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Identification of sex-determination regions and development of male-specific markers for topmouth culter (*Culter alburnus*)

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ABSTRACT

The topmouth culter (*Culter alburnus*), a carnivorous freshwater species in the family Cyprinidae (order Cypriniformes), holds high economic value and displays marked sexual dimorphism in growth, where females exhibit accelerated growth relative to males. Therefore, the study of the sex determination mechanism in topmouth culter is of great significance for monosex breeding (such as all-male or all-female populations) and the conservation of germplasm resources. However, the genetic basis of sex determination of topmouth culter remains unknown. In this study, we performed whole-genome resequencing of female and male topmouth culter against a male reference genome to characterize sex determination and sex chromosomes. Genome-wide association studies (GWAS) showed that male-specific SNPs were significantly enriched on LG4, which was potential sex chromosome of the topmouth culter. Furthermore, potential male-specific sequences were identified on LG4 by screening for sequencing depth differences between males and females. With these sequences, male-specific molecular markers were screened and then validated in three different populations. None of reported sex-determination genes were annotated in these sex-biased regions on LG4. Our results indicated that the topmouth culter employed a male heterogametic (XX/XY) sex determination system. In summary, we successfully developed effective male-specific molecular markers for sex identification and characterized the genetic basis of sex determination in the topmouth culter. Our findings provide valuable genetic markers for sex-controlled breeding programs and lay a theoretical foundation for further exploration of the mechanisms underlying fish sex determination.

1. Introduction

Topmouth culter (*Culter alburnus*), a carnivorous freshwater cyprinid endemic to East Asia, represents a cornerstone of modern aquaculture in China. Valued for its rapid growth, robust adaptability to diverse freshwater ecosystems such as reservoirs, lakes, and ponds, and significant ecological role, this species is integral to regional food security and the economic vitality of rural communities. The market appeal of *C. alburnus* is particularly strong, driven by consumer preference for its tender, palatable flesh with a low number of intermuscular bones, allowing it to command premium prices that are often two to three times higher than those of staple carp species. A key biological characteristic that underpins its aquaculture potential is a pronounced sexual dimorphism in growth rate; females typically grow 20–30 % faster and reach larger ultimate sizes than males (Liu et al., 2017). This disparity presents a clear and compelling economic incentive for the development of monosex (all-female) aquaculture systems, which would substantially optimize yield, and maximize economic returns per unit area.

Historically, achieving monosex populations in aquaculture has relied on methods like gynogenesis, androgenesis, and exogenous hormone administration. While gynogenesis and androgenesis can produce monosex offspring, these techniques are frequently hampered by technical complexities, low survival rates, and the high cost of implementation on a commercial scale (Liu et al., 2025; Kašpar et al., 2022).

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More commonly, sex reversal is induced by treating fry with hormones or aromatase inhibitors (Singh, 2013; Afonso et al., 2001). However, this approach raises significant concerns regarding potential environmental contamination from hormonal residues and perceived risks to food safety, which can negatively impact consumer confidence and market access. A more sustainable, precise, and socially acceptable strategy is Marker-Assisted Selection (MAS) within a controlled breeding program. Such a program would involve producing "pseudo-males" (genetically female, XX, but phenotypically male) and then crossing them with normal females (XX) to yield all-female (XX) progeny (Zhai et al., 2022). The critical prerequisite for this advanced breeding approach is the availability of reliable, accurate, and cost-effective sex-specific molecular markers to distinguish genetic males from pseudo-males. Despite its economic importance, the molecular mechanisms governing sex determination in topmouth culter have remained entirely uncharacterized. Consequently, prior to this investigation, no such markers had been developed, creating a significant bottleneck that has severely impeded the progress of genetic improvement and sex-control technologies for this high-value species.

The challenge of developing these markers is rooted in the broader scientific complexity of sex determination in vertebrates, particularly in teleost fishes. In mammals and birds, sex determination pathways are highly conserved and typically governed by a master-switch gene located on a pair of well-differentiated heteromorphic sex chromosomes (e.g., *SRY* on the Y chromosome in mammals, *DMRT1* on the Z in birds) (Miyawaki et al., 2020; Kuroiwa, 2018). In stark contrast, teleosts exhibit an unparalleled diversity and evolutionary lability in their sex determination mechanisms, which can range from simple monogenic systems (GSD, Genetic Sex Determination) to polygenic systems (PSD), and often involve significant environmental influences (ESD, Environmental Sex Determination) (Heule et al., 2014). This remarkable plasticity is thought to be fueled by the teleost-specific whole-genome duplication event, which provided a wealth of duplicated genes (paralogs) that could be co-opted into novel functions, including sex determination, without compromising their original roles (Glasauer and Neuhauss, 2014). This has led to a phenomenon of rapid turnover of sex-determining genes and chromosomes among fish species, making them exceptional models for studying the evolution of sex (Wang et al., 2024). Even within the Cyprinidae family, a spectrum of mechanisms is observed: goldfish (*Carassius auratus*) sex is determined by both genetic and thermal cues (Wen et al., 2020), while zebrafish (*Danio rerio*) shifted from a WZ/ZZ system in wild populations to a complex polygenic system under domestication (Wilson et al., 2014). This evolutionary dynamism means that sex-determination systems cannot be inferred from closely related species; a species-specific investigation is therefore indispensable to uncover the genetic basis of sex in *C. alburnus*.

This quest for species-specific sex-linked loci has been paralleled by a dramatic evolution in molecular technologies. Early research on fish sex determination relied on conventional marker techniques such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeats (SSR) (Liu, Chen, 2023). These pioneering methods were instrumental in identifying the first sex-linked markers in species like rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*), providing foundational knowledge for the field (Iturra et al., 1997; Lee et al., 2003). However, these technologies were inherently limited by low marker density, which provided only sparse coverage of the genome, and often suffered from issues with reproducibility and low throughput. In many species, these constraints made the identification of tightly linked or definitive sex-specific markers challenging, if not impossible (Rao et al., 2012). The advent of high-throughput sequencing (HTS), or Next-Generation Sequencing (NGS), has fundamentally transformed this landscape. By enabling the rapid and cost-effective generation of massive amounts of genomic data, HTS technologies facilitate comparative genomics strategies with unprecedented power and precision. Approaches such as pooled-sequencing (Pool-Seq) combined with genome-wide association studies (GWAS) allow for the simultaneous scanning of millions of Single Nucleotide Polymorphisms (SNPs) across the entire genome. This makes it possible to pinpoint specific genomic regions—or even entire chromosomes—that exhibit strong differentiation between males and females, as has been successfully demonstrated in grass carp (*Ctenopharyngodon idella*), mandarin fish (*Siniperca chuatsi*), and autotetraploid crucian carp (*Carassius auratus*) (Zhang et al., 2017, 2024; Wen et al., 2022b). These whole-genome NGS approaches have become the gold standard for efficiently identifying sex chromosomes and developing robust sex-specific markers in non-model organisms.

Fortunately, our laboratory had worked out a chromosome-level assembly of male topmouth culter (Unpublished data). Using this male genome as a reference, we performed whole-genome resequencing of male and female pools to characterize sex differentiated regions and sex chromosome. In this study, sex-biased SNPs and fragments were screened and identified from the whole genome. Consequently, potential sex chromosome (LG4) containing sex differentiation regions were identified in topmouth culter. Our results indicated this fish species utilized a male heterogametic system (XX-XY). In addition, male-specific molecular markers were successfully developed and validated in three populations of topmouth culter. Our findings not only provide critical genetic insights into sex determination mechanisms but also promote the progress of sex controlled breeding programs in topmouth culter.

2. Material and methods

2.1. Experimental fish and DNA extraction

The three experimental fish populations used in this study were collected from the Ministry of Education Polyploid Fish Breeding Center of Hunan Normal University in Changsha, the Teqing Family Farm of Hunan province of China, and Hunan Buckwheat Lake High Quality Fish Research Institute Co., Ltd. The caudal fin of each individual was collected and preserved in 1.5 mL EP tubes with 95 % ethanol for DNA extraction. The phenotypic sex of each individual of topmouth culter was determined by examining milt and gonadal observation via dissection. For genomic sequencing, 5 female and 5 male experimental fish from the National Ministry of Education's Polyploid Fish Breeding Center of Hunan Normal University were randomly selected. Then, genomic DNA was extracted using the Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech NO. B518251, China) according to the manufacturer's instructions. The genomic DNA was placed on a 1.3 % agarose gel, and the quality of the extracted DNA was detected by agarose gel electrophoresis. A NanoDrop ND2000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to measure the concentration of the genomic DNA. Finally, the extracted genomic DNA was stored at −80°C for subsequent Illumina sequencing. For genotyping, 23 females and 23 males were sampled from the Ministry of Education's Province Polyploid Fish Breeding Center of Hunan Normal University, 15 females and 15 males were sampled from Teqing Family Farm in Hunan Province, and 20 females and 21 males were sampled from the Hunan Buckwheat Lake High Quality Fish Research Institute Co., Ltd. The caudal fins were cut into pieces using sterilized medical scissors and then treated with 5 % Chelex and 20 mg proteinase K at 55°C for 2 h, followed by denaturation of the proteinase K at 99°C for 2 min. After a brief centrifugation, the supernatant containing genomic DNA (gDNA) was collected into new tubes. Subsequently, the concentration of the genomic DNA was diluted by half and stored at −20°C for subsequent PCR genotyping.

2.2. Whole-genome re-sequencing

The high quality genomic DNA was used to conduct whole-genome resequencing. For each sample, 0.2 μg of genomic DNA was used to construct sequencing libraries according to the manufacturer's protocol

of the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). Briefly, genomic DNA was fragmented into 350 bp segments using a Bioruptor sonicator (Diagenode). The DNA fragments were then end-repaired to generate blunt ends, followed by adenylation at the 3′ ends. Subsequently, specific adapters were ligated to the adenylated fragments, and PCR amplification was performed to enrich the adapter-ligated DNA fragments. The PCR products were purified using the AMPure XP system (Beckman Coulter, Beverly, USA), and the concentration of fragmented DNA was measured with a Qubit® 3.0 Fluorometer (Invitrogen, USA). The size distribution of the libraries was analyzed using the NGS3K/Caliper system, and the libraries were further quantified by real-time PCR (3 nM). Exponential cluster generation was performed using the cBot cluster generation system using the Illumina PE cluster kit (Illumina, USA) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced on an Illumina platform to generate 150 bp paired-end reads.

2.3. Identification of sex differentiated regions

To check genomic differences between males and females from the whole genome, we conducted a genome-wide association study using a male chromosome-level assembly of topmouth culter as a reference genome. Firstly, we made an index of the reference genome in FASTA format using BWA software (version 0.7.17). Then, Illumina sequencing data generated from male pool and female pool were respectively aligned to the reference genome of topmouth culter. Subsequently, the aligned sequences were subjected to sorting, merging, and duplicate removal using Picard tools (version 2.18.2) with default settings. Next, BAM files were filtered using Samtools software (version 1.8), setting the parameters to remove reads with a quality value less than 20 and non-uniquely mapped reads. After that, we generated pileup files using the *mpileup* command of Samtools with the filtered BAM files of both males and females, setting the parameter to disable per-base pairing quality (-B). Then, we converted the pileup files into sync files using *mpileup2sync* of Popoolation2 (version 1.201), setting the minimum quality value to 20. Finally, we used PSASS (https://github.com/SexGenomicsToolkit/PSASS) to calculate pool-specific SNPs, between pools FST, and depth in a sliding window from pileup files. The PSASS parameters were set as follows: the minimum depth for considering sites was set to 10 (min-depth = 10), the allele frequency of SNP heterozygotes in a pool was set to 0.5 ± 0.2 (–freq-het 0.5, –range-het 0.2), the allele frequency of SNP homozygotes in a pool was set to be greater than 0.95 (–freq-hom 1, –range-hom 0.05), the output resolution of the sliding window metrics was 10 Kb, and the sliding window size was 100 Kb. The R software package (http://github.com/RomainFeron/PSASS-vis) and the CMplot package were used to visualize these data (Yin et al., 2021).

2.4. Screening and developing sex-specific molecular markers

Previous studies have shown that topmouth culter used a male heterogametic sex-determining system (XX/XY), as all individuals in a population produced by gynogenesis are females (Jia, 2019). Taking advantage of the male topmouth culter genome as a reference, we searched for Y-potential sequence from whole genome by screening depth difference between male and female. A 100 kb sliding windows was used to calculate the distribution of the sequencing depth of males and females. Potential Y-linked fragments were screened from regions, where few reads enriched in female and approximately half average depth in male. The reads coverages in these regions were manually checked using the Integrative Genomics Viewer (IGV 2.17.1). Then, potential Y-linked fragments from these regions were extracted using IGV. Subsequently, based on these potential Y-linked fragments, a series of sex-specific primers were designed using the online tool Primer3 version 0.4.0 (https://bioinfo.ut.ee/primer3-0.4.0/). These primers were tested using 5 females and 5 males. To ensure the reliability of the markers, we performed gradient PCR assays (ranging from 50°C to 60°C) to optimize the annealing temperature. The primer pair showing the most distinct bands and highest specificity was selected. Finally, the effective sex-specific primer was validated in three different populations. The total volume of the PCR amplification reaction for genotyping was 10 μL, including 5 μL of 2x Taq Master Mix (Vazyme, China), 3.5 μL of ddH_2O, 0.5 μL each of forward and reverse primers (10 μM), and 0.5 μL of DNA. The PCR amplification reaction was carried out as follows: pre-denaturation at 94°C for 5 min; denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min, for a total of 35 cycles; then extension at 72°C for 7 min, and storage at 4°C. Finally, the PCR products were detected on a 1.5 % agarose gel.

3. Results

3.1. Statistics of whole-genome resequencing data

For whole-genome resequencing, 5 females and 5 males were randomly selected to generate male and female genomic sequences individually for genome-wide association study (Table 1). A total of 431,231,486 (64.70 Gb) and 392,990,430 (58.96 Gb) paired-end clean reads was obtained from males and females respectively. The percentage of reads from males and females was approximately 99.64 % and 99.56 % respectively, which aligned onto the male reference genome. The average sequencing depths for males and females were 46.04 and 41.73 respectively. Furthermore, nucleotide statistics revealed that the quality scores of the resequencing data were decent with 97.52 % and 97.50 % for Q20 and 95.51 % and 95.47 % for Q30 in males and females respectively. The average GC content was 38.59 % for both male and female pool (Table 1). These information indicated that our sequencing data was sufficiently large and of high quality.

3.2. Identification of potential sex chromosomes and sex differentiation regions

In teleosts, sex chromosomes (X-Y and Z-W) usually lack obvious morphological differences, making them difficult to be distinguished using karyotype analysis. In topmouth culter, karyotype analysis also did not reveal any morphological differences between its sex chromosomes. Therefore, we performed a whole genome analysis to uncover the genetic differences between males and females of topmouth culter from a microcosmic perspective. By comparing the whole-genome data of males and females, we analyzed the distribution of sex-biased single-nucleotide polymorphisms (SNPs) across the entire genome using a 100 kb sliding window. The results showed that sex-biased SNPs were enriched in linkage group 4 (LG4) (Fig. 1A). In addition, a narrow region enriched with male-biased SNPs was observed on LG4. However,

Table 1
Statistics of whole genome resequencing data.

Sample	Raw reads	Clean reads	Clean Base (Gb)	Q20 (%)	Q30 (%)	GC (%)
Male 1	82,934,864	82,325,444	12.35	97.54	95.53	38.33
Male 2	96,173,334	95,508,778	14.33	97.58	95.62	38.71
Male 3	89,794,586	89,113,518	13.37	97.51	95.50	38.86
Male 4	74,442,716	73,913,710	11.09	97.49	95.46	38.50
Male 5	91,103,410	90,370,036	13.56	97.48	95.45	38.55
Female 1	75,021,070	74,405,770	11.16	97.50	95.48	38.66
Female 2	71,364,820	70,782,366	10.62	97.45	95.37	38.33
Female 3	84,489,322	83,707,640	12.56	97.48	95.43	38.48
Female 4	85,487,774	84,842,894	12.73	97.54	95.55	38.60
Female 5	79,873,750	79,251,760	11.89	97.53	95.52	38.90

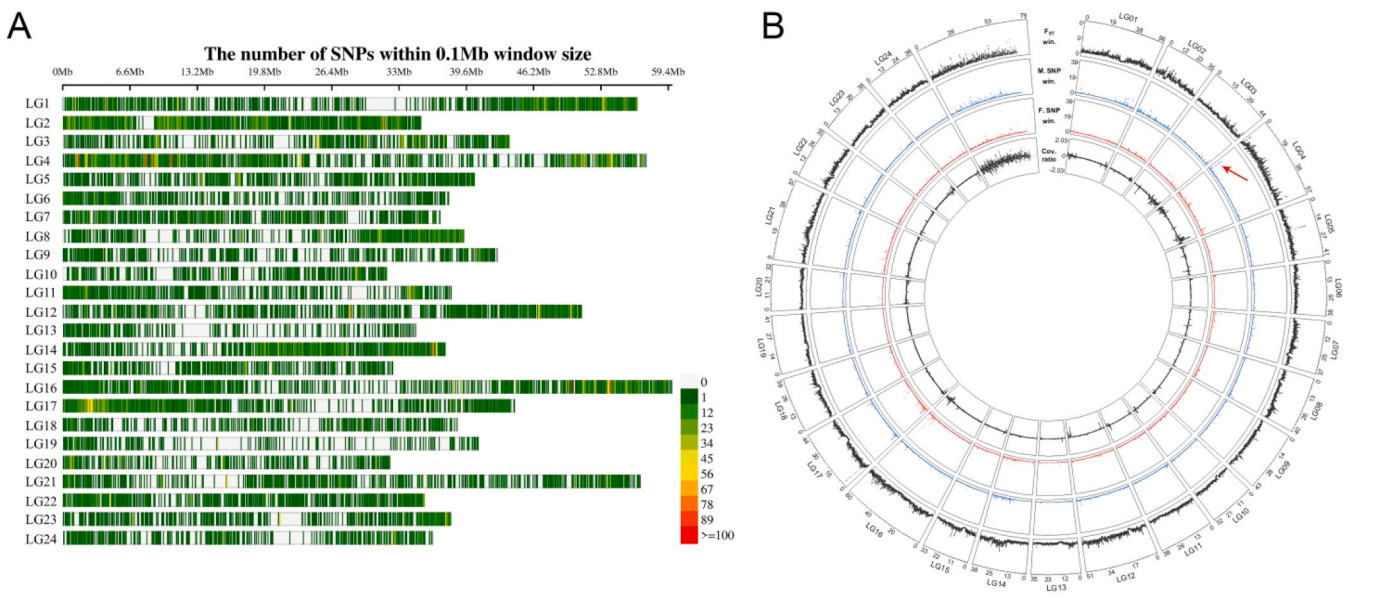


Fig. 1. The distribution of sex-biased SNPs and characterization of genetic differentiation between sexes in the topmouth culter, Using the male topmouth culter genome as a reference, genomic differences between sexes was overall analyzed across all 24 chromosomes of topmouth culter with a 100 Kb sliding window size. A. The distribution of sex-biased SNPs showed that sex-biased SNPs were significantly enriched in linkage group 4 (LG4). B. From outer to inner tracks, the circular plot showed the distribution of overall genetic differentiation index (F_{ST}) between females and males, male-biased SNPs, female-biased SNPs and coverage ratio between males and females respectively.

female-biased SNPs were not enriched on LG4. The distribution of coverage ratio between sexes across whole genome showed that the ratio varied largely at the end of LG4 (Fig. 1B). It indicated the regions that sequencing depth was different between males and females. This narrow region contained a relatively high density of male-biased SNPs was approximately 0.3 Mbp at the beginning of LG4 (Fig. 2B). Meanwhile, we noticed that the sequencing depth at the beginning of LG4 was relatively lower than average depth, implying genomic sequences at this region were complex (Fig. 2C). The complexity of sex differentiated region made the identification of sex-biased genomic sequences difficult. Additionally, the sequencing depth differences between females and males were obviously present at the end of LG4, which indicated potential sex-specific sequences likely existed at LGs (Fig. 2C). F_{ST} is a statistical index used to evaluate genetic differentiation among populations. The F_{ST} value in this region showed a moderate degree of genetic differentiation between females and males, which suggested that genetic differences between sex chromosomes were small (Fig. 2A). These results implied that LG4 in the topmouth culter genome was a potential sex chromosome with a narrow sex differentiated region and few potential male-specific fragments.

3.3. Identification of male-specific molecular markers

As we identified a potential sex chromosome LG4 in topmouth culter, it was reasonable to search for sex-specific fragments on LG4. To identify male-specific fragments, we calculated male-pool and female-pool sequencing depth using a small window 1 kb. Due to sequencing reads were mapped onto a male genome, genomic regions with half average depth in male and no/few in female harbored potential male-specific fragments (Supplemental File 1). We manually checked these potential male-specific fragments using IGV. Then male-specific sequences were extracted from these regions (Fig. 3A), and a batch of male-specific markers were designed based on these sequences. Among them, we screened a primer pair that produced a clear target band exclusively in males and no band in females (Table 2, Fig. 3B). To validate the specificity and universality of this primer pair, we performed PCR-based genotyping in three populations with 59 males and 58 females (Supplemental File 2). Genotyping results showed no bands in all female individuals in three populations. And the male-specific band was presented in all male individuals (n = 23) in population 1. However, the band was absent in 2 out of 15 male individuals in population 2, and the band was absent in 1 out of 21 male individuals in population 3, which may resulted from sex reversal or sampling mistakes. These results illustrated that this male-specific primer pair could be applied as sex marker to effectively identify the genetic sex of topmouth culter (Fig. 4).

3.4. Identification of canditates for sex determining gene in topmouth culter

Sex determining gene usually located on the sex chromosome near sex differentiated regions. We had identified a narrow sex differentiated region and some sex specific fragments on the potential sex chromosome LG4. To search for candidates for sex determining gene, we annotated genes with these genomic sequences using BLAST searches in the NCBI database. “Usual suspect” gene for sex determination such as *dmrt1*, *amh*, *amhr2*, *gdf6*, and *gsdf* were not observed. However, a sry-related high mobility group box (Sox) gene *sox10* was annotated near the sex differentiated region, which played a role in the cascade of gene activation that led to male development by activating the *sox9* gene (Polanco et al., 2010). Further analysis of the resequencing data revealed no significant non-synonymous SNPs within the exons of *sox10* between the male and female pools, implying that its potential function in sex determination may be driven by regulatory variations rather than coding sequence mutations. The phylogenetic analysis showed that the *sox10* protein sequence of *Culter alburnus* (topmouth culter) clusters closely with those of other teleost fishes, particularly within the Cyprinidae family. It shared a more recent common ancestor with species such as *Cyprinus carpio* (common carp) and *Carassius auratus* (goldfish), as evidenced by their proximate branching patterns. This clade was distinct from those of more distantly related fishes such as *Oryzias latipes* (medaka) and *Oreochromis niloticus* (Nile tilapia), as well as from tetrapod vertebrates. Notably, the presence of the *sox10* gene within a sex-differentiated genomic region in *Culter alburnus* (topmouth culter) suggested a potential role in sex determination or sexual development. The high sequence conservation of *sox10* among fish species implied its functional importance, and its location in a sex-related region may

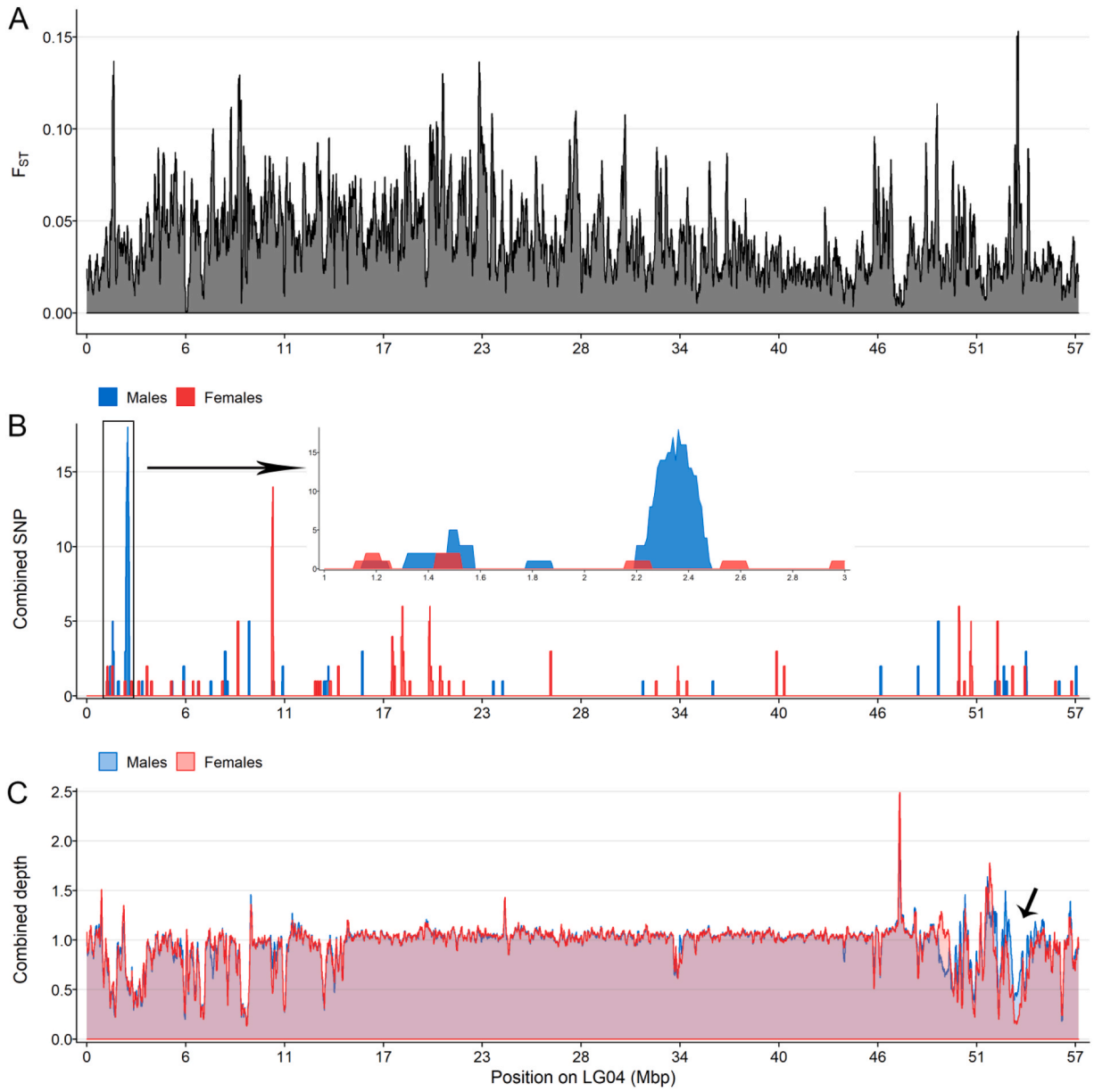


Fig. 2. Identification of male-biased SNPs and fragments on LG4 of topmouth culter, F_{ST} between females and males, sex-biased SNPs, and relative sequencing depth between males and females were calculated using a 100 kb sliding window with an output of 10 kb. (A) The distribution of Genetic differentiation index (F_{ST}) between females and males on LG4. (B) A narrow sex-differentiated region (SDR) enriched male-biased SNPs identified on LG4, indicated by black arrow. (C) Differences of sequencing depth between females and males on LG4. Relative female and male sequencing depth are showed in red and blue respectively.

indicate a lineage-specific neofunctionalization or co-option of this gene in sex differentiation pathways.

4. Discussion

The elucidation of sex determination mechanisms is a cornerstone of genetic research in aquaculture, holding profound implications for broodstock management, monosex culture, and the overall efficiency and sustainability of production systems. This study provides a comprehensive genomic investigation into the sex determination system of the economically important topmouth culter (*Culter alburnus*). Contrary to traditional approaches such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA markers (RAPD), and simple sequence repeats (SSR), next-generation sequencing (NGS) technology have greatly increased the efficiency of sex markers searching and the pace of sex-determination related genetic research. Recently, a batch of sex markers from different fishes were subsequently identified using whole genome resequencing approaches (Zhang et al., 2017; Wen et al., 2023, 2022c; Liu et al., 2018). Through whole-genome resequencing of male and female pools, we have successfully identified a potential sex chromosome, developed a robust male-specific molecular marker, and proposed a candidate gene, *sox10*, that may play a pivotal role in the sex determination pathway. Our findings not only offer practical tools for genetic sex identification but also contribute significantly to the understanding of the evolution of sex chromosomes in teleost fish.

Vertebrates exhibit diverse sex determination systems, encompassing genetic sex determination (GSD) and environmental sex determination (ESD) (Nagahama et al., 2021). GSD primarily relies on sex chromosomes and sex-related genes to establish sexual dimorphism, including male heterogametic system (XX/XY), female heterogametic system (ZW/ZZ), as well as alternative genetic architectures such as the

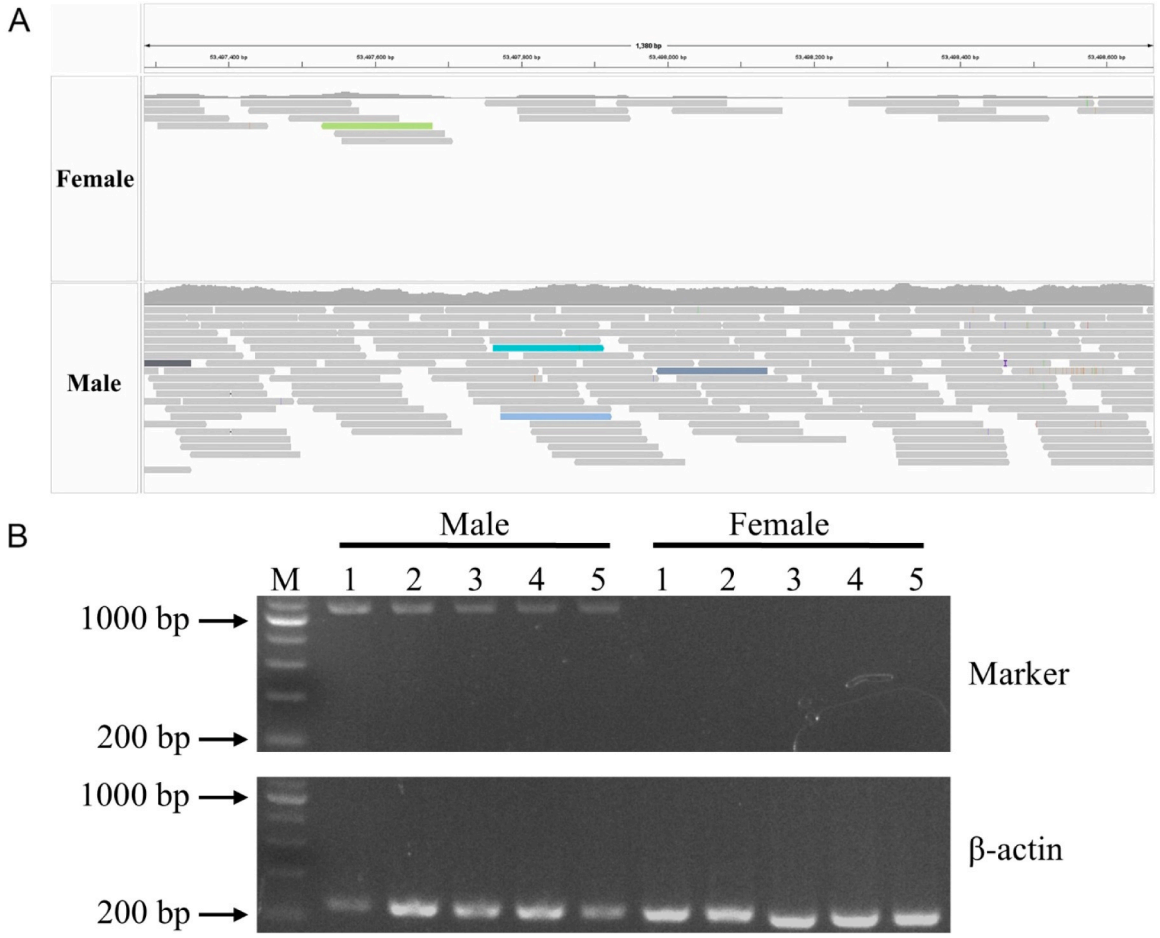


Fig. 3. Identificaton of potential male-specific fragments and development of male-specific markers in topmouth culter. (A) Male-specific genomic fragments identified through genome-wide comparison between males and females were manually checked using IGV. (B) Male-specific molecular markers were Successful developed to identify genetic sex of topmouth culter.

Table 2
One pairs of male-specific primers.

Primers	Forward primer (5′→3′)	Reverse primer	TM (℃)	Product length (bp)
β-actin	TAAAGACCTGTATGCCAACACC	CAGACAGAGTATTTGCGCTCA	56	252
Marker	GCAGTCACATGATCCTTTTTCTC	GGTAACCAAATAGTTGGTGACC	56	1123

XO/XX system and polygenic networks (Zhou et al., 2004). Some other species use more complex systems to determine sex. African cichlid fish possess a polygenic sex determination, where allele interactions generate the male ZZXY genotype and three distinct female genotypes (ZZXX, ZWXX, ZWXY) (Moore et al., 2022). The species *Oplegnathus fasciatus* and *O. punctatus* utilize an X1X2Y sex determination system (Xu et al., 2019). In contrast, ESD is influenced by external environmental factors including temperature, pH levels, and social cues to regulate sexual differentiation rather than relying on direct genetic programming. Previous study reported that progenies of topmouth culter produced through gynogenesis were all female, indicating that this species employed an XX/XY sex determination system (Jia, 2019). In this study, one of the most significant findings of this research is the identification of LG4 as the putative sex chromosome in topmouth culter. The enrichment of male-biased SNPs on LG4, particularly within a narrow region at its beginning, is a classic signature of a nascent sex-determination region (SDR) on a male-heterogametic (XX/XY) system. In such systems, the Y chromosome, harboring the male-determining gene, accumulates sequence differences and structural variations over evolutionary time, which are reflected as clusters of male-specific or male-biased SNPs when compared to the X chromosome. This aligns with findings in other commercially important fish species within the Cyprinidae family and related groups, where such male-heterogametic systems are frequently reported (Wen et al., 2020, 2024).

In our previous researches, bursts of TEs and simple repeat accumulations were observed around sex determination loci (Wen et al., 2020, 2022c). The complexity of SDR in topmouth culter is further highlighted by the coverage ratio and depth analyses. The region exhibiting a lower-than-average sequencing depth suggests the presence of repetitive sequences, structural variations, or regions that are difficult to assemble—a common characteristic of young sex chromosomes. The moderate F_{ST} value observed in this region is particularly insightful. A high F_{ST} would indicate near-complete divergence, as seen in ancient, highly differentiated sex chromosomes like the human Y. A moderate F_{ST}, however, is indicative of a young sex chromosome system where genetic recombination between the X and Y chromosomes has only recently been suppressed, and the sequences are still largely homologous with limited differentiation. This "young" state explains the difficulty in identifying clear, large-scale male-specific sequences through

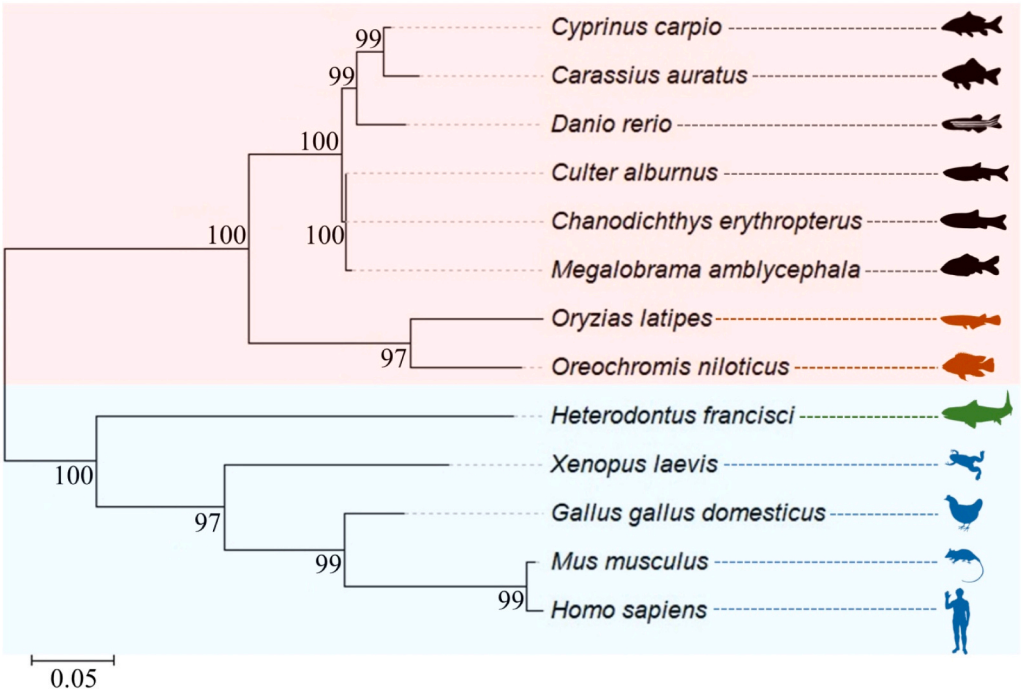


Fig. 4. The *SOX10* phylogenetic tree, The evolutionary history was inferred using the Maximum Likelihood method in MEGA 12. The tree is based on an alignment of SOX10 protein sequences, with bootstrap support tested for 1000 replicates. Branch lengths are scaled to the number of amino acid substitutions per site.

traditional methods and underscores the value of our high-depth resequencing approach in detecting these subtle, yet critical, genomic differences.

Leveraging this genomic insight, we successfully developed a PCR-based male-specific molecular marker. The identification of a small window with half the average depth in males and near-zero depth in females was the critical step that allowed for the extraction of truly male-specific sequences. The subsequent validation of the designed primer pair across three independent populations demonstrated high specificity (100 % in females) and high, though not absolute, universality in males (100 % in Population 1, ~87 % in Population 2, ~95 % in Population 3). This band was not detected in the two male individuals from Population 2 or the one male individual from Population 3, an observation that warrants particular attention. While it could be attributed to technical errors like sampling or DNA quality, it more intriguingly opens the door to biological explanations such as spontaneous sex reversal, a known phenomenon in fish where environmental factors can sometimes override genetic sex, or the presence of rare genetic variants that disrupt the marker binding site. In goldfish, neo-male fish generated by spontaneous sex reversal were also observed (Wen et al., 2020). This finding is of direct practical application for aquaculture. It provides a simple, cost-effective, and non-lethal method for early genetic sex identification in topmouth culter, which is invaluable for establishing monosex breeding populations, a common strategy to control reproduction and improve growth rates, given that one sex often exhibits superior production traits.

Sex control breeding represents a valuable strategy in aquaculture, aimed at enhancing productivity, economic returns, and sustainability through the manipulation of sex ratios or developmental processes. While traditional methods have relied on hormone induction, growing food safety concerns have underscored the need to minimize its use (Mei and Gui, 2015). As a result, sex-specific molecular markers have emerged as a powerful, hormone-free alternative. The efficacy of this approach is demonstrated by several commercially successful varieties, including the "All-male No. 2" yellow catfish (*Pelteobagrus fulvidraco*), the "Taro-excellent No. 1" tongue sole (*Cynoglossus semilaevis*), and the "Yue-Min No. 1" Nile tilapia (*Oreochromis niloticus*) (Liu, Chen, 2023). This technique is particularly advantageous for species like the topmouth culter, which exhibits significant sexual dimorphism: females grow larger and faster than males. Given its late sexual maturity (2–3 years), early sex identification via genetic markers is essential for selective breeding. This study successfully developed a reliable sex-specific marker for the topmouth culter. The application of this marker to facilitate the creation of an all-female population promises to significantly increase aquaculture yield and overall production efficiency for this species.

Teleost fish exhibit remarkable diversity in their sex determination (SD) mechanisms, a series of master regulatory factors were reported in different fish species such as *dmrt1*, *sox3*, *amh*, *amhr2*, *gdf6*, *gsdf*, *sdy* and *bcar1* (Wen et al., 2022a). Specifically, the Sox family genes are well-known to orchestrate reproduction and sex differentiation in bony fishes (Anitha and Senthilkumaran, 2021). For instance, *sox3* has been identified as the master sex-determining gene on the Y chromosome in the fish *Oryzias dancena*, providing direct evidence that Sox members can independently evolve into primary sex-determining triggers (Takehana et al., 2014). Similarly, in topmouth culter, the most promising candidate emerging from our analysis is the *sox10* gene. *Sox10* is a member of the SRY-related HMG-box family of transcription factors, well-known for their critical roles in developmental processes including sex determination and sex differentiation (Hu et al., 2021). While *sox10* is traditionally recognized for its function in neural crest and glial cell development, its presence within a sex-differentiated region in topmouth culter is highly suggestive of a neofunctionalization event. In *Cynoglossus semilaevis*, *Sox10* is a gene with sexually dimorphic expression (Zhang et al., 2019b). Our phylogenetic analysis confirms that the topmouth culter *Sox10* protein is highly conserved and clusters robustly with its orthologs in other cyprinids like common carp and goldfish. This high sequence conservation implies a critical, non-redundant function. The potential role of *sox10* in sex determination is not without precedent. Gene regulatory networks are interconnected, and a mutation placing a key developmental gene like *sox10* under the control of sex-specific regulatory elements or altering its coding sequence could allow it to influence the sex differentiation cascade. A plausible mechanism, as suggested in the literature, is that Sox10 could act as an

upstream regulator of *sox9*, a well-established downstream effector in the male sex differentiation pathway across vertebrates (Da Silva et al., 1996; Polanco et al., 2010; Zhang et al., 2019a). We hypothesize that in topmouth culter, *sox10* may influence sex determination through indirect pathways. Functional validation, such as gene editing, is required to elucidate its potential role.

5. Conclusion

Our study integrates genomics and molecular biology to make significant strides in understanding sex determination in topmouth culter. We have provided strong evidence for an XX/XY system located on LG4, characterized by a young and narrow sex-differentiated region. The development of a highly specific male marker is an immediate practical output that can be directly implemented in breeding programs to enhance aquaculture productivity. Furthermore, the identification of *sox10* as a novel and compelling candidate for the SD gene opens up an exciting new avenue of research. It not only deepens our understanding of sex determination in this specific species but also contributes to the broader picture of the dynamic and repeated evolution of sex-determining mechanisms in vertebrates. The journey from association to causation now requires targeted functional studies to unequivocally confirm the role of *sox10* and to unravel the precise molecular cascade it governs in directing male development in topmouth culter.

CRediT authorship contribution statement

Shaojun Liu: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **wen ming:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation, Conceptualization. **Hong Zhang:** Writing – review & editing, Validation, Data curation. **Quan Shen:** Writing – review & editing. **Min Tao:** Writing – review & editing, Funding acquisition. **Saibin Gong:** Data curation. **Jie Li:** Data curation. **Wenting Rao:** Investigation. **Xiya Fang:** Writing – review & editing. **Yuhua Tang:** Resources, Investigation. **Li Hu:** Investigation. **Jiayi Yang:** Resources, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2026.103463.

Data availability

Data will be made available on request.

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