1 **Prediction of the sex-determination gene in tunas (*Thunnus* fishes)**

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23 **Abstract**

24 Fish species have a variety of sex determination systems. Tunas (genus *Thunnus*) have an XY
 25 genetic sex-determination system. However, the Y chromosome or responsible locus has not yet
 26 been identified in males. In a previous study, a female genome of Pacific bluefin tuna (*T. orientalis*)
 27 was sequenced, and candidates for sex-associated DNA polymorphisms were identified by a
 28 genome-wide association study using resequencing data. In the present study, we sequenced a male
 29 genome of Pacific bluefin tuna by long-read and linked-read sequencing technologies, and explored
 30 male-specific loci through a comparison with the female genome. As a result, we found a unique
 31 region carrying the male-specific haplotype, where a homolog of estrogen sulfotransferase gene was
 32 predicted to be encoded. The genome-wide mapping of previously resequenced data indicated that,
 33 among the functionally annotated genes, only this gene, named *sult1st6y*, was paternally inherited in
 34 the males of Pacific bluefin tuna. We reviewed the RNA-seq data of southern bluefin tuna (*T.*
 35 *maccoyii*) in the public database and found that *sult1st6y* of southern bluefin tuna was expressed in
 36 all male testes, but absent or suppressed in the female ovary. Since estrogen sulfotransferase is
 37 responsible for the inactivation of estrogens, it is reasonable to assume that the expression of
 38 *sult1st6y* in gonad cells may inhibit female development, thereby inducing the individuals to become
 39 males. Thus, our results raise a promising hypothesis that *sult1st6y* is the sex-determination gene in
 40 *Thunnus* fishes, or at least functions at a crucial point in the sex-differentiation cascade.

41

42 **Key words:** bluefin tuna, sex determination, gene duplication, estrogen sulfotransferase

43 **Introduction**

44 Fish species have a substantial variety of sex-determination systems compared to other animals,
45 ranging from environmentally to genetically modulated ones. In the environmental sex determination
46 system, individual sex depends mainly on nongenetic factors such as temperature, growth rate, and
47 density, while in the genetic system, specific genes dominantly control sex differentiation. Regarding
48 sexuality, it is estimated that more than 98% of fish are gonochoristic (Pandian, 2011). Therefore, in
49 most fish, some responsible loci should exist biasedly between males and females or differentially
50 affect gonadal phenotypes, even if the sex is primarily determined by environmental factors. In
51 heterogametic (XY) males or heterogametic (ZW) females, sex chromosomes may be cytologically
52 evident markers as carrying the sex determination loci, but they are often homomorphic in fishes
53 (Devlin and Nagahama, 2002), undistinguishable from autosomes by direct microscopic observation.
54 In other words, fish sex chromosomes are not highly differentiated, implying that turnovers may
55 have often occurred over the course of evolution (Pennell et al., 2018). Recently, sex determination
56 genes have been reported in several fish species, including *DMY/Dmrt1* in medaka (Matsuda et al.,
57 2002; Nanda et al., 2002) and Chinese tongue sole (Cui et al., 2017), *Amhr2* in fugu (Kamiya et al.,
58 2012), *sdY* in rainbow trout (Yano et al., 2012), *Hsd17b1* in yellowtail (Koyama et al., 2019), and
59 *amh* in Patagonian pejerrey (Hattori et al., 2012) and northern pike (Pan et al., 2019). These studies
60 suggest that a master gene for sex determination in fish can be easily replaced by another gene,
61 which may be followed by the above-mentioned turnover of the sex chromosome. In particular, the
62 cases in fugu and yellowtail are prominent examples, where only single nucleotide substitutions are
63 critical for sex determination, suggesting that the replacements of master gene occurred only recently
64 in these lineages. Thus, the sex determination genes of fish are often different for each lineage and
65 are not always identified by deductive approaches from model organisms (that is, case studies are
66 required to identify the genes).

67 Regarding tunas, the fish of the genus *Thunnus* South 1845, it is estimated that Pacific bluefin
68 tuna (*Thunnus orientalis*, PBT) has an XY genetic sex determination system (Agawa et al., 2015).
69 However, the chromosome or locus responsible has not yet been identified. One reason for this is
70 that no distinguishable sex chromosomes were observed in the cytological analysis. It has been
71 reported that little tuna, *Euthynnus affinis*, may have a ZW sex determination system (Yazawa et al.,
72 2019). These observations suggest that the sex determination gene of *Thunnus* species has only
73 recently emerged, and therefore, that the sex chromosomes are not highly divergent. Another reason
74 is the technical difficulty in handling: since tunas are large and difficult fish to culture, *in vivo*
75 experiments for identifying sex-associated loci are costly and time-consuming. On the other hand,
76 because tunas have a high market value worldwide, tuna target fishing and aquaculture have been
77 conducted in many countries. In aquaculture, the control of the sex ratio in tanks or ocean nets is
78 very important for breeding, as keeping more females than males leads to an increased production of
79 fertilized eggs. Since tunas do not show any clear sexual dimorphism, the direct observation of the
80 gonad has been the main technique for sex identification. However, this is a destructive inspection
81 and is not applicable for selectively collecting females or removing males in tanks. Therefore,
82 molecular markers associated with the sex of tuna have been of interest as an alternative for rapid
83 sex identification. In particular, DNA markers have been considered promising, where a small
84 portion of tissue sections, such as the fin or skin, are sufficient for PCR assays. A DNA marker
85 associated with PBT sex was reported in 2015 (Agawa et al., 2015). The marker, Md6, was
86 developed from the DNA samples of cultured PBT individuals and showed an association with sex
87 in the aquaculture population. Regarding the genomic approach, the PBT male genome data were
88 open (Nakamura et al., 2013), but the scaffold sequences were too fragmented for a genome-wide
89 screening of sex-associated loci. Recently, transcriptomics of southern bluefin tuna (*T. maccoyii*)
90 have been conducted, and the differentially expressed genes between male and female gonads (testis

91 and ovary) were examined (Bar et al., 2016). However, it should be noted that a differentially
92 expressed gene between the testis and ovary is not always the sex determination gene, but may be a
93 downstream gene regulated by the master gene.

94 In 2019, Suda et al. sequenced the genome of female PBT, compared the assembled scaffold
95 with that of the male genome, and explored sex-associated regions from the scaffold sequences by
96 resequencing 31 PBT individuals (15 males and 16 females) (Suda et al., 2019). They performed a
97 genome-wide association (GWA) study focusing on sex-associated single nucleotide variants (SNVs)
98 between males and females, and identified several regions carrying male-specific alleles. Finally,
99 they developed three PCR primer pairs for sex identification, namely scaf64_3724604_F/R,
100 scaf64_3726411_F/R, and scaf64_3724591_F/R, based on male-specific heterozygous SNVs and
101 short indels detected in the 6.5-kb region of female scaffold 64 (F64). The PCR primers were
102 designed so that the amplification patterns were different between PBT males and females:
103 scaf64_3724604_F/R and scaf64_3726411_F/R were amplifiable in males (the sizes were 113 bp
104 and 143 bp, respectively) but not in females, and scaf64_3724591_F/R was amplified in both males
105 and females, but the sizes were different (142 bp and 149 bp in males and only 149 bp in females).
106 PCR amplification using 115 individuals (56 males and 59 females) yielded 100% accuracy of sex
107 identification, implying that the PBT sex-determination gene may exist around the 6.5-kb region in
108 F64. They predicted some genes in F64 and discussed the possible involvement in the sex
109 determination of PBT. However, promising genes, such as those related to the cascade, were not
110 identified. The potential problem in the previous study was that they found male-specific
111 heterozygous polymorphisms in multiple scaffolds. Since most sex determinants are often attributed
112 to a single locus, they suspected that multiple candidates occurred due to assembly errors.

113 In this study, we improved the male genome and examined the sex-associated regions using
114 previously published data. We have found a sex-associated gene that is paternally inherited and is

115 involved in estrogen inactivation. The current results provide the most promising candidate for the
116 sex determination gene of tuna.

117 **Materials and Methods**

118 **Genome sequencing and assembly**

119 A three-year-old male F2 individual of Pacific bluefin tuna, which was cultured at the Seikai
 120 National Fisheries Research Institute (currently Fisheries Technology Institute), was collected for
 121 genome sequencing. Frozen heart tissue was prepared from the specimen, and DNA was extracted
 122 using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands). Then, an SMRT-bell template
 123 library was prepared following the manufacturer's protocol and sequenced using the Pacific
 124 Biosciences (PacBio) Sequel platform (P6C4 chemistry) (Pacific Biosciences, Inc. CA). From the
 125 same tissue sample, the DNA was extracted also using the standard phenol-chloroform protocol. A
 126 Chromium 10X library was prepared using Chromium Genome Library Kit v2, Genome Gel Bead
 127 Kit, Genome Chip Kit v2, and i7 Multiplex Kit (10X Genomics, CA) following the manufacturer's
 128 protocol, and the linked reads were sequenced using the Illumina NovaSeq 6000 platform (Illumina,
 129 Inc. CA). The PacBio reads were first assembled by HGAP4 (SMRT Link v5.1.0.26412) (Chin et al.,
 130 2013). The contigs obtained were corrected using NovaSeq linked-reads by Pilon v1.23 (Walker et
 131 al., 2014) and Tigmint v1.1.2 (Jackman et al., 2018), and finally scaffolded by ARKS v1.0.2
 132 (Coombe et al., 2018) in combination with LINKS v1.8.5 (Warren et al., 2015). The scaffold
 133 sequences obtained were submitted to the DNA Data Bank of Japan under the accession nos.
 134 BOUD01000001-BOUD01000948. To examine the phased genomic regions, the linked reads were
 135 assembled using Supernova2 (v2.1.1) (Weisenfeld et al., 2017). The former assembly (HGAP4 +
 136 ARKS) was assessed by Benchmarking Universal Single-Copy Orthologs (BUSCO) version 4.0.5
 137 (Simão et al., 2015) with a set of Actinopterygii orthologs (actinopterygii_odb10). The genes around
 138 the sex-associated region were predicted based on the protein sequences of model fish species in the
 139 Ensembl database (release 99) (Aken et al., 2017), namely *Danio rerio*, *Oryzias latipes*,
 140 *Gasterosteus aculeatus*, *Takifugu rubripes*, and *Tetraodon nigroviridis*, by Exonerate v2.4.0 (Slater

141 and Birney, 2005). The PBT female genome sequence was obtained from the GenBank database
142 (accession no. BKCK00000000). The nucleotide sequence comparison was performed by MUMmer
143 v4 (Marcais et al., 2018). The repeat library of PBT genome was constructed by RepeatModeler
144 Open-1.0, and the repetitive regions were masked by RepeatMasker Open-4.0
145 (<http://www.repeatmasker.org>). The duplicated regions were further detected by self-to-self
146 BLASTN (Altschul et al., 1990) in each of the PBT male and female genomes, and the sequences
147 detected were used also for masking.

148

149 **Genome-wide SNV/CNV analysis**

150 The sequenced reads for 31 PBT individuals (15 males and 16 females) (Suda et al., 2019) were
151 downloaded from the NCBI SRA database (accession no. DRR177387). The read sequences were
152 extracted using SRA Toolkit (<https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software>), and
153 then preprocessed using Trimmomatic v0.36 (Bolger et al., 2014) and ParDRe v2.2.5
154 (Gonzalez-Dominguez and Schmidt, 2016), according to Suda et al. Mapping to the scaffold
155 sequences and single-nucleotide variant calling were conducted using MapCaller v0.9.9.37 (Lin and
156 Hsu, 2019). After filtering out inadequate sites (not biallelic, depth < 20), genome-wide association
157 (GWA) between males and females was estimated through 2×3 contingency tables using the R
158 package, *rrBLUP* (Endelman, 2011), with minor allele frequency > 0.05. Statistical significance was
159 defined as $P < 0.05$ after Bonferroni correction based on the total number of variant sites examined.
160 For relative depth analysis, raw read depth per nucleotide site was counted using Samtools (*samtools*
161 *depth*) (Li et al., 2009), and then divided by the median depth. Copy number variations (CNVs) were
162 detected by CNVcaller (Wang et al., 2017), where the above-mentioned mapping data were scanned
163 by a 500-bp window and regional copy numbers were obtained by merging them in the neighboring
164 windows. The difference in the regional copy number between males and females was tested using

165 the Mann-Whitney U test with Bonferroni correction. Furthermore, the regions carrying either of the
166 three types of copy numbers (0, 0.5, or 1) among the 31 samples were selected, and the GWA was
167 estimated in the same way as the SNV-based GWA scan mentioned above.

168

169 Sequence analysis of sex-associated genes

170 The transcriptome data of southern bluefin tuna (Bar et al., 2016) were downloaded from the NCBI
171 SRA database (accession no. SRP059929). The read sequences were extracted using SRA Toolkit,
172 preprocessed by Trimmomatic, and assembled using Trinity v2.8.4 (Haas et al., 2013). From the
173 contig sequences obtained, protein-coding sequences were predicted using TransDecoder v5.0.0
174 (Haas et al., 2013). Homologs of PBT genes were searched using BLASTP (Altschul et al., 1997)
175 from the contig sequences, and the transcriptional variants were manually checked based on the
176 sequence alignment using MAFFT v7.310 (Katoh et al., 2009). Using the Trinity contig sequences as
177 a reference, gene expression was measured using RSEM v1.3.1 (Li and Dewey, 2011). For molecular
178 phylogenetic analysis, homologs in other fish genomes were downloaded from the Ensembl database.
179 The coding sequences were then aligned using MAFFT, where the deduced protein sequences were
180 aligned and converted into nucleotide sequence alignments. The maximum-likelihood tree was
181 constructed using RAxML-NG v0.9.0 (Kozlov et al., 2019).

182 **Results**

183 **Genome assembly and validation of sex-associated markers**

184 We constructed the genome sequence of male PBT by *de novo* assembly, yielding a total of 948
 185 scaffolds (>1000 bp) and totaling 827 Mb (Table 1). Using BUSCO for quality assessment, this
 186 assembly completely captured 96.3% of Actinopterygii orthologs (3505 out of 3460). On the other
 187 hand, the score for the female genome was 89.3% (3253 out of 3640), consistent with previous
 188 reports. Using the sex-associated PCR primer sequences reported in a previous study, we performed
 189 a BLAST search against the current male genome, and found that two scaffolds, namely M175 and
 190 M44, carried the regions amplifiable by the primer sets (two male-specific sets,
 191 scaf64_3724604_F/R and scaf64_3726411_F/R, and a common primer set, scaf64_3724591_F/R)
 192 (Table 2). In scaffold M175 (232,647 bp in total), the regions amplified by the three primer sets were
 193 closely located. The estimated amplicon sizes were congruent with those previously observed only in
 194 males: 113 bp for scaf64_3724604_F/R, 143 bp for scaf64_3726411_F/R, and 142 bp for
 195 scaf64_3724591_F/R, respectively. Scaffold M44 (4,583,724 bp in total) had a single region
 196 amplified by scaf64_3724591_F/R, and the estimated amplicon size was 149 bp, which was
 197 equivalent to that observed previously in both males and females. Thus, based on the estimated PCR
 198 products, M175 carried all of the male-specific regions and M44 carried the common haplotype to
 199 both males and females. We compared the nucleotide sequences of M175 and M44, and found that
 200 M175 was almost dissimilar to that of M44 (Figure 1), while M44 was fully aligned with female
 201 scaffold F64 (Figure 2), in which the 6.5-kb sex-linked region (SLR) was reported in the previous
 202 study. The 6.5-kb SLR was found also in M44. On the other hand, we found no counterpart of M175
 203 in the female genome, although the 6.5-kb SLR of M44/F64 (SLR_{M44} or SLR_{F64}) was partially
 204 similar to the 10.7-kb region in M175 (SLR_{M175}) (Figure 1). We assembled the male genome using
 205 Supernova2 and obtained scaffolds corresponding to M44 and M175, respectively (Supplementary

Figure 1). The M44-like scaffold carrying SLR_{M44} was phased into two pseudo-haploid scaffolds, but SLR_{M44} itself was homozygotic. On the other hand, the scaffold carrying SLR_{M175} was not phased.

Read depth analysis

We downloaded the resequencing data of 31 PBT individuals (15 males and 16 females) and mapped the reads to the current male genome sequence. As a result, both male and female reads were fully mapped to SLR_{M44} (Figure 3 and Supplementary Figure 2). Regarding M175, the female reads were hardly mapped to SLR_{M175}, in contrast to the male reads. Furthermore, there were consistently fewer male reads mapped to SLR_{M175} than SLR_{M44}. We computed the median read depths for whole scaffolds, and thereby converted the read depths of SLR_{M44} and SLR_{M175} into the relative values. In males, the relative depths of SLR_{M175} were close to 0.5 (Figure 4). On the other hand, the relative depths of SLR_{M44} were close to 1 in both males and females. These observations strongly suggest that, in shotgun sequencing libraries, the DNA content derived from SLR_{M175} in the male samples is always half that derived from the average scaffolds, reflecting the ploidy or copy number. The majority of chromosomal regions, except for highly repetitive ones or those of polyploidized genomes, should exist as singletons in a diploid manner (2n), and the relative depths for many regions would be approximately 1. Therefore, our observations suggest that SLR_{M175}, with a relative depth of ~0.5, should exist in a haploid manner (1n) only in males, while SLR_{M44} should exist in a diploid manner, like many other regions, in both males and females.

Genome-wide association analysis

Since the results obtained until now appeared to be inconsistent with those previously reported (see Discussion), we validated the previous mapping and GWA results based on the female genome. We

230 mapped the 31 resequenced read sets to the female reference genome and re-performed GWA
 231 analysis. As a result, the previous result was roughly reproduced, with significant peaks observed in
 232 multiple scaffolds, including F64 (Figure 5A). However, when the mapped reads between the female
 233 and present male genomes were compared, over 10% of the reads mapped to female SLR_{F64} in the
 234 15 male samples were found to be potentially derived from SLR_{M175} (Figure 6). This observation
 235 implies that the reads derived from the male-specific region, such as SLR_{M175}, would have been
 236 incorrectly mapped to paralogous regions in the female genome in the previous study. To avoid the
 237 effect of such cross mapping, we performed the GWA scan again after the duplicated regions were
 238 thoroughly masked by RepeatMasker and BLASTN. This result was in contrast to that from the
 239 unmasked reference: no significant peaks were observed in the masked sequences (Figure 5B).
 240 Similarly, GWA scans were performed using the current male genome, but no significant peaks were
 241 found in the masked sequences (Supplementary Figure 3).

242 We then tested another approach to identify the regions carrying male-specific haplotypes, such
 243 as SLR_{M175}. Using the mapping data for the 31 individuals, we computed copy number variations
 244 (CNVs) across the male genome and explored the regions whose copy numbers were significantly
 245 different between males and females. As a result, only one candidate was found in M175
 246 (18,001–78,750 bp), very close to SLR_{M175}, and the copy number in males was significantly larger
 247 than that in females (Figure 7A). All of the males had 0.5 copies of the detected region and all of the
 248 females had no copy (i.e., absent), in concordance with the relative depth analysis results (Figure 4).
 249 We computed genome-wide associations for the haploid regions by converting the copy numbers (0,
 250 0.5, and 1) to three quasi-genotypes (0/0, 0/1, and 1/1). As a result, only one significant peak for this
 251 region was detected in scaffold M175 (Figure 7B).

252

253 **Protein-coding genes in scaffold M175**

254 We scanned the M175 sequence and predicted 12 protein-coding genes (Figure 8 and Supplementary
255 Table 1). Of these, two genes, g3 (32,614–64,628 bp) and g4 (76,852–77,963 bp), were located in
256 the haploid region (18,001–78,750 bp) detected by CNV analysis. The deduced amino acid sequence
257 of g3 was similar to that of estrogen sulfotransferase encoded by *sult1st6* (e.g., with ~83% identity to
258 ENSORLP00000007539 of *Oryzias latipes*). Therefore, the homolog of PBT was named *sult1st6y*.
259 In the case of g4, the deduced amino acid sequence was similar to that of ENSTRUP00000072273
260 (gene ID: ENSTRUG00000033234) of *Takifugu rubripes*, with ~70% identity. As a “novel gene” in
261 the Ensembl database, the function was unknown. However, the domain “Integrase, catalytic core”
262 was predicted by Pfam and PROSITE, and found to overlap with the Gene3D domain “Ribonuclease
263 H superfamily.” With regard to the neighboring genes, the protein sequence of g2 was matched to
264 ENSTRUP00000068256 (ENSTRUG00000032453, novel gene), with the Pfam domain “PiggyBac
265 transposable element-derived protein,” and g5 was matched to ENSTRUP00000067783
266 (ENSTRUG00000032600, novel gene) with the Pfam domain “Reverse
267 transcriptase/retrotransposon-derived protein, RNase H-like domain.” Notably, g1 was similar to g5
268 and matched to ENSTRUP00000067783, suggesting that g2–g4 were sandwiched between
269 duplicated genes (g1 and g5). In addition, g6 was matched to ENSORLP00000040498
270 (ENSORLG00000025541, novel gene) with the Pfam domain “Transposase, Tc1-like,” and the other
271 genes, g7–g12, were matched to the same protein, ENSORLP00000044417
272 (ENSORLG00000028531, novel gene) with the Gene3D domain “Immunoglobulin-like fold.” Thus,
273 only g3 was a functionally annotated gene, and the others were novel or related to transposable
274 elements. We found no homologs of *sult1st6y* (g3) in the scaffolds M44/F64. Instead, the paralog
275 was found in male scaffold 30 (M30, 6594208–6602041 bp), named *sult1st6a*, and the nucleotide
276 and protein identities to *sult1st6y* were 92% and 89%, respectively. Male scaffold M30 corresponded
277 to a part of female scaffold 47 (F47) (Supplementary Figure 4), where *sult1st6a* was also found.

278 To determine whether these *sult1st6* genes are present in other tuna species, we downloaded the
 279 RNA-seq data of southern bluefin tuna (SBT), which were obtained from 10 male testis samples and
 280 10 female ovary samples. We assembled the reads using Trinity and conducted a homology search
 281 against the contigs using PBT *sult1st6y/sult1st6a* sequences. As a result, we found the homologs of
 282 *sult1st6y/sult1st6a* in SBT, although the Trinity contigs were originally classified into a single cluster.
 283 We conducted phylogenetic analysis using PBT and SBT *sult1st6* and the homologs in the Ensembl
 284 database (Figure 9 and Supplementary Figure 5). The phylogenetic tree showed that each SBT
 285 homolog formed a clade with each of PBT's *sult1st6y/sult1st6a*, respectively, suggesting that SBT
 286 also had two paralogs of *sult1st6* and the duplication to *sult1st6y* and *sult1st6a* occurred in the
 287 common ancestor of PBT and SBT. Using the transcript contigs as a reference, we mapped the reads
 288 in each of the 20 samples to those and measured the gene expression levels (Figure 10). As a whole,
 289 the expression of SBT's *sult1st6y/sult1st6a* was low compared to that of the control (0–2% of β -actin
 290 in FPKM). In detail, the expression of *sult1st6a* was observed in both the testis and ovary samples,
 291 although it tended to be low in the latter (0–0.01% of β -actin in FPKM) (Figure 10A). The
 292 expression of *sult1st6y* was observed in all 10 testis samples, but none of ovary samples, indicating a
 293 significant bias of *sult1st6y* expression between the testes and ovaries ($P < 0.05$, Fisher's exact test)
 294 (Figure 10B).

295 Discussion

296 In this study, the male genome of Pacific bluefin tuna was sequenced using long-read
 297 sequencing technologies. The scaffold sequences assembled were more contiguous than those
 298 published in 2013, which were obtained from pyrosequencing and short paired-end reads.
 299 Completeness assessment indicates that the current version is better than that of the recent female
 300 genome, although the number of scaffolds (948) is larger than that for the female genome (444).
 301 Therefore, the current genome is expected to be useful for the validation and extension of previous
 302 results regarding the sex identification of PBT. In the previous study, three PCR primer sets were
 303 designed from the sequence of female scaffold F64, so that the amplification patterns were different
 304 between PBT males and females. In this study, we first searched for the locus amplified by the
 305 primer sets in the current male genome, and found two regions: one in scaffold M44 and the other in
 306 M175. Based on the results of the large-scale sequence comparison, M44 was found to be the
 307 counterpart of F64, while M175 was male-specific. Since M44 and F64 were well aligned with each
 308 other, including 6.5-kb SLRs (SLR_{M44}/SLR_{F64}), it is unlikely that large-scale misassemblies are
 309 present in M44/F64. The PCR amplification pattern in SLR_{M44} , estimated using primer sequences,
 310 was identical to that observed in females. Regarding M175, all the PCR amplicons with
 311 male-specific sizes were observed within the 10.7-kb region (SLR_{M175}). It is worth noting that the
 312 PCR primer sets were originally designed based on the SNVs predicted in F64 by GWA analysis.
 313 Therefore, according to the previous results, M44 and M175 should be allelic to each other. However,
 314 these sequences are quite dissimilar to each other. Furthermore, based on the mapping result of
 315 resequenced data, the relative read depths to that for the average scaffold data were (SLR_{M44} ,
 316 SLR_{M175}) $\approx (1, 0)$ in females but (SLR_{M44} , SLR_{M175}) $\approx (1, 0.5)$ in males. These estimates indicate that
 317 SLR_{M44} should exist in a diploid manner in both males and females, whereas SLR_{M175} should exist in
 318 a haploid manner only in males. If SLR_{M44} was allelic to SLR_{M175} in males, the relative read depths

(SLR_{M44}, SLR_{M175}) should be (0.5, 0.5). The result of relative read depth is congruent with that of phased assembly by Supernova2, where two pseudo-haplotype scaffolds carrying homozygotic SLR_{M44} were obtained, but only unphased one was obtained for SLR_{M175}. These results indicate that SLR_{M44} and SLR_{M175} are located at different loci in the male genome, where SLR_{M175} is the male-specific structural variant (i.e., inserted in males or deleted in females) and is inherited paternally. In other words, SLR_{F64} (or SLR_{M44}) reported in the previous study was not associated with PBT sex, although the PCR primer sets designed from SLR_{F64} are still available for sex identification based on the amplification pattern in SLR_{M175}.

Now, the previous result, in which male-specific heterozygous polymorphisms were detected in F64, needs to be reconsidered. The previous GWA scan was performed using the female genome as a reference, focusing on male-heterozygous SNVs or small indels, which may be detectable by the resequenced short reads. However, M175 found in the current study is a large-scale structural variant absent from the female genome and should be undetectable by short-read mapping. We considered the possibility that the reads derived from paralogous loci were cross-mapped to the female reference genome, causing fake heterozygous SNV calls in genotyping prior to GWA analysis. In the previous study, the repeat or paralogous sequences were not masked; therefore, it is likely that the reads derived from the paternal 10.7-kb region (i.e., SLR_{M175}) were mapped to the 6.5-kb region of F64 in the analysis of male DNA samples. This hypothesis was confirmed by checking the mapped reads: at least 10% of the male reads mapped to SLR_{F64} in the previous analysis were estimated to be derived from SLR_{M175}. Furthermore, we compared the GWA results between the two conditions for the female reference sequence: duplicated regions were unmasked (i.e., raw sequences) and masked. The result was clear-cut, where the peaks in multiple scaffolds were roughly reproduced in the unmasked sequences, but no such peaks were observed in the masked sequences. Thus, we conclude that the previous GWA result was an artifact due to cross-mapping, and many of the scaffolds predicted (e.g.,

343 F64) were not associated with the sex of PBT. Since we found no significant GWA peaks in the
344 masked male reference genome, the SLR of PBT would be undetectable from the information of
345 SNVs or small indels. The current results provide a caveat that traditional GWA analysis, which
346 targets SNVs using short reads, may not always be sufficient for the identification of SLR in species
347 with a genetic sex determination system.

348 Instead of SLR_{M44}, we found that SLR_{M175} is a male-specific locus absent from the female
349 genome. It should be noted that this finding is due to a homology search of the primer sequences
350 designed in the previous study. Therefore, it is possible that the current male genome contains other
351 scaffolds carrying sex-associated structural variants. To check this possibility, we conducted a
352 genome-wide scan based on a CNV detection scheme using mapping data. As a result, we observed
353 that only M175 had a 61-kb region (18,001–78,750 bp) where the CNV was significantly different
354 between males and females. Since this region is very close to SLR_{M175}, eventually the candidate
355 sex-associated locus was narrowed down to a single region around or including SLR_{M175}. Regarding
356 the CNV detected, we found that PBT females have no copy, while males have 0.5 copies of the
357 region, indicating that this region exists in a haploid manner in males, or equivalently, that this
358 region is paternally inherited. Thus, we conclude that this region (61-kb region + SLR_{M175}) is the
359 only candidate for the Y-linked locus in PBT. In M175, we predicted a total of 12 protein-coding
360 genes, two of which (g3 and g4) were included in the predicted haploid region, one (g3) of which
361 was annotated as encoding estrogen sulfotransferase (SULT1ST6), while the other (g4) was
362 functionally unknown. We note that g4 has the domain “Integrase, catalytic core” or “Ribonuclease
363 H superfamily” which is a feature of the retroviral integrase superfamily (Nowotny, 2009).
364 Regarding the other 10 predicted genes in M175, the functions are unknown. In particular, g1, g2,
365 and g5 may be involved in transposons according to domain prediction. Therefore, around the
366 male-specific haploid region, only the centermost g3, namely *sult1st6y*, is functionally annotated and

367 sandwiched between possibly transposable elements, implying that the *g3* locus may have been
368 unstable and transposed from the ancestral locus.

369 The current study suggests that *sult1st6y* is the most convincing gene involved in the sex
370 determination or differentiation of PBT. The encoding protein, SULT1ST6, belongs to the family of
371 cytosolic sulfotransferases (SULTs) and has been studied as a detoxifying enzyme in mammals
372 (Suiko et al., 2017). Regarding fish species, the SULT genes have been investigated mainly in
373 zebrafish, and 20 distinct paralogs (9 SULT1s, 3 SULT2s, 5 SULT3s, 1 SULT4, 1 SULT5, and 1
374 SULT6) have been characterized. Among these, SULT1ST6, one of the nine SULT1 paralogs,
375 displays strict substrate specificity for estrogens (e.g., estrone). This gene is thought to be involved
376 in organogenesis, such as the development of the eye and muscle in zebrafish (Yasuda et al., 2005).
377 On the other hand, there is no report about the relationship between estrogen sulfotransferase and sex
378 development. In fish, steroid hormones are considered to be the key factor for sex determination
379 (Yamamoto, 1969; Devlin and Nagahama, 2002; Nakamura, 2010), wherein sex can change
380 depending on the levels of estrogen or androgen production in gonad cells. The gene directly
381 contributing to steroidogenesis has also been reported as a sex-determination gene in yellowtails
382 (Koyama et al., 2019). Since endogenous estrogens are inactivated by sulfation (Raftogianis et al.,
383 2000), it may not be surprising that the function of *sult1st6* is involved in sex development. If this
384 gene is expressed in gonad cells at the initiation of sex development, it may inhibit female
385 development by depleting active estrogens, resulting in male development. In the present study, we
386 found that *sult1st6y* of PBT was paternally inherited in the population. It is unlikely that an
387 estrogen-inactivating gene with such a mode of inheritance is unrelated to the sex development of
388 PBT. In addition, we found that both female and male PBT had a paralog of *sult1st6y*, namely
389 *sult1st6a*, in male scaffold M30 or female scaffold F47. It is worth noting that, in the previous study,
390 F47 was another candidate carrying sex-associated SNVs (Suda et al., 2019), and the locus of

391 *sult1st6a* was close to the artifact GWA peak. This observation is not only attributable to
 392 cross-mapping, as mentioned before, but also indirectly suggests that the *sult1st6*-like gene might be
 393 associated with the sex of PBT. Since *sult1st6a* is inherited regardless of sex in a diploid manner, we
 394 speculate that *sult1st6a* plays a common role in PBT males and females, for example, in
 395 organogenesis at the early developmental stage, as proposed in the model fish. This suggests that
 396 *sult1st6y* could have been immune from the original role of *sult1st6* as a redundant copy: after gene
 397 duplication in the ancestor of PBT, the function of *sult1st6y* may have been spatiotemporally
 398 differentiated from that of *sult1st6a* in the proto-paternal line. In fact, it has been reported that a
 399 copy of duplicated genes may be sex-determination gene in fishes (Hattori et al., 2012; Yano et al.,
 400 2012; Matsuda and Sakaizumi, 2016). We found that homologs of *sult1st6y* and *sult1st6a* were
 401 present in the southern bluefin tuna. Phylogenetic analysis suggested that gene duplication occurred
 402 in the common ancestor of PBT and SBT. The duplication event is specific to the tuna lineage, not
 403 observed in other fishes, except for tandem duplications in some species. According to the
 404 transcriptome, SBT *sult1st6y* was expressed in male's testis but not in female's ovary. Considering
 405 that the expression of *sult1st6a* was at least detectable in females, although at a much lower level
 406 than in males, *sult1st6y* below the detection limit might be originally absent from SBT females like
 407 PBT females. Although this hypothesis will need to be tested by sequencing the entire SBT genome,
 408 our observations suggest that SBT *sult1st6y* may also be involved in sex development like PBT
 409 *sult1st6y*.

410 The results presented in this study provide an attractive hypothesis for *sult1st6y* being a
 411 sex-determination gene in *Thunnus* fishes, or at least functioning at an important point in the
 412 sex-differentiation cascade. Our hypothesis may be tested by further genetic experiments, although
 413 the handling of tuna in tanks is not easy. Recently, genome-editing technology has been applied to
 414 the mutagenesis of tuna (Higuchi et al., 2019). For example, it may be possible to check whether the

415 sex ratio of larvae is heavily biased by the knockout of *sult1st6y* in fertilized eggs. Lastly, it is worth
 416 emphasizing that the CNV-based approach used in this study is very useful for identifying a
 417 trait-associated region using short reads when it is involved in large structural variants. In particular,
 418 when the region contains recently duplicated sequences, the traditional SNV-based approach may not
 419 only miss structural variants but also produce artifacts due to cross-mapping to unassociated regions.
 420 In other fish species, sex-associated SNVs have often been observed in multiple scaffolds (Fowler
 421 and Buonaccorsi, 2016; Star et al., 2016; Dong et al., 2019), some of which might be cases of
 422 cross-mapping of short reads, unless the sexes are polygenically determined. We believe that a
 423 combination of traditional SNV- and CNV-based approaches is useful for the identification of
 424 sex-associated genes in fish species with a genetic sex-determination system.

425 **Data availability statement**

426 The genome sequences of Pacific bluefin tuna obtained in this study were deposited to the DNA

427 Data Bank of Japan. The accession numbers can be found in the article.

428

429 **Author contributions**

430 YN and AF conceived the study. KG coordinated the experiments for sampling, and KH, KK, MY,

431 and TT performed those. AF conducted genome sequencing, and YN conducted bioinformatic

432 analyses. YN drafted the manuscript, and MY helped to improve it. All authors read and approved

433 the final version.

434

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593

594 **Tables**

Table 1. Assembly statistics of PBT genomes

	This study	Previous study*
Number of scaffolds	948	444
Total scaffold size (Mbp)	827	787
N50 scaffold size (Mbp)	13.2	7.9
BUSCO completeness (%)	96.3	89.3
Single copy BUSCOs (%)	93.6	86.1
Duplicated BUSCOs (%)	2.7	3.2

*Accession no. BKCK00000000.

595

596 **Table 2. Amplified sizes of sex identification PCR primer sets**

Primer set	This study		Amplified size in the previous study (bp)	
	M44	M175	Female	Male
scaf64_3724604_F/R	-	113	-	113
scaf64_3726411_F/R	-	143	-	143
scaf64_3724591_F/R	149	142	149	142, 149

597

598 **Figure legends**

599

600 **Figure 1.** Sequence comparison between PBT male scaffolds M175 (X-axis) and M44 (Y-axis). Left
601 panel indicates the full-size comparison (232,647 bp vs. 4,583,724 bp), and the boxed region is
602 enlarged at right panel. In the right panel, SLR_{M44} and SLR_{M175} are highlighted in gray.

603

604 **Figure 2.** Sequence comparison between male scaffold M44 and female scaffold F64. The 6.5-kb
605 SLRs are indicated with arrows, respectively.

606

607 **Figure 3.** Mapped read depths around SLRs in resequenced PBT samples. For each of the female
608 (DRR177382) and male (DRR177383) samples, SLR is highlighted in gray. The read depths for all
609 the 31 samples are shown in Supplementary figure 2.

610

611 **Figure 4.** Comparison of relative read depths between males and females for SLR_{M44}/SLR_{M175}. The
612 mapping of resequenced reads was conducted using an unmasked male reference.

613

614 **Figure 5.** Genome-wide association with the sex of PBT using the female reference sequences. For
615 the unmasked (A and C) and masked (B and D) references, Manhattan plot and Q-Q plot are shown,
616 respectively. In Manhattan plots, genome-wide significance cutoffs based on Bonferroni correction
617 are indicate by a red line, and the significant peak in scaffold F64 is indicated by a triangle. *P*-values
618 lower than 10^{-15} are treated as 10^{-15} for convenience.

619

620 **Figure 6.** Classification of the resequenced reads mapped to SLR_{F64} in the female genome.

621

622 **Figure 7.** Genome-wide association with copy number between PBT males and females. The
 623 mapping of resequenced reads was conducted using a masked male reference. The plots on scaffold
 624 M175 are indicated by triangles. (A) Differences in regional copy number between males and
 625 females are denoted as the values of $-\log_{10}(p)$ in the Mann-Whitney U test. Upward plots indicate
 626 that the copy number is larger in males than in females, while downward plots indicate the inverse.
 627 Genome-wide significance cutoffs based on Bonferroni correction are indicated by dashed lines. (B)
 628 Manhattan plot (left) and Q-Q plot (right) from CNV-based GWA scan. The regions with copy
 629 number 0, 0.5, or 1 were selected (P -values lower than 10^{-15} were treated as 10^{-15}). In the Manhattan
 630 plot, genome-wide significance cutoff based on Bonferroni correction is indicated by a red line.
 631

632 **Figure 8.** Protein-coding regions in scaffold M175. The region predicted with a copy number of 0.5
 633 and 10.7-kb SLR_{M175} are shown in red and black, respectively.
 634

635 **Figure 9.** Phylogenetic tree of *sult1st6* in fish species. For each of the nodes, the bootstrap
 636 probability is indicated when it is >95%. The tree constructed using more homologs linked with the
 637 Ensembl IDs is shown in Supplementary Figure 5.
 638

639 **Figure 10.** Expression of *sult1st6* in southern bluefin tuna's gonad cells. (A) *sult1st6a* and (B)
 640 *sult1st6y*.

Figures

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10