Title

Rapid turnover and evolution of sex-determining regions among *Sebastes* rockfish

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Abstract

Nature has evolved a wealth of sex determination (SD) mechanisms, driven by both genetic and environmental factors. Recent studies of SD in fishes have shown that not all taxa fit the classic paradigm of sex chromosome evolution and diverse SD methods can be found even among closely related species. Here, we apply a suite of genomic approaches to investigate sex-biased genomic variation in eight species of *Sebastes* rockfish found in the Northeast Pacific Ocean. Using recently assembled chromosome-level rockfish genomes, we leverage previously published sequence data to identify disparate sex chromosomes and sex-biased loci in five species. We identify two putative male sex chromosomes in *S. diaconus,* a single putative sex chromosome in the sibling species *S. carnatus* and *S. chrysomelas*, and an unplaced sex determining contig in the sibling species *S. miniatus* and *S. crocotulus.* Our study provides evidence for highly labile SD in a recently speciated genus of bony fish, and sheds light on the diverse origins of sex determination mechanisms present in the animal kingdom.

Introduction

Sexual reproduction is common to nearly all animals, and nature has evolved a great diversity of mechanisms to produce distinct sexes, both within and among animal taxa. The mechanisms underlying sex determination (SD) may be genetic (GSD), environmental (ESD), or some combination of both (Bachtrog et al., 2014). Even among organisms with GSD, there is substantial diversity owing to the many independent origins of sex chromosomes across the tree of life (Renner & Ricklefs, 1995; Mank et al., 2006; Pokorná & Kratochvíl, 2009). Most familiar among GSD mechanisms is heterogametic sex determination (HSD), in which the presence or absence of a particular sex chromosome drives gonadal differentiation. While eutherian mammalian males are heterogametic (XY), in birds and moths (among many others) females are the heterogametic sex (ZW), whereas males are homogametic. These systems have become nearly fixed in their clades, giving the impression of HSD as an ultimate and inevitable outcome of a sex chromosome evolution. Conversely, in many other systems genetic sex determining chromosomes or regions are not fixed and turn over frequently on an evolutionary scale. This lability

37   allows us to understand the origin of SD systems by examining sex determining regions at different
38   stages.

39   The classical philosophy surrounding the evolution of HSD suggests a logical series of steps (Muller,
40   1964; Ohno, 1967; Kratochvil et al., 2021). First among these is the acquisition of a master sex
41   determining (MSD) gene, an allele which may itself play a key role is testis differentiation or modulate
42   expression at other loci. To go from a single mutation differing between sexes to highly differentiated
43   sex chromosomes, recombination between haplotypes containing differing sex determining alleles
44   needs to be suppressed. Without this, any linkage between additional mutations and the MSD, including
45   sexually antagonistic loci, will inevitably decay. Without recombination suppression, genetic
46   differentiation between sexes would remain limited to one or more mutations that directly control sex,
47   rather than a whole sex-determining region.

48   There are several models for how recombination suppression can evolve and what consequences might
49   ensue (Wright et al., 2016). The sexual conflict model, for example, suggests selection against
50   recombination around the MSD and linked genes with sex-specific effects leads to sex-specific
51   supergenes. These gene complexes are passed to offspring as a unit, who then simultaneously inherit a
52   suite of tightly linked, sex-specific adaptations. Another plausible explanation for reduced
53   recombination involves mutations to transcription factor binding sequences, which can rapidly result in
54   highly specific heterochiasmy within populations (Kong et al., 2010). Whatever the mechanism of
55   recombination suppression, the differentiating sex chromosome exhibits weakened selection against
56   deleterious alleles due to reduced effective population size and recombination. The accumulation of
57   mildly deleterious alleles can deactivate genes on the Y chromosome, leading to reduced selection to
58   maintain those regions and an overall size reduction in a process known as Y-degeneration (Bachtrog,
59   2013). This one-way trajectory can result in a relatively stable sex chromosome over short evolutionary
60   time but leaves the degenerated chromosome vulnerable to loss from the genome. One model to
61   explain this loss is Muller's ratchet, summarized by Charlesworth & Charlesworth (2000) as a stepwise,
62   stochastic loss of the Y chromosomes carrying the least deleterious load, in which each loss results in the
63   fixation of one or more deleterious alleles in the population (Muller, 1932). As a result, the Y
64   chromosomes in circulation within the population become increasingly impaired in their function. This
65   mechanism is especially plausible in small populations but may also act over relatively short
66   evolutionary time even when populations are large (Gessler, 1995).

67   While sex determining regions are highly variable, MSD genes are often convergent and are frequently
68   involved in testis differentiation (Graves & Peichel, 2010). Indeed, a 'limited options' hypothesis has
69   been proposed to explain the convergence of SD genes among vertebrates, owing to the small number
70   of players in this role. Among these are the androgen receptor (*ar*), Anti-Mullerian hormone (*amh*),
71   Doublesex and mab-3 related transcription factor (dmrt1), and *sox-3/SRY* genes (Kratochvil et al., 2021;
72   Graves & Peichel, 2010). Their critical role in gonadal development makes these genes susceptible to
73   cooption as MSD, as rare gain-of-function mutations can easily tip the scales by promoting
74   differentiation to one sex or disabling the other.

75   The classical model fits taxa with relatively ancient and conserved HSD but a growing body of evidence
76   in other taxa, without fixed SD, points to more flexibility in the evolution of sex chromosomes

77   (Kratochvil et al., 2021; Li & Gui, 2018). Among vertebrates, teleost fish exhibit the most variability in
78   sex-determination mechanisms (Bachtrog, 2014). Among these are ESD, as in many flatfishes
79   (Luckenbach et al., 2009), socially or environmentally driven sequential hermaphroditism in gobies
80   (Sunobe et al., 2017), variable MSD in several clades including salmonids, halibut, and tuna (McKinney et
81   al., 2020; Chiba et al., 2021; Edvardsen et al., 2022).

82   The strongest evidence for lability in SD regions can be found in taxa with newly or only slightly
83   differentiated sex chromosomes. One such example was recently revealed in cichlid fishes (El Taher et
84   al., 2021), whose SD locus is highly labile on short evolutionary timescales. Cichlids comprise an
85   estimated 3000 species, over two hundred of which underwent analysis for SD mechanisms in the study
86   by El Taher et al. (2021). The study found evidence for frequent turnovers in poorly differentiated sex
87   chromosomes, sex chromosome fusions and other large-scale rearrangements, as well as some
88   convergent and well-conserved sex-linkages in some clades. The highly variable origin, breadth, and
89   position of these sex-linked regions within cichlids demonstrates the ephemeral nature of SD in taxa
90   without a fixed mechanism.

91   *Sebastes*, a highly speciose genus of rockfish, have undergone rapid speciation in the Pacific Ocean
92   (Mangel et al., 2007). Among Northwest Pacific rockfish, three species have been found to possess a
93   duplicated *amh*, acting as MSD gene (Song et al., 2021). The redundancy conferred by a gene
94   duplication is a prime candidate for neofunctionalization (Edgecombe et al., 2021). Indeed, several
95   independent duplications of *amh* among teleost fish have acquired sex-determining function (Hattori et
96   al., 2013), examples include tilapia (Liu et al., 2022), stickleback (Jeffries et al., 2022), and ayu
97   (Nakamoto et al., 2021). The study by Song et al. (2021) notably failed to attribute the same MSD
98   mechanism to a handful of Northeast Pacific rockfish, indicative of variable SD systems within a single,
99   recently speciated genus.

100   Recent genome sequencing of over 80 species of Pacific rockfish (Kolora et al., 2021) permits
101   chromosome mapping and analysis of previously published sequence data. Here, we leverage previously
102   published Restriction site Associated DNA sequencing (RADseq) data for eight species of Pacific rockfish
103   to identify putative regions responsible for SD. We make use of a novel high throughput program, as
104   well as traditional variant calling methods, to identify genomic variation associated with sex.
105   Additionally, we assemble a phylogeny of whole gene sequences for *amh* in Pacific rockfish including 78
106   *Sebastes* spp. and close relatives, to place the duplication in evolutionary context and identify species
107   for further investigation of *amh* as the MSD gene.

108   ## Materials & Methods

109   *Data acquisition*

110   Previously published RADseq data was publicly available from NCBI for eight species of *Sebastes* (Tbl. 1).
111   These included the gopher (*S. carnatus*) and black-and-yellow (*S. chrysomelas*) rockfishes (Fowler &
112   Buonaccorsi, 2016), sunset (*S. crocotulus*) and vermillion (*S. miniatus*) rockfishes (Longo et al., 2021),
113   boccacio (*S. paucispinis*), canary (*S. pinniger*) and yelloweye (*S. ruberrimus*) rockfishes (Andrews et al.,
114   2018) and deacon (*S. diaconus*) rockfish (Vaux et al., 2019).

115 As our analyses relied on sexed sequence data, unsexed samples were omitted. Additionally, the study
116 that examined speciation in *S. crocotulus* and *S. miniatus* included putative hybrid individuals, which
117 were also omitted.

Species	Males	Females	Total	NCBI Accession #
S. carnatus	10	10	20	PRJNA307574
S. chrysomelas	13	7	20	PRJNA307574
S. crocotulus	39	44	83	PRJNA721730
S. diaconus	29	74	103	PRJNA560239
S. miniatus	42	32	74	PRJNA721730
S. paucispinis	9	4	13	PRJNA451040
S. pinniger	24	30	54	PRJNA451040
S. ruberrimus	79	62	141	PRJNA451040

Table 1 | Summary of sample species. Our study comprises eight total species from four previously published datasets. Sample sizes and sex ratios varied between species.

118 *GWAS for sex bias*

119 To identify sex-biased markers, we first used RADSex (Feron et al., 2021; RADSex version 1.2.0). RADSex
120 compares identical RADseq reads to assess presence or absence of markers in each individual and
121 calculates the number and distribution of markers based on read depth. Unaligned sequences in FASTQ
122 format were fed into RADSex alongside a table containing sample accession numbers and sex, with
123 Bonferroni statistical correction disabled. Yates' correction for continuity was left in place for all
124 analyses, as some species had low sample numbers (n < 30). After identification, markers were mapped
125 to species-specific reference genomes (Kolora et al., 2021), except in the cases of *S. crocotulus* and *S.*
126 *chrysomelas*, whose markers were mapped to the genomes of their sibling species, *S. miniatus* and *S.*
127 *carnatus*, respectively. All reference genomes used were generated using Illumina sequence data and
128 scaffolded against the chromosome-level *S. aleutianus* genome using RagTag (Alonge et al., 2021). This
129 common scaffolding means that positions are roughly syntenic between the genomes and allows for
130 easy comparison.

131 For use in a traditional variant calling pipeline, the sequences were mapped to the above reference
132 genomes with the Burrows-Wheeler aligner (Vasimuddin et al., 2019; bwa-mem2 version 2.2.1), read
133 groups were appended with Picard (Broad Institute, 2019; Picard Toolkit version 2.26.3), converted to
134 BAM and sorted with samtools (Danecek et al., 2021; samtools version 1.13).

135 We then used *freebayes* (Garrison & Marth, 2012; freebayes version 1.3.5) to call variants individually
136 for each scaffold, parallelized with *GNU parallel* (Tange, 2018). When calling *S. diaconus* variants, spikes
137 in computational resource allocation required further separation of scaffolds for chromosomes 13, 19
138 and 20 into 5Mb subsections. All intermediate variant call files were then sequentially combined into a
139 single file for each species.

140 Custom Perl scripts were used to compute variation between the sexes using three metrics:
141 presence/absence of individual loci (missing data), allele frequency and heterozygosity at each locus.
142 Our scripts included Pearson's chi-square test of independence with Yates' correction for continuity, for

143   a likewise comparison with RADSex outputs. All scripts used in analysis are included in a git repository
144   (github.com/ntbsykes/rockfish_sex).

145   To visualize the results, we calculated the proportion of markers with a p-value < 0.005 for all test
146   statistics across a sliding 250bp window (Fig. 1). Given our relatively small sample size and high marker
147   number, it is unlikely for any individual marker, in some species, to reach statistical significance using
148   traditional genome-wide association (GWA) thresholds. With the sliding window, we instead identified
149   regions with unusually high concentrations of near-significant markers, which is indicative of sex
150   differentiation.

151   For further investigation of two sex-determining regions in *S. diaconus*, we compiled a list of the most
152   significant loci with a higher allele frequency in males (-log(P) ≥ 20). After isolating these sites using
153   *vcftools* (vcftools version 1.13; Danecek et al., 2011), we used *R* (R version 4.1; R Core Team, 2021) to
154   plot the genotype at each locus and mean number of male-biased alleles for all samples (Fig. 2).

155   *Mapping canary, sunset, and vermillion rockfish data to higher quality genomes*

156   The initial Illumina-sequenced and RagTag-assembled genomes used for alignment provide a common
157   order for identifying shared regions but are lower quality than *de novo* assembled long-read genomes.
158   We leveraged two high quality reference genomes for more accurate alignment of three species'
159   datasets: *S. pinniger* (GCA_916701065.2,) *S. miniatus*, and *S. crocotulus* (the latter two both aligned to
160   GCA_916701275.1). We then used BLAST (Camacho *et al.*, 2009; blast+ version 2.12.0) to compare the
161   nucleotide sequence of observed peaks to detected by the previous alignment. Finally, to characterize
162   the extent of differentiation at these loci, we applied a similar approach to our isolation of sex-biased
163   loci in *S. diaconus* (this time -log(P) ≥ 7; Fig. 3B), extracted read depth at each locus using bcftools
164   (Danecek et al., 2021; bcftools version 1.13), and averaged the read depth by sex across the entire
165   unplaced contig, taking the difference (Fig. 3C).

166   *Assembly of amh phylogeny in Sebastes and their relatives*

167   Coding sequences for *Sebastes amha* and *amhy* were acquired from NCBI (MW591742, MW591743)
168   (Song et al., 2021) and queried against 88 rockfish genomes (Kolora et al., 2021) and three-spine
169   stickleback (GAculeatus UGA version5) using NCBI BLAST in command-line, yielding matches on exons.
170   We parsed the BLAST results in R to determine gene start and end position, as well as coding strand. We
171   then used samtools faidx to extract the whole gene sequence for all copies of *amh* in each species.

172   These were aligned with MUSCLE multiple sequence alignment tool (Edgar, 2003). The alignments were
173   manually checked in BioEdit 7.2.5 (Hall, 1999). Finally, a maximum likelihood phylogeny of all gene
174   copies was produced in iqtree 1.6.2 (Nguyen et al., 2015) with 1000 bootstrapping replicates. The
175   resulting gene phylogeny was visualized in Figtree (Rambaut, 2018; Fig. 4).

176   *BLAST queries for 'usual suspect' sex determination genes*

177   We wanted to determine the location of 'usual suspect' SD genes in our study species and assess
178   whether these genes occurred near any sex-biased regions. To accomplish this, we used command-line

179   BLAST to query FASTA sequences for *ar, dmrt1,* and *sox-3/SRY* genes from three-spined stickleback
180   against all available *Sebastes* genomes.

Results

182   *GWAS for sex bias*

183   Three species display significant enrichment of sex bias across large portions of one or more
184   chromosomes: *S. carnatus*, *S. chrysomelas*, and *S. diaconus* (Fig. 1). These regions contain high enough
185   concentrations of significant markers to be easily detected by our rolling mean of significance.

186
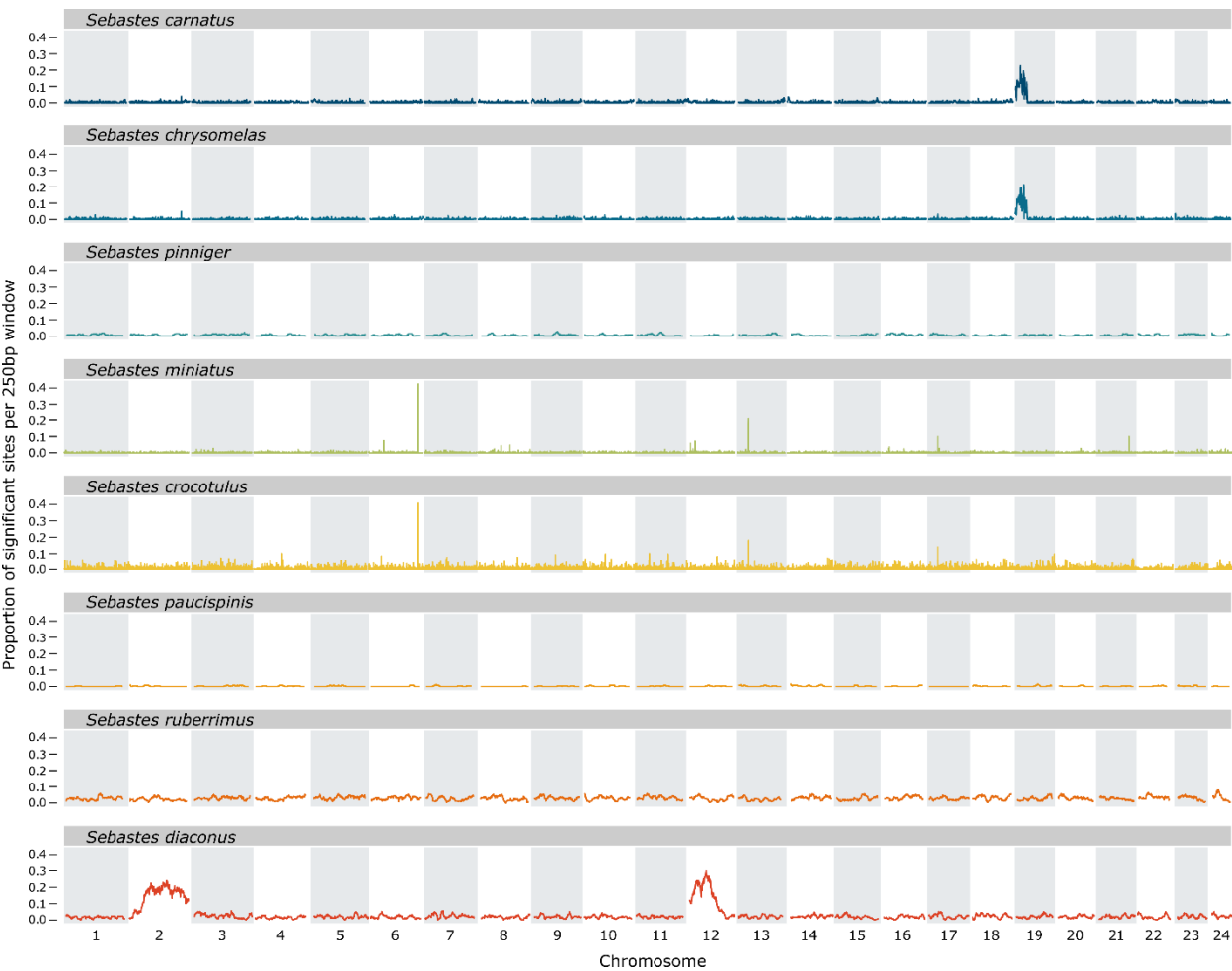
Figure 1 | Whole genome rolling mean of significant SNPs. Markers were characterized as significant or not significant based on a probability threshold of association with sex (P < 0.005). Significant markers were given a value of 1, while insignificant markers were zero and a rolling mean was calculated across windows of 250 SNPs for the whole genome. For clarity, the comparative metrics with clearest signal in each species were selected for visualization: *S. carnatus & S. chrysomelas* – allele frequency, heterozygosity, RADSex; *S. crocotulus & S. miniatus* – missing data, allele frequency, heterozygosity; *S. paucispinis, S. pinniger & S. ruberrimus* – RADSex; *S. diaconus* – RADSex.

187   *Sebastes carnatus* and *S. chrysomelas* have similar concentrations of low p-values on chromosome 19,
188   between 0 and ~8Mbp. The signal across this region was detected by *RADSex* as well as our test of allele
189   frequency and heterozygosity differences (Figure S1-S2).

190   *Sebastes diaconus* has a very high concentration of sex-biased SNPs on both chromosomes 2 and 12 (Fig.
191   1). On chromosome 2, significant sex-bias extends from ~5Mbp to the end of the chromosome at just
192   over 40Mbp and is detectable by RADSex and our allele frequency and heterozygosity metrics (Fig. S3).
193   Similar enrichment of significant sex-bias on chromosome 12 was observed in a region spanning 0 to
194   ~20Mbp (Fig. S4). This unique pattern male-biased alleles across two chromosomes warranted further
195   investigation.
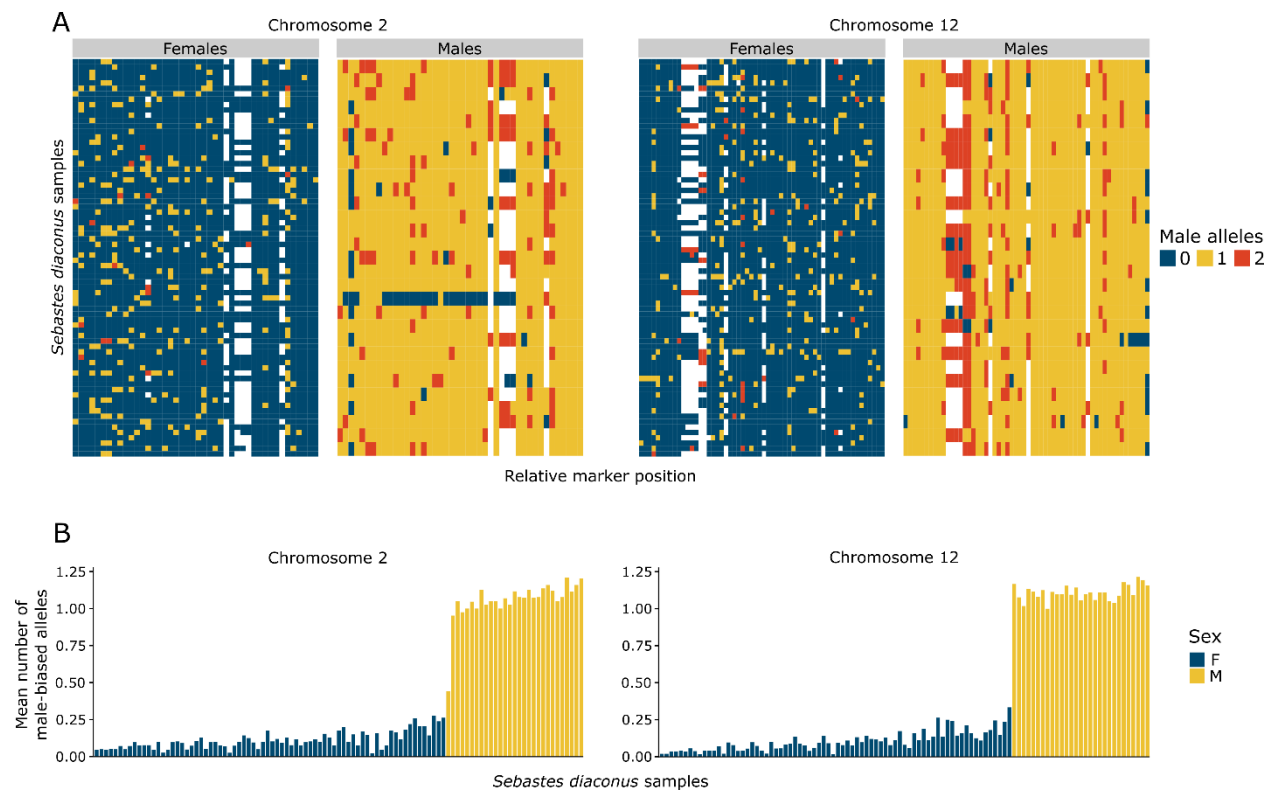
Figure 2 | A) Comparison of per-sample male-biased alleles in *Sebastes diaconus*. Significantly male-biased alleles (-log(P) ≥ 20) are present on both chromosomes 2 and 12 for nearly all samples. The sex-determining locus likely lies in a region with alleles shared by all males. **B) Per-sample mean number of significantly male-biased alleles on both sex chromosomes.** Females, shown in blue, possess significantly fewer male alleles at these sites on average

196   While most *S. diaconus* males possess the full suite of significantly male-biased alleles on both
197   chromosomes 2 and 12, a single male sample, SRR9968840, lacks these male alleles on the majority of
198   chromosome 2 (Figure 2A). Figure 2B reveals that although males and females differ significantly in
199   allele frequency in these regions, no alleles are unique to one sex.

200   Alignment to the commonly scaffolded *S. miniatus* genome suggests that sibling species *S. crocotulus*
201   and *S. miniatus* share three narrow regions of sex-bias: ~33.9Mbp on chromosome 6, ~8.25Mbp on

202   chromosome 13, and ~7.30Mbp on chromosome 17 (Fig. 1). A comparison of mean sample depth
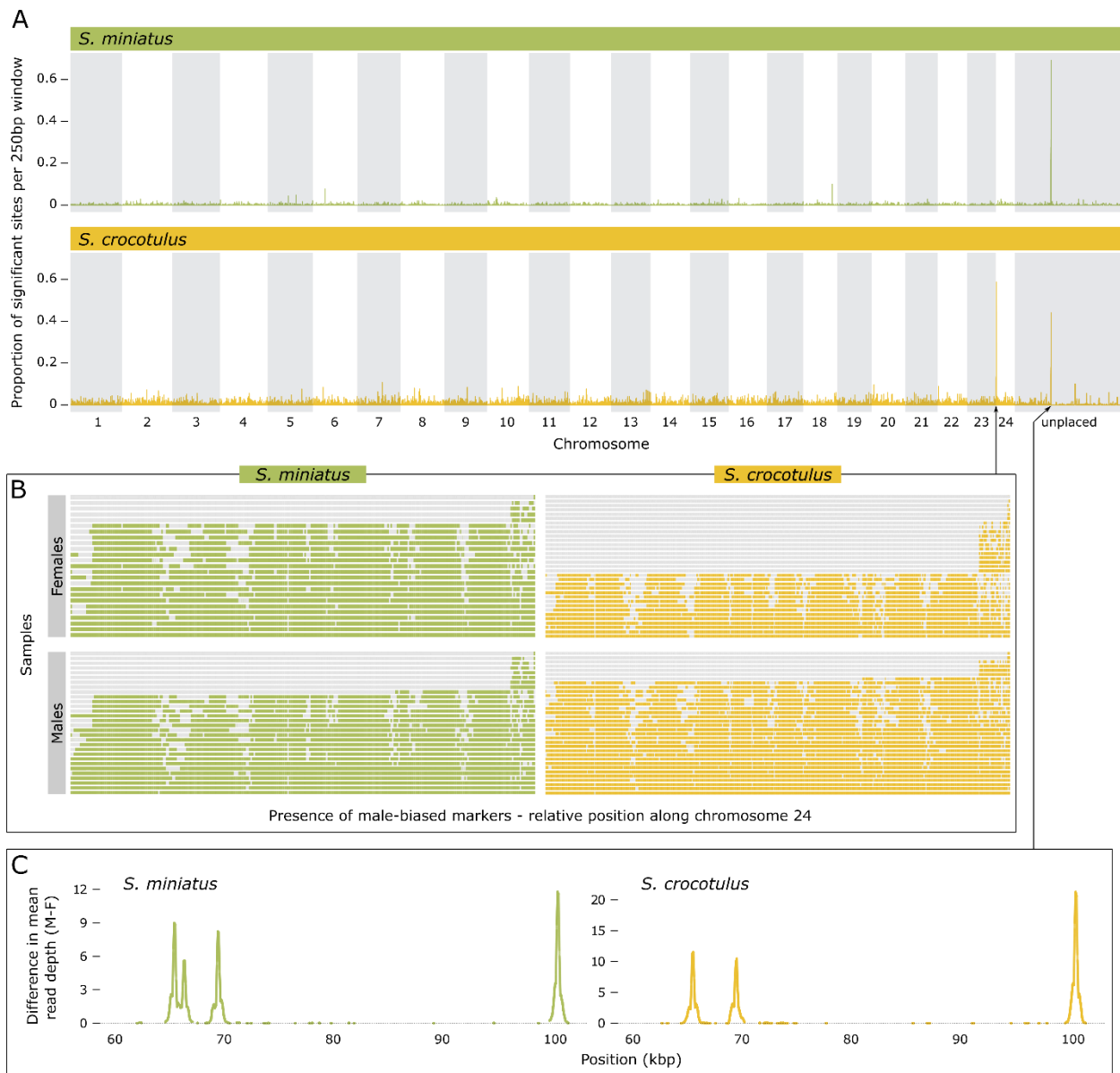203   between sexes in this region revealed significant male bias (Fig. S3).

204

Figure 3 | A) High quality genome alignment of *S. miniatus* and *crocotulus* reveals regions significantly associated with maleness. Both species' data were mapped against the *S. miniatus* genome. One sex-biased region, shared by both species, maps to an unplaced contig. Significant sex bias was detected in *S. crocotulus* along the first megabase of chromosome 24. **B) Missing data among *S. crocotulus* females leads to significant sex bias on chromosome 24.** Distinct sample groupings may represent patterns of recombination. **C) Difference in mean read depth between the sexes along the entirety of a sex-biased, unplaced contig.** Loci are homologous to those on chromosomes 6, 13, and 17 in the Figure 1 ragtag assemblies.

205   Only two significant markers in *S. ruberrimus* align to chromosome-level contigs: on chromosomes 4
206   (36.54 Mbp) and 5 (17.35 Mbp), while four more map to unplaced contigs. These markers are
207   significantly biased toward males; their distribution between sexes, as revealed by RADSex, is visible in
208   supplementary figure 6. Since these markers are not neighboured by other significant markers, as
209   would be expected by a sex determining region, these may represent misplaced reference sequence. No
210   significant sex bias was detected in either *S. pinniger* or *S. paucispinis*.

211   *Mapping canary, sunset, and vermillion rockfish data to higher quality genomes*

212   While this analysis yielded no novel insight into sex-bias in *S. pinniger*, narrow peaks on an unplaced
213   contig (CAKALS010000047.1) were shared by *S. miniatus* and *S. crocotulus* (Fig. 3A). BLAST results
214   indicate that these are homologous to the peaks previously mapped to RagTag assembly chromosomes
215   6, 13, and 17. Males had consistently higher read depth along this contig which, considering our likewise
216   analysis of the RagTag alignment, was expected (Fig. 3C; Fig. S5). Another region of sex bias was
217   detected in *S. crocotulus*, along the first megabase of chromosome 24. We found that this
218   differentiation is due to large swathes of missing data in *S. crocotulus* females (Fig. 3B; Fig. S9).

219   *Assembly of amh phylogeny in Sebastes and their relatives*

220   We found that the *amhy* gene in *S. schlegelii* forms a monophyletic group with copies of *amh* found
221   primarily on chromosome 4, and includes representatives from *Sebastes* sister genus, *Hozukius.* This
222   supports an early origin of *amhy* prior to the split of *Sebastes-Hozukius* but after the divergence of
223   *Sebastiscus* (Fig. 4A).

224   The initial characterization of *amhy* identifies two insertions in intron 4 and used them as markers to
225   separate the gene copies (Song et al., 2021). By comparing the outgroup sequence in *Sebastolobus,* we
226   found that these are instead deletions in *amh*, rather than insertions in *amhy*. We also found that these
227   deletions are not monomorphic in all orthologs of *amh,* and individual may possess one, both or neither
228   deletion (Fig. 4B).

229   Our BLAST search results reveal that the locations of orthologous common SD genes are highly
230   conserved within *Sebastes*, and none overlapped regions enriched for sex-biased markers. In our study
231   species, only the *amh* locus in *S. miniatus* lies on a different chromosome from the rest (Fig. 4, Table S1).

Discussion

233   By employing a thoughtful array of analytical metrics, we find evidence of a wide diversity of sex
234   determining mechanisms within just a handful of *Sebastes* species in the Pacific Ocean. Further, the
235   varied metrics permit characterization of the type and degree of differentiation within sex-determining
236   regions. Diversity among SD regions, origins, and mechanisms within a dataset comprising only eight
237   species exemplifies the lability of SD systems in the recently speciated genus (Fig. 5).
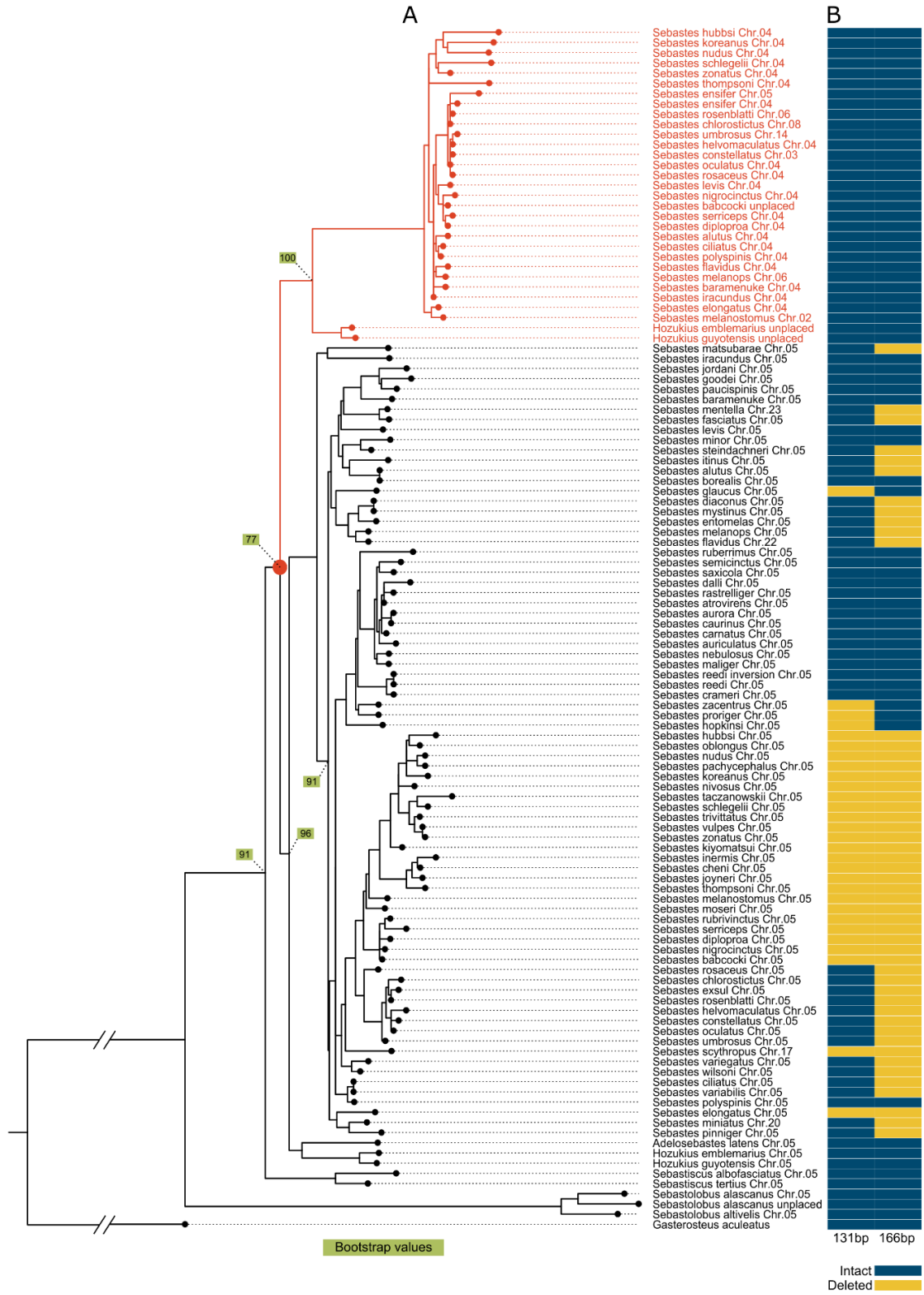
Figure 4 | A) Maximum likelihood phylogeny for *amh* and *amhy* in *Sebastes*, with outgroups. The *amh-amhy* duplication event is shown with a red circle and *amhy* copies are highlighted in red. Bootstrap values, highlighted in green, show confidence in sorting at nodes of particular interest. **B) Table of intron 4 deletions in *amh*.** Both deletions are derived in the autosomal *amh* and are divided variously among clades of *Sebastes*.
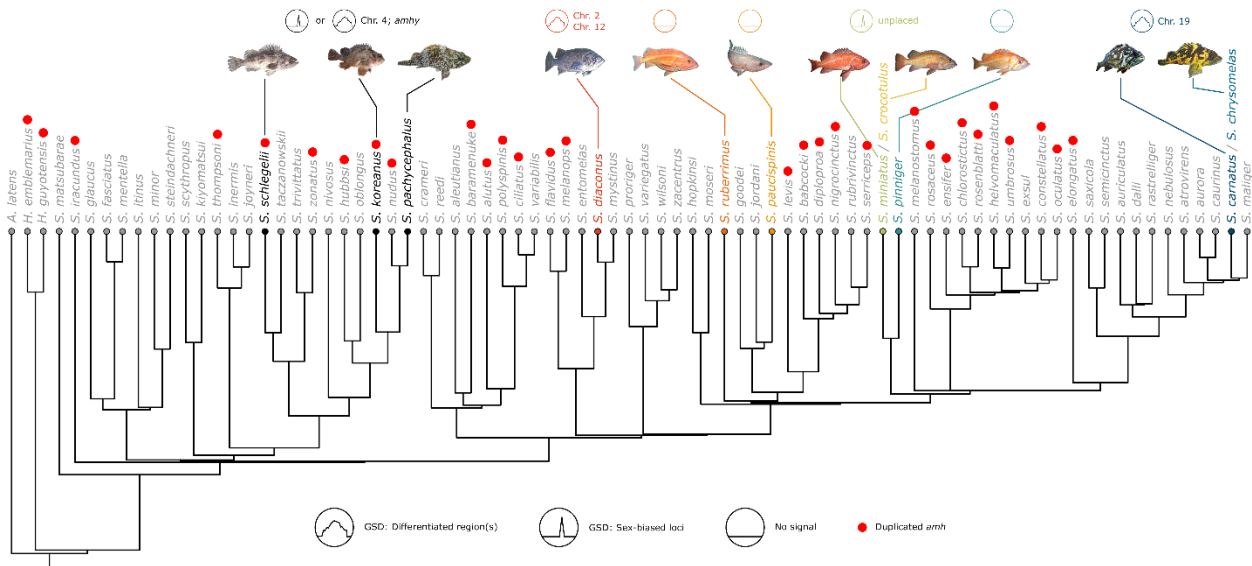
Figure 5 | A graphical summary of our findings. Sex-determining regions in *Sebastes* are highly variable in their location and degree of differentiation. The duplication of *amh* predates the *Hozukius*/*Sebastes* split but may not persist in all extant *Sebastes*. Cooption of the duplicated *amhy* as MSD appears to be monophyletic, specific to Northwest Pacific species.

239

Methods to study sex determination

241 Many different methodological approaches have been employed to identify genomic regions that differ
242 between sexes (Grayson et al., 2022). Depending on the type and degree of differentiation, different
243 analytical methods yield different results. For example, newly differentiating homomorphic sex
244 chromosomes may retain much the same genomic backdrop, their differentiation limited to allele
245 frequency, whereas sex-specific regions are easily recognized by a presence/absence bias or vast
246 differences in sample read depth. We employ three analytical metrics to cover these bases: site allele
247 frequency and heterozygosity inform differentiation before degeneration, whereas presence/absence
248 comparison suggests a deletion in one sex or insertion in the other. Our comprehensive suite of genomic
249 analyses provides both fine-scale resolution of sex-biased markers and in-depth characterization of sex
250 chromosome differentiation.

251 New methods and programs are constantly in development to address this issue. Here, we compare the
252 effectiveness of one such program (RADSex) to a traditional variant calling pipeline and our various
253 genomic analyses. While RADSex did detect large scale genomic variation in *S. carnatus, S. chrysomelas,*
254 and *S. diaconus,* this high throughput method did not register significant differences at a finer scale in *S.*
255 *miniatus* and *S. crocotulus*. Our presence/absence metric revealed a much higher density of sex-specific
256 markers at these sites, which, when analyzed for mean sample depth reveal a highly differentiated ~120
257 kbp contiguous segment of DNA, almost exclusive to males. This difference in resolution is likely due to
258 the way RADSex requires identical reads. In traditional RADseq, the start of read one is anchored by a

259   restriction digest site but read two are variable, due to random shearing of the other end of the
260   fragment. This means the second reads are unlikely to start at the same position, thus making them non-
261   identical even if they don't have any genetic polymorphism. As a result, while RADSex only showed two
262   differentiated markers, individually called variants showed that this difference was spread across a
263   larger region, including three peaks in male read depth covered by second reads (Fig. 3C). This gave us
264   more confidence that this represented a true difference rather than an isolated mis-mapping of two
265   reads.

266   One advantage of RADSex is the identification of sex biased markers is reference-free. Although in our
267   study we have quality reference genomes for all species, that is not always the case. This also provides
268   an advantage in cases where the reference genome is the homogametic sex. In this case, read mapping
269   will be especially poor for reads from the sex determining region (e.g. Y chromosome) since the true
270   mapping location is not present and may lead to spurious sex biased regions across the genome. In our
271   study, the samples used to construct reference genomes were not sexed, therefore we do not know if
272   sex determining regions are missing. From RADSex, we don't find a large number of unmapped sex
273   biased markers, suggesting that our reference-based analyses are not hampered by this issue.

274   The advent of streamlined programs to study sex chromosomes provides many useful tools. It is crucial
275   to note, however, that each analytical method comes with a specific set of strengths and weaknesses.

276   *Two putative sex chromosomes in deacon rockfish*

277   We have identified two putative sex chromosomes in *S. diaconus*: chromosomes 2 and 12. The breadth
278   of these sex-biased regions indicate that the differentiation is in a mature stage. However, many of
279   these markers are still present in some proportion of females (Figure S6). Indeed, the sex-bias on both
280   chromosomes is in allele frequency and heterozygosity, with very few sites specific to one sex (Figures
281   S3-S4).

282   According to the classic paradigm, sex chromosomes accumulate differences through mutation before
283   recombination suppression can lead to weakened selection and subsequent loss of genetic material
284   (Kratochvil et al., 2021). The broad observed male bias in allele frequency and heterozygosity on these
285   two chromosomes then presents a paradox. The lack of signal in missing data suggests a relatively young
286   sex determining region, which has not accumulated sex-specific genes or degenerated, but the
287   differentiated region is very large and encompasses the equivalent of a whole chromosome. Under this
288   model, recombination suppression evolves gradually, therefore the size of the differentiated region
289   would suggest an old sex determining region.

290   The male bias in allele frequency and heterozygosity may suggest that though male-biased mutations
291   have begun to accumulate, there are few or no significantly female-antagonistic loci linked to these sex-
292   determining alleles. Without selection against genes linked to male-specific alleles, homomorphy in
293   these chromosomes may persist between the sexes. If the male alleles were limited to a single
294   chromosome, this would be consistent with early male heterogametic SD in a newly differentiating sex
295   chromosome, before degeneration takes place. Indeed, as degeneration is dependent on recombination
296   suppression (Bachtrog, 2013; Charlesworth & Charlesworth, 2000) homomorphic sex chromosomes

297   which can still readily recombine will be unlikely to undergo degeneration – which is especially true
298   when sex-determining loci have poor or no linkage to nearby alleles.

299   However, out of all samples, we only see evidence of recombination between sex-biased haplotypes in
300   one individual (Fig. 2), despite the size of the region, which suggests very strong and broad
301   recombination suppression. Additionally, while the classic model of sex chromosome differentiation can
302   account for the state of differentiation on these chromosomes, the presence of two sex chromosomes in
303   this species makes analysis more nuanced. There are examples in nature of large-scale chromosomal
304   rearrangements leading to novel sex chromosomes, including sex chromosome fusions in stickleback
305   (Sardell et al., 2021) and a reciprocal translocation in common frogs, which resulted in two physically
306   separate but coinherited sex chromosomes (Scott, 2019). A third explanation may be found in Chinook
307   salmon, in whom the sex-determining locus is readily translocated between two chromosomes
308   (McKinney et al., 2020). In *S. diaconus*, we find no evidence for missing data on either chromosome,
309   which makes a Y fusion to one or both chromosomes unlikely; we would expect the fused Y to be absent
310   in females. In this case, we postulate that recombination suppression may have been achieved through
311   a chromosomal translocation involving a proto-sex chromosome and an autosome. This can explain the
312   division of male alleles between two chromosomes and provide a mechanism for recombination
313   suppression; recombination between translocated chromosomes produces unbalanced gametes, which
314   means that a single translocation can produce recombination suppression over a large region.

315   Most male *S. diaconus* possess at least one copy of the male allele at each of the loci of interest on both
316   sex chromosomes, but one sample lacks these alleles at several sites on chromosome 2. This suggests
317   that the gene(s) most directly responsible for SD in *S. diaconus* reside either on chromosome 12, or in
318   the terminal region of chromosome 2. In either case, while the actual mechanism underlying sex
319   determination in this species remains unknown, this result points to the possibility of coinheritance of
320   many male-adaptive alleles across two chromosomes.

321   *Chromosome 19 is the sex chromosome in gopher and black-and-yellow rockfishes*

322   Our analysis identifies chromosome 19 as a putative sex chromosome in the sibling species gopher (*S.*
323   *carnatus*) and black-and-yellow (*S. chrysomelas*) rockfishes. While the smaller sample sizes (n = 20 in
324   each species) used for study by the original authors somewhat hinder our statistical power, it appears
325   that several markers on this chromosome are male-specific. The differences in presence/absence
326   analyses of both species indicate that *S. carnatus* has a higher degree of heteromorphism (Fig. S1-S2).

327   These species have a very recent common ancestor (Fowler & Buonaccorsi, 2016) and since they have
328   inherited the same sex chromosome, offer an opportunity to compare differentiation between the
329   species. As in *S. diaconus*, allele frequency and heterozygosity are the main signal of sex differences,
330   indicative of a sex chromosome in the early stages of differentiation. In comparing the two species, we
331   can see slightly more male-specific markers are present in *S. carnatus* than in *S. chrysomelas* (Fig. S1-S2).
332   This may indicate that *S. carnatus* is experiencing a faster rate of sex chromosome differentiation, in
333   which males have acquired insertions or females have undergone deletions of male-associated loci.

334   The presence of a single sex-biased region restricted to a single chromosome lends support to the classic
335   paradigm of differentiating sex chromosomes in these species. Indeed, the central peak of significant
336   marker density which declines outward in both species is consistent with typical patterns of linkage
337   disequilibrium. Notably, a BLAST query of common SD genes against the *S. carnatus* and *S. chrysomelas*
338   genomes did not provide any hits on chromosome 19, thus we do not know the actual genetic
339   mechanism. The sex of our reference genomes samples is not known; it is possible that the reference
340   genomes for these species are female and so our BLAST query would not find any male-specific genes.

341   *An unplaced sex-determining region in sunset and vermillion rockfishes*

342   We have identified an unplaced, putative sex-determining region in the sibling species *S. miniatus* and *S.*
343   *crocotulus*. Differences in mean sample depth reveal distinct peaks of sex differentiation in these
344   species: four in *S. miniatus* and three in *S. crocotulus*. The shape of these peaks however, while
345   suggestive that the degree of male bias tapers outward from a single locus, is an artifact of RADseq
346   technology, where read depth is highest near the first read cut site. All peaks are on a single unplaced
347   contig, and all RADtags on that region have a significant male bias, suggesting the entire contig is a male-
348   specific haplotype.

349   Mapping of the sunset and vermillion rockfish data to a higher quality reference genome was critical in
350   accurate characterization of the SD region; the RagTag-assembled alignment scattered the signal of sex
351   differentiation onto different chromosomes. Although the reference genomes we used in our initial
352   analyses are at the chromosome level, they were assembled using Illumina short-read data and then
353   scaffolded with a chromosome-scale relative. Under this scenario, it is more likely that individual contigs
354   of a highly diverged SD region are randomly scaffolded due to a lack of obvious synteny.

355   The significant differentiation observed on chromosome 24 of *S. crocotulus* presents an intriguing
356   possibility: that our unplaced sex-determining region could be at the start of chromosome 24. As we
357   established, both the unplaced SD region and the differentiation along the first megabase of
358   chromosome 24 are characterized by missing data in females. At the start of chromosome 24, there are
359   two distinct haplotypes, one with large amounts of missing data for some SNPs and one without.
360   Interestingly, we see some evidence of limited recombination between the haplotypes, which suggests
361   that the haplotypes can recombine, but also that recombination is very rare (Fig. 3B). We found that
362   both species have the deletion haplotype, but in *S. crocotulus* it is more likely to occur in males. Taken
363   together, this supports a scenario where the deletion haplotype is older than the speciation of *S.*
364   *crocotulus* and *S. miniatus* and has either become recently linked or unlinked to the SDR in one species.

365   *Weak support for GSD in yelloweye rockfish; no indication of GSD in boccacio and canary rockfish*

366   Significant sex-biased markers are detected in *S. ruberrimus* (Figure S6), but while the markers exhibit
367   significant male bias, none are exclusive to males. Only a single marker could be mapped to
368   chromosome 4, while the others are all on unplaced scaffolds. It is possible that, like *S. miniatus* and *S.*
369   *crocotulus,* the seemingly disparate sex-biased markers all originate from the same region but were
370   incorrectly scaffolded. Unfortunately, the small number and placement of these markers do not permit
371   further analysis without mapping to a higher quality genome.

372   No outlying sex-biased markers were detected by our analyses of *S. paucispinis* and *S. pinniger*. As *S.*
373   *paucispinis* was the species with the fewest samples in this study (n = 13), we have limited confidence in
374   this assessment pertaining to that species.

375   A lack of genetic markers associated with sex suggests SD by abiotic environmental factors, or social sex
376   ratio pressures. While ESD is perhaps best known in reptiles, it has also been demonstrated in bony
377   fishes, with temperature as the primary environmental determinant of sex (Yamamoto et al., 2019).
378   Other abiotic factors, such as salinity (Saillant et al., 2003) and pH (Reddon & Hurd, 2013) have also been
379   shown to induce skewed sex ratios in fishes. Further investigation into the forces underlying ESD in
380   these and other species is warranted, as warming and acidifying oceans leave species relying on ESD
381   much more vulnerable to impacts on sex ratios.

382   *Duplication of amh before speciation of Sebastes*

383   Our analysis found that the duplication of *amh* found by Song et al. (2021) predates the
384   *Hozukius/Sebastes* speciation event. Therefore, we might expect this duplication to be present in all
385   extant *Sebastes* species. While our phylogeny detects whole gene copies in only 28 *Sebastes* species, it is
386   possible that the duplication is male-specific and would not be detectable on genomes assembled from
387   female samples. As Kolora et al. (2021) did not sex their samples before assembly, we are unable to
388   make that determination. Further, when the duplicated *amhy* in *S. schlegelii* may have acquired its SD
389   function remains in question. Our phylogeny groups *S. schlegelii amhy* with that of *S. thompsoni, S.*
390   *hubbsi, S. koreanus, S. zonatus,* and *S. nudus,* each with a range limited to the Northwest Pacific. While
391   we have confirmed the presence of *amhy* in these species, its role in sex determination is unknown.

392   There are two duplications in *Sebastes* that stand out as evidence for the lability of SD genes in this
393   genus. In *Sebastes reedi*, the entire sequence of *amh* on chromosome 5 is found in an inverted
394   duplication immediately adjacent to the original gene copy. *Sebastes ensifer* possesses two copies of
395   *amhy*, one in the typical chromosome 4 position and one on chromosome 5, the typical position for
396   *amh.* This may suggest a translocation or gene conversion that overwrote the original *amh*, although we
397   cannot rule out that is a bioinformatic artifact during assembly.

398   *Areas for further research*

399   We have demonstrated that SD among Northeast Pacific *Sebastes* is highly diverse and, due to the
400   relatively undifferentiated sex chromosomes we uncovered, highly labile. However, as our study
401   comprises only eight species, our evolutionary inferences are severely limited. Further studies involving
402   sexed sequence data from geographically diverse *Sebastes* may shed light on the evolutionary trajectory
403   of SD systems in this genus. This could reveal shared mechanisms of GSD, gain-of-function events,
404   translocations, and other genomic rearrangements underpinning the lability of SD.

405   Deeper investigation into the genes underlying SD is required. While our study has discovered sex
406   chromosomes in three species, and potential SD loci in two more, the precise genetic mechanisms at
407   work are yet unknown. Until recently, most eukaryotic genomes sequences published were haploid
408   representations of a diploid individual. For species with partially differentiated SD regions, the SD region
409   is often shunted to an unplaced scaffold. Fully phased genomes for the heterogametic sex allows for

appropriate read mapping and an accurate assessment of gene level differences in the SD region (Carey et al., 2022).

Ultimately, while genomic variation gives us a place to look, genes must be expressed to play a role in sex determination. While sampling is underway, thorough sexing also provides an opportunity for isolation of the gonads, which can later be used to assemble a transcriptome. Characterization of the mRNA transcribed in gonads would significantly narrow the search for SD gene candidates (Chen et al., 2016; Gao et al., 2022).

Conclusion

Sex determination systems in nature are myriad and dynamic. While patterns do emerge in the evolution of SD mechanisms in animals, ever more analyses of systems without fixed modes of SD challenge the classic paradigm underlying sex chromosome differentiation. Here, we demonstrate that *Sebastes* rockfish exhibit a wide diversity of SD mechanisms of independent origins. In this recently speciated genus, we find evidence for heterogametic, multi-locus, and environmental SD mechanisms, in only a handful of study species.

Further study into the diversity of SD in this highly speciose genus is essential to enhance our understanding of the selective and stochastic forces underlying sex determination. Indeed, our results contribute to a growing body of evidence that our current understanding of SD evolution is lacking. As studies into previously overlooked taxa reveal the wealth and lability of SD systems in nature, we stand to better characterize the genetic and ecological drivers of sex differentiation, a fundamental trait spanning the tree of life.

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