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ORIGINAL ARTICLE

# Identification of Sex-Linked SNPs and Sex-Determining Regions in the Yellowtail Genome

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**Abstract** Unlike the conservation of sex-determining (SD) modes seen in most mammals and birds, teleost fishes exhibit a wide variety of SD systems and genes. Hence, the study of SD genes and sex chromosome turnover in fish is one of the most interesting topics in evolutionary biology. To increase resolution of the SD gene evolutionary trajectory in fish, identification of the SD gene in more fish species is necessary. In this study, we focused on the yellowtail, a species widely cultivated in Japan. It is a member of family Carangidae in which no heteromorphic sex chromosome has been observed, and no SD gene has been identified to date. By performing linkage analysis and BAC walking, we identified a genomic region and SNPs with complete linkage to yellowtail sex. Comparative genome analysis revealed the yellowtail SD region ancestral chromosome structure as medaka-fugu. Two inversions occurred in the yellowtail linage after it diverged from the yellowtail-medaka ancestor. An association study using wild yellowtails and the SNPs developed from BAC ends identified two SNPs that can reasonably distinguish the sexes. Therefore, these will be useful genetic markers for yellowtail breeding. Based on a comparative study, it was suggested that a PDZ domain containing the GIPC protein might be involved in yellowtail sex determination. The homomorphic sex chromosomes widely observed in the Carangidae suggest that this family could be a suitable marine fish model to investigate the early stages of sex chromosome evolution, for which our results provide a good starting point.

**Keywords** Sex-determining locus · Yellowtail · Linkage analysis · Association scan

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## Introduction

Although sexual dimorphism is a widely conserved phenomenon in extant multicellular organisms, sex determination mechanisms are divergent among taxonomic classes and even among closely related species. In contrast to most mammals and birds, where the heterogametic sex with a well-differentiated sex chromosome is extremely conserved, teleost fishes show a wide variety of sex-determining (SD) modes among closely related species and different populations within the same species, e.g., male- (XY) or female-heterogametic (ZW) gonochorism, hermaphroditism, unisexuality, and environmental dependency (Mank et al. 2006; Kikuchi and Hamaguchi 2013; Marshall Graves and Peichel 2010).

In addition to divergent SD modes, teleost fish SD genes are also divergent among species. To date, SD genes have been identified in medaka (*DMY*; Matsuda et al. 2002, *Gsdf*$^{Y}$; Myosho et al. 2012, *Sox3*; Takehana et al. 2014), patagonian pejerrey (*amhy*; Hattori et al. 2012), fugu (*Amhr2*; Kamiya et al. 2012), rainbow trout (*sdY*; Yano et al. 2012), and probably in half-smooth tongue sole (*dmrt1*; Chen et al. 2014). Based on empirical evidence in which the same master SD gene is repeatedly used in animals, it is suggested that

there are constraints on the type of genes that can be co-opted as master SD genes (Bachtrog et al. 2014). However, this hypothesis remains unproven because of the limited number of SD genes identified to date. Therefore, further studies across a broader range of species are required.

In vivo functional analysis using either knockout or knockdown transgenic technologies or genetic analysis is usually required to identify SD genes. Because of their clarity and universality, in vivo functional analyses are ideal and are thus widely used in fish SD gene studies (Matsuda et al. 2007; Myosho et al. 2012; Hattori et al. 2012; Yano et al. 2012; Takehana et al. 2014). However in fish, especially marine fishes, in vivo functional analysis is quite difficult because their eggs are small, and maintenance of hatched embryos is labor-intensive. However, breeding a pedigree family is relatively easy, and so genetic analysis can be applied to a broader range of marine fishes. Indeed, genetic analyses of SD locus have been applied to many marine fishes, such as fugu (Kikuchi et al. 2007; Kamiya et al. 2012), half-smooth tongue sole (Chen et al. 2007), turbot (Martínez et al. 2009; Viñas et al. 2012), halibuts (Galindo et al. 2011; Palaiokostas et al. 2013), and rock bream (Xu et al. 2013). Nevertheless, genetic analysis also has disadvantages, e.g., if the species of interest has a heteromorphic sex chromosome where recombination is absent (Kikuchi and Hamaguchi 2013). Given these facts, a phylogenetic group where no highly differentiated sex chromosomes are observed is considered most suitable for SD gene identification via genetic analysis.

The family Carangidae includes approximately 32 genera and 140 species, of which, 27 species from 13 genera have been karyologically investigated (Chai et al. 2009). According to several studies, no heteromorphic sex chromosome has been observed in Carangidae species, suggesting that genetic analysis might identify SD genes from several Carangidae taxa (Chai et al. 2009; Caputo et al. 1996; Sola et al. 1997).

For these reasons, we have begun to investigate SD genes in yellowtail fish, *Seriola quinqueradiata*, a member of the Carangidae and a highly consumed fish in Japan. We have found that yellowtail sex is genetically determined by the ZZ-ZW sex-determination system and a sex-linked locus is located in Linkage Group (LG) 12 (Fuji et al. 2010). However, the genetic markers we developed were located far from the SD gene, and therefore, further improvement was required. In this study, we increased the resolution of a genetic map around the SD locus, constructed a BAC-based physical map, developed sex-linked SNP markers using a wild population DNA panel, and sought yellowtail candidate SD genes based on synteny between the yellowtail and other fish genomes.

## Materials and Methods

### SD Locus Estimation

#### *Yellowtail SD Locus Linkage Analysis*

We first re-performed the linkage analysis with additional SSR markers developed from the yellowtail BAC library and five pedigrees (families A to F) previously used (Fuji et al. 2010; Fuji et al. 2014). Genotype data were obtained according to Fuji et al. (2014). All SSR markers used here are listed in supplementary table 1. The linkages between the SD locus and each marker were estimated using the *scanone* function with the EM method and binary model implemented in R/qtl (Broman et al. 2003). LOD thresholds were determined by permutation tests with 1000 replicates. Bayes credible intervals were obtained for interval estimates.

#### *Development of Pedigree Family and Genetic DNA Markers for Breakpoint Analysis*

Another family named family S, which was composed of 512 progenies, was newly developed according to our previous study (Fuji et al. 2010). The sex phenotypes were determined by histological examination of gonads after anesthetizing the individuals by cooling with ice and recorded as binary traits. The tail fin was clipped from each fish and stored in absolute ethanol until use. Genomic DNA was extracted using a DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions.

In addition to the markers used above, we isolated new SSR markers using the syntenic relationship between the yellowtail and three other teleost genomes. Based on homology with partial BAC sequences mapped on the yellowtail LG 12, Chromosomes (Chr) 3, 8, and XI in *Tetraodon nigroviridis*, *Oryzias latipes*, and *Gasterosteus aculeatus*, respectively, were homologous to the yellowtail LG 12. The gene catalogue for each Chr was retrieved from the Ensembl Genome Browser with a one-to-one orthologous relationship to another fish Chr (http://asia.ensembl.org/index.html). In total, three gene catalogues (*T. nigroviridis*–*O. latipes*, *T. nigroviridis*–*G. aculeatus*, and *O. latipes*–*G. aculeatus*) were aligned, and the genes shared by those three chromosomes were identified. The protein sequences of the shared genes were also retrieved from Ensembl and used for tBLASTn searches (*e*-value<0.01) against yellowtail ESTs in GenBank. As *Sox9*, which is a critical Sry target gene and plays a pivotal role in early testis development, was also found in *T. nigroviridis* Chr 3 and our in-house EST database, we included it in the subsequent BAC library screening too (Uhlenhaut et al. 2009). In total, 19 cDNA fragments (18 ESTs and *Sox9*) were subjected to PCR-based two-step BAC screening. In the first PCR step, BAC DNA pools composed of six plates were used as

templates to identify a positive batch of plates. In the second step, the pools representing each plate, row, and column were amplified and positive BAC clones were then identified. In each BAC PCR screening reaction, 10 μl of PCR mixture containing 2 pmol each of BES specific primer, 0.25 U of Ex Taq polymerase (Takara), and 10 ng of pooled BAC DNA were amplified under the following conditions; an initial denaturation step for 3 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, appropriate annealing temperature for 1 min, 72 °C for 3 min, and a final extension step at 72 °C for 5 min. All EST IDs and primers used for the screening and positive BAC clone IDs are listed in supplementary table 1.

In SSR isolation, the positive BAC clone end sequences were first obtained using BigDye® Terminator v3.1 and an ABI 3130xl Genetic Analyzer (Life Technologies) to check if SSRs could be identified in the BAC-end sequences (BESs) according to the manufacturer's instructions. If no SSR was found in the BES, BAC-derived SSR markers were developed according to Glenn and Schable (2005). We separated 300–1000 bp DNA fragments made from *Afa*I-digested BAC DNAs on 1.5 % agarose gels; these were then purified from the gel and ligated with an adaptor generated from a set of oligo nucleotides (top oligo: 5′-GTCACTGACTCGTAGACT GCGTACT-3′, bottom oligo: 5′-AGTACGCAGTCTACGA GTCAG-3′). The fragmented DNAs were amplified with an adaptor-specific primer (5′-TGACTCGTAGACTGCGTACT-3′) under the following conditions; an initial denaturation step for 3 min at 95 °C, followed by 15 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR amplicons were then hybridized with biotinylated $(CA)_{10}$ and $(CT)_{10}$ probes in 3× SSC/0.1 % SDS at 68 °C for 1 h following denaturation at 95 °C for 5 min and incubated with approximately 100 μg of Dynabeads® MyOne™ Streptavidin C1 beads (Life Technologies) at room temperature for 30 min. The beads were washed sequentially in 3× SSC/0.1 % SDS at room temperature, 1.5× SSC/0.1 % SDS at 60 °C, and 3× SSC at room temperature twice; DNAs were eluted in TE at 95 °C for 10 min. The eluted DNAs were subjected to a second amplification as described above and cloned into a pGEM-T Easy Vector (Promega). Sixteen clones for each BAC clone were sequenced, and a primer pair was designed for each unique sequence.

The SSR markers developed from the BAC clones were assigned to the yellowtail genetic map to identify the markers in LG 12 using our mapping panel (Fuji et al. 2014). PCR amplifications were performed according to Schuelke (2000). Each PCR mixture containing 0.4 pmol of M13-tailed gene specific primer, 1.6 pmol each of another gene specific primer and 5′TET-labeled M13 primer, 1 μg of BSA (New England BioLabs), 0.25 U of Ex Taq polymerase (Takara) and template was amplified under the following conditions; step 1: 95 °C for 2 min, step 2: 27 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, step 3: 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, final step: 72 °C for 10 min. All sequence accession numbers and primer sequences are shown in supplementary table 1. The amplicons were size separated on acrylamide gel and visualized using an FLA-9000 image scanner (GE Healthcare). Genotype data were analyzed along with the other SSR marker genotype data obtained by Fuji et al. (2014) in R/qtl with an LOD score threshold of 4.0 and the Kosambi function for estimation of genetic distance (Broman et al. 2003).

### *Construction of Recombinant DNA Panel and Breakpoint Analysis*

The genotypes of the ten SSR markers (Sequ0021TUF, 0776TUF, 0782TUF, 0845TUF, 0900TUF, 0362BAC, 0485BAC, 0793TUF, 2379BAC, and 2384BAC) closest to the SD locus according to the linkage analysis above were obtained from the family S DNA panel. Of the family S progenies, recombinants among those marker loci were picked up and considered a recombinant DNA panel of the SD locus. The SSR markers isolated from the BAC clones and assigned to the yellowtail LG 12 as described above were also subjected to genotyping using the recombinant DNA panel.

All genotype data were manually aligned based on the theory that double recombination is quite rare for short distances.

## Construction of BAC-Based Physical Map, Isolation, and Mapping the Genetic Markers

A BAC-based physical map of the SD locus was constructed by the PCR screening method described above. The BESs of the positive BAC clones were obtained by sequencing, and primers were designed for each BES clone by clone. In cases where multiple positive BAC clones were obtained, their order was determined by PCR. Genotypes of the recombinant DNA panel for each BES were determined by PCR direct sequencing. The BES specific primers and genomic DNA were mixed, and the target fragments were amplified as described in the BAC screening method above. The amplicons were processed with illustra™ ExoStar™ (GE Healthcare) following the manufacturer's instructions and sequenced at Operon Biotechnologies (Tokyo, Japan). All BESs are deposited in GenBank (AG999763-AG999982).

## Wild Population Association Scan

The SNPs identified in the SD locus were used to assess how well they distinguished the sexes in a natural population. Forty seven each of male and female wild yellowtails were caught at Goto Island in Nagasaki Prefecture, Japan (The number of alleles: min. 4, max. 21, mean 14.75: Heterozygosity: min.

0.54, max. 0.92, mean 0.805). SNP genotypes were determined by PCR direct sequencing as described above. The significance of each SNP was estimated by Fisher's exact probability test.

### Comparative In Silico Analysis of the Yellowtail SD Locus

The teleost fish genome sequences were retrieved from Ensembl (medaka, zebrafish; http://asia.ensembl.org/index.html), USCS Genome Browser (fugu, stickleback; http://genome.ucsc.edu/), and GenBank (half-smooth tongue sole; CM002373.1–CM002394.1) and used for database construction. BLASTn searches against the five genomes were performed with the BESs in the BAC contig as a query with a cutoff *e*-value $e^{-10}$ (Camacho et al. 2009). The top hit query-subject pairs were extracted and modified for Oxford grid constructions using our in-house perl scripts used in our previous study (Edwards 1991; Fuji et al. 2014).

## Results

### SD Locus Estimation

We first obtained the genotypes of the genetic maker developed by Fuji et al. (2014) from families A to F and reanalyzed the SD locus. The results revealed that six SSR marker loci were adjacent to the SD locus (Fig. 1a). According to the genetic map developed by Fuji et al. (2014), ten markers (Sequ0021TUF, 0776TUF, 0782TUF, Sequ0845TUF, 0900TUF, 0362BAC, 0485BAC, 0793TUF, 2379BAC, and 2384BAC) are located in this region (Fig. 1b), and of those, the loci Sequ0485BAC and 2379BAC were homozygous and the other eight marker loci were informative in family S (Fig. 2). In terms of the genotypes of the eight informative marker loci, ten recombinants comprised of four females and six males were identified (Fig. 2). Additionally, among the new SSR markers developed by synteny-based BAC cloning, we identified five SSR markers around the SD locus and subsequently obtained the genotypes of these from the recombinant DNA panel of the SD locus for breakpoint analysis (Figs. 2 and S1). *Sox9*, represented by ssr254n21, located proximal to the SD locus, but a recombination was still observed (Figs. 2, 3, and S1). All of the marker genotypes, including SNP marker 045p01t7_SNP, which was found in a BAC clone adjacent to the locus Sequ0485BAC, revealed that the SD locus resided between loci ssr254n21/ssr253c12 and 045p01t7_SNP approximately 0.6 cM (3/512×100) away from each other, and the locus ssr263g21 was completely linked to sex (Fig. 2).

### Development of BAC-Based Physical Map and Completely Linked Genetic Markers from BESs

According to the breakpoint analysis results, BAC walking and genotyping using BES-derived SNPs were

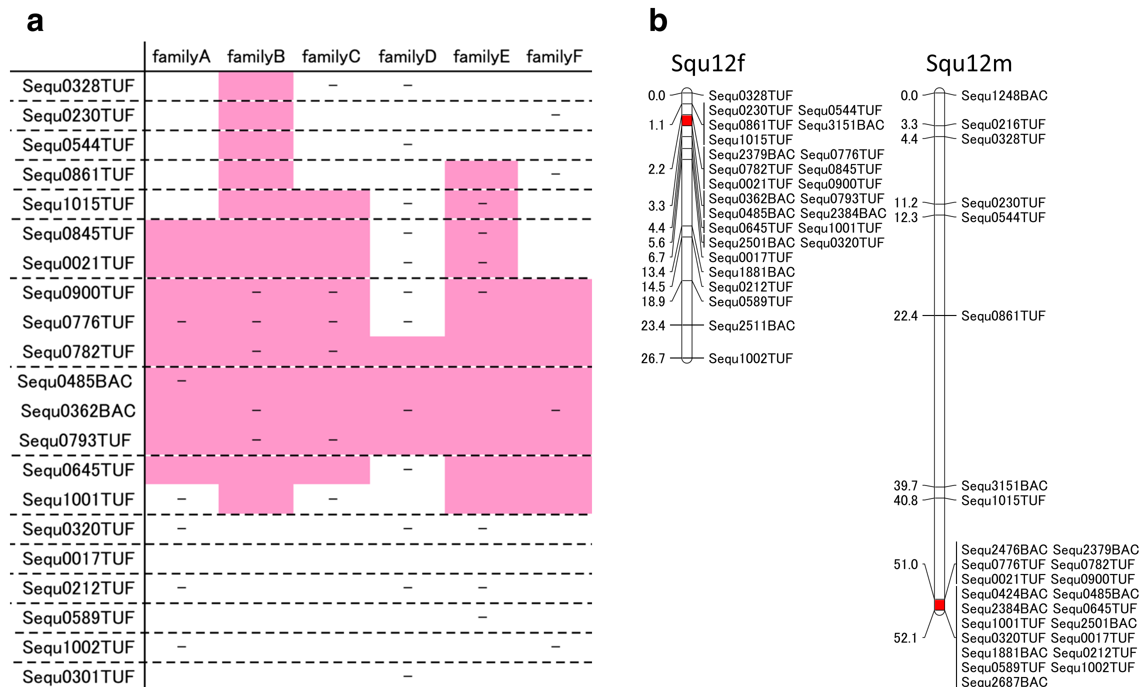


**Fig. 1** Linkage analysis results. **a** Schematic representation of the results. *Left column* gives the SSR marker names used in the linkage analysis. Regions highlighted in *red* show 95 % Bayes credible intervals. *Dash* indicates missing genotype. *Dotted line* indicates an interval where segregation has been observed in this or our previous study. **b** High-density genetic linkage map of the yellowtail linkage group 12 in males (Squ12m) and females (Squ12f) (Fuji et al. 2014). 95 % Bayes credible intervals are highlighted in *red*

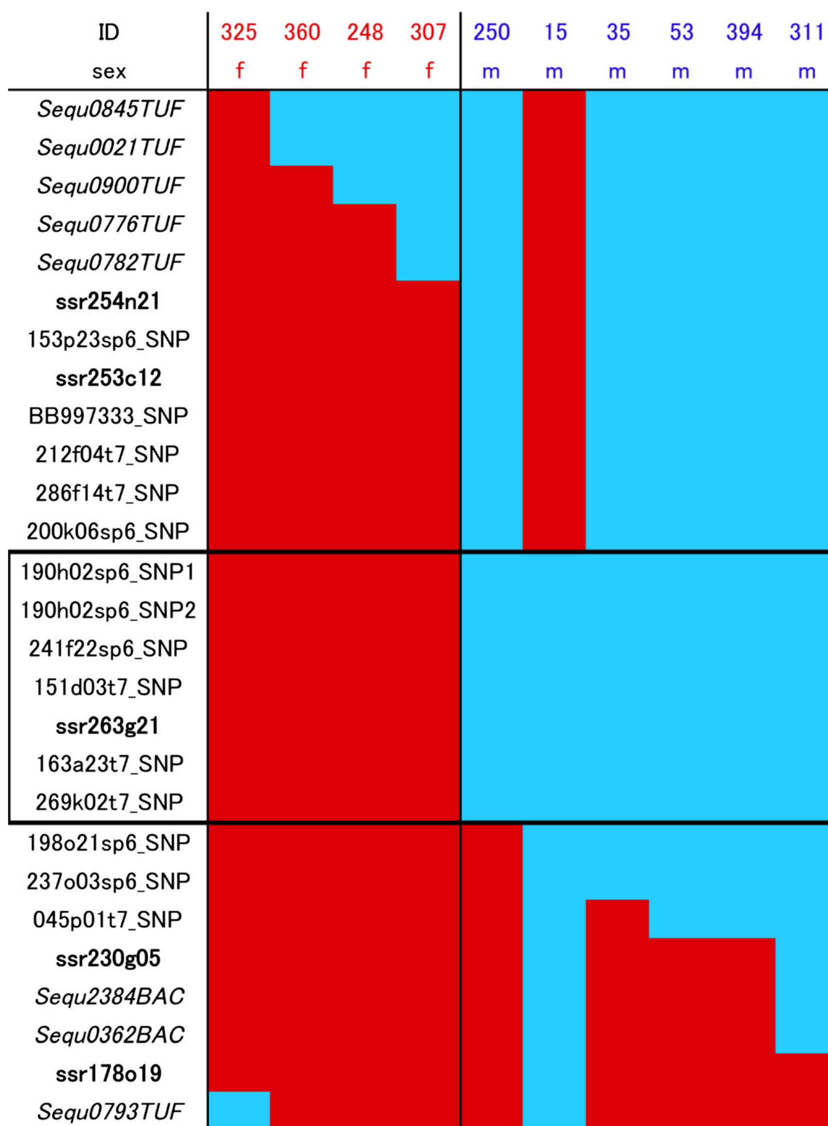


**Fig. 2** Family S yellowtail SD locus genetic map. *Italicized* SSR markers are those developed in our previous study (Fuji et al. 2014). *Bolded* markers were isolated using syntenic relationships. Others with '_SNP' at the end are SNP markers developed by BAC walking

initiated from the loci ssr253c12, ssr254n21, ssr263g21, and 045p01t7_SNP. The minimal tiling path of the SD region consisted of ten BAC clones (Fig. 3). Unfortunately, the resulting BAC contig had a gap between ssr254n21 and ssr263g21, and thus did not cover the entire SD region. In addition to the SSR marker ssr263g21, four SNP loci were identified from the BESs in the SD region (Figs. 2 and 3).

### Wild Population Association Scan

To evaluate whether or not the SD gene is included in the BAC contig, and which SNP marker is most useful for sex identification, we tested the SNP markers for sex association using the wild population DNA panel and identified one nonsignificant (190h02sp6_SNP2) and two significant (163a23t7_SNP and 269k02t7_SNP) W-derived SNPs in the sex-linked region (Table 1, Fig. 3). In particular, the SNP marker 269k02t7_SNP exhibited the strongest association where the sex of 99 % of the individuals could be identified by their genotype.

### Comparative In Silico Analysis of Yellowtail SD Locus

The BLAST searches identified homologous relationships between the yellowtail SD locus and fugu Chr 5, medaka Chr 8, stickleback Chr XI, and half-smooth tongue sole Chr 17, but not zebrafish (Figs. 4 and S2–6). Since chromosome structure is well conserved between fugu Chr 5 and medaka Chr 8, we speculate that the ancestral state of the yellowtail SD region is fugu-medaka type with two inversions occurring in the yellowtail lineage following divergence from a medaka-yellowtail ancestor (Figs. 4 and S2–4).

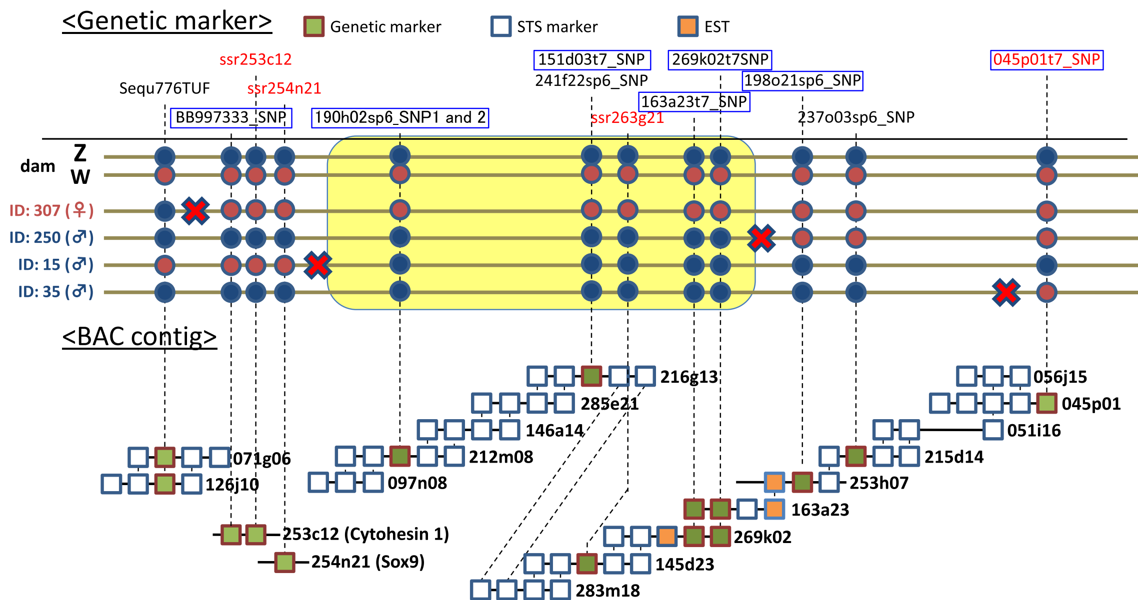


**Fig. 3** Schematic diagram of the yellowtail SD locus with genetic markers and BAC contig. *Blue boxes* and *red letters* indicate SNP markers used in the wild population association scan and BAC walking starting point, respectively. *Red crosses* indicate breakpoints identified in breakpoint analysis. The genomic region showing complete linkage to sex determination is highlighted in *yellow*

## Discussion

We previously located a yellowtail SD locus between loci Sequ0021TUF and 0017TUF on LG 12 (Fuji et al. 2010). These SSR markers are useful for sex identification in pedigree individuals but not in wild populations because of the nature of SSR markers and linkage equilibrium between them and the SD locus. In this study, we re-performed linkage analysis using markers on our recent dense genetic map, constructed a BAC-based physical map, and isolated potential sex-linked SNP markers from BAC ends to increase the SD locus resolution and identify SNP markers for sex identification in wild populations (Fuji et al. 2014). Although the BAC contig does not include the entire genomic region tightly linked to sex, we identified one nonsignificant (190h02sp6_SNP2) and two significant (163a23t7_SNP and 269k02t7_SNP) associations from W-derived SNPs, clearly indicating that the yellowtail SD gene is included in the BAC contig (Table 1, Fig. 3). The most significant associations were found in the SNPs 163a23t7_SNP and 269k02t7_SNP (Table 1). In particular, 269k02t7_SNP identified individual sexes with 99 % accuracy. Therefore, this SNP marker will be a useful genetic marker for sex identification in current yellowtail breeding programs in Japan.

As mentioned in the "Introduction", several SD gene studies have suggested that these genes play critical role in

**Table 1** Results of the wild population association scan

| SNP name | Allele 1 | Allele 2 | Sex | No. of 1/1 | No. of 1/2 | No. of 2/2 | *p* value |
|---|---|---|---|---|---|---|---|
| BB997333_SNP | A | T | Male | 22 | 20 | 3 | 0.13 |
| | | | Female | 32 | 13 | 1 | |
| 190h02sp6_SNP2 | G | A | Male | 14 | 24 | 9 | 0.59 |
| | | | Female | 10 | 25 | 12 | |
| 151d03t7_SNP | C | T | Male | 0 | 14 | 33 | 1.004e-06 |
| | | | Female | 4 | 32 | 9 | |
| 163a23t7_SNP | A | G | Male | 44 | 3 | 0 | <2.2e-16 |
| | | | Female | 1 | 45 | 0 | |
| 269k02t7_SNP | A | C | Male | 46 | 1 | 0 | <2.2e-16 |
| | | | Female | 0 | 46 | 0 | |
| 198o21sp6_SNP | C | G | Male | 0 | 2 | 45 | 0.5028 |
| | | | Female | 0 | 0 | 36 | |
| 045p01t7_SNP | G | A | Male | 8 | 18 | 19 | 0.1525 |
| | | | Female | 7 | 9 | 25 | |

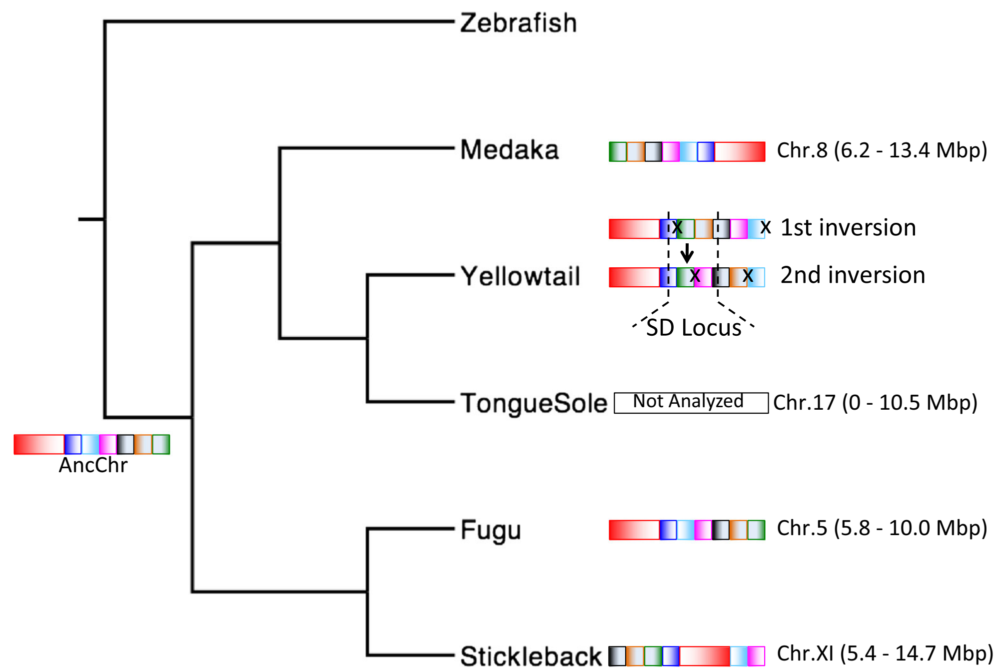


**Fig. 4** Evolutionary changes in chromosome structure at the yellowtail SD locus. The phylogenetic tree was reconstructed based on Near et al. (2013). 'AncChr' shows the estimated ancestral chromosome structure

gonadal differentiation and tend to be recruited to the top of the SD cascade, although an exception to this has been recorded in rainbow trout (Yano et al. 2012). Moreover, *Dmrt1* and its paralogs, and members of the transforming growth factor-β (TGF-β) signaling pathway such as *Gsdf*, *amhy*, and *Amhr2* have been identified as sex determiners in several vertebrates (Matsuda et al. 2002; Yoshimoto et al. 2008; Smith et al. 2009; Chen et al. 2014; Hattori et al. 2012; Kamiya et al. 2012; Myosho et al. 2012). One of these known SD genes could also determine sex in the yellowtail. Comparative analysis using isolated BESs revealed that the yellowtail SD locus was homologous to fugu Chr 5 (8.0–9.9 Mbp), medaka Chr 8 (6.2–9.8 Mbp), stickleback Chr XI (5.5–8.4 and 14.60–14.63 Mbp), and half-smooth tongue sole Chr 17 (0.01–0.17, 2.3–3.5, 10.0–10.4 Mbp). Furthermore, although two inversions have been observed in the yellowtail genome, micro-synteny of this genomic region is relatively well conserved except in half-smooth tongue sole, suggesting that we might be able to infer a potential yellowtail SD gene from the information we have on representative regions in the other fish genomes (Figs. 4 and S2–5). Therefore, we first sought known fish SD genes by a tBLASTn search on representative regions in the fugu, medaka, and stickleback genomes; however, no significant hits were obtained. We next sought other members of the TGF-β signaling pathway and found a PDZ domain containing the GIPC1 protein in all three fishes (supplementary data 1–3). PDZ domains are one of the most abundant protein-protein interaction modules found in all metazoans. Proteins that contain the PDZ domains play critical roles in quite broad biological processes such as cell migration, tissue growth and differentiation, and embryonic development by scaffolding their interacting partners (Ye and Zhang 2013). GIPC (GAIP-interacting protein, C terminus) was originally identified as a protein that binds to the C terminus of the RGS (G protein signaling regulator) protein GAIP (RGS19), a GTPase-activating protein (GAP) for G$\alpha_i$ subunits (De Vries et al. 1998). Three GIPC proteins, GIPC1, GIPC2, and GIPC3, have been found in mammals, all of which consist of a GIPC homology (GH1) domain, PDZ domain, and GH2 domain; where the GH1 and GH2 domains are involved in dimerization and interaction with myosin VI (MYO6), respectively (Katoh 2013). The GIPC1 PDZ domain is involved in direct interactions with a variety of PDZ ligands, such as RGS19, NRP1, GLUT1, SEMA4C, SDC4, and IGF1R (Katoh 2013). It has been reported that one of the PDZ ligands, endoglin, interacts with GIPC and specifically enhances TGF-β1-induced phosphorylation of Smad1/5/8 (Lee et al. 2008). Yet, the role of endoglin in the reproductive system has not been shown. Testicular TGF-β1 modulates Leydig cell steroidogenesis, the organization of peritubular myoid cells, testis development, and spermatogenesis (Rocio et al. 2013). Hence, PDZ domains containing GIPC proteins might play a critical role in the SD cascade and probably determine sex in the yellowtail. Another possible sex determiner might be *sox9*. *Sox9* is a member of SRY-box (Sox)-family and plays a pivotal role in inducing male developmental program in mice, such as Sertoli cell differentiation, Müllerian duct regression and suppression of ovarian pathway (Tanaka and Nishinakamura. 2014). *Sox9* has long-range regulatory elements within the its 5′ control region and the translocations and inversions of the genomic region proximal to *sox9* disrupt *cis*-acting regulation of *sox9* and cause the

semilethal skeletal malformation syndrome campomelic dysplasia with associated XY sex reversal (Bagheri-Fam et al. 2001). In contrast, in medaka, it has been shown that the two co-orthologous copies of *sox9*, *sox9a*, and *sox9b* were not involved in testis determination but *sox9b* affected germ cell proliferation and/or survival (Nakamura et al. 2012). Although the yellowtail *sox9* is identified outside the SD region and medaka *sox9* co-orthologues do not directly regulate testis determination, it is still worth considering participation of yellowtail *sox9* in sex determination because the facts that, according to the synteny, *sox9* we found in yellowtail LG12 should be *sox9b* and heterozygous XX mutants of medaka *sox9b* (a half-dose of functional *sox9b*) resulted in male secondary sex characteristics and upregulation of testis-marker gene (Fig. 3) (Nakamura et al. 2012). In addition, although we did not find any known SD genes in yellowtail SD region, we cannot exclude them at this time because sex chromosome turnovers by transposition of an existing SD gene might occur in teleost fishes (Ross et al. 2009). In any case, genomic sequence of the SD region is obviously required to fully understand the yellowtail SD system.

Apart from the SD gene itself, the frequent recombination observed in the yellowtail SD region and the widely distributed homomorphic sex chromosomes in the Carangidae also attract interest in sex chromosome evolution. A sex chromosome evolution scenario has been proposed in which genetic linkage between sex determiners and sexually antagonistic mutations is favored following SD gene acquisition in the early stages of differentiation (Bachtrog 2013). Although this scenario is theoretically well characterized, there is little empirical evidence to support it (Kirkpatrick and Guerrero 2014). Our results clearly indicate that the yellowtail sex chromosome is at an early stage in its evolution. Therefore, it might be possible to observe the early stages of sex chromosome differentiation in this or another Carangidae species in future studies.

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**Conflict of Interest** A patent regarding sex-linked SNP markers is pending (Japanese Unexamined Patent Application Publication No. 2014-180233).

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