_m among closely related species than among more distantly related species. Assessing the effect of ancient polymorphism on our estimate of α_m in salmonid fish is difficult as α_m can not be estimated in more distantly related *Oncorhynchus* species (simply because the *GH-2* gene is not duplicated in these. However, we note that the expected effect of ancient polymorphisms would be that of underestimating α_m. Our estimate is thus conservative in this sense.

Outside mammals α_m has been estimated at 2–6 in birds, a system with ZW female heterogamety (Ellegren and Fridolfsson 1997; Kahn and Quinn 1999; Carmichael et al. 2000). These observations have been important for demonstrating that the difference in evolutionary rate between differentiated sex chromosomes can not only be explained by a chromosome-specific reduction in the mutation rate, as this would give values of α_m of less than 1 in birds (i.e. that Z would evolve slower than W whereas the opposite is found). To our knowledge, the only additional observation among animals, besides mammals and birds, comes from *Drosphila*. Comparing substitution rates between X-linked and autosomal sequences of D. *melanogaster* and *D. simulans,* Bauer and Aquardo (1997) found no indication of a sex-bias in the mutation rate. The rarity of studies revealing sex-specific mutation rates in a broader range of organisms probably reflects the fact that very few conserved target sequences present on both X and Y, or on Z and W, are available from less well characterized genomes.

Our observation of $\alpha_m = 5.35 - 6.60$ in salmonid fish adds evidence from another vertebrate class of a male bias to the mutation rate. This estimate is not affected by a possible chromosome-specific reduction in the X chromosome mutation rate (McVean and Hurst 1997) as we used an autosome–Y chromosome comparison. While the number of gametes produced by male fish generally vastly exceeds that produced by females, the difference in the number of germ cell generations that proceed the formation of gametes in spermatogenesis and oogenesis has rarely been examined in detail. Both coho and chinook salmon are anadromous, i.e. they spawn just once, at the age of 2–7 years. Females may lay in the order of 10,000 eggs. In the related rainbow trout, the number of spermatogonia produced by males is estimated at 250 million (R. Billard, cited in Lebrun et al. 1982). We thus note that a significant sex-difference in the mutation rate should be predicted from germline biology given that the number of cell divisions plays a role for mutagenesis. While we confirm this to be the case, we can not quantitatively assess whether our estimate of α_m ties in with the ratio of male to female germline cell divisions. An important question for further research will be to investigate, in any organism group, if α_m actually covaries perfectly with the ratio of male to female germline cell divisions. An indication of that this may very well be the case is the fact that

in *Drosophila,* where $\alpha_m \approx 1$, males and females seem to produce equal numbers of gametes (Bauer and Aquardo 1997).

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