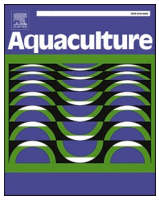
Aquaculture 582 (2024) 740559

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Identification of sex locus and a male-specific marker in blunt-snout bream (*Megalobrama amblycephala*) using a whole genome resequencing method

Ming Wen [1], Siyu Wang [1], Chunchun Zhu [1], Yuxin Zhang, Zheng Liu, Chang Wu, Shi Wang, Yude Wang, Li Ren, Min Tao, QinBo Qin, Chun Zhang, Fangzhou Hu, Qingfeng Liu, Chenchen Tang, Shaojun Liu [*]

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, China

ARTICLE INFO

Keywords:
Blunt-snout bream
Resequencing
Sex determination
Sex chromosome
Sex-marker

ABSTRACT

Fish sex is often associated with its economic traits, such as growth and body color. The mechanism of fish sex determination is always a hot spot for scientific research. Fish is one of the most diverse groups of vertebrates, and its sex determination mechanism is diverse and complex. Blunt-snout bream (BSB), belonging to Cyprinidae and the genus *Megalobrama*, is an important economic fish in China. Ordinary consumers are more inclined to buy male fish that does not carry eggs, hence the preparation of this all-male group of BSB meets the needs of the industry. In this study, we applied a whole genome resequencing approach to search sex-linked markers and better characterized sex determination in this fish. Our results confirmed that BSB uses a male heterogametic (XX/XY) system. Individuals from two populations including 10 males and 10 females were sequenced using a whole genomic sequencing method. A large potential sex differentiated region with approximately 6 Mb at the beginning of linkage group (LG) 9 of female BSB genome was identified using genome comparative analysis. This region contained a high density of male-biased genetic polymorphisms and harbored 275 genes, whereas reported sex-related genes didn't exist in this region. With the help of male genome, some male-biased sequences were identified using genome-wide associated analysis, and subsequently a male-specific marker was developed. Our results exhibited that BSB had a considerably large sex locus on LG9, which was likely sex chromosome of this species. Moreover, we developed a male-specific marker for BSB. Our study not only is of great practical significance to the development of sex-controlled breeding technology, but also is of theoretical significance to further explore the sex determination mechanism in fish.

1. Introduction

Fish is one of the most important protein sources for human beings. The total amount of aquatic products has continued to increasing in the past several decades in China, providing a guarantee for food safety and promoting the development of aquaculture industry (Hu et al., 2021a). Blunt-snout bream (BSB), belonging to the order Cyprinidae and the genus *Megalobrama*, is one of the most important economic fish in China, and its output in 2019 reached 760,000 tons. Due to inbreeding, overfishing and the destruction of living environment, the degradation of wild germplasm resources of BSB become more and more serious. Therefore, accelerating the cultivation of new strains and expanding the supply of aquatic products will help to ensure the supply of animal meat protein for human being, and also facilitate to the sustainable development of fisheries.

Fish sex is usually related to the economic traits (such as size and body color), which can be applied to improve aquatic yield and farming efficiency (Mei and Gui, 2015). The growth rate and body size of many fish show a significant correlation to sex. Parthenogenesis is one of the diverse fish reproductive strategies, and its utilization has created a typical example for fish genetics and breeding (Mei and Gui, 2015). The development of sex-specific molecular markers and sex control biotechnology provide an important way to improve fish germplasm and promote the innovation of breeding technology. With the development of high-throughput sequencing technology, the sex determination mechanism of various economic fishes has been studied, such as rainbow

* Corresponding author.
E-mail address: lsj@hunnu.edu.cn (S. Liu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.aquaculture.2024.740559
Received 26 September 2023; Received in revised form 4 January 2024; Accepted 7 January 2024
Available online 8 January 2024

trout (*Oncorhynchus mykiss*) (Yano et al., 2012), half-smooth tongue sole (*Cynoglossus semilaevis*) (Cui et al., 2017), tilapia (*Oreochromis niloticus*) (Li et al., 2015), and channel catfish (*Iclalurus punctatus*) (Bao et al., 2019). This further contributes to promoting the production and quality upgrade of these economic fish species. Therefore, the rapid development of basic research on fish sex determination mechanism laid a solid theoretical basis for aquaculture.

Sex determination has been suggested to be a relatively conserved trait, related to reproduction and Darwinian fitness. However, compared with mammals and birds, the sex determination mechanism of fish is remarkably diverse, including single gene regulation, polygenic regulation, environmental factors regulation, hermaphroditism and dioeciousness. The evolutionary variability of sex determination in fish is consistent with the rapid turnover of different modes, which makes teleost ideal model animals for studying the evolutionary adaptation of sex determination (Mank and Avise, 2009). In cypriniformes, cyprinidae is the most widely distributed and has the largest species, with nearly 3700 species. However, there are relatively few studies on sex determination in Cyprinidae, which is probably a result of the complexity and instability of their genome. Even zebrafish, the most representative model animal of cyprinid fish, lost its original sex determination factors after long-term domestication in the laboratory (Wilson et al., 2014). Therefore, the exploration of the sex determination mechanism of cyprinid fish also provides some reference materials for us to understand the sex evolution of fish. From this point of view, the study of fish sex determination can not only have application value for the development of aquaculture, but also contribute to revealing the evolutionary mechanism of fish sex determination.

Sex determination is a developmental process in which gonad primordia with bidirectional potentiality can develop into ovaries or testes. This process is determined by environmental factors (temperature, pH, etc.) or genetic material, or both (Herpin and Schartl, 2009). To elucidate the mechanism of gonad development, it is vital to identify master sex-determining gene as it is a major factor for triggering sex differentiation. Since the discovery of the sex-determining gene Sry in vertebrates, people tend to believe that sex-determining mechanisms are conserved in genetic evolution. In 2002, the first sex-determining gene DMY in fish was reported, leading to a new understanding of the mechanism of sex determination (Matsuda et al., 2002; Nanda et al., 2002). With the development of high-throughput sequencing technology, the pace of fundamental research on the genetic mechanism of fish sex determination has also been greatly promoted in recent years (Bachtrog, et al., 2014). The master sex-determining gene of a variety of fish has been successively identified, such as rainbow trout (Yano et al., 2012), puffer fish (*Takifugu rubripes*) (Kamiya et al., 2012), Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2012), sablefish (*Anoplopoma fimbria*) (Rondeau et al., 2013), half smooth tongue sole (Cui et al., 2017), striped catfish (*Pangasianodon hypophthalmus*) (Wen et al., 2022d), and channel catfish (Bao et al., 2019).

Furthermore, the sex-determining genes of some closely related species are also diverged. For example, Japanese medaka (*Oryzias latipes*), Luzon medaka (*Oryzias luzonensis*) and Ganges medaka (*Oryzias dancena*) have evolved different master sex-determining genes of DMY (Matsuda et al., 2002), GsdfY (Myosho et al., 2012) and Sox3Y (Takehana et al., 2014). In addition to the mechanism of sex determined by a single gene, sex mechanism of some fish is regulated by multiple genes, such as the zebrafish (*Danio rerio*) (Liew et al., 2012) and *Astatotilapia burtoni* (Roberts et al., 2016). Besides, it is known that approximately 270 fish species have heterozygous sex chromosomes, most (70%) of them are male heterozygous (XX-XY) and 30% are female heterozygous (ZZ-ZW) (Bao et al., 2019). These findings suggested that fish sex determination mechanisms are highly plastic from an evolutionary perspective.

Sex marker is a critical signal to search master sex determining gene through whole genome, and it is also a useful tool to distinguish fish genetic sexes. Numerous sex-specific or sex chromosome-specific markers were successfully identified using different techniques including AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and SNP (single nucleotide polymorphism) (Mei and Gui, 2015). With the completion of whole genome sequencing of a large number of species, a series of sex markers were identified by whole genome comparison between two sexes, such as bighead carp (Liu et al., 2018), largemouth bass (Wen et al., 2022b), mandarin fish (Wen et al., 2022c) and channel catfish (Wen et al., 2022a). It is efficient to distinct genetic sexes, especially at early developmental stages, with the help of sex specific markers (Bao et al., 2019; Wen et al., 2020). In addition, sex specific markers are of great benefit to sex control breeding (Liu et al., 2013). In BSB, sex specific markers failed to be developed, when utilizing the approaches of AFLP, SSR, and SNP (Gao et al., 2012; Rao et al., 2012). Obviously, these markers have laid a solid foundation for the identification of fish genetic sex and sex chromosomes, and provided an efficient technical approach for large-scale production of all-male or all-female populations.

Fortunately, two versions of female chromosome-level genomes of BSB were available in the public database (Liu et al., 2017; Ren et al., 2019). In our laboratory, we generated a male draft genome of BSB, and the male genome was still being further optimized. Taking these advantages, we conducted genome-wide comparative analysis between male and female of BSB to identify sex-linked genetic polymorphisms/specificity. In this study, we identified a large sex differentiated region, and developed a Y-linked molecular marker. Our experiments revealed the genetic basis and mechanism of sex determination of BSB, and also provided a basis for aquaculture application.

2. Materials and methods

2.1. Experimental fish and DNA extraction

Three populations of experimental fish used in this study for resequencing were sampled from three locations, the National Education Ministry Breeding Center of Polyploidy Fish of Hunan Normal University of Changsha City, Xuefeng Mountain Fish Breeding Valley of Wugang City, and Longhu Special Aquaculture Farm in Shuangjiangkou Town of Ningxiang City in China. All matured fish, with two-years old, were sampled at the breeding season during June. Fish phenotypic sex of each individual was identified by checking the semen and the roughness of the pectoral fins. Tail fin clip of each individual was collected and stored in a 1.5 ml tube with 90% alcohol for DNA extraction and genotyping.

For genome resequencing, we used TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 (Code No. 9765) to extract genomic DNA according to the manufacturer's instructions. After extraction, genomic DNA was run in a 1% agarose gel to check its degradation and contamination. The concentration of genomic DNA was assayed by a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and a Qubit® DNA Assay Kit in Qubit® 3.0 fluorometer (Invitrogen, Carlsbad, CA). Finally, genomic DNA was stored at −80 °C for Illumina sequencing. For genotyping, tail fins clips were treated at 55 °C for 2 h with 5% Chelex and 20 mg Proteinase K, followed by denaturation of Proteinase K at 99 °C for 2 min. After a brief centrifugation, the supernatant containing genomic DNA (gDNA) was collected into a new tube. Subsequently, genomic DNA was diluted to half and stored at −20 °C for PCR.

2.2. Whole genome resequencing of BSB from two populations

In order to minimize genetic differences among populations, samples were selected from two populations of BSB. And 5 males and 5 females were sampled from each population for whole genome resequencing. For each sample, a total amount of 0.2 μg genomic DNA was used to generate sequencing library with the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the operating manual.

Table 1
Summary of the resequencing data of male and female from two populations.

Populations	Samples	Raw reads	Clean reads	Q20 (%)	Q30 (%)	GC (%)
P1	F1	37,388,204	37,173,174	96.71	91.39	38.22
	F2	35,952,846	35,771,594	96.75	91.50	36.87
	F3	40,079,051	39,881,291	96.83	91.62	37.83
	F4	37,991,957	37,713,591	96.03	90.33	36.85
	F5	42,263,549	41,931,495	96.19	90.73	36.70
	M1	39,171,750	38,949,615	96.81	91.57	37.63
	M2	35,293,208	35,101,923	96.90	91.78	38.06
	M3	38,400,128	38,178,245	96.84	91.65	37.79
	M4	34,536,715	34,352,004	96.94	91.90	37.75
	M5	34,459,527	34,273,820	96.79	91.56	37.49
P2	F1	67,123,552	66,483,002	97.15	92.36	41.39
	F2	69,246,924	68,637,938	97.02	92.09	40.90
	F3	66,214,154	65,715,726	97.23	92.46	39.20
	F4	69,680,388	68,933,362	96.69	91.41	41.19
	F5	105,998,654	104,432,730	96.17	90.52	42.19
	M1	66,403,352	65,916,914	96.75	91.41	38.68
	M2	65,983,078	65,515,478	96.89	91.65	39.05
	M3	85,804,350	85,171,098	96.70	91.31	39.10
	M4	66,547,924	66,078,142	96.81	91.45	39.21
	M5	74,966,974	73,987,462	96.50	91.08	41.48

Briefly, genomic DNA samples were sonicated using a Bioruptor (Diagenode) to generate a size of 350 bp fragmented DNA. Then the DNA fragments were endpolished to yield short blunt-ended DNA fragments. Subsequently, the short DNA fragments were adenylated and ligated to specific adapters, followed by further PCR amplification. After purification of PCR products using AMPure XP system (Beckman Coulter, Beverly, USA), the concentration of fragmented DNA was measured by Qubit®3.0 Flurometer (Invitrogen, USA). Libraries were analyzed for size distribution by NGS3K/Caliper, and libraries were further qualified by real-time PCR (3 nM). The index-coded was clustered using a cBot Cluster Generation System using Illumina PE Cluster Kit (Illumina, USA) following the operating manual. After cluster generation, the DNA libraries were sequenced on Illumina platform and 150 bp paired-end reads were generated.

2.3. Characterization of sex chromosome and sex differentiated region

Using BWA mem version 0.7.17 with default settings, Illumina reads from the male and female were mapped separately into a female chromosome-level assembly [GCF_018812025.1]. Then, for each sample, the generated BAM file was sorted by coordinate using SortSam of Picard tool version 2.18.2. Subsequently, all reads of the sorted BAM file were assigned into a new read-group using AddOrReplaceReadGroups of Picard. BAM files of all female and all male were, respectively, merged into a single file using MergeSamFiles of Picard with default settings. Afterwards, duplicate reads originated from PCR amplification were removed using MarkDuplicates of Picard. Additionally, BAM files were filtered using Samtools version 1.8 to remove reads that mapping quality less than 20 and multiple mapping. Subsequently, a pileup file was generated using the two sexes BAM files with samtools. With this Mpileup file, a sync file was generated using popoolation mpileup2sync version 1.201, with minimum quality set to 20. The sync file contained the nucleotide composition of each sex for each position in the reference. Total SNPs at all reference positions and window coverage between male and female were calculated using PSASS (https://github.com/SexGenomicsToolkit/PSASS), respectively. High-density sex-biased SNP regions were identified with a 100 kb sliding window with output points every 10 kb, using PSASS. PSASS was set as follows: the minimum depth of considered sites was set to 10 (−min-depth = 10), and the frequency range of sex-linked SNPs in heterogametic sexes was set to 0.5 ± 0.1, the frequency of sex-linked SNPs in homogametic sexes was set to be greater than 0.95. An R package that supports analysis (http://github.com/RomainFeron/PSASS-vis) was used to present the data visually.

2.4. Identification of Sex-specific primers and population genotyping

Previous studies suggested that BSB used a male heterogametic system (XX-XY), as all progenies derived from BSB gynogenesis are females with normal ovary structure (Zhang et al., 2015). A male draft genome assembly (unpublished data) was used as reference, and male and female reads were mapped onto it. The overall depth of male and female were counted with a 10 kb sliding window. The Y-potential specific fragments were identified, in which exhibited half of overall depth in male and less than 2× depth in female. Regions with coverage differences between male and female were manually checked using IGV (Thorvaldsdottir et al., 2013). These Y-potential specific fragments in the regions with mapped few female reads and half average depth reads in male were extracted using IGV (Fig. 3A). Then, based on these Y-potential specific fragments, we designed a series of sex-specific primers using an online tool Primer3 version 4.1.0 (http://primer3.ut.ee). The reaction recipe of PCR for genotyping was as bellow: for each reaction, 0.25 units of Ex Taq, 1 μl of 10× PCR Buffer, 100 μM dNTP mixture, 1 μl of 50 ng/μl genomic DNA, 0.1 μM of each primer in a total volume of 25 μl. The conditions of PCR were denatured at 94 °C for 30s, annealed at 56 °C for 30s and extended at 72 °C for 30s for 35 cycles. At last, PCR products were run on 1.5% agarose gel.

3. Results

3.1. Statistics of whole genome resequencing data

To minimize genetic differences among populations, we sampled experimental fish from two populations, and in each population 5 males and 5 females of BSB were randomly selected. Whole genome sequencing of each sample was conducted using Illmina platform. A total of 541,567,006 and 571,939,279 paired-end clean reads were generated from 10 males and 10 females (Table 1). Approximately, the percentages of reads mapped to BSB female genome reference were approximately 94.86% and 93.69% respectively. The mean depth of male and female were 57.37 and 59.20 respectively. It exhibited that our sequencing data were large enough and of high quality. In addition, nucleotide statistics showed that the averages of Q20 of the resequencing data were 96.68% and 96.79%, and the averages of Q30 were 91.40 and 91.57 of the two populations respectively. The average of GC contents of the two populations was 37.52% and 40.24%. The GC content differences between the two populations indicated that there were genetic differences between the two populations (Table 1).

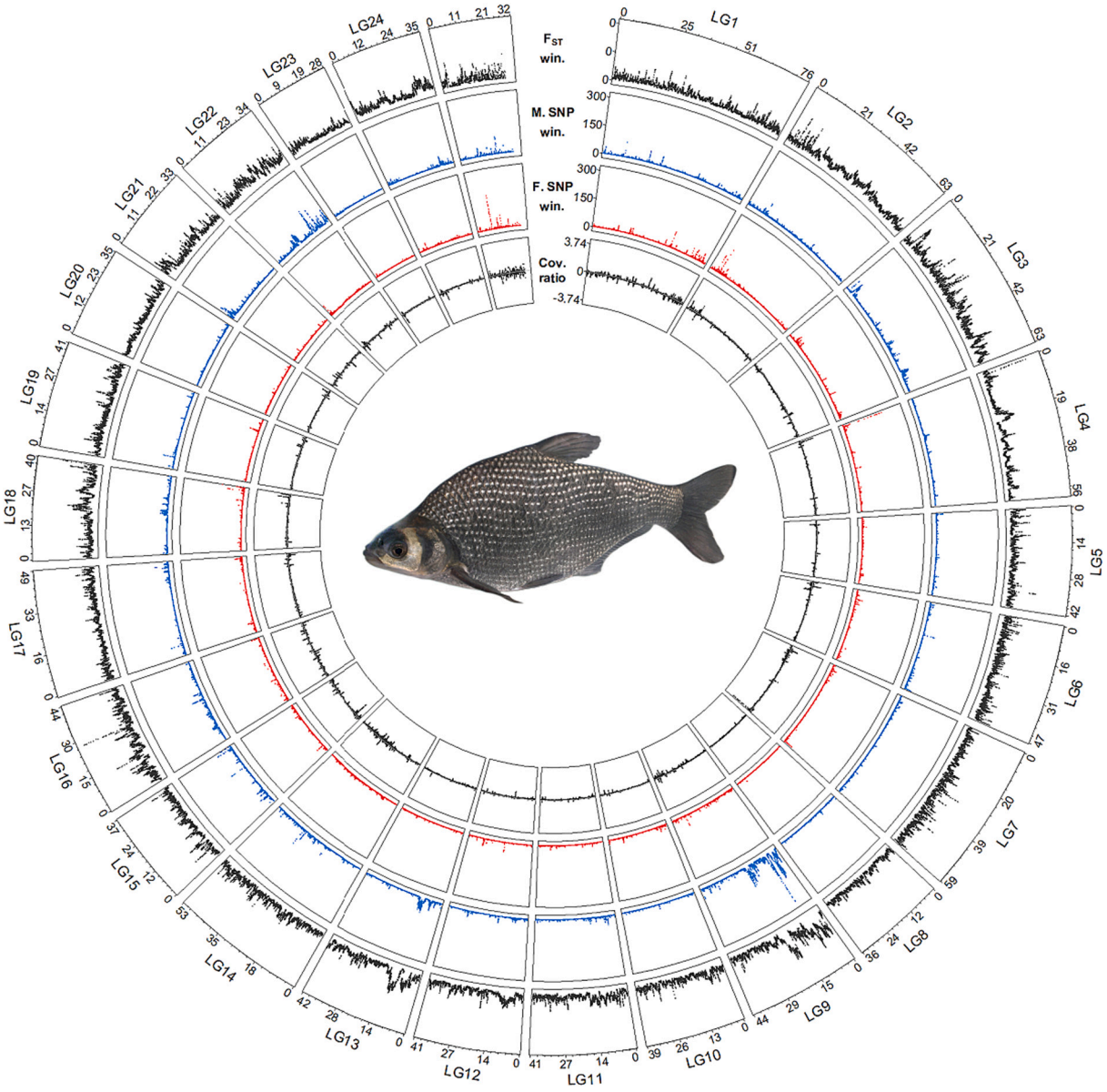


Fig. 1. Sex chromosome identified in blunt snout bream using whole genome resequencing strategy.
The resequencing reads of 10 males and 10 females were mapped onto the female genome respectively. Then the distributions of F_{ST}, SNPs, and depth were calculated using a 100 kb sliding window with an output point every 10 kb. A circular plot presented the statistics of whole genome of the sequencing analysis. All the 24 linkage groups (LGs) were labelled with their LG number with all unplaced scaffolds combined together. From the outer to inner tracks were respectively: the window F_{ST}, the male-specific SNPs, the female-specific SNPs, and the reads depth ratio between males and females.

3.2. Potential chromosome and sex-differentiated region identification using a female genome of BSB as reference

Usually, it is unlikely to distinguish sex chromosomes by karyotype, because there is no obvious morphological difference between sex chromosomes, i.e. X-Y and *Z*-W, in most teleost fish. In chromosome karyotype analysis, we did not observe morphological differences between sex chromosomes in BSB. Therefore, to identify genetic differences between male and female, we aligned male and female reads to a chromosome-level female assembly ASM1881202v1 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_018812025.1/), generated from a gynogenesis female fish. The overall sex-biased Single Nucleotide Polymorphism (SNP) was calculated throughout the whole genome by comparing the male and female genome-wide mapping data. The distribution of overall SNPs calculated using a 10 kb sliding window showed that there was a significant sex-linked signal on LG9, in which male-biased SNPs were enriched (Fig. 1). At the corresponding region, the value of F_{ST} was also relatively high, implying genetic difference between male and female at this region was significant (Fig. 1 and Fig. 2A). This non-recombination region was in a size of approximately 6 Mb (Fig. 2B). And coverage difference between male and female was not observed on LG 9, it is reasonable as the mapping reference is a female genome without Y chromosome. Our results suggested that LG9 from the female BSB genome assembly was potentially the sex chromosome with sex differentiated region. Due to the sex determination system of BSB is XX-XY, the genome assembly of gynogenesis fish merely contained X chromosome without Y chromosome. Using the female genome as reference, it is impossible to identify potential male-specific fragments.

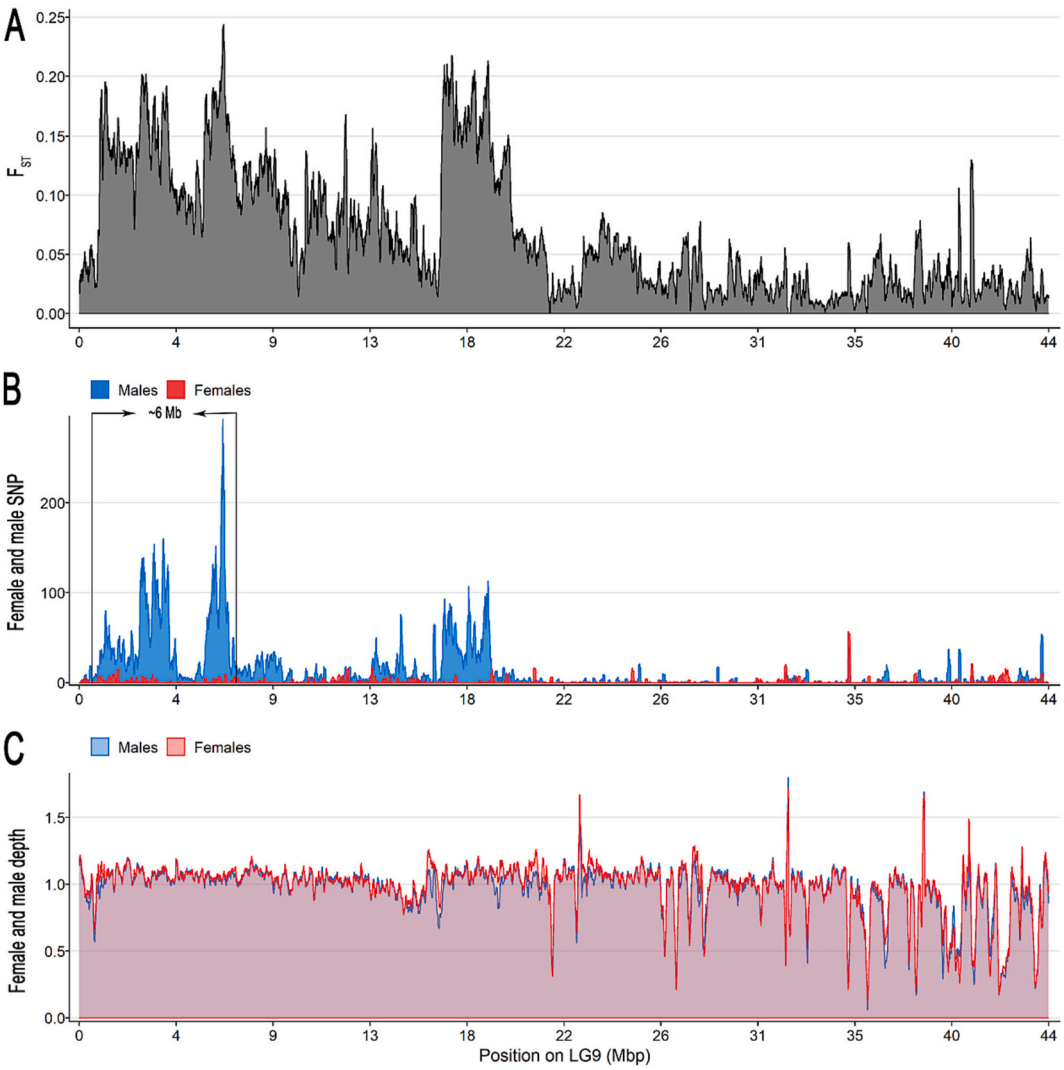


Fig. 2. A distinct sex differentiated region identified on LG9.
F_{ST}, SNPs and coverage in LG9 were calculated using 100 kb sliding window with an output point every 10 kb. The fixation index (FST) is a measure of the differentiation between two sexes due to genetic structure. (A) At the beginning of LG9, the value of F_{ST} was higher than the average of whole genome, implying great differences were present at this region. (B) An approximately 6 Mb sex-differentiated region was identified on LG9, which is highlighted with a black box. The female- and male-specific SNPs were respectively indicated by red and blue color. (C) Regions with coverage differences between male and female were not observed in LG9. The depths in female and male were respectively indicated by red and blue line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Identification and validation of a Y-linked specific marker using a male genome of BSB as reference

To identify male-specific markers in BSB, male and female Illumina reads were, respectively, mapped onto a draft and unpublished BSB male genome assembly. In this genome assembly, the X and Y chromosome was assembled onto a mixture of linkage group. Using this draft male genome assembly as reference, overall coverage of male and female was calculated using 1000 bp window with an output point every 500 bp. Fragments with coverage more than 30 in male and less than 5 in female were screened out, which distributed in 12 scaffold sequences (Supplemental excel file 1). These fragments were potentially male-specific sequences. Coverage differences between male and female were checked using Integrative Genomics Viewer (IGV), and fragments with half the overall depth in male and few reads mapped in female were potentially Y-linked specific sequences. These potential Y-linked sequences were extracted using IGV, and a batch of male-specific markers was designed based on these sequences. We screened out a pair of primers, which only generated a clear band in male with a size of 575 bp, but no band in female. The sequences of this pair of primers were: forward primer was TTCAGCACACAGACCAAGGATT and reverse primer was TGTTCCGAAGATGAGCGAATGA. Alignment flanking sequences of the male potential specific sequence to the female genome showed a high identity to LG15, rather than the potential sex chromosome LG9. It indicated that these male potential specific sequences may be anchored on the wrong linkage group, and the unpublished male assembly required to be further optimized to improve its accuracy. To validate the specificity and universality of this pair of primers, a PCR based genotyping was conducted in three populations with a total of 60 males and 65 females. The genotyping results showed that this pair of primers was tightly linked to male and absent in female in all of the three populations (Fig. 3). It confirmed that this male specific primer was efficient in identifying genetic sex in BSB.

3.4. Annotation of candidate sex determining genes in BSB

As an approximately 6 Mb potential sex differentiated region was observed at the beginning of LG9, it is reasonable to search the master

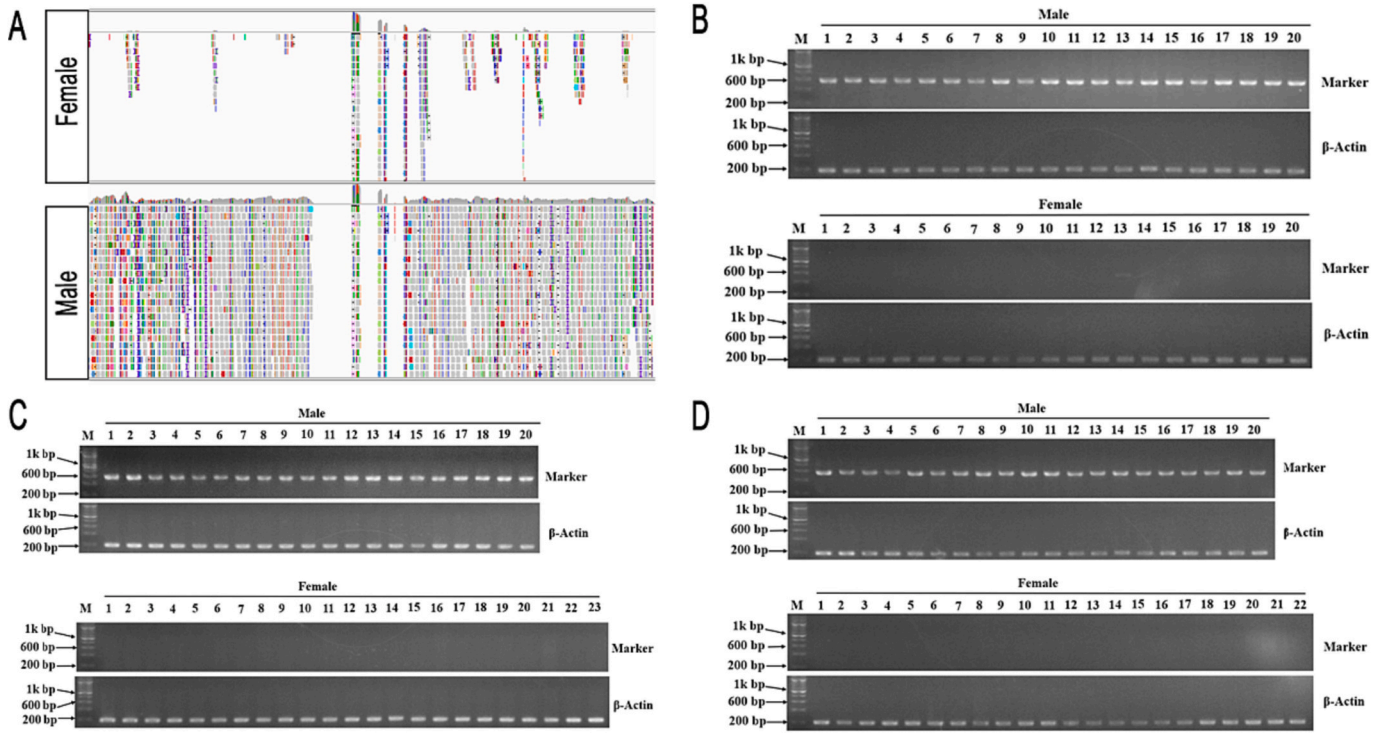


Fig. 3. Potential male specific fragments identified and male specific marker developed in BSB.
Male biased genomic regions and male specific marker validation. (A) Male biased genomic sequences identified from population genome comparison between male and female were manually checked using IGV. (B). Male specific marker verified using Changsha population. (C) Male specific marker verified using Wugang population (D). Male specific marker verified using Ningxiang population.

sex determining gene in this region. From the beginning 8 Mb of Chr7 spanning SDR, 275 genes were annotated (Supplemental excel file 2). Meanwhile, statistics of SNP distribution on these genes of male and female were also calculated (Supplemental excel file 2). Sox family genes play vital roles on sex determination and differentiation (Hu et al., 2021b). A sox gene, sox4a, was located on the sex differentiated region, whereas there were no SNPs observed on its coding and non-coding sequences (Supplemental excel file 2). Annotation of potential Y-specific sequences showed that most of these sequences were annotated to transposons and no reported sex-related genes were found. It indicated that to search the sex determining gene in BSB, and it was better to generate a high-quality and contiguous genome assembly.

4. Discussion

Sex determination in fish is intricate and diverse in contrary to mammals and birds. The predominant mechanism in many fish involves sex determination through X and Y chromosome, as observed in species such as yellow catfish (Wang et al., 2009) and Largemouth bass (Wen et al., 2022b). Conversely, a significant portion of other fish relies a female heterogametic system (ZZ-ZW), such as blue tilapia (Melard, 1995) and half-smooth tongue sole (Chen et al., 2007). A few of fish species utilize even more intricate sex determining systems including XX/XO, XX/XY1Y2, X1X2X1X2/ X1X2Y, X1X2X1X2/X1X2X1, ZZ/ZO and ZZ/ZW1W2 (Gong et al., 2022; Mei and Gui, 2015). Blunt-snout bream (BSB), belonging to the order of Cypriniformes and the family of Cyprinidae, stands out as an herbivorous and economically important fish. Sex of cyprinid fish is genetically determined with limited influences from environmental factors (Goto-Kazeto et al., 2006; Liew et al., 2012). A prior study indicated showed that BSB progenies generated from gynogenesis were exclusively females, implying BSB had a male heterogametic sex determining system (XX-XY) (Zhang et al., 2015). In this study, sex determining genetic mechanism of BSB was explored by comparing genetic differences between male and female using individual resequencing strategy, conclusively affirming the utilization of a XX-XY sex-determining system in BSB. Despite the limited documentation on the sex determination of BSB, our genotyping results indicated an unequivocal concordance between phenotypic and genetic sexes, implying environmental factors likely exert minimal or no effect on sex determination of BSB.

Sex-specific markers are an efficient tool for sex control technology, facilitating the generation of mono-sex population. Establishment of mono-sex population can increase aquaculture production and value especially for some economic fish with sexual dimorphisms, such as body size and color. With the assistance of sex-specific marker, sex control technology was successfully applied in some important economic fish (Cui et al., 2017; Dan et al., 2013; Yano et al., 2012). While BSB is gonochoristic and lacks sexual dimorphism in terms of significant growth differences between males and females，consumers preferences favor male fish due to their lack of eggs. In the process of breeding, there is often a higher demand for female parents. In addition, rearing male and female BSB separately offers the advantage of reducing the heterogeneity in the size of the cultured population caused by natural breeding, thus effectively cutting down on farming costs. Therefore, the preparation of all-male and all female BSB populations holds the potential to significantly boost the aquaculture industry. This study presents the development of a Y-specific marker, enabling the distinction between genetic female (XX) and male (XY or YY) BSB. Genotyping utilizing this marker showed the presence of a band in males and the absence of a band in females. This marker offers the possibility of selecting neomale fish (XX) to establish an all-XX population through mating with normal female (XX).

In teleost fish, distinguishing morphological difference between sex chromosomes such as W to Z and Y to X through karyotype analysis is often challenging (Bao et al., 2019). However, genetic differences could be easily identified through genome-wide association analysis (Geng et al., 2015; Zhou et al., 2021). In our study, an approximately 6 Mb potential sex differentiated region was identified on LG9 in BSB,

implying that this linkage group is likely the BSB sex chromosome. To date, sex differentiated region of numerous fish species have been unveiled using genome-wide association analysis including goldfish, *Sarotherodon melanotheron*, *Oreochromis niloticus*, channel catfish, yellow perch and common seadragon (Bao et al., 2019; Feron et al., 2020; Gammerdinger et al., 2014; Gammerdinger et al., 2016; Qu et al., 2021; Wen et al., 2020). These findings indicated great genetic differences between X/Z and Y/W chromosome at this region, resulting in recombination suppression. Transposable elements typically occurred concomitantly in the flanking regions of this differentiated region (Kabir et al., 2022; Wen et al., 2022d). Recombination suppression subsequently contributes to the structural and functional differentiation of sex chromosomes.

In teleost fish, paralogs derived from several common genes such as dmrt1, amh, sox3, gdf6, amhr2 were identified as master sex determining genes in different species (Pan et al., 2016). Hundreds of genes located in the sex differentiated region of BSB including sox4a. Sox family genes, known for involving in sex determination and differentiation (Anitha and Senthilkumaran, 2021), played crucial roles in these processes. $Sox3^{Y}$ was co-opted as male determining factor in a medaka related fish, *oryzias dancena*. Similar to most sox family genes, the expression of sox4a was significantly upregulated in ovary and testis in *Collichthys lucidus* (Liu et al., 2022). Despite its association with sex-related functions in other species, no sequence differences were observed in the coding and non-coding region of sox4a between male and female genomes in BSB. To determine whether sox4a participated in sex determination of BSB, combined omics experiments and high-quality male genome assembly should be included in the future investigations.

5. Conclusion

In this study, we confirmed that sex determination of BSB was regulated by genetic factors, and that its genetic sex determination system was male heterogametic (XX/XY). A notably extensive sex differentiated region (~6 Mb) was characterized on LG9 of BSB, implying it was likely to be the sex chromosome. However, we annotated 275 genes within this region, containing no obvious candidate for potential master sex determining gene. In addition, we have successfully identified some male-biased sequences, and a Y-linked marker was developed and verified in three distinct BSB populations. Our findings not only laid a solid foundation for unveiling the mystery of sex determination mechanism, but also hold the potential to advance the field of sex control technology in BSB.

CRediT authorship contribution statement

Ming Wen: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Funding acquisition, Conceptualization. **Shaojun Liu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

All these individual re-sequencing data of blunt-snout bream have been submitted in the database of Sequence Read Archive (SRA) under BioProject PRJNA951457.

Acknowledgements

This work was supported by the National Key Research and Development Plan Program (Grant No. 2022YFD2400604), the National Natural Science Foundation of China (Grant No. 32002372), the National Natural Science Foundation of China (Grant No. 32293252, U19A2040), the Training Program for Excellent Young Innovators of Changsha (Grant No. kq2107006), the Youth Science and Technology Talents Lifting Project of Hunan Province (Grant No. 2023TJ-N02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.740559.

References

Anitha, A., Senthilkumaran, B., 2021. Role of sox family genes in teleostean reproduction-an overview. Reprod. Breed. 1, 22–31.
Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T.L., Hahn, M.W., Kitano, J., Mayrose, I., Ming, R., Perrin, N., Ross, L., Valenzuela, N., Vamosi, J.C., Tree of Sex, C, 2014. Sex determination: why so many ways of doing it? PLoS Biol. 12, e1001899.
Bao, L., Tian, C., Liu, S., Zhang, Y., Elaswad, A., Yuan, Z., Khalil, K., Sun, F., Yang, Y., Zhou, T., Li, N., Tan, S., Zeng, Q., Liu, Y., Li, Y., Li, Y., Gao, D., Dunham, R., Davis, K., Waldbieser, G., Liu, Z., 2019. The Y chromosome sequence of the channel catfish suggests novel sex determination mechanisms in teleost fish. BMC Biol. 17, 6.
Chen, S.L., Li, J., Deng, S.P., Tian, Y.S., Wang, Q.Y., Zhuang, Z.M., Sha, Z.X., Xu, J.Y., 2007. Isolation of female-specific AFLP markers and molecular identification of genetic sex in half-smooth tongue sole (Cynoglossus semilaevis). Mar. Biotechnol. (N.Y.) 9, 273–280.
Cui, Z., Liu, Y., Wang, W., Wang, Q., Zhang, N., Lin, F., Wang, N., Shao, C., Dong, Z., Li, Y., 2017. Genome editing reveals dmrt1 as an essential male sex-determining gene in Chinese tongue sole (Cynoglossus semilaevis). Sci. Rep. 7, 42213.
Dan, C., Mei, J., Wang, D., Gui, J.F., 2013. Genetic differentiation and efficient sex-specific marker development of a pair of Y- and X-linked markers in yellow catfish. Int. J. Biol. Sci. 9, 1043–1049.
Feron, R., Zahm, M., Cabau, C., Klopp, C., Roques, C., Bouchez, O., Eche, C., Valiere, S., Donnadieu, C., Haffray, P., Bestin, A., Morvezen, R., Acloque, H., Euclide, P.T., Wen, M., Jouano, E., Schartl, M., Postlethwait, J.H., Schraidt, C., Christie, M.R., Larson, W.A., Herpin, A., Guiguen, Y., 2020. Characterization of a Y-specific duplication/insertion of the anti-Mullerian hormone type II receptor gene based on a chromosome-scale genome assembly of yellow perch, *Perca flavescens*. Mol. Ecol. Resour. 20, 531–543.
Gammerdinger, W.J., Conte, M.A., Acquah, E.A., Roberts, R.B., Kocher, T.D., 2014. Structure and decay of a proto-Y region in Tilapia, *Oreochromis niloticus*. BMC Genomics 15, 975.
Gammerdinger, W.J., Conte, M.A., Baroiller, J.-F., D'cotta, H., Kocher, T.D., 2016. Comparative analysis of a sex chromosome from the blackchin tilapia, *Sarotherodon melanotheron*. BMC Genomics 17, 808.
Gao, Z., Luo, W., Liu, H., Zeng, C., Liu, X., Yi, S., Wang, W., 2012. Transcriptome analysis and SSR/SNP markers information of the blunt snout bream (Megalobrama amblycephala). PLoS One 7, e42637.
Geng, X., Sha, J., Liu, S., Bao, L., Zhang, J., Wang, R., Yao, J., Li, C., Feng, J., Sun, F., Sun, L., Jiang, C., Zhang, Y., Chen, A., Dunham, R., Zhi, D., Liu, Z., 2015. A genome-wide association study in catfish reveals the presence of functional hubs of related genes within QTLs for columnaris disease resistance. BMC Genomics 16, 196.
Gong, J., Li, B., Zhao, J., Zhou, Z., Ke, Q., Zhu, Q., Xu, D., Zhou, T., Xu, P., 2022. Sex-specific genomic region identification and molecular sex marker development of rock bream (Oplegnathus fasciatus). Mar. Biotechnol. (N.Y.) 24, 163–173.
Goto-Kazeto, R., Abe, Y., Masai, K., Yamaha, E., Adachi, S., Yamauchi, K., 2006. Temperature-dependent sex differentiation in goldfish: establishing the temperature-sensitive period and effect of constant and fluctuating water temperatures. Aquaculture. 254, 617–624.
Hattori, R.S., Murai, Y., Oura, M., Masuda, S., Majhi, S.K., Sakamoto, T., Fernandino, J.I., Somoza, G.M., Yokota, M., Strüssmann, C.A., 2012. A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. Proc. Natl. Acad. Sci. 109, 2955–2959.
Herpin, A., Schartl, M., 2009. Molecular mechanisms of sex determination and evolution of the Y-chromosome: insights from the medakafish (Oryzias latipes). Mol. Cell. Endocrinol. 306, 51–58.
Hu, F., Zhong, H., Wu, C., Wang, S., Guo, Z., Tao, M., Zhang, C., Gong, D., Gao, X., Tang, C., Wei, Z., Wen, M., Liu, S., 2021a. Development of fisheries in China. Reprod. Breed. 1, 64–79.
Hu, Y., Wang, B., Du, H., 2021b. A review onsoxgenes in fish. Rev. Aquac. 13, 1986–2003.
Kabir, A., Ieda, R., Hosoya, S., Fujikawa, D., Atsumi, K., Tajima, S., Nozawa, A., Koyama, T., Hirase, S., Nakamura, O., Kadota, M., Nishimura, O., Kuraku, S., Nakamura, Y., Kobayashi, H., Toyoda, A., Tasumi, S., Kikuchi, K., 2022. Repeated translocation of a supergene underlying rapid sex chromosome turnover in Takifugu pufferfish. Proc. Natl. Acad. Sci. U. S. A. 119, e2121469119.
Kamiya, T., Kai, W., Tasumi, S., Oka, A., Matsunaga, T., Mizuno, N., Fujita, M., Suetake, H., Suzuki, S., Hosoya, S., Tohari, S., Brenner, S., Miyadai, T., Venkatesh, B., Suzuki, Y., Kikuchi, K., 2012. A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, Takifugu rubripes (fugu). PLoS Genet. 8, e1002798.

Li, M., Sun, Y., Zhao, J., Shi, H., Zeng, S., Ye, K., Jiang, D., Zhou, L., Sun, L., Tao, W., Nagahama, Y., Kocher, T.D., Wang, D., 2015. A tandem duplicate of anti-Müllerian hormone with a missense SNP on the Y chromosome is essential for male sex determination in Nile Tilapia, *Oreochromis niloticus*. PLoS Genet. 11, e1005678.
Liew, W.C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K.R., Orban, L., 2012. Polygenic sex determination system in zebrafish. PLoS One 7, e34397.
Liu, H., Guan, B., Xu, J., Hou, C., Tian, H., Chen, H., 2013. Genetic manipulation of sex ratio for the large-scale breeding of YY super-male and XY all-male yellow catfish (Pelteobagrus fulvidraco (Richardson)). Mar. Biotechnol. (N.Y.) 15, 321–328.
Liu, H., Chen, C., Gao, Z., Min, J., Gu, Y., Jian, J., Jiang, X., Cai, H., Ebersberger, I., Xu, M., 2017. The draft genome of blunt snout bream (Megalobrama amblycephala) reveals the development of intermuscular bone and adaptation to herbivorous diet. GigaScience. 6, gix039.
Liu, H., Pang, M., Yu, X., Zhou, Y., Tong, J., Fu, B., 2018. Sex-specific markers developed by next-generation sequencing confirmed an XX/XY sex determination system in bighead carp (Hypophthalmichehys nobilis) and silver carp (Hypophthalmichthys molitrix). DNA Res. 25 (3), 257–264.
Liu, F., Zhou, L., Zhang, J., Wang, Y., Wang, Z., Liu, X., Cai, M., 2022. Genome-wide identification and transcriptome-based expression profiling of the sox gene family in the spinyhead croaker (Collichthys lucidus). J. Fish Biol. 100, 15–24.
Mank, J.E., Avise, J.C., 2009. Evolutionary diversity and turn-over of sex determination in teleost fishes. Sex. Dev. 3, 60–67.
Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimizu, N., 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature. 417, 559.
Mei, J., Gui, J.F., 2015. Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish. Sci. China Life Sci. 58, 124–136.
Melard, C., 1995. Production of a high percentage of male offspring with 17α-ethynylestradiol sex-reversed Oreochromis aureus. I. Estrogen sex-reversal and production of F2 pseudofemales. Aquaculture. 130, 25–34.
Myosho, T., Otake, H., Masuyama, H., Matsuda, M., Kuroki, Y., Fujiyama, A., Naruse, K., Hamaguchi, S., Sakaizumi, M., 2012. Tracing the emergence of a novel sex-determining gene in medaka, Oryzias luzonensis. Genetics. 191, 163–170.
Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., Schartl, M., 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proc. Natl. Acad. Sci. U. S. A. 99, 11778–11783.
Pan, Q., Anderson, J., Bertho, S., Herpin, A., Wilson, C., Postlethwait, J.H., Schartl, M., Guiguen, Y., 2016. Vertebrate sex-determining genes play musical chairs. C. R. Biol. 339, 258–262.
Qu, M., Liu, Y., Zhang, Y., Wan, S., Ravi, V., Qin, G., Jiang, H., Wang, X., Zhang, H., Zhang, B., Gao, Z., Huysseune, A., Zhang, Z., Zhang, H., Chen, Z., Yu, H., Wu, Y., Tang, L., Li, C., Zhong, J., Ma, L., Wang, F., Zheng, H., Yin, J., Witten, P.E., Meyer, A., Venkatesh, B., Lin, Q., 2021. Seadragon genome analysis provides insights into its phenotype and sex determination locus. Sci. Adv. 7, eabg5196.
Rao, H., Deng, J., Wang, W., Gao, Z., 2012. An AFLP-based approach for the identification of sex-linked markers in blunt snout bream, Megalobrama amblycephala (Cyprinidae). Genet. Mol. Res. 11, 1027–1031.
Ren, L., Li, W., Qin, Q., Dai, H., Han, F., Xiao, J., Gao, X., Cui, J., Wu, C., Yan, X., Wang, G., Liu, G., Liu, J., Li, J., Wan, Z., Yang, C., Zhang, C., Tao, M., Wang, J., Luo, K., Wang, S., Hu, F., Zhao, R., Li, X., Liu, M., Zheng, H., Zhou, R., Shu, Y., Wang, Y., Liu, Q., Tang, C., Duan, W., Liu, S., 2019. The subgenomes show asymmetric expression of alleles in hybrid lineages of Megalobrama amblycephala × Culter alburnus. Genome Res. 29, 1805–1815.
Roberts, N.B., Juntti, S.A., Coyle, K.P., Dumont, B.L., Stanley, M.K., Ryan, A.Q., Fernald, R.D., Roberts, R.B., 2016. Polygenic sex determination in the cichlid fish Astatotilapia burtoni. BMC Genomics 17, 835.
Rondeau, E.B., Messmer, A.M., Sanderson, D.S., Jantzen, S.G., von Schalburg, K.R., Minkley, D.R., Leong, J.S., Macdonald, G.M., Davidsen, A.E., Parker, W.A., 2013. Genomics of sablefish (Anoplopoma fimbria): expressed genes, mitochondrial phylogeny, linkage map and identification of a putative sex gene. BMC Genomics 14, 452.
Takehana, Y., Matsuda, M., Myosho, T., Suster, M.L., Kawakami, K., Shin, I.T., Kohara, Y., Kuroki, Y., Toyoda, A., Fujiyama, A., Hamaguchi, S., Sakaizumi, M., Naruse, K., 2014. Co-option of Sox3 as the male-determining factor on the Y chromosome in the fish Oryzias dancena. Nat. Commun. 5, 4157.
Thorvaldsdottir, H., Robinson, J.T., Mesirov, J.P., 2013. Integrative genomics viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. 14, 178–192.
Wang, D., Mao, H.L., Chen, H.X., Liu, H.Q., Gui, J.F., 2009. Isolation of Y- and X-linked SCAR markers in yellow catfish and application in the production of all-male populations. Anim. Genet. 40, 978–981.
Wen, M., Feron, R., Pan, Q., Guguin, J., Jouanno, E., Herpin, A., Klopp, C., Cabau, C., Zahm, M., Parrinello, H., Journot, L., Burgess, S.M., Omori, Y., Postlethwait, J.H., Schartl, M., Guiguen, Y., 2020. Sex chromosome and sex locus characterization in goldfish, Carassius auratus (Linnaeus, 1758). BMC Genomics 21, 552.
Wen, M., Pan, Q., Larson, W., Eche, C., Guiguen, Y., 2022a. Characterization of the sex determining region of channel catfish (Ictalurus punctatus) and development of a sex-genotyping test. Gene 146933.
Wen, M., Zhang, Y., Wang, S., Hu, F., Tang, C., Li, Q., Qin, Q., Tao, M., Zhang, C., Zhao, R., Wang, S., Liu, Q., Tang, C., Liu, S., 2022b. Sex locus and sex markers identification using whole genome pool-sequencing approach in the largemouth bass (Micropterus Salmoides L.). Aquaculture. 559.
Wen, M., Zhang, Y., Wang, S., Wang, Y., Qin, Q., Tao, M., Zhang, C., Zhao, R., Wang, S., Hu, F., Liu, Q., Wei, Z., Gu, Q., Tang, C., Liu, S., 2022c. Characterization of sex locus and sex-specific sequences in the mandarin fishes. Aquaculture. 561.
Wen, M., Pan, Q., Jouanno, E., Montfort, J., Zahm, M., Cabau, C., Klopp, C., Iampietro, C., Roques, C., Bouchez, O., Castinel, A., Donnadieu, C., Parrinello, H., Poncet, C., Belmonte, E., Gautier, V., Avarre, J.C., Dugue, R., Gustiano, R., Ha, T.T. T., Campet, M., Sriphairoj, K., Ribolli, J., de Almeida, F.L., Desvignes, T., Postlethwait, J.H., Floi Bucao, C., Robinson-Rechavi, M., Bobe, J., Herpin, A., Guiguen, Y., 2022d. An ancient truncated duplication of the anti-Mullerian hormone receptor type 2 gene is a potential conserved master sex determinant in the Pangasiidae catfish family. Mol. Ecol. Resour. 22, 2411–2428.
Wilson, C.A., High, S.K., McCluskey, B.M., Amores, A., Yan, Y.L., Titus, T.A., Anderson, J. L., Batzel, P., Carvan 3rd, M.J., Schartl, M., Postlethwait, J.H., 2014. Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. Genetics. 198, 1291–1308.
Yano, A., Guyomard, R., Nicol, B., Jouanno, E., Quillet, E., Klopp, C., Cabau, C., Bouchez, O., Fostier, A., Guiguen, Y., 2012. An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. Curr. Biol. 22, 1423–1428.
Zhang, X., Gao, Z., Luo, W., Wen, J., Song, W., Wang, W., 2015. Studies on morphological characteristics and genetic analysis of the gynogenesis blunt snout bream (Megalobrama amblycephala). Acta Hydrobiol. Sin. 39, 126–132.
Zhou, Z., Wang, M., Yang, J., Liu, B., Li, L., Shi, Y., Pu, F., Xu, P., 2021. Genome-wide association analysis reveals genetic variations and candidate genes associated with growth-related traits and condition factor in Takifugu bimaculatus. Reprod. Breed. 1, 89–99.