Ph. D. Thesis

Studies on genome evolution and gain of sex determination system in *Seriola* species

ブリ類のゲノム染色体進化と性決定様式の獲得に関する研究

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CONTENTS

Abstract

Fishes are the vertebrate group which appeared first on the earth and are the most diverse taxon in many aspects of evolution. This high level of biodiversity is a powerful basis for studying genome evolution. In this study, genome structures and correlations between genetic variation and phenotype of greater amberjack (*Seriola dumerili*) were investigated. The amberjacks are economically very important, and its ecology is much different from model fish species such as Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Therefore, the knowledge of amberjack genome is invaluable in terms of aquaculture and understanding fish genome evolution. To investigate chromosome evolution in fish species, newly 181 markers were mapped, which allowed the construction of yellowtail radiation hybrid (RH) physical map with 1,713 DNA markers, which was much denser than a previous map, and the de novo assembled sequences were anchored onto the RH physical map. Finally, a total of 13,977 expressed sequence tags (ESTs) were mapped on a genome sequence assembly aligned with the physical map. Using the high-density physical map and anchored genome sequences, the yellowtail genome structure was compared in detail with those of five model fishes to characterize the yellowtail genome. Between yellowtail and Japanese medaka, almost all regions of the chromosomes were conserved and some blocks comprising several markers were translocated. Using the genome information of the spotted gar (*Lepisosteus oculatus*) as a reference, syntenic relationships and chromosomal rearrangements that occurred during evolution in four other acanthopterygian species (Japanese medaka, zebrafish, spotted green pufferfish (*Tetraodon nigroviridis*) and three-spined stickleback (*Gasterosteus aculeatus*)) were documented. The evolutionary chromosome translocation frequency was 1.5–2 times higher in yellowtail than in medaka, pufferfish, and stickleback. Sex determination system of fish is highly diverse compared with that of mammal. Thus, investigations using nonmodel fish species helps the understanding of sex determination

systems of fish. In this study, sex-associated SNPs of greater amberjack were identified using SNP information of 10 males and 10 females by an association test. Sex-associated SNPs were detected on chromosome 12 and mainly covered with two scaffolds, c168 and c43. Genotypes of sex-associated SNPs indicated that greater amberjack has female heterogametic sex determination system (ZZ/ZW). Furthermore, the genomic structure of greater amberjack was compared with those of yellowtail and Japanese medaka. Whole genome alignment and synteny analysis indicated that sex determination system of greater amberjack is markedly different from that of medaka and inferred that the sex determination region on chromosome 12 is conserved in the *Seriola* species.

Chapter 1: Introduction

Fishes were the vertebrate group which appeared first on the earth and are the most diverse taxon in many aspects of evolution. Fishes comprise more than 50% of all vertebrate species and are characterized by the diversities of morphology, behavior and habitat use (Nelson *et al*., 2016). This high level of biodiversity is very useful for studying genome evolution.

Genome duplication plays an important role in the process of evolution, and two times of whole-genome duplication (WGD) are considered to have led to the increase of size and complexity of vertebrate genomes (Ohno, 1970). Teleosts are the most diversified group in fishes. They are overwhelmingly the dominant group of bony fishes, with nearly 30,000 recognized species comprising about 96% of all the extant fish species. In the common ancestor of teleost lineage, an additional polyploidization, called the teleost-specific WGD, occurred 226–350 MYA (Vandepoele *et al*., 2004; Christoffels *et al*., 2004; Hoegg *et al*., 2004; Hurley *et al*., 2007). This polyploidization event influenced the evolution of structural and functional gene diversity of teleosts (Braasch and Postlethwait, 2012), because one of the duplicate genes acquired a new function (neofunctionalization) or both copies were subfunctionalized. Additional WGD events occurred independently in many species, including higher-level taxa of teleosts and other ray-finned fishes (for example, salmonids, carp and sturgeon). Thus, some fish lineages experienced at least four times of WGD since the origin of vertebrates. Therefore, teleost genomes are good models for understanding the significance of WGD during vertebrate evolution (Berthelot *et al*., 2014).

The representatives of teleost species in genome studies are spotted green pufferfish (Jaillon *et al*., 2004), Japanese medaka (Kasahara *et al*., 2007), three-spined stickleback (Jones *et al*., 2012), zebrafish (Howe *et al*., 2013), Atlantic salmon (*Salmo salar*) (Lien

et al., 2016) and common carp (*Cyprinus carpio*) (Zhao *et al*., 2013; Xu *et al*., 2014). Chromosome-level genome assemblies of these five species are available in the NCBI Genome Database (National Center for Biotechnology Information,). Chromosome-level assemblies or assembled genome sequences integrated with gene maps, termed 'chromonomes,' are powerful tools that enable analyses of conserved syntenies among species (Braasch *et al*., 2015). The detection of conserved syntenies can provide valuable information about chromosomal rearrangements that occurred in evolution. Hence, these five genome assemblies have been used in comparative genomic studies of teleost species.

In non-teleost fishes, a chromosome-level genome assembly of has been reported from the spotted gar (*Lepisosteus oculatus*) (Braasch *et al*., 2016). Spotted gar is a primitive fish species of Actinopterygii, the lineage of which diverged from the teleost lineage before the teleost-specific WGD event (Amores *et al*., 2011). Hence, the spotted gar is an ideal outgroup for the investigation of teleost genes and genome evolution. Whereas gars are good genomic and laboratory models, other groups of non-teleost ray-finned fishes (i.e., bichirs, sturgeons, paddlefish, and bowfin) are less useful, due to specialized morphologies, lineage-specific polyploidizations and/or difficult husbandry (Braasch *et al*., 2015).

Amberjacks in the family Carangidae are a commercially important group of marine teleosts and several species of the genus *Seriola* have become the basis for prosperous aquaculture. However, genome information is lacking in *Seriola* species and its relatives in genome structure. The Japanese amberjack *Seriola quinqueradiata*, also called yellowtail, is native to the northwest Pacific, especially around the Korean Peninsula and parts of Japan. Juveniles of yellowtail exist with macroalgae floating on ocean currents along the coastline, where they are caught for a thriving aquaculture industry in Japan. Ecological feature of yellowtail, such as their habitats and life cycle, differs from those of other teleosts such as Japanese medaka and pufferfish, in which information of genome

sequences are available. Hence, in this study, yellowtail genome was examined to acquire new information about fish genome evolution.

Gene map is essential for ordering shotgun genome sequences and evolutionary analysis. Chromosome level assembly is the ultimate form of a physical map. However, it cannot be completed by just sequencing genome with current methods. A very dense genetic or physical map are indispensable to construct chromosome level assembly because current methods of DNA sequences produce contigs or scaffolds of limited length containing many gaps.

There exist two types of gene maps, that is, genetic and physical maps. Genetic map is based on the use of genetic techniques including cross-breeding experiments or examination of family history (Brown, 2002). Therefore, this method cannot locate genes on regions where crossovers are absent, as seen in sex chromosomes, and wide recombination hotspots are present even though these regions are physically narrow on chromosomes. Moreover, genetic map is insufficient for directing the sequences phase of a genome project because of its limited accuracy and resolution (Brown, 2002). These problems can be resolved with physical mapping method. One of the physical mapping, radiation hybrid mapping, was developed as a general approach for constructing long-range maps of mammalian chromosomes (Gross and Henry, 1975; Cox *et al*., 1990). In this method, a high dose of x-rays is used to break chromosomes of the target species into fragments (Cox *et al*., 1990). These broken chromosomal fragments are recovered in rodent cells, and hybrid clones are analyzed for the presence or absence of specific DNA markers (Cox *et al*., 1990). By estimating frequency of breakage between markers, it is possible to determine their physical distance and order. In addition, this method can provide a map at 100-500 kb level of resolution.

Radiation hybrid (RH) physical map and genetic linkage map have been developed in many species because of their economic importance (Aoki *et al*., 2014; Fuji *et al*., 2014).

On physical map, intervals between genes show physical distance, unlike on linkage map, and so scaffolds of genome sequences can be easily lined up using physical map. Hence, in this study, dense physical map of yellowtail was developed using an RH mapping panel.

In the chapter 2, genome sequences of yellowtail were lined up using an updated RH physical map, and 13,977 expressed genes were mapped on the physical sequence map. Thus, synteny relationships between yellowtail chromosomes and chromosomes of four model fish species were compared in detail. Here, the yellowtail 'chromonome,' which denotes genome sequencing integrated with an RH map to identify the chromosomal order of the scaffolds was determined. Next, the yellowtail genome structure was compared with that of the four model fishes and the spotted gar in order to identify characteristics of the yellowtail genome. Using the spotted-gar genome as a reference, synteny relationships and evolutionary chromosomal rearrangements in four acanthopterygian species (yellowtail, Japanese medaka, spotted green pufferfish, and three-spined stickleback) were clarified. It was found that the yellowtail chromosome structure is the most closely related to that of Japanese medaka, and that the evolutionary chromosome translocation frequency in yellowtail was 1.5–2 times higher than in those of the other fish species studied.

Sex determination systems of fish is highly diversified, while sex is determined by the *Sry* gene on the Y chromosome in most mammals (Waters *et al*., 2007). Although sex is primarily determined by genes in many fish species, it is also affected by environmental factors (e.g., exogenous steroids and temperature) and some fish change sex with growth. Various genes have been identified as master sex genes in fish, for example, *Dmy* of Japanese medaka (Matsuda *et al*., 2002), *GsdfY* of *Oryzias luzonensis* (Myosho *et al*., 2012), *Sox3* of *Oryzias dancena* (Takehana *et al*., 2014), *Amhy* of *Odontesthes hatcheri* (Hattori *et al*., 2013), *Amhr2* of torafugu (*Takifugu rubripes*) (Kamiya *et al*., 2012), *Dmrt1* of tongue sole (*Cynoglossus semilaevis*) (Cui *et al*., 2017), and *SdY* of rainbow trout

(*Oncorhynchus mykiss*) (Yano *et al*., 2012).

In salmonids, *SdY* is conserved as a male-specific Y-chromosome sequence in *Oncorhynchus* as well as many other salmonid species in the Salmoninae, Thymallinae, and Coregoninae, while these species do not share any Y-chromosome conserved synteny (Yano *et al*., 2013). Although medaka species own both male-heterogametic (XY) and female-heterogametic (ZW) sex determination systems, their sex-determining (SD) loci are located on different chromosomes (Takehana *et al*., 2007b, 2007a, 2008; Tanaka *et al*., 2007; Nagai *et al*., 2008). While *O. latipes* and *O. curvinotus* have *Dmy* (Matsuda *et al*., 2002, 2003), other *Oryzias* species lack it (Takehana *et al*., 2007a, 2007b, 2008, 2014; Tanaka *et al*., 2007; Nagai *et al*., 2008). This suggests that sex determination mechanism of medaka species is regulated by different genes among species, which appeared not in old years in *Oryzias* evolution (Takehana *et al*., 2014). Domesticated zebrafish have lost a sex-determinating gene that wild zebrafish possesses. However, domesticated strains have an alternative sex determination system, which is inferred to have evolved recently in the process of domestication (Wilson *et al*., 2014).

As mentioned above, various taxa of fish have evolved diversified sex determination systems. Therefore, the investigation of these systems is valuable for our understanding of sex determination systems in fish and their evolution. Knowledge of the sex determination process is also valuable given the importance of controlling the sex ratio of fished targeted to aquaculture. *Seriola* species in the family Carangidae are important edible fish around the world. Two *Seriola* species, yellowtail (*Seriola quinqueradiata*) and greater amberjack (*Seriola dumerili*), are the main species for aquaculture production in Japan. It have been reported that sex-determining gene of yellowtail is located at the end of linkage group 12 (Koyama *et al*., 2015). Moreover, the draft genome sequence and transcriptome sequence of yellowtail were recently published (Aoki *et al*., 2015; Kawase *et al*., 2018). Greater amberjack is more widely distributed in tropical and temperate

waters of the world than yellowtail and its genome sequence was also recently published (Araki *et al*., 2018). These two *Seriola* species are genetically close and the information of their genome and transcriptome sequences is available (Aoki *et al*., 2015; Araki *et al*., 2018; Kawase *et al*., 2018). Furthermore, comparison with nonmodel fish is expected to help our understanding of the highly diverse sex determination systems in fish. This study focused this issue and examined specifically whether the same gene acts as an SD gene in yellowtail and greater amberjack.

In chapter 3, sex-associated SNPs of greater amberjack was identified and most of the SNPs were present on chromosome 12. Furthermore, the genomic structure of greater amberjack was compared with those of medaka and yellowtail and indicated that the sex determination system of greater amberjack differs from that of medaka. However, it was elucidated that the SD region is conserved in the two *Seriola* species.

Chapter 2: Constructing a 'Chromonome' of Yellowtail (*Seriola quinqueradiata*) for Comparative Analysis of Chromosomal Rearrangements

1.1. Introduction

A high-quality gene map of densely spaced markers is necessary for affirming the correct placement of scaffolds on chromosomes and for deciding the order of markers within the assemblies because current methods of DNA sequence assembly produce contigs and scaffolds of limited length, leaving scores, or in some cases, containing thousands of sequence gaps (Lewin *et al.*, 2009). Assembled genome sequences integrated with gene maps, termed 'chromonomes,' are powerful tools that enable the comparison of chromosome structures among species (Braasch *et al*., 2015). In this chapter, RH map of yellowtail denser than the previous map (Aoki *et al.*, 2015) was made and integrated with the genome assembly to construct a 'chromonome'. Then, the 'chromonome' was compared with other model fishes for its characterization.

1.2. Materials and Methods

Obtaining yellowtail genome and transcriptome sequences

The National Research Institute of Aquaculture, Japan (NRIA) has been developing *de novo* whole-genome sequences and transcriptome sequences of yellowtail. Genome sequences (BDMU01000001–BDMU01000384) and transcriptome sequences (IACH01000001–IACH01013125) of yellowtail were obtained from the DNA Data Bank of Japan (DDBJ).

Designing the primer pairs for RH mapping

To orient the genomic scaffolds, primer pairs were designed on from the information of genome sequence for RH mapping. The first primer pairs (product size 150–200 bp) were designed from the sequences, and the nested primer pairs (product size 100–150 bp) were designed from the first-round PCR products using Primer3 (Untergasser *et al.*, 2012) for expression analysis with the BioMark HD system (Fluidigm, CA, USA).

Construction of an RH map

The RH panel, comprising 93 RH cell lines and positive and negative controls, has been already published by Aoki et al. (Aoki *et al.*, 2014). DNA from each cell line was extracted using a Blood and Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany). Genotyping reactions were carried out on a Fluidigm platform using the BioMark 96.96 Dynamic Array for gene-expression analysis, as described in XX (Aoki *et al.*, 2014).

CarthaGène software (de Givry *et al.*, 2005) was used to perform two-point linkage analyses and to determine marker order and inter-marker distances in centiRays (cRs). CarthaGène analyzes multiple populations' maximum-likelihood consensus maps using a fast expectation–maximization algorithm for maximum-likelihood estimation and powerful ordering algorithms. The group command at a logarithm of the odds (LOD) threshold of 4.0 and a distance threshold of 50 determined the linkage groups, and the previous RH map of yellowtail was used as a reference (Aoki *et al.*, 2015).

Construction of the 'chromonome'

A nucleotide-to-nucleotide BLAST (blastn) search of yellowtail expressed sequence tags (ESTs) was performed against the yellowtail genome sequences with the cut-off e-value 1e-10. If the e-value of the top hit was greater than half the e-value of the second hit, the combinations of sequences were removed from the subsequent analyses. The

position and direction of each of the genome sequences was determined based on the RH map and the result of a BLAST search between ESTs and the genome sequences.

Comparative genomic analysis

The location of ESTs was positioned on the genome sequences of the RH map, and the mapped ESTs were used for comparative analysis. The complementary DNA (cDNA) sequences of Japanese medaka, spotted green pufferfish, three-spined stickleback, and spotted gar were obtained from Ensembl (Yates *et al.*, 2016). Translated-nucleotide-to-translated-nucleotide BLAST (tblastx) searches were performed against the cDNA sequences of yellowtail, medaka, spotted green pufferfish, three-spined stickleback and spotted gar to detect orthologs, using an e-value cut-off of 1e-5 and reciprocal best hits. The orthologs were aligned on the genetic map of each species to identify syntenic relationships. Oxford grids (EDWARDS, 1991) and Circos plots (Krzywinski *et al.*, 2009) were constructed to compare the genomic data of yellowtail and four other teleost species (Japanese medaka, spotted green pufferfish, three-spined stickleback, and zebrafish).

The orthologs between spotted gar and the other species were aligned on the genetic map of spotted gar (Braasch *et al.*, 2016). Syntenic relationships of each of the four species was compared with spotted gar to find chromosomal rearrangements in the ray-finned fishes (Acanthopterygii). The regions showing different syntenic relationships were considered to be the regions containing chromosomal rearrangements. If conserved syntenic relationship with spotted gar is observed in medaka chromosome but not in yellowtail chromosome, the segment in the region was considered to have undergone chromosomal rearrangement in the yellowtail lineage.

1.3. Result

Constructing the yellowtail 'chromonome'

Two-point analysis was performed with a distance threshold of 50 and a logarithm of the odds (LOD) score of 4.0 resulted in 117 RH groups. 181 markers were newly mapped on the updated RH map. 61 RH groups, containing a total of 1,713 markers, were assigned to 24 linkage groups (LGs). Thus, the RH map was constructed with a set of 1,713 markers. In each group, the RH map ranged from 645.3 to 1,874.5 cR, with an average of approximately 1,263.1 cR. The combined size of all the RH groups was 30,314.5 cR.

The sequences of mapped markers of yellowtail were used for BLAST searches against the yellowtail genome sequence assembly, and 200 scaffolds with a total length of 601 Mb (93.9% of the total size of the genome scaffolds) were anchored on the RH map (Figure 1, Table 1). The number of anchored scaffolds per LG ranged from 2 to 18. A total of 13,977 ESTs (21.2 Mbp) were mapped on the anchored genome sequences. With these results, a high-density physical map was constructed with 13,977 ESTs (1 EST/46 kbp).

Comparative analysis of chromosomal rearrangements

Synteny analysis with the 13,977 of mapped ESTs was performed using tblastx searches against the cDNA sequences of five species (medaka, spotted green pufferfish, three-spined stickleback, zebrafish and spotted gar) (Table 2). A total of 6919, 6379, 7378, 6882, and 6522 yellowtail sequences had orthologs in medaka, pufferfish, stickleback, zebrafish and gar, respectively.

Between yellowtail and medaka, 5,665 of the orthologs were located in the 24 LGs of yellowtail and 24 chromosomes of medaka, and 92.3% of them was contained in major conserved groups shown in an Oxford grid (Figure 2). The chromosomes of these two

species showed one-to-one correspondence (Figure 6). The remaining 1,254 orthologs were mapped to un-anchored scaffolds or were not mapped to scaffold sequences. Almost all regions of the yellowtail and medaka chromosomes were conserved, and some blocks comprising several markers were translocated (Figure 2, Figure 6).

Between yellowtail and spotted green pufferfish, 4,581 of the orthologs were located in the 24 LGs of yellowtail and 21 chromosomes of pufferfish, and 90.0% of them were contained in conserved groups shown in an Oxford grid (Figure 3). The chromosomes of these two species showed mostly one-to-one correspondence, but three pairs of two-to-one correspondence (Figure 7).

Between yellowtail and three-spined stickleback, 6,147 of the orthologs were located in the 24 LGs of yellowtail and 21 chromosomes of stickleback, and 95.2% of them contained groups shown in an Oxford grid (Figure 4). The chromosomes of these two species showed one-to-one correspondence, but three pairs of two-to-one correspondence (Figure 8).

Spotted green pufferfish and three-spined stickleback have 42 (2n) chromosomes, while yellowtail and Japanese medaka have 48 (2n) chromosomes. Therefore, in comparisons between yellowtail and the fishes with 42 chromosomes, there can be one-to-two correspondence. The chromosome combinations between pufferfish (Tni) and yellowtail (Squ) were: Tni1 – Squ4 / Squ18, Tni2 – Squ6 / Squ7, and Tni3 – Squ11 / Squ12 (Figure 3). The combinations between stickleback (Gac) and yellowtail (Squ) were: Gac11 – Squ11 / Squ14, Gac4 – Squ5 / Squ18, and Gac7 – Squ19 / Squ22 (Figure 4).

Between yellowtail and zebrafish, 6111 of the orthologs were located in the 24 LGs of yellowtail and 25 chromosomes of zebrafish. Some clusters consisting of a large number of orthologs were present in each chromosome. Eight chromosomes in each species showed one-to-one correspondence, and three chromosomes showed a one-to-two

relationship (Figure 5, Figure 9). The chromosome combinations between zebrafish (Dre) and yellowtail (Squ) were: Dre5 – Squ15 / Squ17, Dre6 – Squ1 / Squ4, Dre7 – Squ9 / Seq22, Dre8 – Squ4 / Squ16 / Seq24, Dre10 – Squ17 / Squ19, Dre13 – Squ8 / Seq20, Dre18 – Squ3 / Squ9 / Squ14, Dre21 – Squ17 / Squ19, and Dre22 – Squ1 / Squ4 (Figure 5).

Between yellowtail and spotted gar, 5586 of the orthologs were located in the 24 LGs of yellowtail and 29 chromosomes of spotted gar. Some combinations of chromosomes in each species showed almost complete two-to-one correspondence, and others showed four-to-one (or more) correspondence (Figure 10, Figure 11).

Publicly available spotted-gar genome information was used as an outgroup to infer the genome evolution of teleosts. The regions showing different syntenic relationships with spotted gar were considered to have undergone chromosomal rearrangements.

The common ancestor of the four species (yellowtail, medaka, spotted green pufferfish and three-spined stickleback) is inferred to have had 48 chromosomes, because most extant acanthopterygian species have 48 chromosomes, and three of the chromosomes in the species with 42 chromosomes (i.e., spotted green pufferfish and three-spined stickleback) possess combinations of two entire chromosomes in different patterns. The inferred distribution of ancestral chromosomes in yellowtail, medaka, spotted green pufferfish, and three-spined stickleback is shown in Figure 12. The translocations were distributed widely on each chromosome (Figure 12). The number of inter-chromosomal translocations in yellowtail was 1.5–2-times higher than those in medaka, pufferfish, and stickleback (Table 3), which had similar numbers of inter-chromosomal translocations (Table 3).

1.4. Discussion

Constructing the yellowtail 'chromonome'

The final RH map constructed in this study had a higher marker density than other available physical maps (Sarropoulou *et al.*, 2007; Guyon *et al.*, 2010, 2012). Whole-genome *de novo* sequencing of yellowtail was developed at NRIA, utilizing the Illumina HiSeq 2500 and PacBio RS II platforms. The project produced 149 Gbp of data by Hiseq2500, and 14.5 Gbp by PacBio RS II, with total coverage at about 200-fold (the sequence data deposited in the DDBJ). The final *de novo* genome assembly was 639.3 Mbp long, with 384 scaffolds, and an N50 scaffold size of 5.6 Mbp. These results imply a value of 1 cR = 22.1 kbp (i.e., 639 Mbp/30,314.5 cR).

200 scaffolds with a total length of 601 Mb (93.9% of the total size of the genome scaffolds) were anchored on the RH map and a high-resolution physical map with 13,977 ESTs (1 EST/46 kbp) was constructed. The RH map was very useful to anchor the assembled genome sequences and thereby to construct a high-density physical map.

A linkage map of yellowtail was constructed by Fuji et al. (Fuji *et al.*, 2014). When the updated RH map in this study and the linkage map by Fuji et al. (Fuji *et al.*, 2014) were compared, some regions narrow in the linkage map were wide in the RH map. The inconsistency in these regions may reflect the rare occurrence of recombination events, which may indicate that the regions correspond to centromeres. The yellowtail karyotype consists of one set of metacentric, one set of submetacentric and 22 pairs of acrocentric chromosomes (Aoki *et al.*, 2014). Therefore, LG12 and LG13 are considered to be a submetacentric and a metacentric chromosome, respectively.

Comparative analysis of chromosomal rearrangements

The newly constructed ultra-high-density yellowtail physical map has enabled more-accurate comparisons of the syntenic relationships between yellowtail and the other fishes. Between yellowtail and Japanese medaka, the chromosomes showed one-to-one

correspondence (Figure 2 and 6). This result accorded with a previous phylogenetic study (Betancur-R *et al.*, 2013), which showed that yellowtail and medaka are phylogenetically close, in spite of the remarkable difference in body form, size and habitat.

In comparisons between yellowtail and the two fishes, spotted green pufferfish and three-spined stickleback, the chromosomes showed one-to-two correspondence. These chromosomes of pufferfish and stickleback probably underwent Robertsonian translocations after the divergence of each lineage. However, almost all the syntenic relationships were conserved in the four species These results suggest that the chromosome structures are well conserved in these fish species derived from a common ancestor.

Zebrafish (*Danio rerio*) is a tropical freshwater fish of Indian origin, belonging to the subfamily Rasborinae in the order Cypriniformes. It has 50 (2n) chromosomes. Yellowtail LG1, LG4 and LG17 contained parts of three zebrafish chromosomes, namely Dre6, Dre11, and Dre22, and many small parts of the zebrafish chromosomes were distributed in multiple yellowtail chromosomes. Clearly, the relationship of the chromosome structure between yellowtail and zebrafish is very complex. These results reflect that zebrafish is farther from yellowtail than the other model fishes in terms of phylogeny.

Spotted gar is a primitive fish species, which lineage diverged from the teleost lineage before the teleost-specific WGD event (Amores *et al.*, 2011). Therefore, its lineage represents the unduplicated sister group of teleosts. Thus, syntenic structures between yellowtail and spotted gar, and between spotted gar and four other acanthopterygian species were compared. Many chromosome blocks were conserved between yellowtail and spotted gar, but some yellowtail chromosome blocks were assembled into chromosomes of spotted gar. Spotted-gar chromosome (Loc) 11 corresponded to Squ10 and Squ16, Loc18 to Squ1 and Squ24, Loc23 to Squ3 and Squ9, Loc25 to Squ1 and Squ24, and Loc27 to Squ3 and Squ9. These spotted-gar chromosomes showed an overall

one-to-two double-conserved syntenic relationship with the yellowtail chromosomes. The other spotted-gar chromosomes corresponded to several yellowtail chromosomes. These data demonstrate the overall one-to-two double-conserved syntenic relationship of spotted gar to a post-teleost WGD.

A high-density physical map with 13,977 ESTs was constructed in this study and yellowtail chromosome rearrangements were compared with four other fish species using syntenic relationships. Using spotted-gar genome information as an outgroup to infer the genome evolution of teleosts, the regions showing different syntenic relationships with spotted gar were considered to have undergone chromosomal rearrangements. In the four species (yellowtail, Japanese medaka, spotted green pufferfish and three-spined stickleback), translocation was widely distributed on each chromosome (Figure 12). Therefore, in the past, illegitimate recombination seems to have occurred randomly in entire chromosome regions in each lineage. The number of inter-chromosomal translocations in yellowtail was 1.5–2 times higher than those in Japanese medaka, pufferfish and stickleback (Table 3), which had similar numbers of inter-chromosomal translocations (Table 3). The difference of the frequency of inter-chromosomal translocations between yellowtail and the other species may be caused by striking differences in the habitat, population size, and/or life history of yellowtail (Fan and Meyer, 2014; Chain and Feulner, 2014).

Chromosome rearrangements and gene duplications play an important role in evolution (Vandepoele *et al.*, 2004; Christoffels *et al.*, 2004; Berthelot *et al.*, 2014). In a gene with two variations on two distant sites, a crossover between the sites generate a new allele and gain the genetic variation. A robertsonian fusion, which decrease the number of chromosomes by a fusion of two acrocentric chromosomes, induces reproductive isolation (Slijepcevic, 1998). Gene duplications can promote gene evolution such as neofunctionalization and subfunctionalize by generating redundancy of functional

genes and can produce persistent heterozygosity by putting one of the two alleles to the different locus on the genome (Berthelot *et al.*, 2014). In the yellowtail linage, the functions of chromosome rearrangements and gene duplications seem to affect the persistence of this species. In any case, these mechanisms require isolations of small population or genetic bottlenecks (Furlan *et al.*, 2012). In future studies, it is worthwhile investigating whether the characteristics of genome structures of yellowtail correlate with the results of population genetics.

Chapter 3: Identification of sex-associated SNPs of greater amberjack (*Seriola dumerili*)

2.1. Introduction

Sex determination systems of fishes is highly diverse, while sex is exclusively determined by the *Sry* gene on the Y chromosome in most mammals (Waters *et al.*, 2007). Although sex is primarily determined by genes in many fish species, it is also affected by environmental factors (e.g., exogenous steroids and temperature) and some fish change sex with growth. Various taxa of fishes evolved remarkably different sex determination systems. Therefore, investigation of these systems in fish species is valuable for understanding of sex determination systems and their evolution in fish. Knowledge of the sex determination process is also valuable given the importance of controlling the sex ratio of commercially important species in aquaculture. *Seriola* species in the family Carangidae are important edible fish around the world. Two *Seriola* species, yellowtail and greater amberjack, are the main species for aquaculture production in Japan.

In this chapter, sex-associated SNPs of greater amberjack were identified, and it was clarified that most of these SNPs are present on chromosome 12. Furthermore, genomic structure of greater amberjack was compared with those of medaka, yellowtail and

California yellowtail (*Seriola lalandi dorsalis*). Sex determination system of greater amberjack was found to be markedly different from that of medaka. SD region was conserved among the *Seriola* species.

2.2. Materials and Methods

Data collection

Genomic sequences of greater amberjack and yellowtail were obtained from DDBJ (greater amberjack: BDQW01000001–BDQW01034655, yellowtail: BDMU01000001–BDMU01000384). Genome scaffold sequences of greater amberjack and yellowtail were combined into each chromosome based on a previously published physical genetic map (Kawase *et al.*, 2018). Transcriptome data of greater amberjack were also obtained from DDBJ (greater amberjack: IACO01000001–IACO01045109, yellowtail: IACH01000001–IACH01013125, FX884179–FX890203). The sequence variation data of greater amberjack were obtained from a preceding study (Araki *et al.*, 2018). cDNA sequences of medaka were obtained from Ensembl (Aken *et al.*, 2017). The chromosome-level assembly of medaka was obtained from NCBI Assembly (ASM31367v1) (Kasahara *et al.*, 2007). The genome assembly of California yellowtail was obtained from GenBank (GCA_002814215.1).

Synteny analysis

To identify orthologs, a nucleotide-to-nucleotide BLAST (blastn) search was performed between cDNA sequences of greater amberjack and yellowtail using an e-value cut-off of 1e－10 and reciprocal best hits. A translated-nucleotide-to-translated-nucleotide BLAST (tblastx) search was also performed between cDNA sequences of greater amberjack and Japanese medaka, using an e-value cut-off of 1e－5 and reciprocal

best hits. The orthologs were aligned on the genetic map of each species to identify syntenic relationships. Then, Circos plots (Krzywinski *et al.*, 2009) were constructed to illustrate the syntenic relationships.

Whole-genome alignments

Interspersed repeats and low-complexity DNA sequences of each genome assembly were masked using Tandem repeats finder (Benson, 1999) and Repeatmasker (Smit *et al.*, 2013) with the following option: species "teleost fish." Then, whole-genome alignments of each orthologous chromosome between greater amberjack (Sdu) and yellowtail (Squ) were performed using LASTZ (Harris, 2007) with the following options: no transition, step=20, and chain. LASTZ is a program for aligning DNA sequences and it is originally designed to handle sequences the size of human chromosomes. Whole-genome alignments of each orthologous chromosome between greater amberjack and Japanese medaka (Ola) were also performed using LASTZ (Harris, 2007) with the following options: transition, step=20, and chain. Genomic sequences alignments between Sdu chromosome 12 and the scaffold sequence (PEQF01098998.1) with hsd17b1 gene of California yellowtail (Sdo) were performed using LASTZ with the following options: no transition, step=20, and chain. After removing alignments shorter than the thresholds (Sdu vs. Squ and Sdu vs. Sdo: 400, Sdu vs. Ola: 150) from Rdotplot files generated from LASTZ, the alignment blocks were plotted by R.

Sex-determining SNP identification

Biallelic SNP information of 10 males and 10 females was obtained from the sequence variation data of greater amberjack. Those SNPs with a minor allele frequency of less than 0.2 or missing data for more than two individuals were filtered out. An association test was performed by GWASpoly with the 1-dom model and false discovery rate level = 0.01. Haplotypes were estimated by PHASE v2.1.1 (Stephens *et al.*, 2001; Stephens and

Scheet, 2005), which is a program implementing the method for reconstructing haplotypes from population data, from the genotype data included in the result of GWASPoly. To identify the proteins into which the transcripts in greater amberjack are translated, a translated nucleotide-to-protein BLAST search (blastx) of the greater amberjack transcriptome sequences against protein sequences of RefSeq vertebrate other (release 69) (O'Leary *et al.*, 2016) was performed with an e-value cut-off of 0.01.

It was reported that the sex-determining locus of California yellowtail has female-specific 61 nucleotide deletion and estradiol 17-beta-dehydrogenase 1 (*Hsd17b1*) is the putative sex-determining gene (Purcell *et al.*, 2018). To check whether greater amberjack has the female-specific deletion or not, indel variations near *Hsd17b1* (between 50kb upstream and downstream) was extracted from the variation data of greater amberjack and existence of a female-specific deletion was checked.

2.3. Results

Synteny analysis

Between greater amberjack and yellowtail, 10,258 entries were identified as orthologs. The chromosomes thought to be orthologous in the two species retained the order of those orthologs. In addition, inter-chromosomal translocation was observed at low frequency (Figure 13). Between greater amberjack and medaka, 14,037 entries were identified as orthologs. The chromosome structure is generally conserved in the two species, but inter-chromosomal rearrangements have occurred more frequently than in the comparison of greater amberjack and yellowtail (Figure 14).

Whole-genome alignments

Pairwise whole-genome alignments of greater amberjack were performed against

yellowtail and Japanese medaka with LASTZ (Harris, 2007). The plots of alignments were shown in Figure 15 (greater amberjack vs. yellowtail) and Figure 16 (greater amberjack vs. Japanese medaka). The alignments between greater amberjack and yellowtail indicate that the chromosome structure is well conserved between the two species and homology of the genome sequences is retained in almost all regions of the genome (Figure 15). Then the same number was assigned to the orthologous chromosomes of the two species using the linkage group numbers in a previous genetic map study (Aoki *et al.*, 2015). In greater amberjack and Japanese medaka, the sequences could be aligned in a large range in some regions (e.g., Sdu2: 4.5–17.8 Mb vs. Ola1: 25.4–8.4 Mb, Sdu3: 7.0–16.7 Mb vs. Ola6: 5.6–16.3 Mb and Sdu4: 17.8–26.0 Mb vs. Ola4: 14.0–23.6 Mb), but chromosomal inversions and intra-chromosomal translocations were frequently observed (Figure 16). Genomic sequence alignments between Sdu12 and the scaffold sequence (PEQF01098998.1) of California yellowtail showed completely linear correspondence (Figure 17).

Sex-associated SNP identification

After SNP pruning, 1,797,184 SNPs were used for subsequent analysis. The 1-dom model in GWASpoly was considered to be an appropriate statistical model because minor alleles control sex in both XX/XY and ZZ/ZW sex determination systems. The loci significantly associated with sex in the 1-dom test by GWASpoly were listed in Table 4. and Manhattan plots of the 1-dom-alt and 1-dom-ref models were shown in Figure 18. In 1-dom-alt and 1-dom-ref, it is assumed that alternative and reference alleles are both dominant. SNPs associated with sex were mainly covered with two scaffolds, contig43 and contig168, and we found one high-score locus, c168_4648290, associated with sex on contig168. The genotypes of sex-associated SNPs were shown in Table 5. Although eight loci (c16_12746979, c43_1018703, c43_1944077, c43_4403196, c168_3069620,

c168_4823950, c168_5414906, and c387_222985) were heterozygous in the male and homozygous in the female, the other loci were heterozygous in the female and homozygous in the male (Table 5). The former corresponds to the genotypes of XX/XY and the latter to those of ZZ/ZW, which are localized on chromosome 12. The region containing significant SNPs on chromosome 12 was defined as SD in this paper. This region ranges from the nucleotide position of 56,399bp to 4,405,455bp on contig43 and from 338,405bp to 5,457,861bp on contig168 (Table 4). In this SD region on chromosome 12, there are genes that encode proteins associated with sex hormones, such as G-protein-coupled estrogen receptor 1 (GPER), estradiol 17-beta-dehydrogenase 1, 17-beta-hydroxysteroid dehydrogenase 14, and transcription factors SOX8 and SOX9.

The reconstructed haplotypes estimated by PHASE from the genotype data were shown in Table 6. All females have haplotypes unique to females, while five male individuals (M5, M6 M7, M8, M10 in Table 7) had two haplotypes that appear to be specific to females. Indel variations near *Hsd17b1* were extracted from the sequence variation data of greater amberjack. Although the possibility of a female-specific deletion was checked, its feature could not be detected.

2.4. Discussion

Sex determinant location

By genome-wide sex association analysis, sex-associated markers were detected on chromosomes (chr)12, 24, 1, 15, and 22 in decreasing order of the number of detected markers on each chromosome. A total of 31 of the 51 sex-associated SNPs were localized on chr12. Sex-associated linkage analysis of yellowtail showed that sex determinants are present on the tip of linkage group 12 (Koyama *et al.*, 2015). Thus, part of chr12 acts as a sex chromosome in greater amberjack, as well as yellowtail. Greater amberjack's SD

loci were present at scaffolds 168 and 43. These sequences were located in the middle of chr12 and the range of the SD region was approximately 7.1 Mb, ignoring the gaps between scaffolds.

Comparative analysis

Whole-genome alignments and synteny analysis between yellowtail and greater amberjack showed a well-conserved chromosome structure (Figure 15). Yellowtail has the female heterogametic sex determination system (ZW/ZZ) and its SD segment is located on Squ12 (Koyama *et al.*, 2015). Sex-associated markers of greater amberjack were also located at Sdu12, which is a chromosome orthologous to Squ12. The plots of alignment of Sdu12 vs. Squ12 showed a linear pattern (Figure 19). Research on the sex-linked SNPs of yellowtail indicated that *Gipc1* and *Sox9* are present near the SD region (Koyama *et al.*, 2015), which also applies to greater amberjack. The SD region is present on the tip of the linkage map in yellowtail (Koyama *et al.*, 2015), whereas it is present in the middle of the chromosome in greater amberjack. This is due to the recombination suppressed in the sex chromosome and the small length of the SD region in the linkage map of yellowtail.

The alignment of Sdu12 vs. the scaffold sequence (PEQF01098998.1) of California yellowtail also demonstrated well-conserved sequences. Hence, it is inferred that the three *Seriola* species share the same sex determination mechanism because these *Seriola* species exhibit female heterogamety and have the same genes in orthologous genomic regions. Although Japanese medaka has the SD gene on chromosome 1 (Ola1) (Matsuda *et al.*, 2002; Wilson *et al.*, 2014), whole-genome alignment showed remarkable homology between Ola8 and Sdu12 (Figure 20). Medaka exhibits male heterogamety and its master SD gene is *Dmy*. Accordingly, the *Seriola* species and medaka clearly have different SD mechanisms. It is considered that SD systems independently evolved in each species after

the divergence of those species from the common ancestor.

ZW sex determination system in amberjack

Various sex determination mechanisms have been reported in fish species. For example, zebrafish (Wilson *et al.*, 2014) and tongue sole (Chen *et al.*, 2014) have the ZZ/ZW system, while Japanese medaka (Matsuda *et al.*, 2002), guppy (*Poecilia reticulata*) (Tripathi *et al.*, 2009), and torafugu (Kamiya *et al.*, 2012) have the XX/XY system. In tilapia species, some species have the XX/XY system while others have the ZZ/ZW system (Cnaani *et al.*, 2008). In most of the fish species, however, sex chromosomes have not been found (Devlin and Nagahama, 2002).

In most of the genotypes in sex-associated SNPs, it was indicated that greater amberjack females are heterozygous (Table 5) and all females have haplotypes unique to females (Table 6). Therefore, it is likely that greater amberjack has a heterogametic sex determination system (ZZ/ZW). SNPs between loci c43_4405455 and c43_1782220 were heterozygous in the male of M7 in Table 5 and homozygous in the female of F6 in Table 5. Therefore, the segment could have undergone recombination. Hence, this SD region may tolerate small-scale recombination, which means not a complete sex chromosome.

Among the organisms with a female heterogametic sex-determination system other than fish, clawed frog (*Xenopus laevis*) has been well studied. In this species, *DM-W* is the SD gene on the W chromosome (Yoshimoto *et al.*, 2008). *DM-W* is a paralog of *Dmrt1*, which was suggested to be related to testis differentiation in mouse and chicken (Raymond *et al.*, 1999, 2000), but has no significant sequence similarity with the transactivation domain of *Dmrt1* (Yoshimoto *et al.*, 2008). In chicken, *Dmrt1* is Z-linked and its higher expression in ZZ than in ZW embryos suggests the possibility that *Dmrt1* may be a dose-sensitive masculinizing gene (Raymond *et al.*, 1999).

In the SD region, there were genes encoding proteins associated with sex hormones,

such as G-protein-coupled estrogen receptor 1 (GPER), estradiol 17-beta-dehydrogenase, 1 and 17-beta-hydroxysteroid dehydrogenase 14. *Sox9* and *Sox8* were also present in the SD region, so they might be sex determinants. GPER is activated by estrogen, the main female sex hormone, and plays an important role in female development. In some teleosts, 17-beta estradiol plays a critical role in ovarian differentiation (Piferrer *et al.*, 1994; Miyata and Kubo, 2000; Kobayashi *et al.*, 2003; Rashid *et al.*, 2007). Hence, if the *Gper* gene or the estradiol 17-beta-dehydrogenase 1 gene in the SD region affects the female pathway and is involved with promoting sex differentiation in greater amberjack, the W chromosome is supposed to induce female differentiation.

Recently, Purcell *et al.* (2018) identified sex-determining region of California yellowtail using genome assembly and re-sequences and suggested that the estradiol 17-beta-dehydrogenase 1 gene is a putative sex-determining gene (Purcell *et al.*, 2018). Accordingly, it is inferred that the three *Seriola* species may share the same SD region that their common ancestor acquired, and the same gene might be involved in sex determination.

GIPC (PDZ domain containing protein, which interacts specifically with the C terminus of RGS-GAIP) was originally identified as a protein binding to the C terminus of the RGS (G protein signaling regulator) protein GAIP (RGS19), a GTPase-activating protein (GAP) for Gαi subunits (De Vries *et al.*, 1998). Endoglin, which is one of the PDZ ligands, interacts with GIPC and specifically enhances TGF-β1-induced phosphorylation of Smad1/5/8 (Lee *et al.*, 2008). Testicular TGF-β1 modulates Leydig cell steroidogenesis, the organization of peritubular myoid cells, testis development, and spermatogenesis (Rocio *et al.*, 2013). Therefore, although the role of endoglin in the reproductive system could not been shown, GIPC1 might play a role in the sex determination of greater amberjack.

SOX9 and SOX8 are group E SOX proteins. These transcription factors contain,

besides a DNA-binding HMG domain and a transactivation domain, a DNA-dependent dimerization domain, unique among SOX proteins. In mammals, *Sox9* is activated by SRY in pre-Sertoli cells and induces Sertoli cell and testis cord differentiation (Barrionuevo and Scherer, 2010). SOX9 binds to a SOX binding site within the *Amh* promoter and interacts with SF1 to synergistically activate *Amh* expression (De Santa Barbara *et al.*, 1998). SOX8 has the same mechanism of action as SOX9, but acts less efficiently (Schepers *et al.*, 2003).

Some teleosts, such as zebrafish and three-spine stickleback, have two *Sox9* genes, termed *Sox9a* and *Sox9b* (Chiang *et al.*, 2001; Cresko *et al.*, 2003). Gene phylogeny and genetic mapping have shown that these are co-orthologs of the tetrapod *Sox9* gene (Chiang *et al.*, 2001). *Sox9a* was found to be expressed in many tissues including brain, muscle, fin and testis, whereas its expression is restricted to previtellogenic oocytes of the ovary (Chiang *et al.*, 2001).

Japanese medaka, tilapia and rice field eel have two *Sox9a* genes, *Sox9a1* and *Sox9a2* (Zhou *et al.*, 2003; Nakamoto *et al.*, 2005; Ijiri *et al.*, 2008). The two *Sox9a* genes of rice field eel have similar structures and both are expressed in testis, ovary and ovotestis. The close similarity in gene structure and expression patterns of the two *Sox9a* genes in rice field eel suggests that they have similar roles in gonadal differentiation during sex reversal in this species and arose in recent gene duplication events (Zhou *et al.*, 2003). In Japanese medaka, *Sox9a2* is expressed in testis, not in ovary (Nakamoto *et al.*, 2005). Therefore, *Sox9a2* is expected to be involved in male gonadogenesis at the early stages of development (Paul-Prasanth *et al.*, 2011) and the later development of testicular tubules in medaka (Nakamoto *et al.*, 2005).

As noted above, the *Sox9* gene plays an important role in the male differentiation of fish, as well as other vertebrates. In greater amberjack, *Sox9* and *Sox8* are present in the SD region, so they might be sex determinants. Although some genes related to the sex

differentiation in the SD region were found in this study, their molecular mechanisms in sex differentiation could not be elucidated.

General discussion

In this study, a high-density RH map of yellowtail was constructed and 200 scaffolds with a total length of 601 Mb were anchored on the RH map.

Comparisons of the genome structure between yellowtail and model fishes showed that chromosome structure of yellowtail is similar to medaka, spotted-green pufferfish and three-spined stickleback. In the *Seriola* species, yellowtail and greater amberjack, Genomic alignments including non-coding region indicated remarkable similarity. The similarity of chromosome structure is consistent with the phylogenetic relationships of the species, that is, the closer the species is in phylogeny, the higher level of similarity the chromosome structure has. Therefore, chromosome rearrangements might occur and fix in a population by chance like genetic drift.

Sex-associated SNPs of greater amberjack were identified on chromosome 12. Genotypes of sex-associated SNPs indicated that females are the heterogametic sex determination system (ZZ/ZW). In the SD region of greater amberjack, there are genes encoding proteins associated with sex hormones, such as G-protein-coupled estrogen receptor 1 (GPER), estradiol 17-beta-dehydrogenase, 1 and 17-beta-hydroxysteroid dehydrogenase 14. Yellowtail also has the female heterogametic sex determination system (ZW/ZZ). Research on the sex-linked SNPs of yellowtail indicated that *Gipc1* and *Sox9* are present near the SD region, and same genes are present near the SD region of greater amberjack. Furthermore, it has been identified that sex-determining region of California yellowtail and has suggested that the estradiol 17-beta-dehydrogenase 1 gene, which is present in the SD region of greater amberjack, is a putative sex-determining gene (Purcell *et al.*, 2018). Thus, the SD region are conserved in the three *Seriola* species.

Sex determining systems of fish is very diverse (Devlin and Nagahama, 2002). However, the SD system of *Seriola* seems to be stable in contrast to other taxonomic groups because these *Seriola* species exhibit female heterogamety and have the same

genes in orthologous genomic regions. Determining which genes is the key for the sex determination of greater amberjack and investigating why the sex determining systems are conserved in *Seriola* will help the understanding of evolution of sex determining mechanisms,

Conclusion

Fishes are not only the most primitive vertebrate which first appeared on the earth, but also are an extremely diversified taxon in morphology, behavior and habitat use, comprising more than 50% of all vertebrate species. This high level of biodiversity is a powerful basis for the study of genome evolution.

In chapter 2, 181 markers were mapped to the RH map of yellowtail to increase the resolution and this map was integrated with the scaffold-level genome assembly to construct the 'chromonome'. Then, the chromosome structure of yellowtail was compared with that of model fishes. Consequently, between yellowtail and Japanese medaka, it is revealed that almost all regions of the chromosomes were conserved, and that some blocks comprising several markers were translocated. Using the genome information of spotted gar as a reference, syntenic relationships and chromosomal rearrangements occurring in evolution of four other acanthopterygian species (Japanese medaka, zebrafish, spotted green pufferfish and three-spined stickleback) were documented. The evolutionary chromosome translocation frequency was 1.5–2 times higher in yellowtail than in medaka, pufferfish and stickleback.

In chapter 3, sex-associated SNPs of greater amberjack were identified, using SNP information of 10 males and 10 females by an association test. Sex determination systems of fishes is highly diversified, compared with that of mammals. Thus, investigations using nonmodel fish species helps our understanding of the sex determination system of fish. Since greater amberjack is one of the commercially important fish and the information of its sex determination system is economically useful in aquaculture, Sex-associated SNPs were determined on chromosome 12 of greater amberjack, mainly covered with two scaffolds, c168 and c43. Genotypes of sex-associated SNPs indicated that females are the heterogametic sex determination system (ZZ/ZW). In the SD region of greater amberjack,

there are genes encoding proteins associated with sex hormones, such as G-protein-coupled estrogen receptor 1 (GPER), estradiol 17-beta-dehydrogenase, 1 and 17-beta-hydroxysteroid dehydrogenase 14. *Sox9* and *Sox8* were also present in the SD region of greater amberjack. Therefore, they might to be sex determinants. Furthermore, genomic structure of greater amberjack was compared with those of yellowtail and Japanese medaka. Whole genome alignments and synteny analysis indicated that the sex determination system of greater amberjack is much different from that of medaka and suggested that the sex determination region is conserved among the *Seriola* species.

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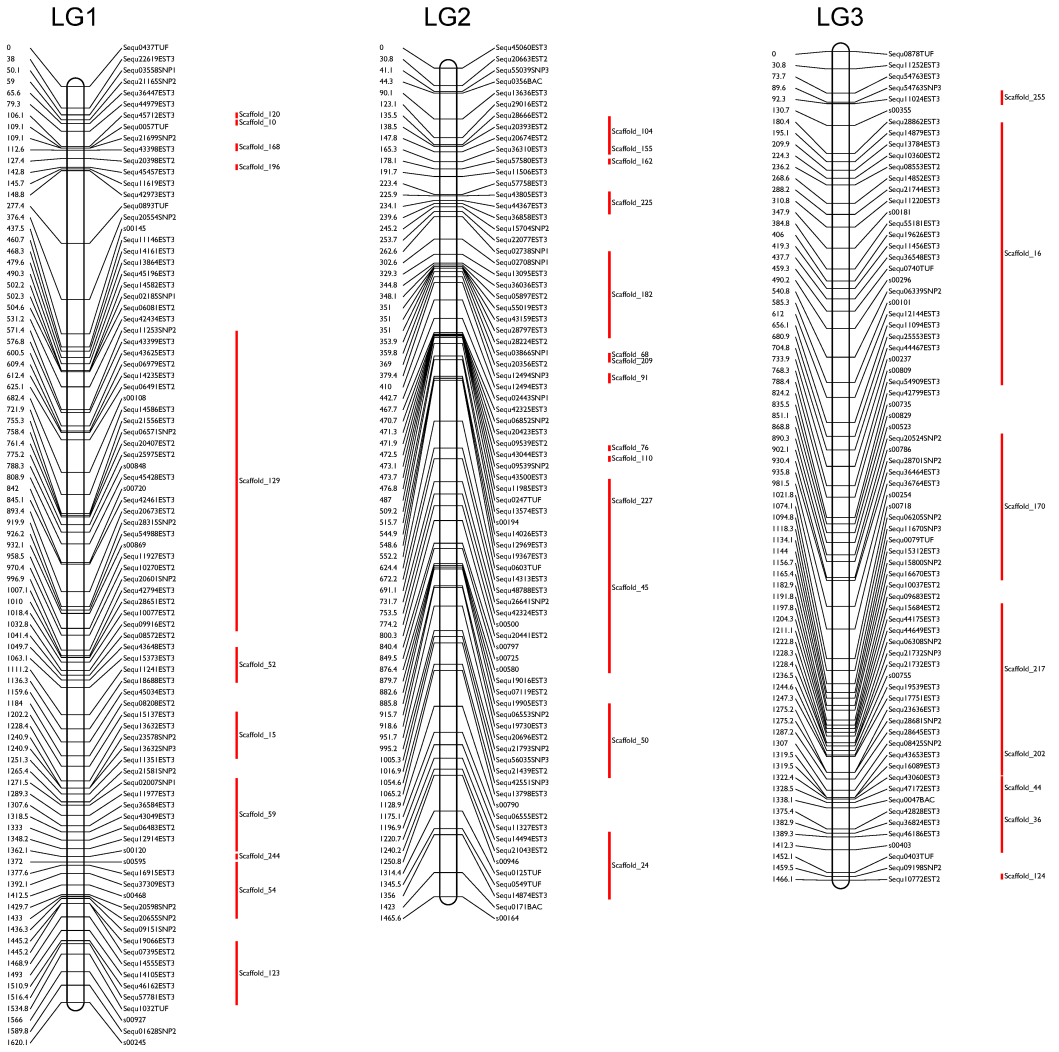
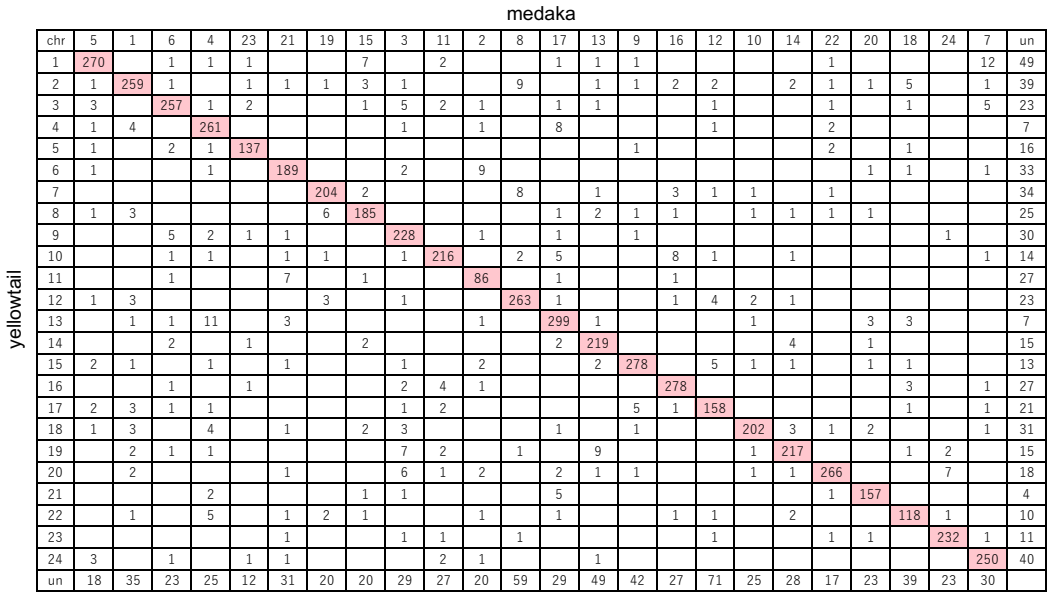


Figure 1. Examples of the fine radiation-hybrid physical map used and the anchored scaffold sequences in this study. Marker names are indicated on the right sides of each chromosome-pattern diagram; red bars indicate the anchored scaffold sequences. The marker distances in centiRays (cRs) are indicated on the left sides of each diagram.



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3	3		257	1	2			1	5	2	1		1	1			1			1		1		5	23
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5	1		2	1	137										1					2		1			16
6	1			1		189			2		9										1	1		1	33
7							204	2				8		1		3	1	1		1					34
8	1	3					6	185					1	2	1	1		1	1	1	1				25
9			5	2	1	1			228		1		1		1									1	30
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15	2	1		1		1			1		2			2	278		5	1	1		1	1			13
16			1		1				2	4	1					278						3		1	27
17	2	3	1	1					1	2					5	1	158					1		1	21
18	1	3		4		1		2	3				1		1			202	3	1	2			1	31
19		2	1	1					7	2		1		9				1	217			1	2		15
20		2				1			6	1	2		2	1	1			1	1	266			7		18
21				2				1	1				5							1	157				4
22		1		5		1	2	1			1		1			1	1		2			118	1		10
23						1			1	1		1					1			1	1		232	1	11
24	3		1		1	1				2	1			1										250	40
un	18	35	23	25	12	31	20	20	29	27	20	59	29	49	42	27	71	25	28	17	23	39	23	30	

Figure 2. Oxford grids showing conservation of synteny between yellowtail *Seriola quinqueradiata* and Japanese medaka. Rows correspond to yellowtail linkage groups. Columns correspond to chromosomes of medaka. The number in each square indicates the number of orthologs. Rows and columns labelled ‘un’ indicate the number of unlocated orthologs. White squares indicate 0–14 orthologs, blue squares 15–30 orthologs, and pink squares >30 orthologs.

spotted green pufferfish

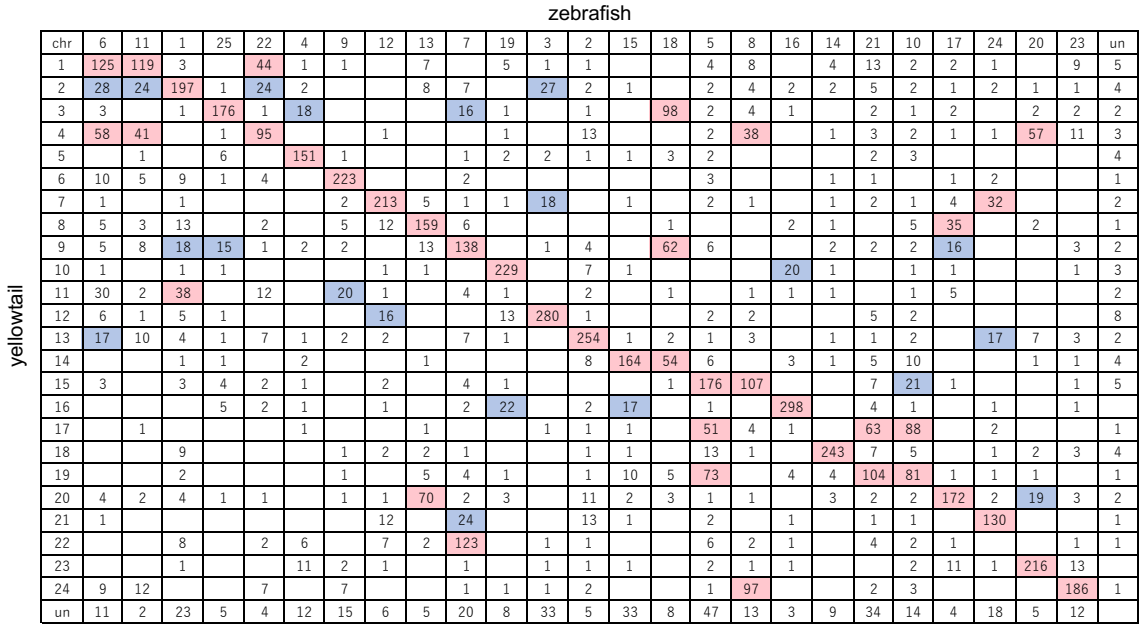
yellowtail	11	18	13	1	19	2	17	5	21	3	15	16	12	8	4	7	10	6	20	14	9	un
1	242						8			1			2								6	64
2		219		1	2	1	3			2		1				1	1			1		80
3	4		248	1	1			4			1		1								6	33
4		4		232				1		1	6				1		1					53
5	1		2		112		1			1			1		1	1	1					40
6		1				205		1		1					1	1	1	1		1	1	19
7						167	4	1		4	17			1				1				35
8	1	5		1		2	176	1			1		2			3						39
9	1		3			1		204	1	1	1		2							1		57
10			1	1		1		1	84					4			2		1			80
11			1			4				114					1							4
12	1	7	1	3	1	13				156	1			1	1		1					101
13			1	10		2					170	1						3			1	68
14											3	134			1	4	15					75
15	3			2		2	1	1					227		5	1						55
16		1	4	2		2	1	1	2					194		3	2	3	1	1		100
17		1							1				4		150							48
18	1	4		178		1		2					1			3	2		19	1		25
19							1	7				2	1		1	207				2		32
20				2		1		4				1					250			4		33
21				1						1	1					2		107				55
22		4		5		1	1								1	1	1		28			96
23						2		1	1					1	2	1	2			211		22
24	2				1	1	1	1				1		1			1	1	1		199	83
un	13	24	23	42	10	40	12	14	15	35	13	34	37	14	63	26	28	10	4	22	22	

Figure 3. Oxford grids showing conservation of synteny between yellowtail and spotted green pufferfish. Rows correspond to yellowtail linkage groups. Columns correspond to chromosomes of spotted green pufferfish. The number in each square indicates the number of orthologs. Rows and columns labelled ‘un’ indicate the number of unlocated orthologs. White squares indicate 0–14 orthologs, blue squares 15–30 orthologs, and pink squares >30 orthologs.

three-spined stickleback

yellowtail	17	9	19	8	4	16	5	6	2	10	1	11	3	13	20	14	7	15	21	18	12	un
1	234	1						9		4				2	1						6	122
2		319			4			2				4		2			2		2	1		11
3			304	1	3				3	1	1		1	1				1			6	7
4		4		275	2				1				5									24
5	2		2	1	149												1					28
6						223			1	2	3	2							1			20
7							240	2	1			4			2							33
8	1	4		1			2	219	1						3		1			1		12
9			2		2	1			282		2									2		7
10			1						1	238			1		4			1	1			21
11						6					118								1			12
12		2					7					299			1	6		1				6
13				5		1					1	1	318	1			3		2			11
14				1				1			239	1	3				3					10
15	3	1				1		2			1			314	1	4						4
16			7						1	6					319		1					16
17	1	2			1					1				7	2	173						25
18		2		5	255	2			2		1		1				3					1
19		2		1	1		1	2	7	1	1			1	4		241	1		2		11
20		1		1			1		4		2						1	285		1	1	13
21		1		1							1		4						157			20
22	1	1		5													116	1				39
23						2			1						1	1		3		268		4
24	1				1	1				1								1	1		266	44
un	17	38	32	32	59	32	20	15	24	17	60	66	25	29	32	87	56	30	15	21	23	

Figure 4. Oxford grids showing conservation of synteny between yellowtail and three-spined stickleback. Rows correspond to yellowtail linkage groups. Columns correspond to chromosomes of three-spined stickleback. The number in each square indicates the number of orthologs. Rows and columns labelled ‘un’ indicate the number of unlocated orthologs. White squares indicate 0–14 orthologs, blue squares 15–30 orthologs, and pink squares >30 orthologs.



chr	6	11	1	25	22	4	9	12	13	7	19	3	2	15	18	5	8	16	14	21	10	17	24	20	23	un
1	125	119	3		44	1	1		7		5	1	1			4	8		4	13	2	2	1		9	5
2	28	24	197	1	24	2			8	7		27	2	1		2	4	2	2	5	2	1	2	1	1	4
3	3		1	176	1	18				16	1		1		98	2	4	1		2	1	2		2	2	2
4	58	41		1	95			1			1		13			2	38		1	3	2	1	1	57	11	3
5		1		6		151	1			1	2	2	1	1	3	2				2	3					4
6	10	5	9	1	4		223			2						3			1	1		1	2			1
7	1		1				2	213	5	1	1	18		1		2	1		1	2	1	4	32			2
8	5	3	13		2		5	12	159	6					1			2	1		5	35		2		1
9	5	8	18	15	1	2	2		13	138		1	4		62	6			2	2	2	16			3	2
10	1		1	1				1	1		229		7	1				20	1		1	1			1	3
11	30	2	38		12		20	1		4	1		2		1		1	1	1		1	5				2
12	6	1	5	1				16			13	280	1			2	2			5	2					8
13	17	10	4	1	7	1	2	2		7	1		254	1	2	1	3		1	1	2		17	7	3	2
14			1	1		2			1				8	164	54	6		3	1	5	10			1	1	4
15	3		3	4	2	1		2		4	1				1	176	107			7	21	1			1	5
16				5	2	1		1		2	22		2	17		1		298		4	1		1		1	
17		1				1			1			1	1	1		51	4	1		63	88		2			1
18			9				1	2	2	1			1	1		13	1		243	7	5		1	2	3	4
19			2				1		5	4	1		1	10	5	73		4	4	104	81	1	1	1		1
20	4	2	4	1	1		1	1	70	2	3		11	2	3	1	1		3	2	2	172	2	19	3	2
21	1							12		24			13	1		2		1		1	1		130			1
22			8		2	6		7	2	123		1	1			6	2	1		4	2	1			1	1
23			1			11	2	1		1		1	1	1		2	1	1			2	11	1	216	13	
24	9	12			7		7			1	1	1	2			1	97			2	3				186	1
un	11	2	23	5	4	12	15	6	5	20	8	33	5	33	8	47	13	3	9	34	14	4	18	5	12	

Figure 5. Oxford grids showing conservation of synteny between yellowtail and zebrafish. Rows correspond to yellowtail linkage groups. Columns correspond to chromosomes of zebrafish. The number in each square indicates the number of orthologs. Rows and columns labelled ‘un’ indicate the number of unlocated orthologs. White squares indicate 0–14 orthologs, blue squares 15–30 orthologs, and pink squares >30 orthologs.

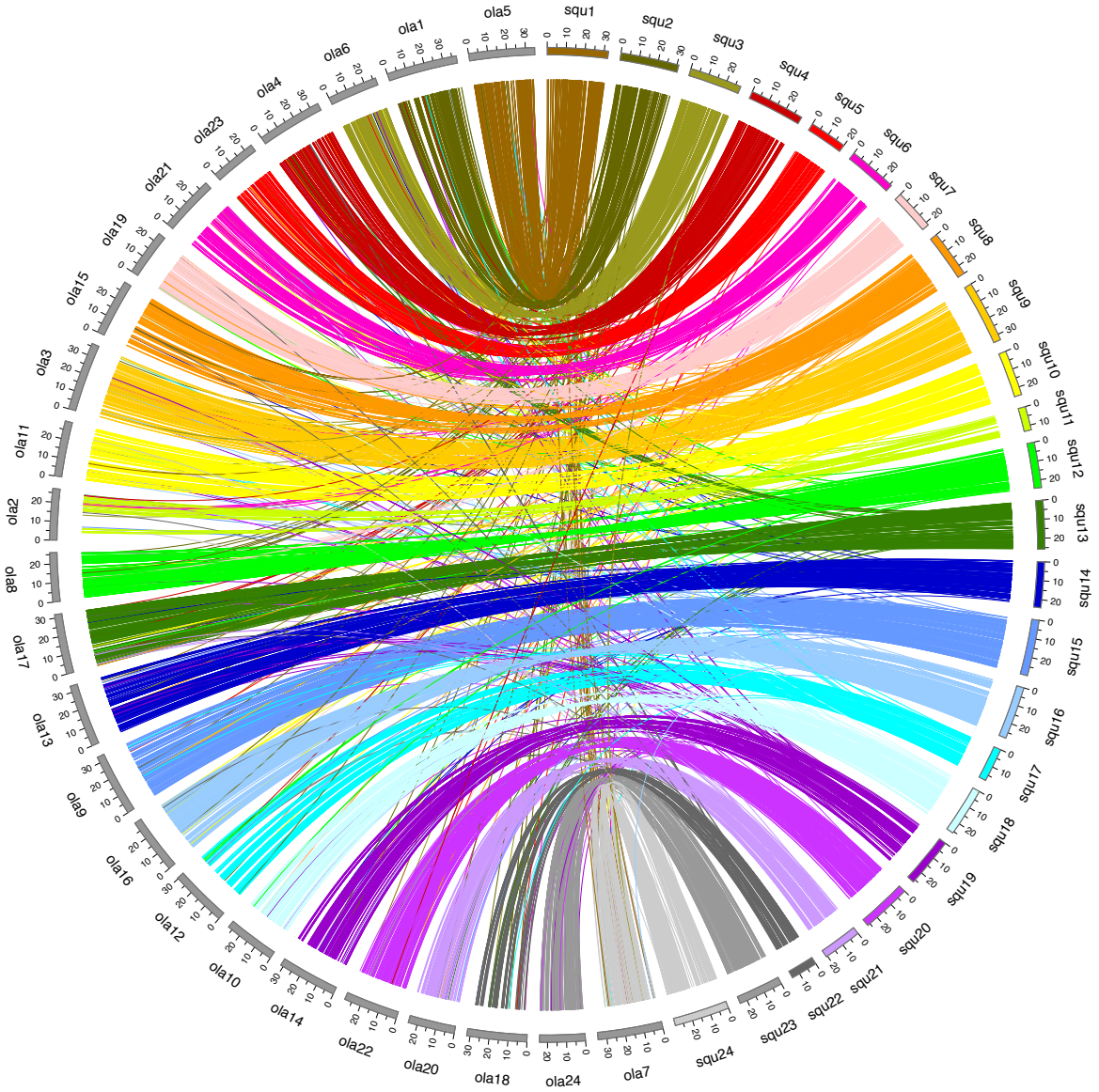


Figure 6. Circos plots showing conservation of synteny between yellowtail (right side, multicolored) and Japanese medaka (left side, grays). “squ” and “ola” indicate *Seriola quinqueradiata* linkage group and *Oryzias latipes* chromosome, respectively.

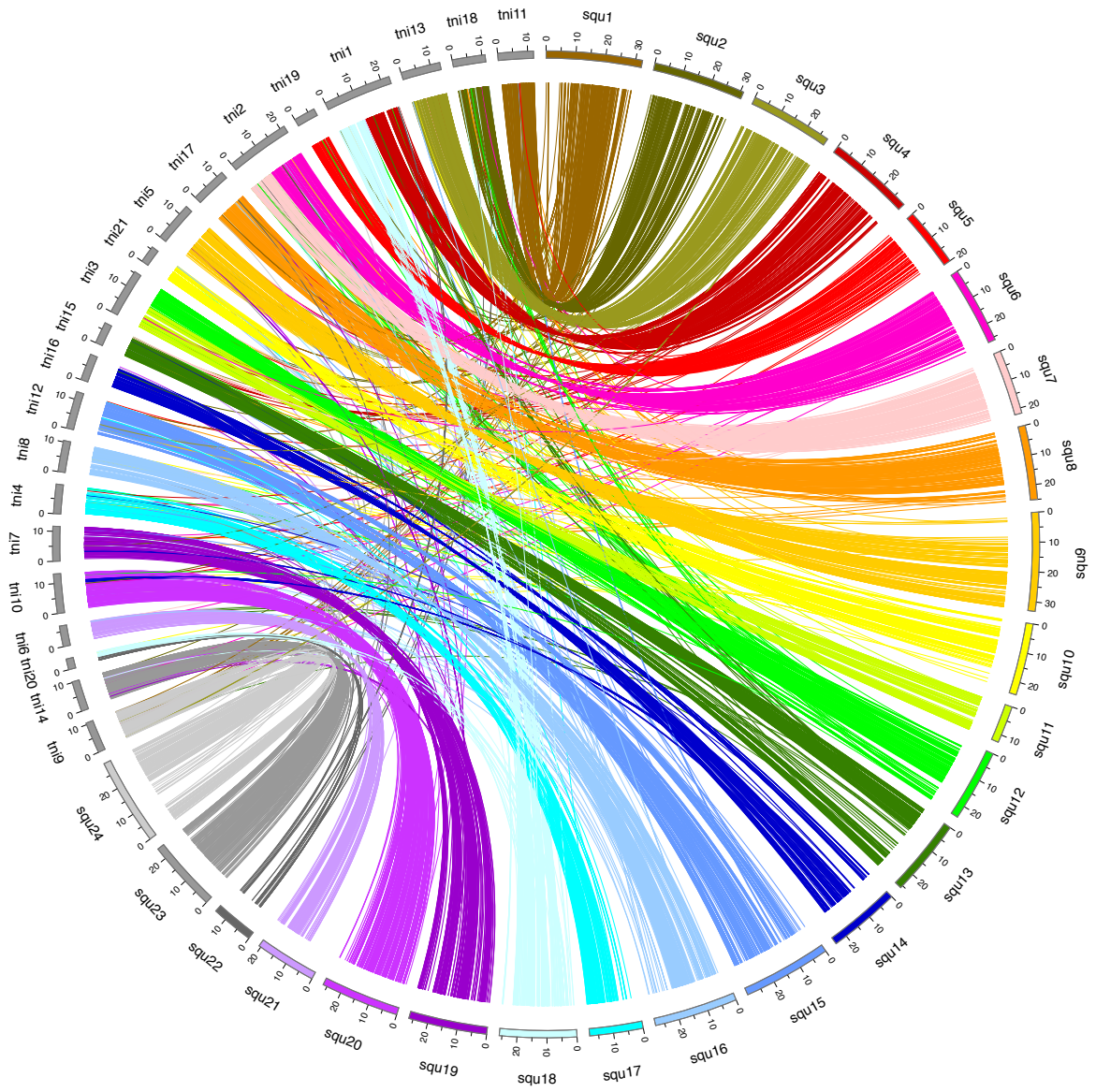


Figure 7. Circos plots showing conservation of synteny between yellowtail (right side, multicolored) and spotted green pufferfish (left side, grays). “squ” and “tni” indicate *Seriola quinqueradiata* linkage group and *Tetraodon nigroviridis* chromosome, respectively.

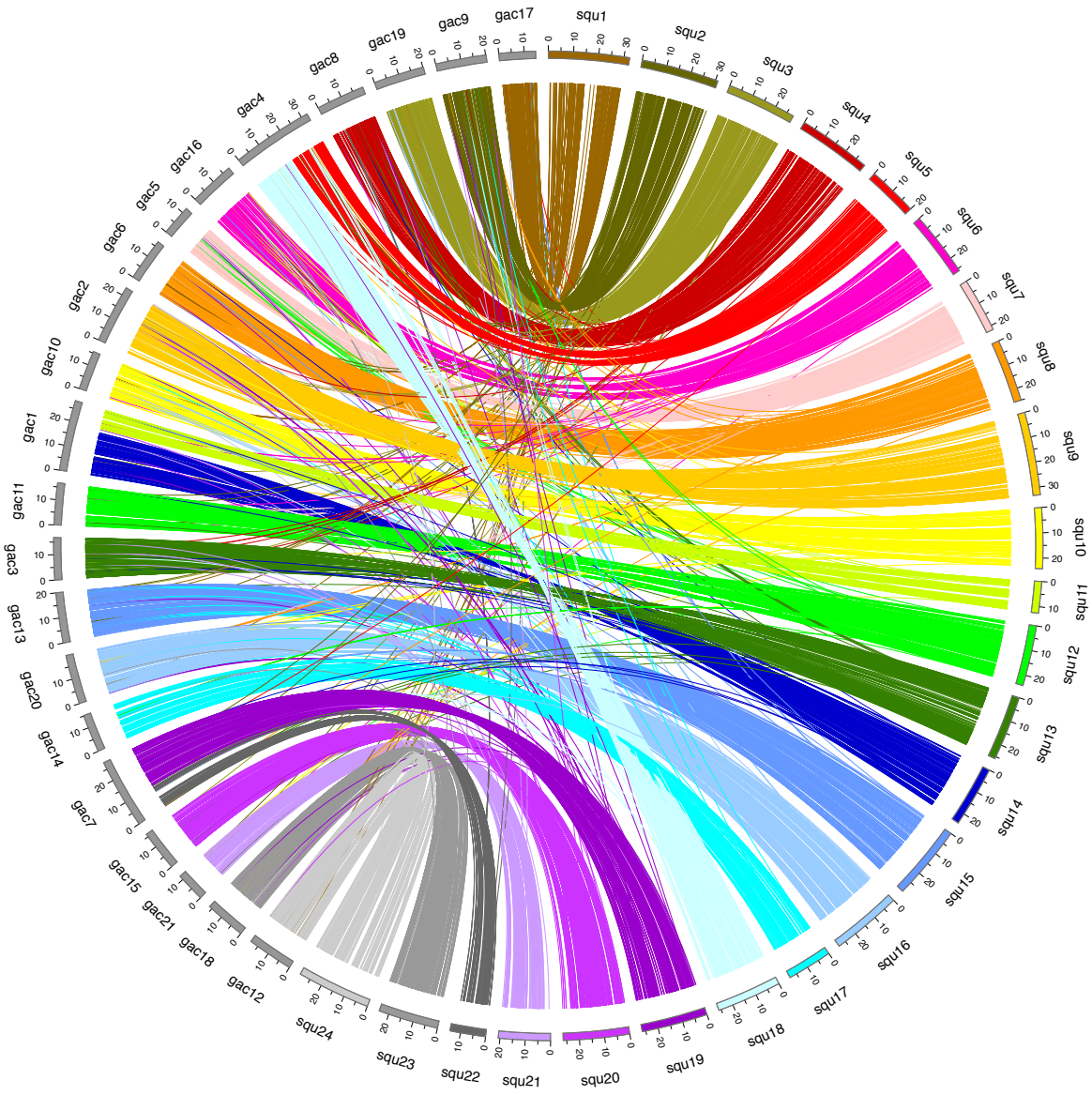


Figure 8. Circos plots showing conservation of synteny between yellowtail (right side, multicolored) and three-spined stickleback (left side, grays). “squ” and “gac” indicate *Seriola quinqueradiata* linkage group and *Gasterosteus aculeatu* chromosome, respectively.

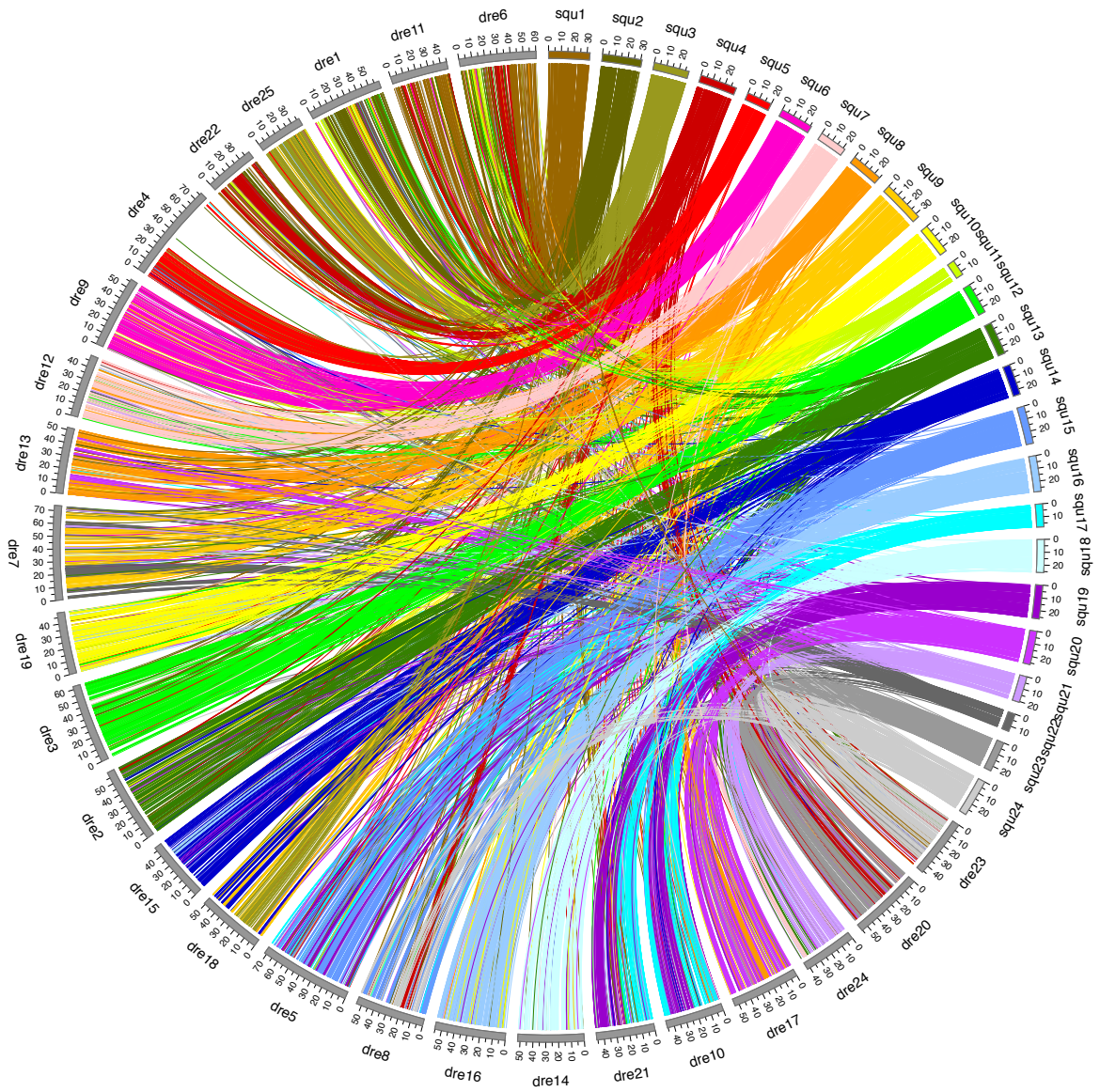
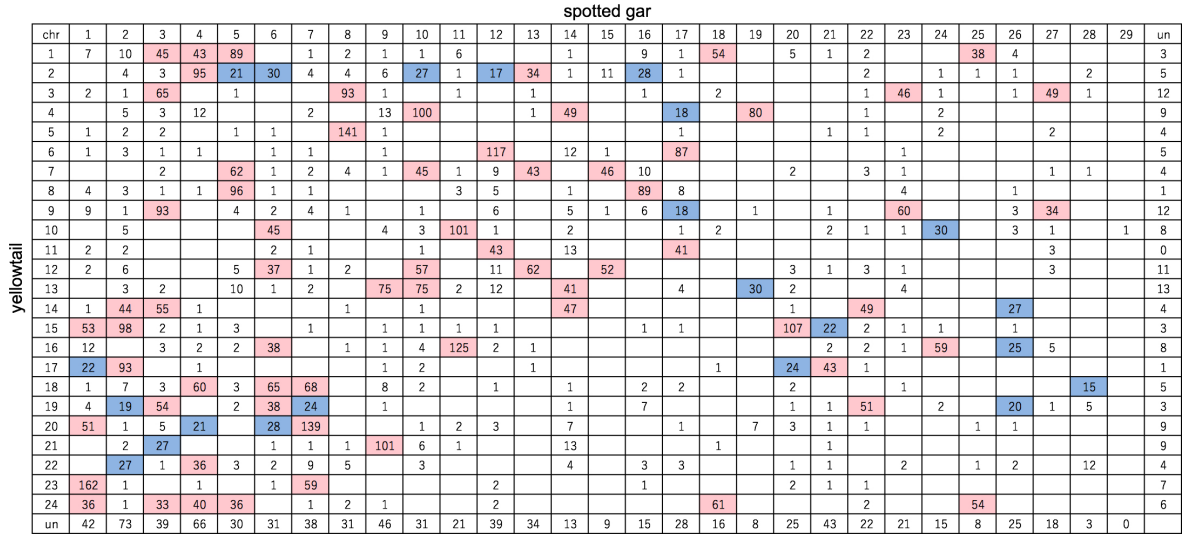


Figure 9. Circos plots showing conservation of synteny between yellowtail (right side, multicolored) and zebrafish (left side, grays). "squ" and "dre" indicate *Seriola quinqueradiata* linkage group and *Danio rerio* chromosome, respectively.



chr	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	un
1	7	10	45	43	89		1	2	1	1	6			1		9	1	54		5	1	2			38	4				3
2		4	3	95	21	30	4	4	6	27	1	17	34	1	11	28	1					2		1	1	1		2		5
3	2	1	65		1			93	1		1		1			1		2				1	46	1		1	49	1		12
4		5	3	12			2		13	100			1	49			18		80			1		2						9
5	1	2	2		1	1		141	1								1				1	1		2			2			4
6	1	3	1	1		1	1		1			117		12	1		87						1							5
7			2		62	1	2	4	1	45	1	9	43		46	10				2		3	1				1	1		4
8	4	3	1	1	96	1	1				3	5		1		89	8						4			1				1
9	9	1	93		4	2	4	1		1		6		5	1	6	18		1		1		60			3	34			12
10		5				45			4	3	101	1		2			1	2			2	1	1	30		3	1		1	8
11	2	2				2	1			1		43		13			41										3			0
12	2	6			5	37	1	2		57		11	62		52					3	1	3	1				3			11
13		3	2		10	1	2		75	75	2	12		41			4		30	2			4							13
14	1	44	55	1				1		1				47						1		49				27				4
15	53	98	2	1	3		1		1	1	1	1				1	1			107	22	2	1	1		1				3
16	12		3	2	2	38		1	1	4	125	2	1								2	2	1	59		25	5			8
17	22	93		1					1	2			1					1		24	43	1								1
18	1	7	3	60	3	65	68		8	2		1		1		2	2			2			1					15		5
19	4	19	54		2	38	24		1					1		7				1	1	51		2		20	1	5		3
20	51	1	5	21		28	139			1	2	3		7			1		7	3	1	1			1	1				9
21		2	27			1	1	1	101	6	1			13				1			1									9
22		27	1	36	3	2	9	5		3				4		3	3			1	1		2		1	2		12		4
23	162	1		1		1	59					2				1				2	1	1								7
24	36	1	33	40	36		1	2	1			2						61				2			54					6
un	42	73	39	66	30	31	38	31	46	31	21	39	34	13	9	15	28	16	8	25	43	22	21	15	8	25	18	3	0	

Figure 10. A: Oxford grid showing conservation of synteny between yellowtail and spotted gar. Rows correspond to yellowtail linkage groups. Columns correspond to spotted-gar chromosomes. The number in each square indicates the number of orthologs. Rows and columns labelled ‘un’ indicate the number of unlocated orthologs. White squares indicate 0–14 orthologs, blue squares 15–30 orthologs, and pink squares >30 orthologs.

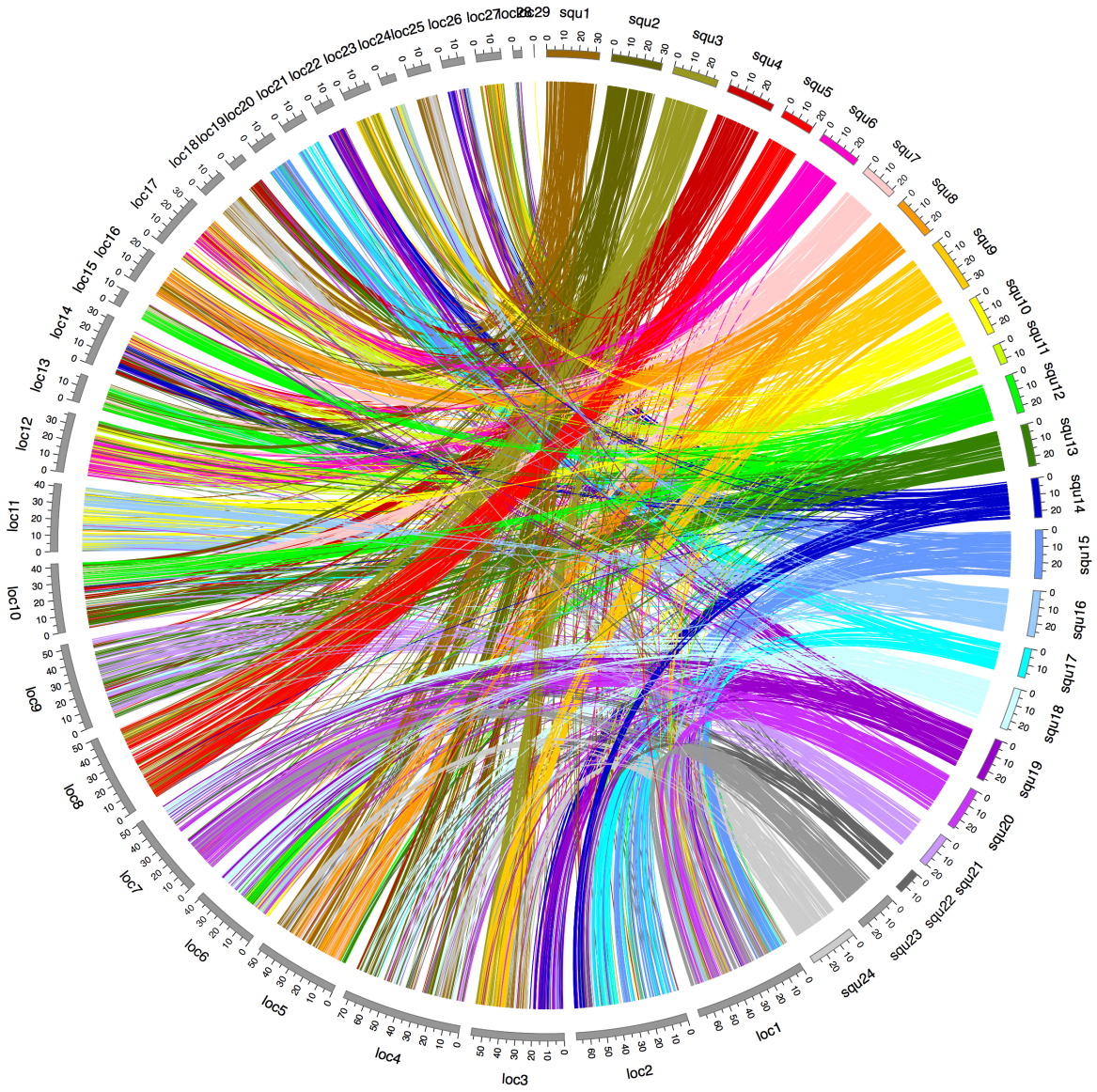


Figure 11. Circos plot showing positional relations of orthologs between yellowtail (right side, multicolored) and spotted gar (left side, grays). “squ” and “loc” indicate *Seriola quinqueradiata* linkage group and *Lepisosteus oculatus* chromosome, respectively.

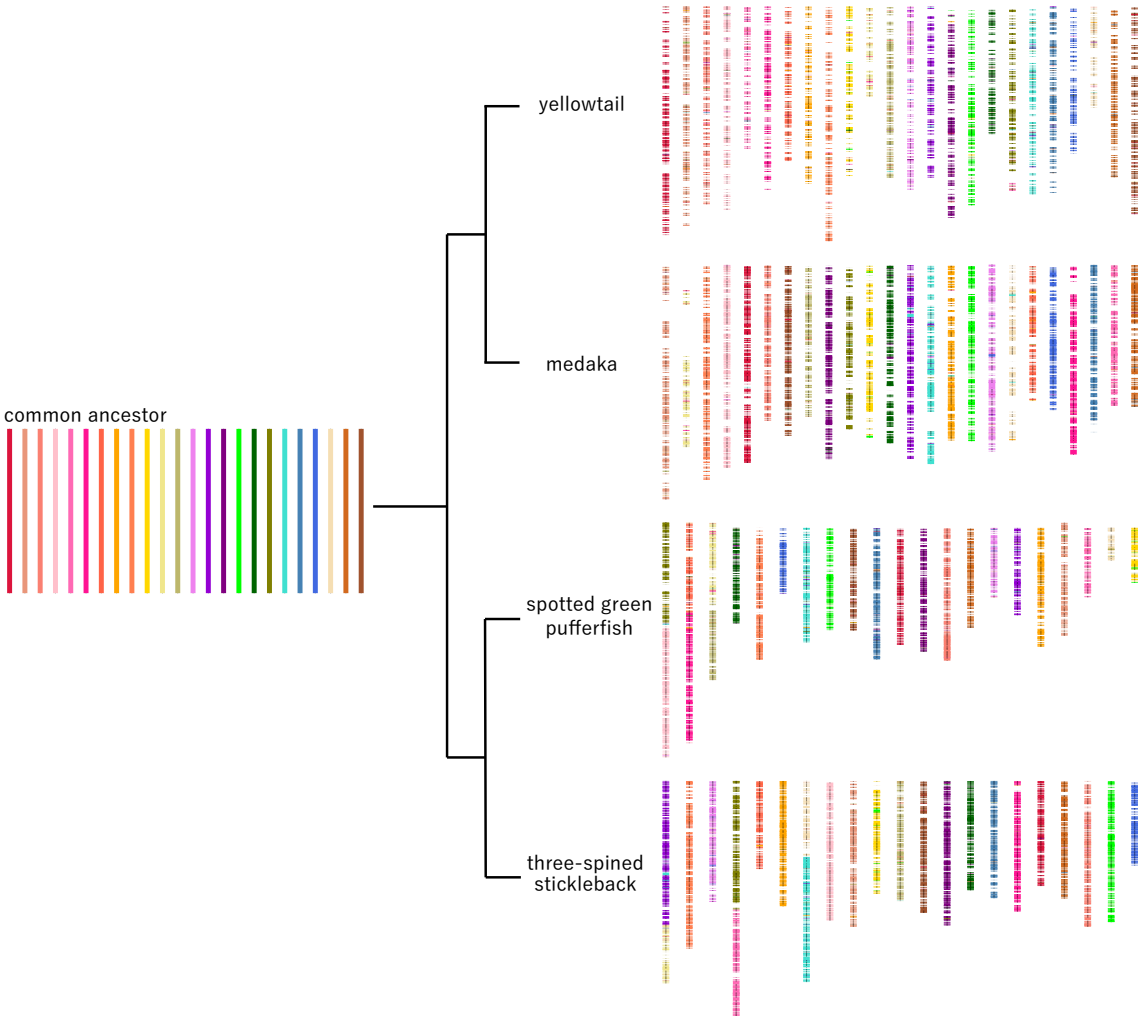


Figure 12. Predicted chromosomal rearrangements of four acanthopterygian species. The 24 putative ancestral chromosomes are represented by the colored bars on the left side; the distribution of the ancestral chromosome segments in the four species is shown on the right side.

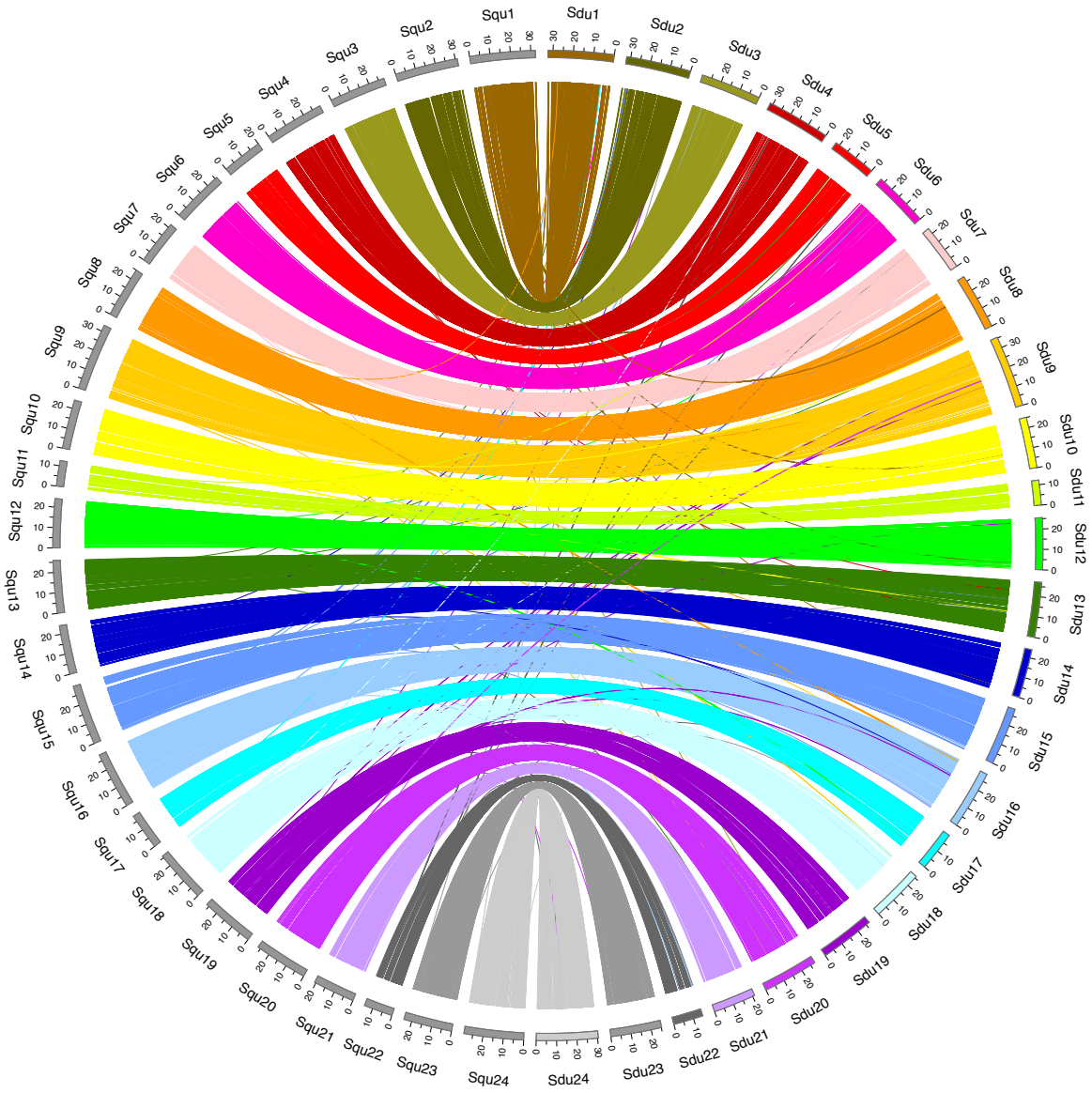


Figure 13. Circos plots showing conservation of synteny between greater amberjack (right side, multicolored) and yellowtail (left side, gray). “Sdu” and “Squ” indicate *Seriola dumerili* linkage group and *Seriola quinqueradiata* linkage group, respectively.

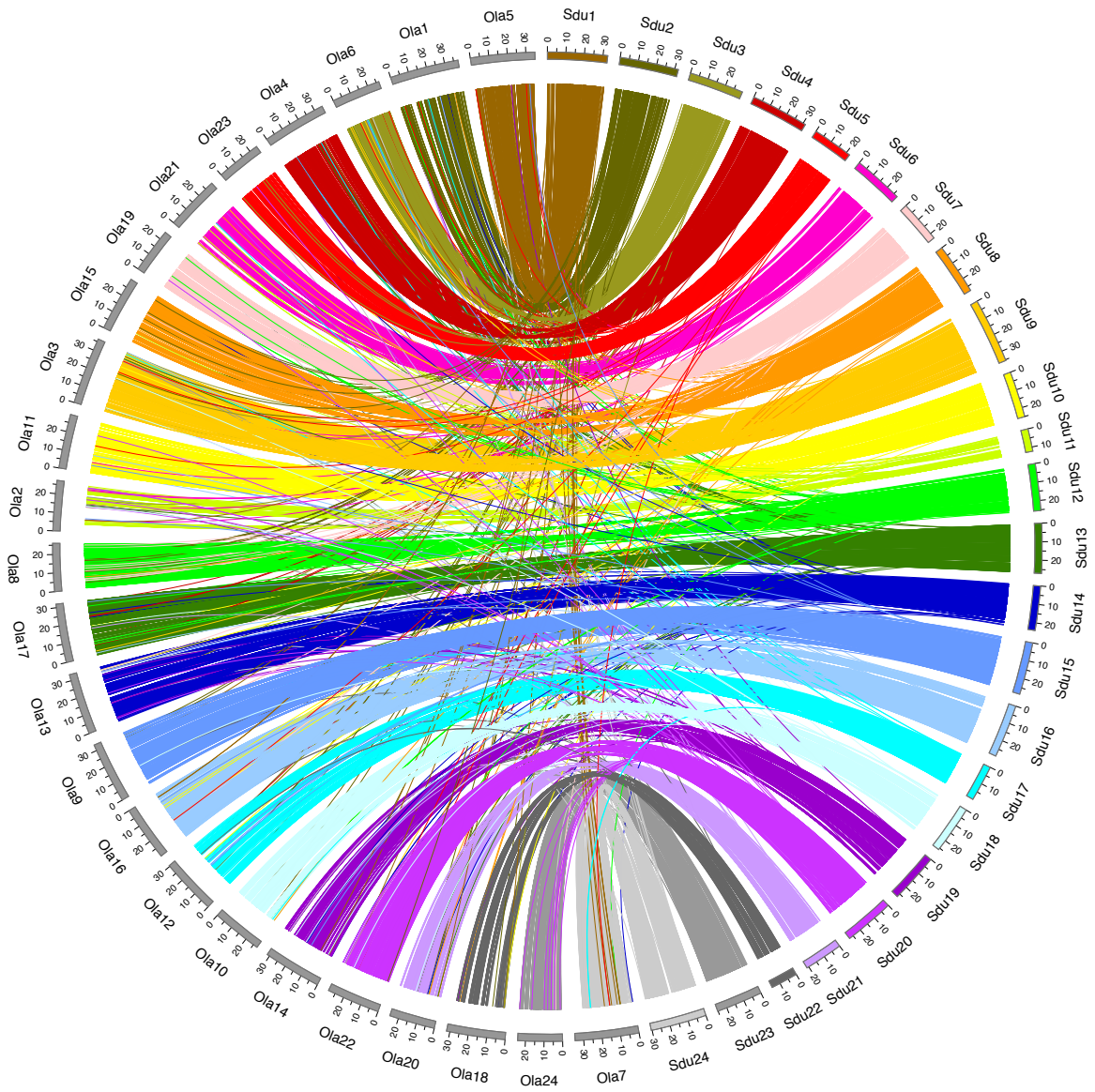


Figure 14. Circos plots showing conservation of synteny between greater amberjack (right side, multicolored) and medaka (left side, gray). “Sdu” and “Ola” indicate *Seriola dumerili* linkage group and *Oryzias latipes* chromosome, respectively.

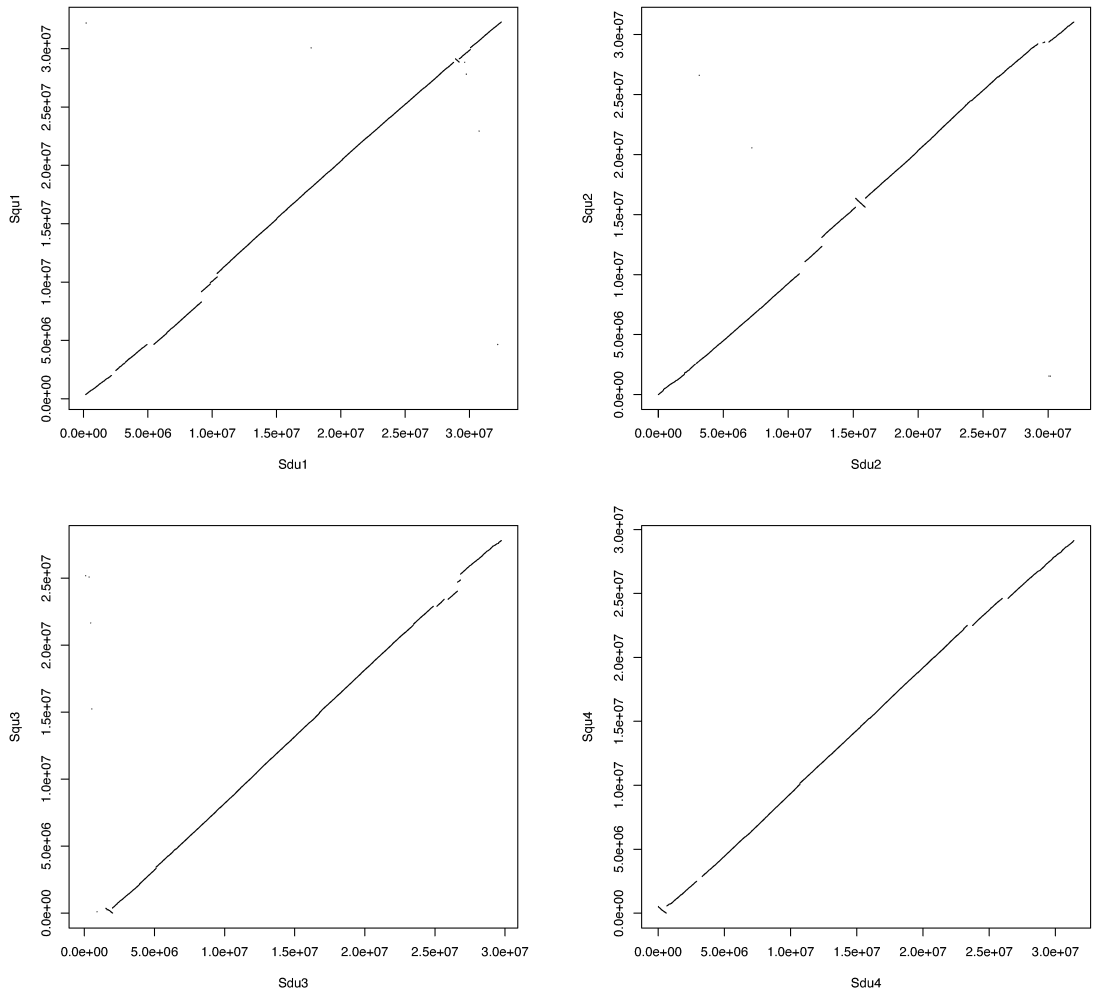


Figure 15. Plots showing the alignment of each orthologous chromosome pair. The horizontal axis and the vertical axis indicate the positions (base pair) of greater amberjack (Sdu) and yellowtail (Squ), respectively. The alignments indicate that the chromosome structure is well conserved between the two species.

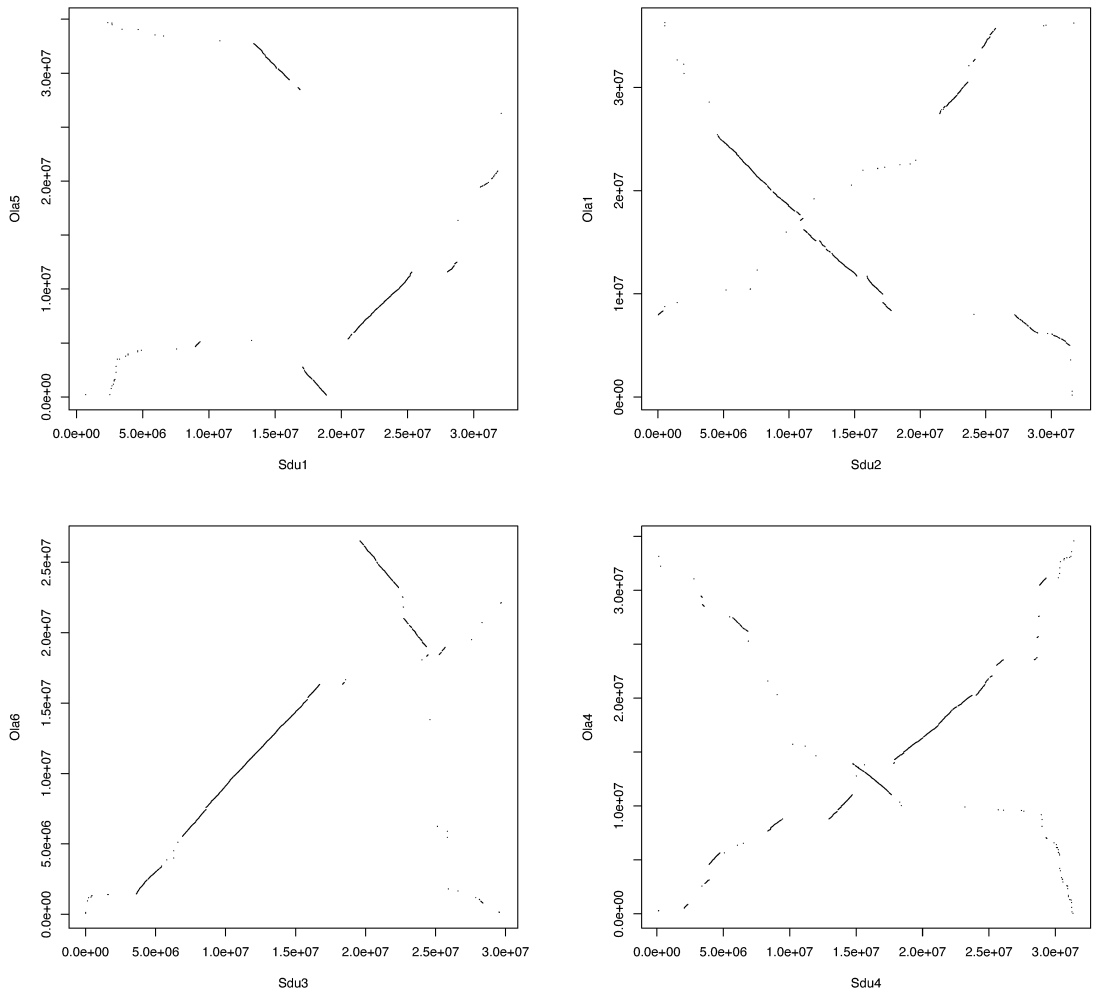


Figure 16. Plots showing the alignment of each orthologous chromosome pair. The horizontal axis and the vertical axis indicate the positions (base pair) of greater amberjack (Sdu) and medaka (Ola), respectively. The sequences could be aligned in a large range in some regions, but chromosomal inversions and intra-chromosomal translocations were frequently observed.

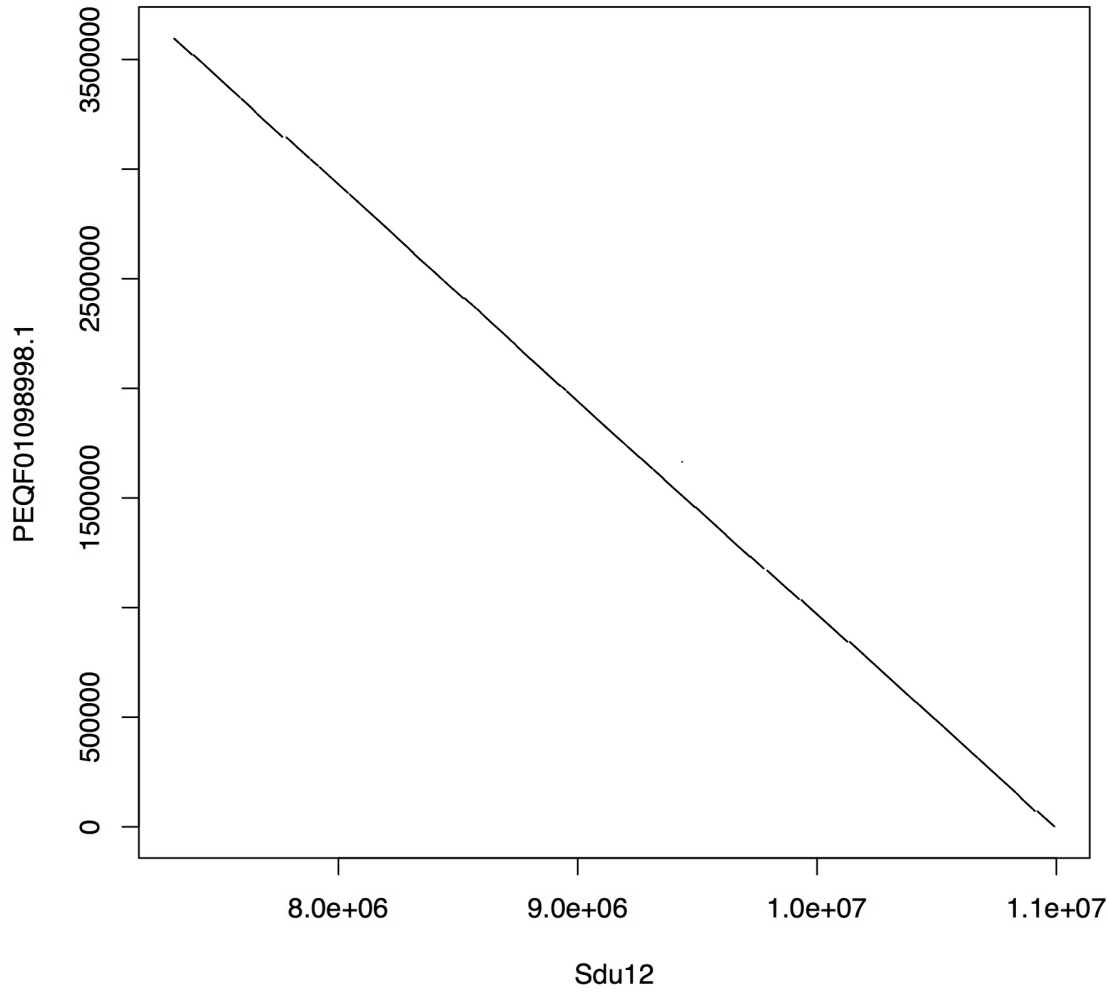


Figure 17. Plots showing the alignment between Sdu12 and the scaffold sequence (PEQF01098998.1) that contains the Hsd17b1 gene of California yellowtail. This sequence alignments showed completely linear correspondence.

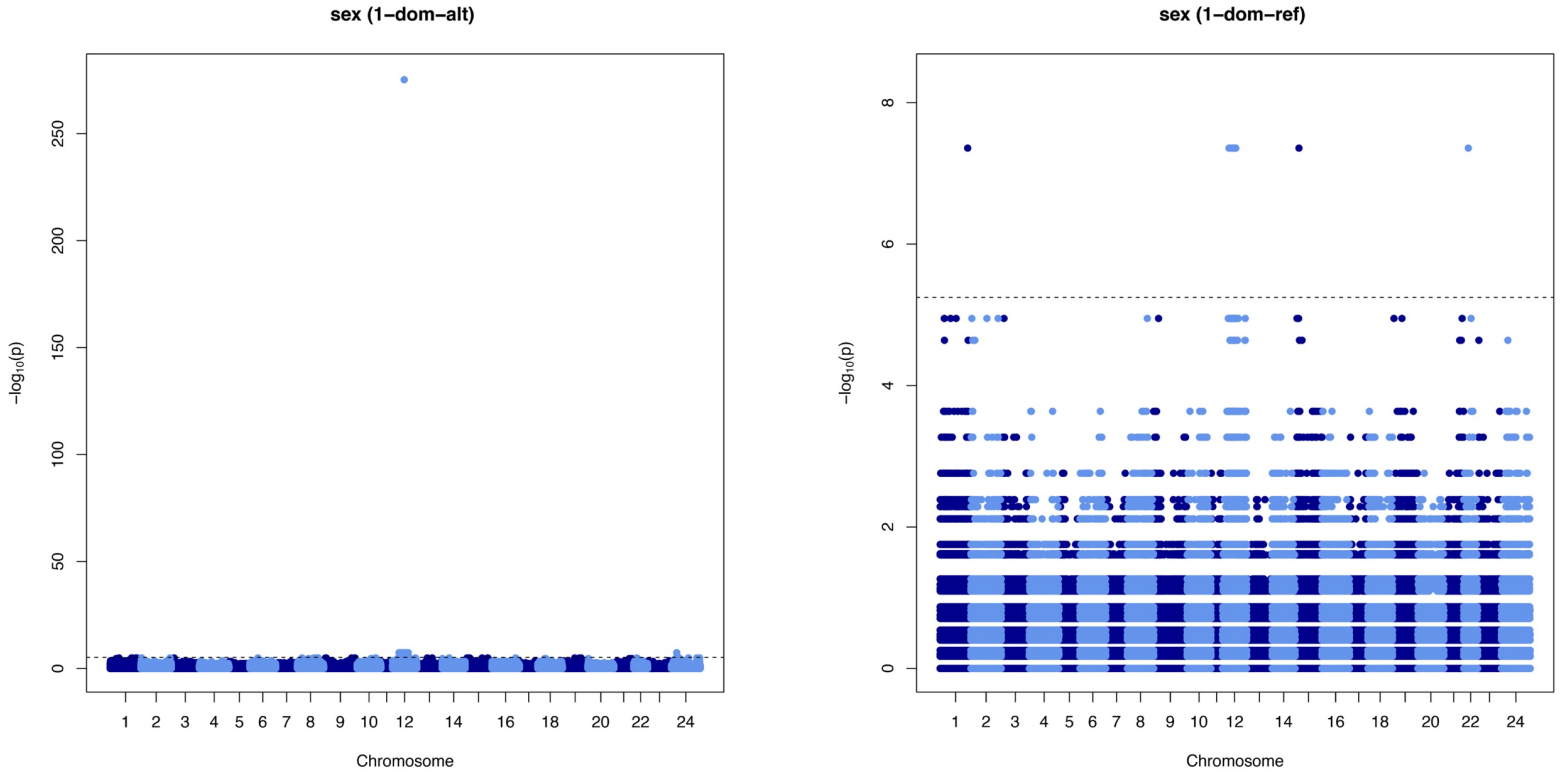


Figure 18. Manhattan plots displaying the result of the 1-dom test by GWASpoly. The horizontal axis indicates the chromosome number and the position of each SNP. The vertical axis indicates the negative logarithm of the P-value for each SNP. Each dot signifies an SNP. The broken line indicates the threshold of FDR level 0.01.

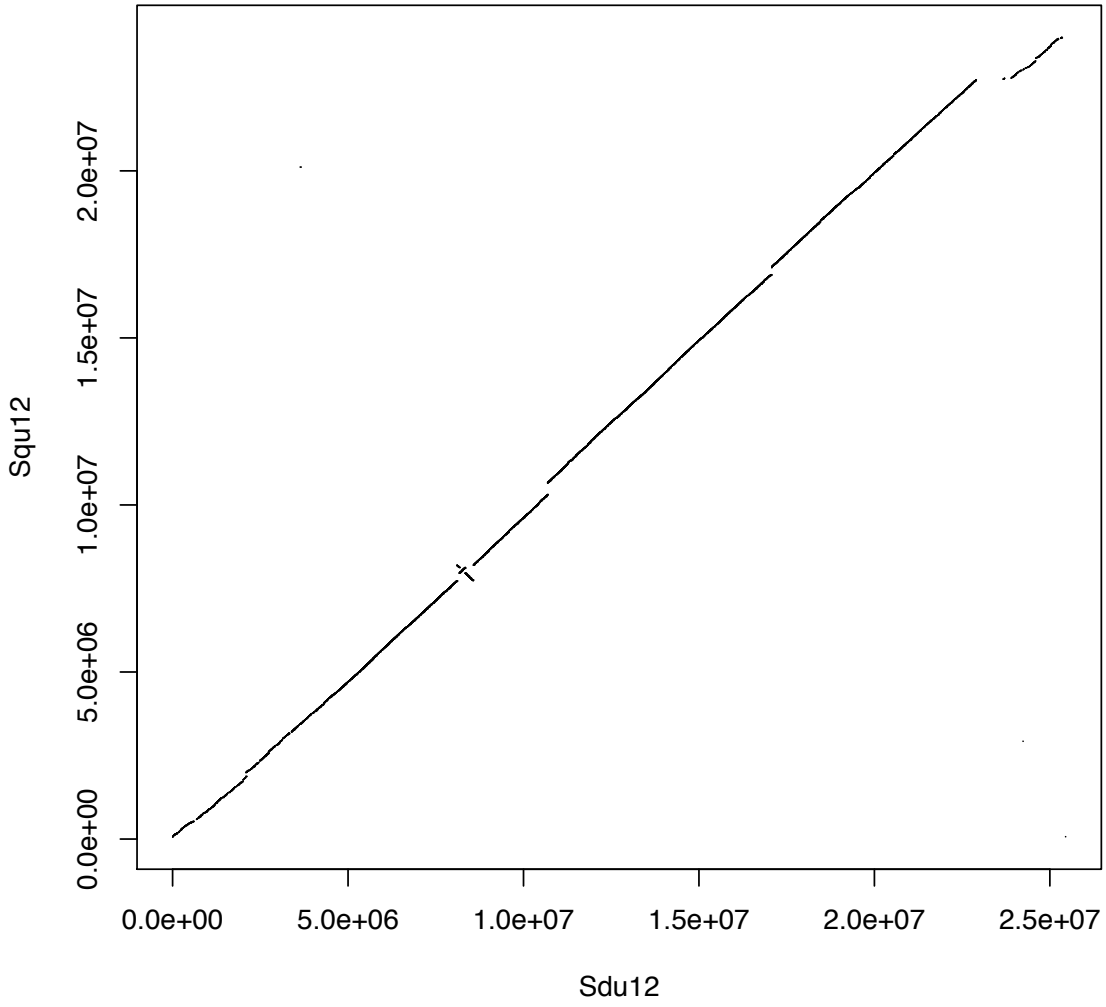


Figure 19. Plots showing the alignment between Sdu12 and Squ12. This alignment showed a linear pattern.

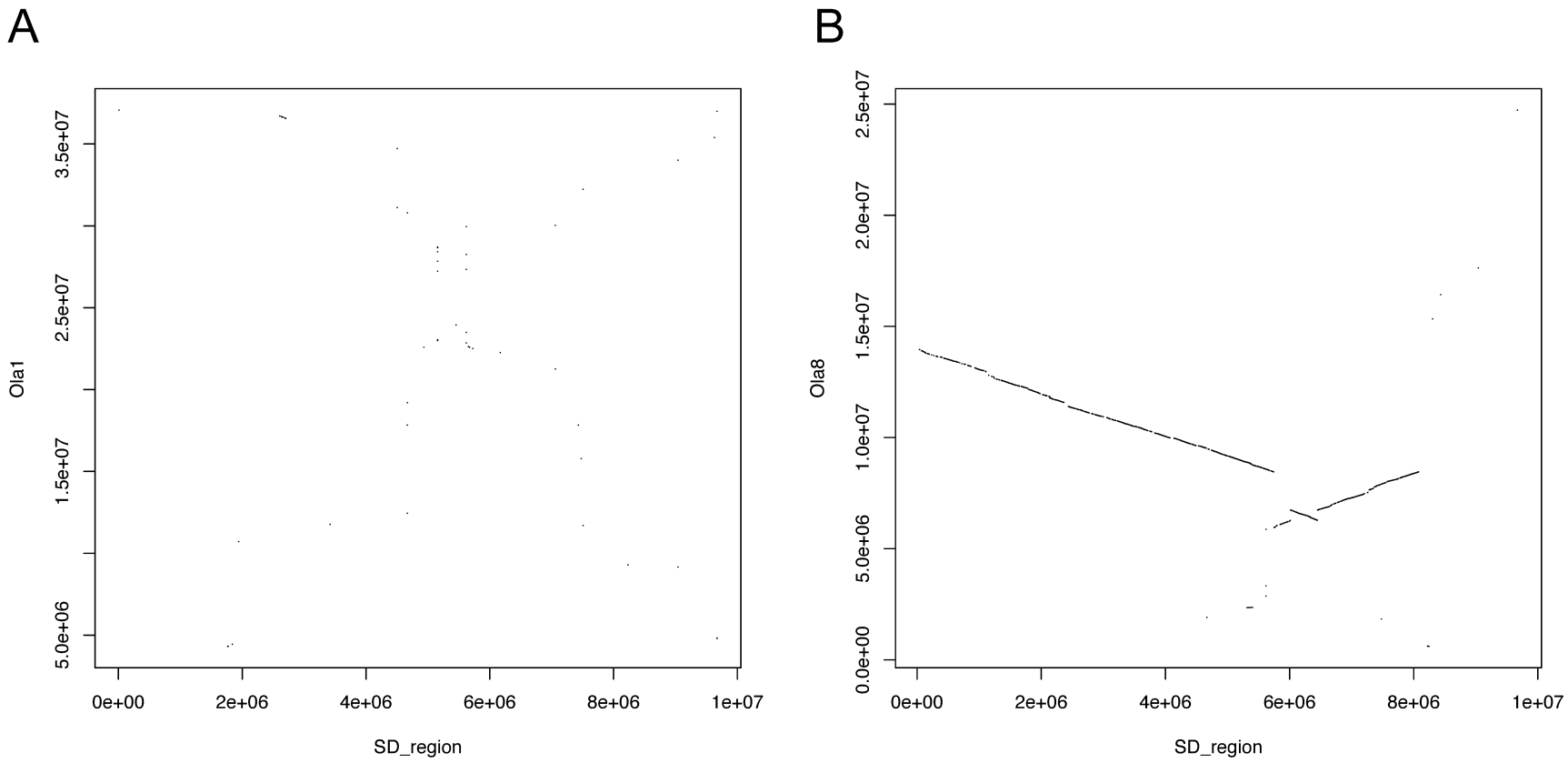


Figure 20. Plots showing the alignments between the sequence of the SD region in greater amberjack and (A) medaka chromosome 1 (Ola1) and (B) medaka chromosome 8 (Ola8). Although Japanese medaka has the SD gene on Ola1, whole-genome alignment showed remarkable homology between Ola8 and Sdu12.

Table 1. Summary statistics of scaffold mapping onto the yellowtail radiation hybrid physical map

chromosome name	the number of markers	the number of scaffolds	the number of bases
LG1	89	11	32,286,024
LG2	78	14	31,041,142
LG3	74	8	27,823,149
LG4	84	8	29,237,640
LG5	49	2	20,020,530
LG6	73	3	26,000,068
LG7	66	2	21,781,103
LG8	71	7	25,028,737
LG9	84	6	33,152,221
LG10	74	7	24,257,723
LG11	38	6	12,614,074
LG12	106	11	24,187,425
LG13	69	9	25,900,440
LG14	65	17	24,241,979
LG15	88	8	29,758,787
LG16	71	15	28,118,061
LG17	78	18	18,004,360
LG18	67	6	25,909,367
LG19	73	6	26,430,848
LG20	64	3	26,418,531
LG21	64	8	20,712,578
LG22	54	8	14,276,494
LG23	64	2	23,995,207
LG24	94	15	29,390,724
total	1737	200	600,587,212
average	72.4	31.7	23,785,580

Table 2. Summary of each BLAST search (tblastx). Reciprocal BLAST searches were executed to detect Reciprocal Best Hit (RBH).

	yellowtail		RBH
	target	query	
medaka	19,743	13,403	6,919
pufferfish	18,965	13,172	6,379
stickleback	22,639	13,879	7,378
zebrafish	39,265	10,897	6,882
spotted gar	17,888	13,490	6,522

Table 3. Summary of inter-chromosomal translocations in four Acanthopterygii species (LG = linkage group)

gar LG	yellowtail	medaka	yellowtail	pufferfish	yellowtail	stickleback	medaka	pufferfish	medaka	stickleback	pufferfish	stickleback
1	10	5	10	4	12	8	13	13	19	10	13	7
2	15	7	14	5	19	6	11	12	17	5	15	5
3	21	14	19	10	15	7	16	19	18	17	15	16
4	16	11	10	7	16	7	12	4	17	7	11	12
5	7	13	9	9	12	5	8	14	16	8	11	5
6	3	5	7	7	5	9	8	12	7	21	12	11
7	10	6	14	8	18	5	11	9	14	19	10	11
8	5	7	9	5	10	4	17	13	16	7	15	14
9	2	7	5	3	12	3	10	8	14	15	7	5
10	4	23	14	7	1	22	11	14	9	19	13	13
11	8	4	3	3	9	3	5	13	13	7	11	0
12	5	10	5	9	5	11	5	6	14	10	10	7
13	8	3	4	4	8	4	3	9	2	7	8	6
14	10	3	8	4	6	3	7	10	8	9	4	2
15	1	4	1	3	2	3	5	2	1	2	1	6
16	8	1	5	2	7	8	3	7	7	11	8	8
17	7	4	11	5	8	5	8	8	7	11	9	9
18	5	4	2	0	8	3	4	4	3	3	3	1
19	8	5	5	5	7	7	4	6	2	9	5	5
20	10	4	7	4	10	2	6	7	3	5	7	6
21	2	1	1	0	3	1	4	2	4	6	0	2
22	10	2	10	4	9	1	4	11	6	7	11	9
23	5	2	2	0	5	2	3	0	6	3	1	2
24	7	1	2	0	8	2	2	1	0	4	2	3
25	1	3	1	1	2	1	1	0	2	4	1	3
26	2	0	3	0	2	0	3	3	7	1	4	2
27	6	2	6	4	5	3	4	3	6	3	5	5
28	0	1	0	0	1	0	1	0	0	0	0	1
29	0	0	0	0	0	0	0	0	0	0	0	0
Total	192	94	159	97	202	111	189	210	238	230	212	176

Table 4. Output from GWASpoly. The softoware was run with 1-dom model and FDR level = 0.01

Trait	Model	Threshold	Marker	Chrom	Position	Ref	Alt	Score	Effect
sex	1-dom-alt	5.25	c43_4405455	12	6857868	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_3763669	12	7499654	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_3697080	12	7566243	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_3027521	12	8235802	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_1782220	12	9481103	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_1229848	12	10033475	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_1221491	12	10041832	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_1018703	12	10244620	0	1	7.36	-0.91
sex	1-dom-alt	5.25	c43_826937	12	10436386	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_573705	12	10689618	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_470587	12	10792736	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_358726	12	10904597	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_330973	12	10932350	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_317236	12	10946087	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_316756	12	10946567	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_190404	12	11072919	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_96832	12	11166491	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_94819	12	11168504	0	1	7.36	0.91
sex	1-dom-alt	5.25	c43_56399	12	11206924	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_5414906	12	11661323	0	1	7.36	-0.91
sex	1-dom-alt	5.25	c168_5007150	12	12069079	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_5006505	12	12069724	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_4823950	12	12252279	0	1	7.36	-0.91
sex	1-dom-alt	5.25	c168_4823685	12	12252544	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_4648349	12	12427880	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_4648290	12	12427939	0	1	275.24	1
sex	1-dom-alt	5.25	c168_4536097	12	12540132	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_4147190	12	12929039	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_3641661	12	13434568	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_3376458	12	13699771	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_3287014	12	13789215	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_3160230	12	13915999	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_3109625	12	13966604	0	1	7.36	0.91
sex	1-dom-alt	5.25	c168_338405	12	16737824	0	1	7.36	0.91
sex	1-dom-alt	5.25	c82_71361	24	5556130	0	1	7.36	0.91
sex	1-dom-alt	5.25	c82_71360	24	5556131	0	1	7.36	0.91
sex	1-dom-alt	5.25	c82_71357	24	5556134	0	1	7.36	0.91
sex	1-dom-ref	5.25	c65_3467901	1	29094626	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c43_4403196	12	6860127	0	1	7.36	0.91
sex	1-dom-ref	5.25	c43_4287549	12	6975774	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c43_2438162	12	8825161	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c43_1944077	12	9319246	0	1	7.36	0.91
sex	1-dom-ref	5.25	c43_827115	12	10436208	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c43_362303	12	10901020	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c168_5457861	12	11618368	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c168_4996348	12	12079881	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c168_3444410	12	13631819	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c168_3116350	12	13959879	0	1	7.36	-0.91
sex	1-dom-ref	5.25	c168_3069620	12	14006609	0	1	7.36	0.91
sex	1-dom-ref	5.25	c16_12746979	15	4013786	0	1	7.36	0.91
sex	1-dom-ref	5.25	c387_222985	22	4833093	0	1	7.36	0.91

Table 5. Genotype data of SNPs detected by GWASpoly. Homozygous is indicated by one letter. M1, M2, .. M10 are male IDs. F1, F2, .. F10 are female IDs.

			Genotype of each sample																			
Marker	chr	position	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
c43_4405455	12	6857868	A	A	A	A	A	A	AG	A	A	A	G	AG	G	AG	AG	G	AG	AG	AG	AG
c43_3763669	12	7499654	T	T	T	T	T	T	TG	T	T	T	G	TG	G	TG	TG	G	TG	TG	TG	TG
c43_3697080	12	7566243	G	G	G	G	G	G	GA	G	G	G	A	GA	A	GA	GA	A	GA	GA	GA	GA
c43_3027521	12	8235802	G	G	G	G	G	G	GA	G	G	G	GA	GA	GA	GA	GA	A	GA	GA	GA	GA
c43_1782220	12	9481103	G	G	G	G	G	G	GT	G	G	G	T	GT	GT	GT	GT	T	GT	GT	GT	GT
c43_1229848	12	10033475	CT	C	C	C	C	C	C	C	C	C	CT	T	CT	CT	CT	CT	CT	CT	CT	CT
c43_1221491	12	10041832	T	TC	T	T	T	T	T	T	T	T	TC	TC	TC	TC	TC	TC	TC	TC	TC	TC
c43_1018703	12	10244620	AG	G	AG	AG	G	AG	AG	AG	AG	AG	A	A	A	A	A	A	A	A	AG	A
c43_826937	12	10436386	T	T	T	T	T	T	T	T	T	T	TA	TA	T	TA	TA	TA	TA	TA	TA	TA
c43_573705	12	10689618	TA	T	T	T	T	T	T	T	T	T	TA	TA	A	TA	TA	TA	TA	TA	TA	TA
c43_470587	12	10792736	A	A	A	A	A	A	A	A	A	A	AC	A	C	AC	AC	AC	AC	AC	AC	AC
c43_358726	12	10904597	C	C	C	C	C	C	C	C	C	C	C	CA	CA	CA	CA	CA	CA	CA	CA	CA
c43_330973	12	10932350	CA	C	C	C	C	C	C	C	C	C	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
c43_317236	12	10946087	A	A	A	A	A	A	A	A	A	A	AG	AG	AG	AG	A	AG	AG	AG	AG	AG
c43_316756	12	10946567	T	T	T	T	T	T	T	T	T	T	TC	TC	TC	T	TC	TC	TC	TC	TC	TC
c43_190404	12	11072919	A	A	A	AT	A	A	A	A	A	A	T	AT	AT	AT	AT	AT	AT	AT	AT	AT
c43_96832	12	11166491	C	C	C	C	C	C	C	C	C	C	CT	CT	CT	C	CT	CT	CT	CT	CT	CT
c43_94819	12	11168504	T	T	T	T	T	T	T	T	T	T	TC	TC	TC	T	TC	TC	TC	TC	TC	TC
c43_56399	12	11206924	T	T	T	T	T	T	T	T	T	T	TC	TC	TC	T	TC	TC	TC	TC	TC	TC
c168_5414906	12	11661323	TC	TC	TC	TC	C	TC	TC	TC	TC	TC	T	T	T	T	T	T	T	T	TC	T
c168_5007150	12	12069079	A	G	G	G	G	G	G	G	G	G	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA
c168_5006505	12	12069724	G	A	A	A	A	A	A	A	A	A	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
c168_4823950	12	12252279	A	GA	GA	GA	A	GA	GA	GA	GA	GA	G	G	G	G	G	G	G	G	GA	G
c168_4823685	12	12252544	T	T	T	T	T	T	T	T	T	T	TC	C	TC	T	TC	TC	TC	TC	TC	TC
c168_4648349	12	12427880	G	G	G	G	G	G	G	G	G	G	GA	G	GA	GA	GA	GA	GA	GA	GA	GA
c168_4648290	12	12427939	A	A	A	A	A	A	A	A	A	A	AT	AT	AT	T	AT	AT	AT	AT	AT	AT
c168_4536097	12	12540132	A	A	A	A	A	A	A	A	A	A	A	AG	AG	AG	AG	AG	AG	AG	AG	AG
c168_4147190	12	12929039	C	C	C	C	C	C	C	C	C	C	C	CT	CT	CT	CT	CT	CT	CT	CT	CT
c168_3641661	12	13434568	G	G	G	G	G	G	G	G	G	G	GA	GA	GA	G	GA	GA	GA	GA	GA	GA
c168_3376458	12	13699771	A	A	A	A	A	A	A	A	A	A	AT	AT	AT	A	AT	AT	AT	AT	AT	AT
c168_3287014	12	13789215	C	C	CT	C	C	C	C	C	C	C	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT
c168_3160230	12	13915999	C	CT	C	C	C	C	C	C	C	C	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT
c168_3109625	12	13966604	G	G	G	G	G	G	G	G	G	G	G	GA	GA	A	GA	GA	GA	GA	GA	GA
c168_338405	12	16737824	C	C	C	C	C	C	C	C	T	C	CT	CT	CT	CT	T	CT	CT	CT	CT	CT
c82_71361	24	5556130	A	A	A	A	AG	A	A	A	A	A	AG	AG	AG	AG	AG	AG	AG	AG	G	AG
c82_71360	24	5556131	A	A	A	A	AC	A	A	A	A	A	AC	AC	AC	AC	AC	AC	AC	AC	C	AC
c82_71357	24	5556134	A	A	A	A	AG	A	A	A	A	A	AG	AG	AG	AG	AG	AG	AG	AG	G	AG
c65_3467901	1	29094626	G	G	G	G	G	G	C	G	G	G	C	CG	C	CG	CG	CG	CG	C	CG	CG
c43_4403196	12	6860127	A	AG	AG	AG	A	AG	AG	AG	AG	AG	G	G	G	G	G	G	G	G	AG	G
c43_4287549	12	6975774	C	C	C	C	C	C	TC	C	C	C	T	TC	TC	TC	TC	T	TC	TC	TC	TC
c43_2438162	12	8825161	G	G	G	G	G	G	AG	G	G	G	A	AG	AG	AG	AG	A	AG	AG	AG	AG
c43_1944077	12	9319246	TC	T	TC	TC	T	TC	TC	TC	TC	TC	C	C	C	C	C	C	C	C	TC	C
c43_827115	12	10436208	A	A	A	A	A	A	A	A	A	A	GA	GA	A	GA	GA	GA	GA	GA	GA	GA
c43_362303	12	10901020	A	A	A	TA	A	A	A	A	A	A	T	TA	TA	TA	TA	TA	TA	TA	TA	TA
c168_5457861	12	11618368	C	C	T	C	C	C	C	C	C	C	T	TC	TC	T	TC	TC	TC	TC	TC	TC
c168_4996348	12	12079881	TA	A	A	A	A	A	A	A	A	A	TA	TA	T	T	TA	TA	TA	TA	TA	TA
c168_3444410	12	13631819	GC	C	C	C	C	C	C	C	C	C	GC	GC	G	GC	GC	GC	GC	GC	GC	GC
c168_3116350	12	13959879	C	C	C	C	C	C	C	C	C	C	GC	C	GC	GC	GC	GC	GC	GC	GC	GC
c168_3069620	12	14006609	TG	TG	TG	TG	T	TG	TG	TG	TG	TG	G	G	G	G	G	G	G	G	TG	G
c16_12746979	15	4013786	TA	TA	TA	TA	TA	T	T	TA	TA	TA	A	A	A	A	A	A	A	A	TA	A
c387_222985	22	4833093	GA	GA	G	GA	GA	GA	GA	GA	GA	GA	A	A	A	A	A	A	A	A	A	GA

Table 6. Haplotypes estimated by PHASE software.

No.	haplotypes	count
1	AACTGGGTGCTGATTAACCATACTTCCGAAATGAACGCACCCGTC	9
2	AACTGGGTGCTGATTAACCATACTTCCGAAATGAACGCACCCGTT	1
3	AACTGGGTGCTGATTAACCATACTTTCGAAATGAACGCATCCGTC	1
4	AACTGGGTGCTGATTAACAATACTTCCAGAATGAACGCACCCGTC	1
5	AACTGGGCGTTAATAAACCATACTTCTAGTATGAACGGACCCGGC	1
6	AGCTGGGTGCCGATTAACCATACTTCTGAAGTGAACGCACTCGGC	1
7	AGCTGGGCGCTAATTAACCATACTTCTGAAGTGAACGCACCCGGC	6
8	AGCTGGGCGCTAATTAACCATACTTCTGAAGTGAACGCACCCGGT	2
9	AGCTGGGCGCTAATTAACCATACTTTTGATGTGTACGCACCCAGC	1
10	AGCTGGGCGCTAATTAACCATACTTTTGAAGTGAACGCACCCGGC	1
11	AGCTGGGCGCTAATTATCCATTCTTCTGAAGTGAACGCACCCGGC	1
12	AGCTGGGCGTTAATTAACCATACTTCTGAAGCGAACGCACCCGGC	1
13	GGCGAAACTTCAATACTAAGCTTCCTTAGTGCATGTAGTTTGAGT	1
14	GGTGAGGCGCTAATACACCATACTTCTGATGTGAACGGACCCGGC	1
15	GGTGAGACTTCAGAACTCAGCTTCCTTAGTGCATACAGTTTGGGT	1
16	GGTGAAACTCTAATTAACCATACTTCTGAAGTGAACGCACCCGGC	2
17	GGTGAAACTCTAATTATCCATTCTTTTGAAGTGAACGCACCCGGC	1
18	GGTGAAACTTCAGAAATAAGCTTCCTTAGTGCGTGTAGTTTCAGT	1
19	GGTGAAACTTCAGAACTAAACTTCCTTAGTGCATGTAGTTTGAGT	1
20	GGTGAAACTTCAGAACTAAGTTCTTTTAGTGTATGTGGATTGAGT	1
21	GGTGAAACTTCAGAACTAAGCTTCCTTAGTGCATGTAGTTTGAGT	5

Table 7. Haplotype combinations of each individual estimated by PHASE software. The number of haplotype corresponds to table3.

ID	haplotype combinations
M1	(4,5)
M2	(1,6)
M3	(3,10)
M4	(1,11)
M5	(1,1)
M6	(1,7)
M7	(1,16)
M8	(1,7)
M9	(2,8)
M10	(1,7)
F1	(15,17)
F2	(12,18)
F3	(13,14)
F4	(9,20)
F5	(8,19)
F6	(16,21)
F7	(7,21)
F8	(7,21)
F9	(1,21)
F10	(7,21)