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Original Research

Genome-Wide Identification, Sequence Alignment, and Transcription of Five Sex-Related Genes in Largemouth Bass (*Micropterus Salmoides*)

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Abstract

Background: Largemouth bass (*Micropterus Salmoides*) is an economically important fish species in China. Most research has focused on its growth, disease resistance, and nutrition improvement. However, the sex-determining genes in largemouth bass are still unclear. The transforming growth factor-beta (TGF-β) gene family, including *amh*, *amhr2* and *gsdf*, plays an important role in the sex determination and differentiation of various fishes. These genes are potentially involved in sex determination in largemouth bass. **Methods**: We performed a systematic analysis of 5 sex-related genes (*amh*, *amhr2*, *gsdf*, *cyp19a1*, *foxl2*) in largemouth bass using sequence alignment, collinearity analysis, transcriptome, and quantitative real-time polymerase chain reaction (qRT-PCR). This included a detailed assessment of their sequences, gene structures, evolutionary traits, and gene transcription patterns in various tissues including gonads, and at different developmental stages. **Results**: Comparative genomics revealed that the 5 sex-related genes were highly conserved in various fish genomes. These genes did not replicate, mutate or lose in largemouth bass. However, some were duplicated (*amh*, *amhr2* and *gsdf*), mutated (*gsdf*) or lost (*amhr2*) in other fishes. Some genes (e.g., *gsdf*) showed significant differences in genomic sequence between males and females, which may contribute to sex determination and sex differentiation in these fishes. qRT-PCR was applied to quantify transcription profiling of the 5 genes during gonadal development and in the adult largemouth bass. Interestingly, *amh*, *amhr2* and *gsdf* were predominantly expressed in the testis, while *cyp19a1* and *foxl2* were mainly transcribed in the ovary. All 5 sex-related genes were differentially expressed in the testes and ovaries from the 56th day post-fertilization (dpf). We therefore speculate that male/female differentiation in the largemouth bass may begin at this critical time-point. Examination of the transcriptome data also allowed us to screen out several more sex-related candidate genes. **Conclusions**: Our results provide a valuable genetic resource for investigating the physiological functions of these 5 sex-related genes in sex determination and gonadal differentiation, as well as in the control of gonad stability in adult largemouth bass.

Keywords: largemouth bass; sex-related gene; transcriptome sequencing; gonadal development; differentially expressed genes

1. Introduction

The study of sex determination in animals is a challenging but critical task. The sex of diverse animals is determined by a hierarchical gene network, and is considered to be one of the most variable processes in evolution [1,2]. Fish are at the intermediate stage of evolution between invertebrates and higher vertebrates. As such they show a much more diverse pattern of sex determination than higher vertebrates, exhibiting both genetic and environmental sex determination systems [3]. For example, arapaima (*Arapaima gigas*) [4] and tiger pufferfish (*Takifugu rubripes*) [5] have an XX/XY sex determination system, while greater amberjack (*Seriola dumerili*) [6] and half-smooth tongue sole (*Cynoglossus semilaevis*) [7] have a ZZ/ZW sex determination system. Barred knifejaw (*Oplegnathus fasciatus*) [8] has an X1X1X2X2/X1X2Y sex determination pattern. The sex of some fish is also affected by the environment [9–11]. Almost all types of vertebrate sex determination patterns have been reported in fish [12–17].

In vertebrates, sex determination and differentiation are often highly plastic and influenced by a range of different genetic and environmental factors [18–21]. The transforming growth factor-beta (TGF-β) signaling pathway is

mainly involved in the formation of tissues and organs, as well as in reproductive development by regulating the growth, proliferation, and differentiation of various cell types [22–24]. Many recent studies have shown that the TGF-β signaling pathway is inextricably linked to sex determination in a diverse array of fish [25]. So far, about 30 sex-determining genes have been reported in fish, of which 20 belong to the TGF-β signaling pathway. These genes are mainly involved in sex determination through gene replication or mutation [2]. In particular, anti-Mullerian hormone (*amh*) induces the degeneration of Mullerian ducts in mammalian males [26]. *amh* was shown to be involved in sex determination in Japanese medaka (*Oryzias latipes*) [27], European perch (*Dicentrarchus labrax*) [28], Nile tilapia (*Oreochromis niloticus*) [29], Northern pike (*Esox lucius*) [30], and black porgy (*Acanthopagrus schlegeli*) [31]. Anti-Mullerian hormone type-II receptor gene (*amhr2*) encodes an important receptor for *amh* and mediates the degeneration of Mullerian ducts [28]. Interestingly, some species have lost *amhr2* [26,32]. However, an extra copy of *amhr2*, named *amhr2y* and located on Y chromosome, was recently identified in several fishes and is believed to act as a master male-determining gene in yellow perch (*Perca flavescens*) [33], tiger puffer [5], Southern catfish (*Silurus meridionalis*) [34], and ayu (*Plecoglossus altivelis*) [35]. Gonadal soma derived factor *(gsdf)*, another member of the TGF-β superfamily, is ubiquitous in bony fishes but has been lost in many tetrapods [36]. Knockout of *gsdf* in male medaka leads to a sex reversal from male to female [37], suggesting that it plays an important role in male sex determination.

Previous studies have reported that the TGF-beta signaling pathway is also involved in regulating the expression of cytochrome P450 family 19 subfamily A member 1 (*cyp19a1*) and forkhead box L2 (*foxl2*). For example, doublesex and mab-3 related transcription factor 1 (*dmrt1*) can directly inhibit *cyp19a1* transcription in Nile tilapia. Overexpression of *dmrt1* resulted in decreased expression of *cyp19a1* and reduced serum estrogen levels in female tilapia, thereby leading to sex reversal [38]. Knockdown of *amhy* in the embryo of male Patagonian silverside (*Odontesthes hatcheri*) leads to the upregulation of *cyp19a1* and *foxl2* mRNAs and to ovarian development [39]. In orange-spotted grouper (*Epinephelus coioides*), *foxl2* recombinant protein increases the expression of *cyp19a1* mRNA, while *foxl3* recombinant protein down-regulates the transcription of *cyp19a1* but up-regulates the transcription of *cyp11b*, which is related to androgen synthesis [40]. In tilapia, *foxl2* up-regulates the expression of *cyp19a1 in vivo*. In female fish, *foxl2* mutation therefore results in sex reversal, decreased expression of *cyp19a*, and reduced serum 17ß-estradiol (E2) level [41,42]. However, a systematic analysis of these 5 sex-related genes (*amh*, *amhr2*, *gsdf*, *cyp19a1*, and *foxl2*) has not yet been reported in largemouth bass.

Largemouth bass is one of the most economically valuable species in the Chinese aquaculture industry, with a production of over 600,000 tons in 2020. It presents a sexual growth dimorphism during early development, with females often living longer and reaching a larger size than males [43]. Our group recently published a draft genome assembly [44] and whole-genome resequencing [45] of largemouth bass. Several previous studies have reported that largemouth bass may have an XX/XY sex determination system [46,47]. These basic studies are very helpful for us to study the sex-determining genes of largemouth bass.

Transcriptome is also an effective technique for identifying sex-determining genes. Several studies have in fact identified sex-related genes in various fishes such as in channel catfish [48], platyfish [49] and rainbow trout [50] through the use of transcriptome analysis. Transcriptomic comparisons could also allow prediction of new sex-related genes in largemouth bass. In the present study, we carried out detailed assessments of the sequences, gene structures, evolutionary traits, and gene expression patterns of 5 sex-related genes (*amh*, *amhr2*, *gsdf*, *cyp19a1*, and *foxl2*). These findings of this work would improve our understanding of the biological roles of these genes in largemouth bass. They should also contribute substantially to in-depth knowledge of sex-related genes in closely related species, such as smallmouth bass (*Micropterus dolomieu*).

2. Materials and Methods

2.1 Genome-Wide Identification of Five Sex-related Genes in Eleven Fish Species

Related protein sequences for *amh*, *amhr2*, *cyp19a1*, *foxl2* and *gsdf* from zebrafish, large yellow croaker, and European seabass were first downloaded from the USA National Center for Biotechnology Information (NCBI). Next, whole genome sequences of 10 representative fishes (Table 1) were downloaded from the NCBI, and the genome dataset of Mandarin fish (*Siniperca chuatsi*) was downloaded from China National Gene Bank Database (CNGBdb). Nucleotide sequences for the 5 sex-related genes were extracted from these genomes using BLAST v.2.2.26 (http://www.ncbi.nlm.nih.gov/blast) (e-value $<1 \times 10^{-5}$), solar v.0.9.6 (The Beijing Genomics Institute (BGI) development, Shenzhen, Guangdong, China), and exonerate v.2.2.0 (https://www.ebi.ac.uk/about/vertebrate-genomics/software/exonerate) with default parameters.

2.2 Phylogenetic and Comparative Genomic Analyses of the Five Sex-Related Genes

A phylogenetic analysis was conducted using the downloaded or extracted amino acid sequences. Multiple protein sequences were aligned using the MUSCLE v.3.7 software (Roque Moraes Drive, Mill Valley, CA, USA) [51] with default parameters. Subsequently, a phylogenetic tree was constructed using IQTREE v.1.6.12 (Center for Integrative Bioinformatics Vienna, Vienna, Austria) [52] based on the maximum likelihood (ML) method and Jones-Taylor-Thornton (JTT+G4) model, with 1000 replicates for

Table 1. Sources of genomic data for identification and extraction of target protein sequences.

Species	Database	Assembly accession	Sex	Common name
Collichthys lucidus	NCBI	GCA_004119915.2	Female	Big head croaker
Dicentrarchus labrax	NCBI	GCA_905237075.1	Male	European seabass
Lateolabrax maculatus	NCBI	GCA_004023545.1	Female	Spotted seabass
Lates Calcarifer	NCBI	GCA_001640805.2	Male	Asian Seabass
Oreochromis niloticus	NCBI	GCA_001858045.3	Female	Nile tilapia
	NCBI	GCA_013350305.1	Female	
	NCBI	GCF_000188235.2	Female	
	NCBI	GCA_922820385.1	Male	
	NCBI	SRA: ERR7448120	Male	
Perca flavescens	NCBI	GCA_004354835.1	Male	Yellow Perch
Larimichthys crocea	NCBI	GCA_900246015.1	Female	Large yellow croaker
Siniperca chuatsi	CNGB	CNP0000961	Female	Mandarin fish
Danio rerio	NCBI	GCA_000002035.4	Unknown	Zebrafish
Micropterus dolomieu	NCBI	GCA_021292245.1	Male	Smallmouth bass
Micropterus salmoides	NCBI	GCA_014851395.1	Female	Largemouth bass
	NCBI	GCA_019677235.1	Male	

NCBI, the USA National Center for Biotechnology Information; CNGB, China National Gene Bank.

evaluation of the branch supports. Detailed locations of upstream and downstream neighboring genes to any sex-related gene on various chromosomes were compared.

2.3 Fish and Sample Collection

One-year-old largemouth bass (five females: body length of 26.6 $\pm$ 4.29 cm and body weight of 457.44 $\pm$ 45.37 g; five males: body length of 28.8 $\pm$ 6.17 cm and body weight of 492.5 $\pm$ 40.45 g) were obtained from a local aquaculture base (Huaxuan Aquatic Co. Ltd., Guangzhou, Guangdong, China). Sampled individuals included only mature specimens with a visible ovary or testis. Fish were anesthetized with tricaine methanesulfonate (MS-222, Fuyu Biotechnology Co., Ltd, Shanghai, China, 70–80 mg/L) and then several tissues including brain, muscle, liver and ovary/testis were dissected, immediately frozen in liquid nitrogen, and stored at –80 °C until use.

Sexually mature largemouth bass were harvested for culture in aerated tanks. The general natural spawning, fertilization, and husbandry procedures were performed as previously described [53]. Approximately 15–20 fry were collected at each time-point from 13 days post-fertilization (dpf) to 56 dpf (8 time points: 13, 18, 23, 28, 33, 45, 50 and 56 dpf). Fry were euthanized with MS-222 (20–30 mg/L for 2 mins) before sample collection. Each fry was cut into two portions (head and trunk), which were immediately frozen in liquid nitrogen and then stored at –80 °C until use. Male and female samples were distinguished using published molecular markers [43,47] that identify male-allele carrying fish at early, undifferentiated stages (**Supplementary Table 1**).

2.4 RNA Extraction

Total RNA was extracted from each sample (total of 72 samples, 0.5–1 cm^3 of tissue per sample) using the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and after RNase-free DNase I (Qiagen) treatment of genomic DNA. The RNA concentration and integrity were examined with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Only those RNA samples with OD260/280 $\geq$1.8 and RNA integrity $\geq$7.0 were selected for transcriptome sequencing.

2.5 Transcriptome Sequencing and Analysis

cDNA libraries from three female and three male gonads were constructed as per the manufacturer's recommendations and then sequenced on an Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) to generate 150-bp paired-end reads. Raw reads were filtered with SOAPnuke v.1.5.6 (BGI, Shenzhen, China) [54] to remove adaptor sequences, those reads with >5% unknown nucleotides, and other low-quality reads. High-quality clean reads from each sample were then mapped to the reference female largemouth bass genome [44] using HISAT2 v.2.1.0 (CCB at JHU, Baltimore, Maryland, USA) [55] with default parameters. RSEM v.1.2.31 (UW-Madison, Madison, WI, USA) [56] was employed to estimate the expression abundance of transcripts. The fragments per kilobase of transcript per million mapped reads (FPKM) method [57] was applied to quantify gene transcription levels. Differentially expressed genes (DEGs) were determined by edgR (https://bioconductor.org/packages/release/bioc/html/edgeR.html) [58] with

default filtering criteria of absolute $\log_2$ (ratio) $\geq$1 and false discovery rate (FDR) $\leq$0.01. DEGs were subjected to Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using DAVID (https://david.ncifcrf.gov/) [59].

2.6 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed to validate data generated by high-throughput sequencing. Tissue-specific expression patterns for the 5 sex-related genes were compared. qRT-PCR was carried out using gene-specific primers (**Supplementary Table 1**) designed by routine Primer6 software (PREMIER Biosoft International, San Francisco, CA, USA).

qRT-PCR was performed on an Applied Biosystems 7300 machine (Applied Biosystems, Foster City, CA, USA). The amplification conditions were set as follows: 95 °C for 3 min and 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. All qRT-PCRs were conducted with three biological replicates of three individuals. *β-actin* was used as the internal reference gene, and the $2^{-\Delta\Delta Ct}$ method [60] was applied to calculate relative transcription values. All data were expressed as the mean $\pm$ standard deviation (SD). The Student's *t* test was applied for statistical analysis, with $p < 0.05$ for considering as statistical significance.

3. Results

3.1 Ubiquitous Presence of the Five Sex-Related Genes in Eleven Representative Bony Fishes

We studied the 5 sex-related genes in the genomes of 11 representative bony fishes. As in most species, largemouth bass was found to contain all the 5 genes with a single copy, whereas gene loss or duplication was found in some other species (Figs. 1,2,3). For example, two *gsdf* genes were identified in European perch (Fig. 2B), which is consistent with previous reports [61]. An earlier study reported a Y-specific duplicate of *amh* (referred to as *amhy*) on the Y chromosome of male Nile tilapia [62]. However, only one *amh* was identified in this published male genome assembly. This inconsistency prompted us to reassemble a haplotype-resolved genome using recently released HiFi reads (NCBI SRA: ERR7448120) in order to validate the *amh* gene (**Supplementary Table 2**). Interestingly, one *amh* gene was identified in haplotype X, whereas two *amh* (*amhy* and *amhΔy*) were characterized in haplotype Y (Fig. 1). This suggests that the previously published assembly of Nile tilapia [63] may have certain defects.

3.2 Phylogenetic and Synteny Analyses

To elucidate the evolutionary history of each sex-related gene, we characterized and compared adjacent genomic regions of each gene locus in representative fishes. This synteny analysis indicated that *amh*, *amhr2*, *cyp19a1*, *gsdf* and *foxl2* are conserved throughout the fish tree of life, with only slight modifications of upstream and downstream neighboring genes (Figs. 1,2,3).

The neighbors for *amh* are *peak1* and *oaz1* (upstream), and *dot1l* and *ell* (downstream) (Fig. 1A). In Nile tilapia, the synteny comparison of X and Y chromosomes confirmed a 10-kb Y-specific insertion within the downstream region of *amhy*. Annotation of this insertion revealed a single gene that is a duplicate copy of *amhy* (termed *amhΔy*; from 2,314,315 bp to 2,317,144 bp in Fig. 1B). *amh*, *amhy*, and *amhΔy* each consists of 7 exons, but *amhΔy* has an early stop codon in the last exon, resulting in a shorter encoded protein sequence (428 aa) compared to its two counterparts (514 aa; Fig. 1C,D).

Most species have only one *gsdf* gene in their genomes (Fig. 2), although the European perch genome contains two *gsdf* genes located on the same chromosome (CAJNNU010000010.1) and sharing 85.71% sequence similarity, but with different neighboring genes (Fig. 2B). Interestingly, in Nile tilapia the protein sequence of *gsdf* (Fig. 2C) differs between the multiple male and female genomes (three females and two males; Table 1), suggesting its potential participation in sex determination. Compared to other species, the genus *Micropterus* (including largemouth bass and its relative smallmouth bass) had less conserved upstream and downstream regions around the *gsdf* gene, with *mmr1* located on other chromosomes (Fig. 2A).

As a receptor for *amh*, *amhr2* plays an important role in sex determination and sex differentiation [64]. A Y-specific insertion of the *amhr2* gene (*amhr2by*) has been identified in yellow perch and is thought to be a candidate sex-determining gene [33]. As in zebrafish, *amhr2* is lost in other cyprinid fishes [32], possibly due to chromosome rearrangement during fish evolution (Fig. 3A). Synteny analyses indicate that both *cyp19a1* and *foxl2* are conserved in the examined species (Fig. 3B,C). However, the *foxl2* neighboring genes are less conserved in zebrafish, implying that it is more distantly related to other fish species.

3.3 Expression Profiling of the Sex-Related Genes in the Developmental Gonads and Adult Tissues of Largemouth Bass

qRT-PCR was performed to characterize the transcription patterns of *amh*, *amhr2*, *gsdf*, *cyp19a1*, and *foxl2* in the liver, muscle, brain, and gonad (testis or ovary) tissues of mature individuals. All genes showed relatively higher transcription levels in the gonad, while low levels or no expression in the other tissues (Fig. 4). This result suggests that these genes are involved in gonad development and maintenance of gonad stability.

amh, *amhr2* and *gsdf* were predominantly transcribed in the testis and less in the ovary, whereas *cyp19a1* and *foxl2* were predominantly expressed in the ovary. The transcription levels of *cyp19a1* and *foxl2* were elevated during gonadal development, with much higher transcription levels observed in female (XX) gonads compared to male (XY) gonads at each of the 8 developmental stages and in the

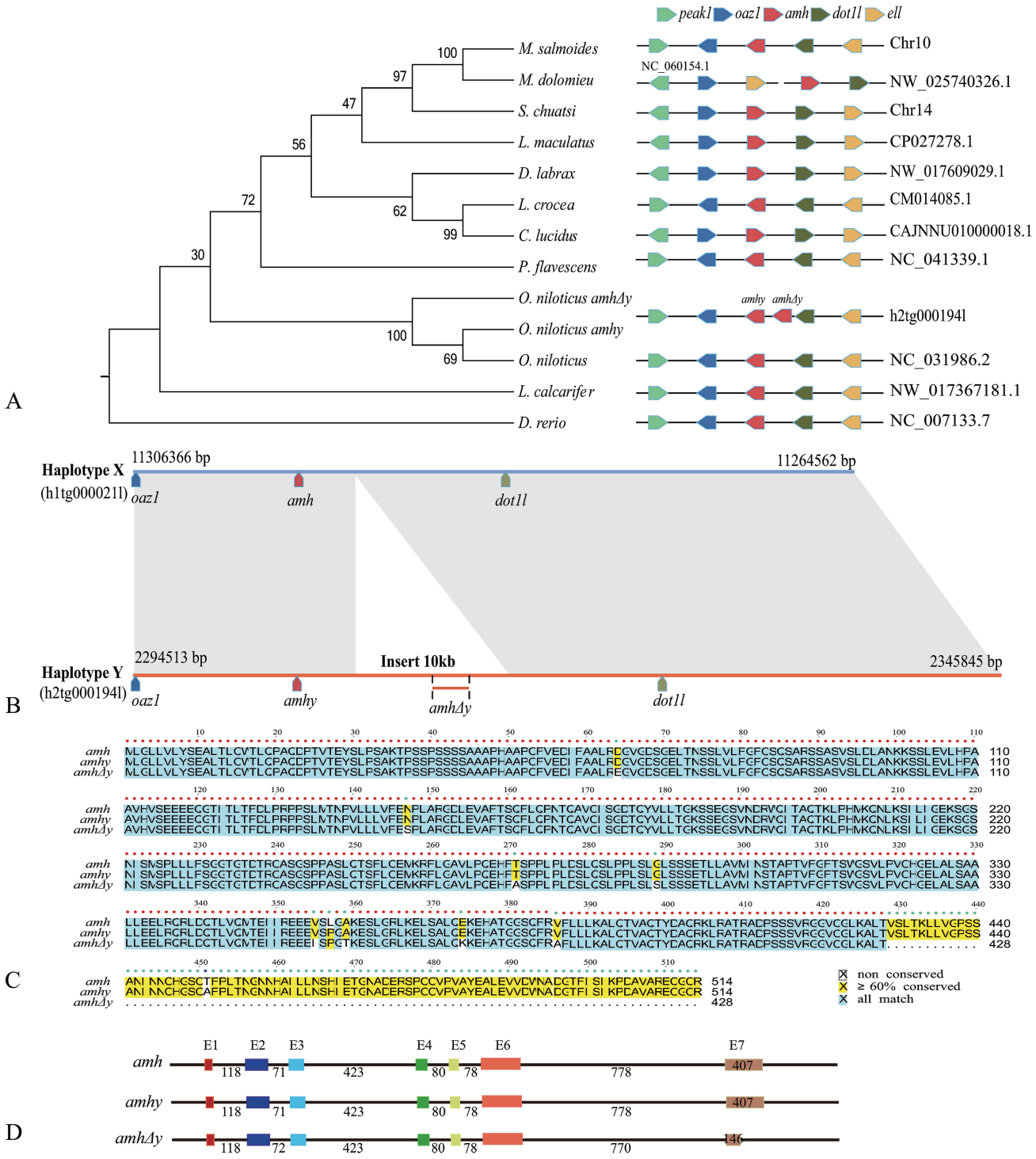

Fig. 1. Characterization of a Y-specific duplication or insertion of the *amhy* gene in the male Nile tilapia genome. (A) The phylogenetic tree and synteny comparison of *amh* in various fishes. The maximum likelihood (ML) tree (left panel) was constructed by IQTREE. Bootstrap values are shown on branches. The right panel presents the synteny of the *amh* gene in each fish species. (B) A synteny comparison between X and Y contigs. Note the Y-specific insertion at the 10-kb region. (C) ClustalW alignments of *amh*, *amhy* and *amhΔy* proteins. (D) Comparison of gene structures among *amh*, *amhy* and *amhΔy*. Rectangles represent exons and lines represent introns. Abbreviations: *dot1l*, DOT1-like histone-lysine N-methyltransferase; *ell*, RNA polymerase II elongation factor ELL; *oaz1*, Ornithine decarboxylase antizyme 1; *peak1*, pseudopodium-enriched atypical kinase 1.

mature gonads. The transcription levels of *amh*, *amhr2* and *gsdf* were elevated during gonadal development, with much higher levels in XY gonads than XX gonads at the 8 developmental stages and in the mature gonads (Fig. 5). The 5

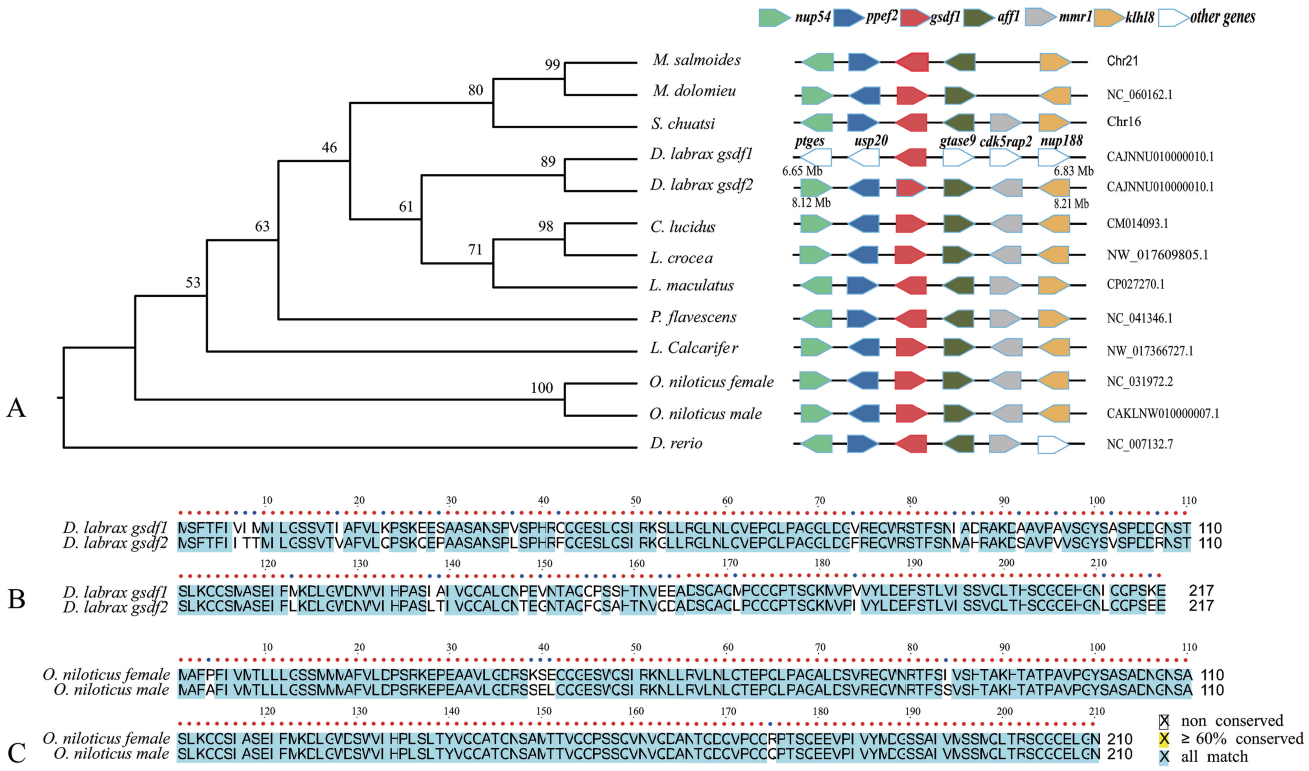

Fig. 2. Comparison of *gsdf* among various fishes. (A) Phylogenetic tree (left) and synteny comparison (right) of *gsdf*. (B,C) ClustalW alignments of *gsdf* proteins in European perch and Nile tilapia. Abbreviations: *aff1*, AF4/FMR2 family member 1; *cdk5rap2*, CDK5 regulatory subunit associated protein 2; *gtase9*, beta-1,3-galactosyltransferase 9; *klhl8*, kelch-like protein 8; *mmr1*, macrophage mannose receptor 1; *nup54*, nucleoporin p54; *ppef2*, serine/threonine-protein phosphatase with EF-hands 2; *ptges*, prostaglandin E synthase; *usp20*, ubiquitin specific peptidase 20; *nup188*, nucleoporin 188.

sex-related genes showed significant differences in expression between males and females, especially at 56 dpf, suggesting this is a critical time-point for the initiation of sex differentiation. In adult gonads, the relative transcription of the 5 sex-related genes (Fig. 4) was consistent with the transcriptome data (Table 2).

3.4 Comparative Transcriptomic Analysis of Testes and Ovaries

A differential expression analysis was performed between testes and ovaries to screen for other sex-related candidate genes. A total of 15,962 differentially expressed genes (DEGs) were predicted. The testis showed 9072 up-regulated DEGs and 6890 down-regulated DEGs compared to the ovary (**Supplementary Figs. 1,2**). To identify genes potentially associated with sexual differentiation and gonadal development, we performed sex-related GO annotation and KEGG pathway analysis of these DEGs. This analysis revealed 7656 DEGs, of which 3636 were matched to 264 KEGG subcategories and to 52 GO terms (**Supplementary Fig. 3**). Many DEGs were predicted to be associated with sex-related GO terms, such as sex differentiation, gamete generation, acrosome assembly, fertilization, and spermatogenesis (Table 3). The transcription of many spermatogenesis-related DEGs, such as sperm surface protein Sp17 (*sp17*) and spermatogenesis-associated protein 22 (*spata22*), was significantly upregulated in the testis, whereas the transcription of some oogenesis-related genes, such as forkhead box protein N5 (*foxn5*) and bone morphogenetic protein 15 (*bmp15*), was markedly upregulated in the ovary.

The DEGs enriched in the sex-related KEGG pathways included the TGF-beta signaling pathway (ko04350), steroid hormone biosynthesis (ko00140), FoxO signaling pathway (ko04068), steroid biosynthesis (ko00100), estrogen signaling pathway (ko04915), Wnt signaling pathway (ko04310), and ovarian steroidogenesis (ko04913) (Table 4). Interestingly, the transcription levels of five *cyp* members (*cyp2k1*, *cyp1a1*, *cyp2u1*, *cyp2j2* and *cyp2f2*) were significantly upregulated in the ovary, whereas the transcription levels of *cyp20a1* and *cyp27c1* were significantly upregulated in the testis. Four *bmp* members (*bmp1a*, *bmp7*, *mbp8a* and *bmp15*) were preferentially expressed in the ovary.

4. Discussion

Most of the master sex-determining genes or candidate genes discovered recently in various fishes belong to the TGF-beta signaling pathway. This pathway appears

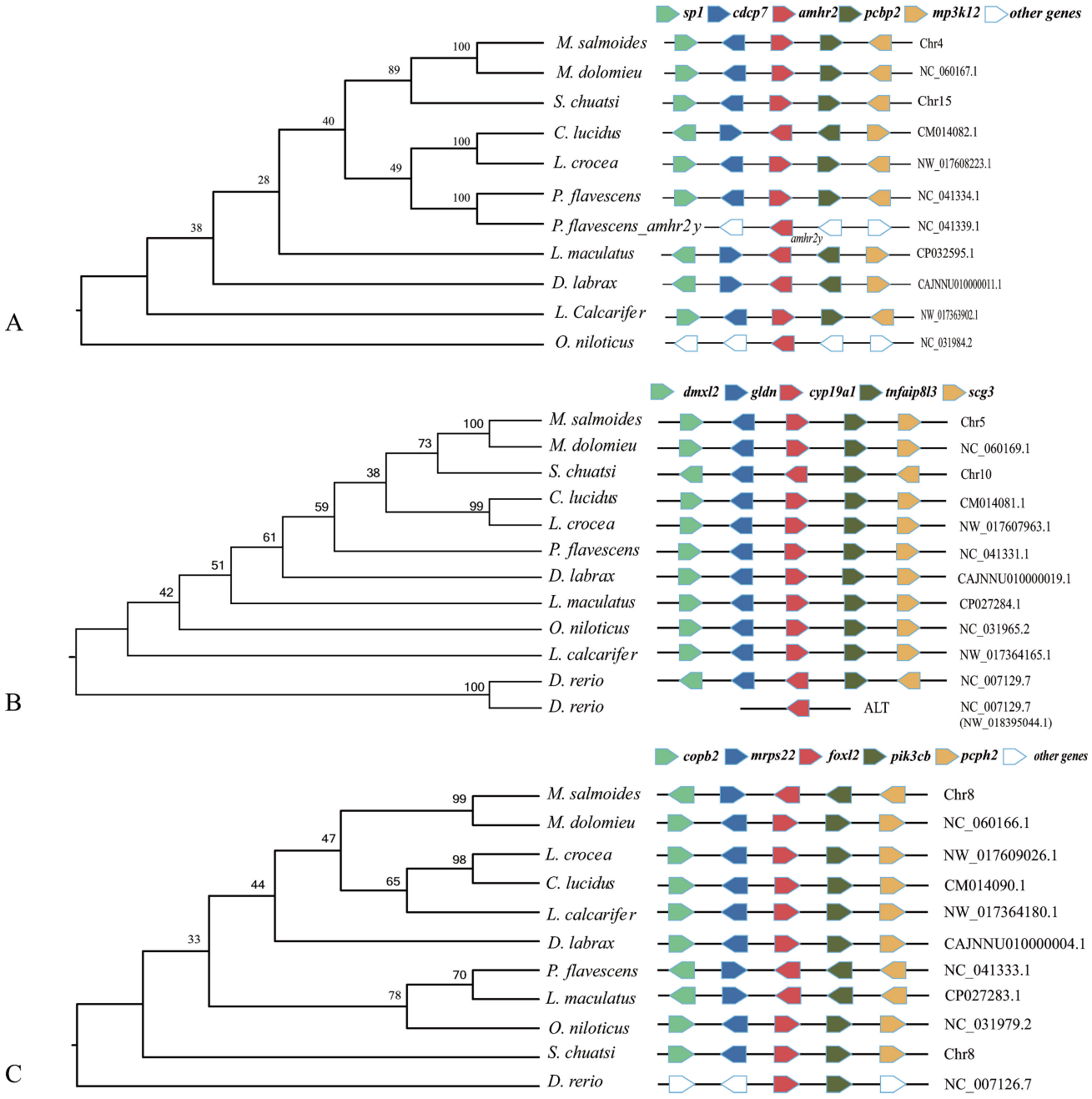

Fig. 3. Phylogenetic trees and synteny comparisons of *amhr2* (A), *cyp19a1* (B) and *foxl2* (C) in various fishes. Abbreviations: *cdcp7*, cell division cycle-associated protein 7; *copb*, coatomer subunit beta; *dmxl2*, dmX-like protein 2; *gldn*, gliomedin; *mp3k12*, Mitogen-activated protein kinase kinase kinase 12; *pcbp2*, Poly(rC)-binding protein 2; *pcph2*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta; *pik3cb*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta; *scg3*, secretogranin-3; *mrps22*, 28S ribosomal protein S22; *sp1*, transcription factor Sp1; *tnfaip8l3*, tumor necrosis factor alpha-induced protein 8-like protein 3.

Table 2. FPKM values of the five sex-related genes in largemouth bass gonads.

Gene	Female 1	Female 2	Female 3	Male 1	Male 2	Male 3	log_2FC (XY/XX)
amh	2.95	3.23	2.65	90.08	125.77	140.61	4.51
amhr2	0.08	0.14	0.12	42.72	44.12	39.5	8.75
gsdf	14.27	7.68	4.33	2002.02	2679.3	2934.29	7.34
cyp19a1	0.25	0.23	0.34	0.05	0.10	0.03	–2.03
foxl2	5.67	4.23	6.78	0.04	0.13	0.04	–6.04

FPKM, fragments per kilobase of transcript per million mapped reads; FC, fold change.

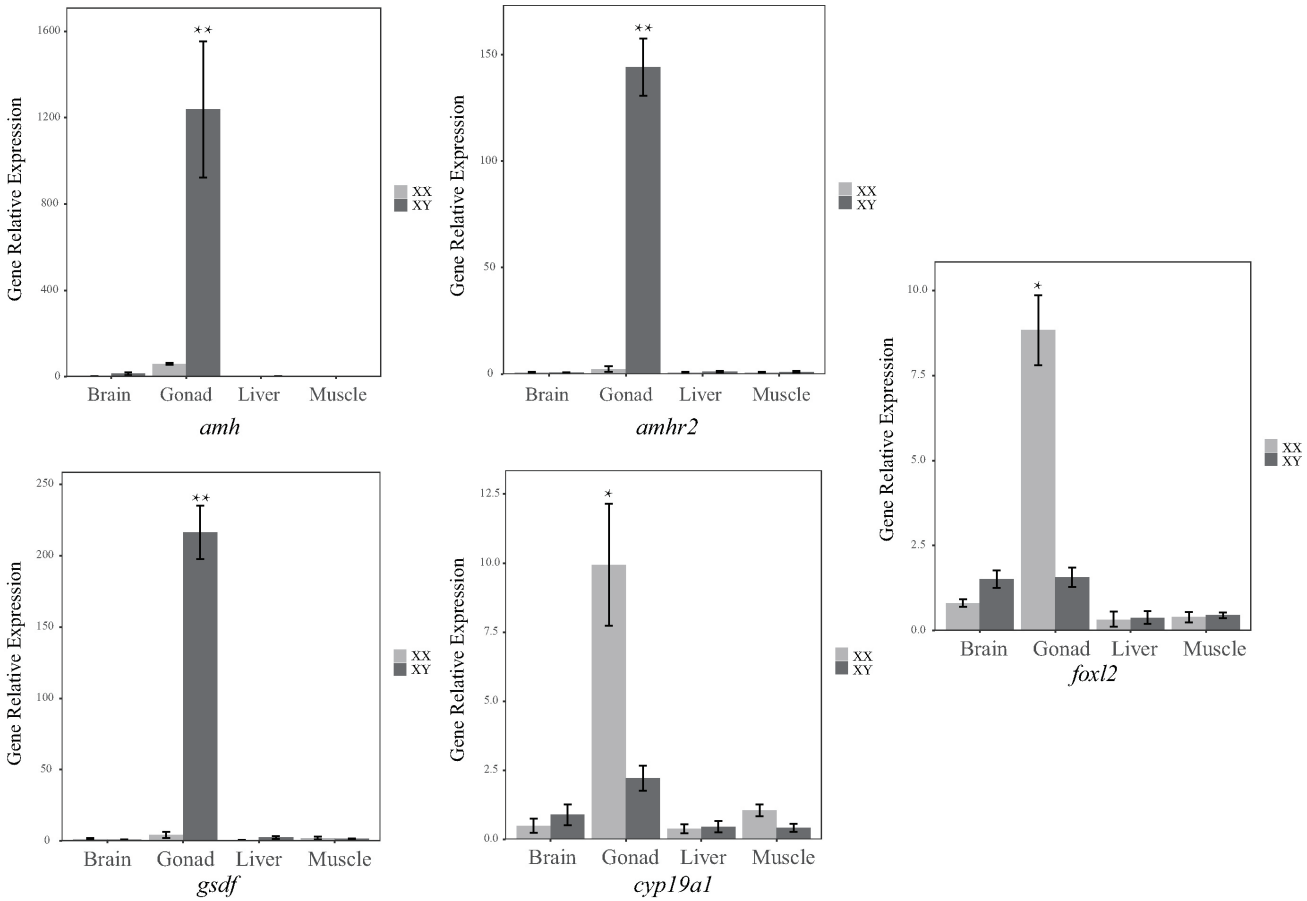

Fig. 4. Relative transcription of the five sex-related genes in different tissues of adult largemouth bass. Data were normalized to *ß-actin*. Values are expressed as relative gene expression (mean ± SD). Significance: * $p < 0.05$, ** $p < 0.01$. SD, standard error.

Table 3. Representative DEGs involved in sex-related GO terms.

GO term	Annotation	Gene name	Log_2FC (XY/XX)	Sex biased
Gamete generation (GO: 0007276)	progesterone receptor	*pgr*	5.31	testis
	membrane-associated progesterone receptor	*mpgr*	1.07	testis
	deleted in azoospermia like	*dazl*	4.71	testis
Fertilization (GO: 0009566)	Sperm surface protein Sp17	*sp17*	3.42	testis
	sperm acrosome membrane-associated protein 4	*samp4*	–15.5	ovary
Sex differentiation (GO: 0007548)	sex determining region Y box 9 protein	*sox9*	1.75	testis
	follistatin related protein	*fsrp*	4.35	testis
	doublesex and mab-3-related transcription factor 1	*dmrt1*	12.76	testis
	bone morphogenetic protein 15	*bmp15*	–6.67	ovary
	doublesex- and mab-3-related transcription factor B1	*dmrtb1*	14.20	testis
	forkhead box protein N5	*foxn5*	–13.16	ovary
Spermatogenesis (GO:0007283)	kelch-like protein 10	*klhl10*	8.92	testis
	spermatogenesis-associated protein 22	*spata22*	4.23	testis
	cilia- and flagella-associated protein 73	*cfap73*	8.76	testis
	coiled-coil domain-containing protein 103	*ccdc103*	6.54	testis
	meiosis expressed gene 1 protein homolog	*meig1*	7.08	testis
Acrosome assembly (GO: 0001675)	zona pellucida sperm-binding protein 3	*zp3*	–12.02	ovary

DEGs, differentially expressed genes; GO, Gene Ontology.

to play a critical role in the process of sex determination and sex differentiation in diverse fishes. For example, *amh* is the sex-determining gene for three-spined stickleback (*Gasterosteus aculeatus*), mandarin fish and Black

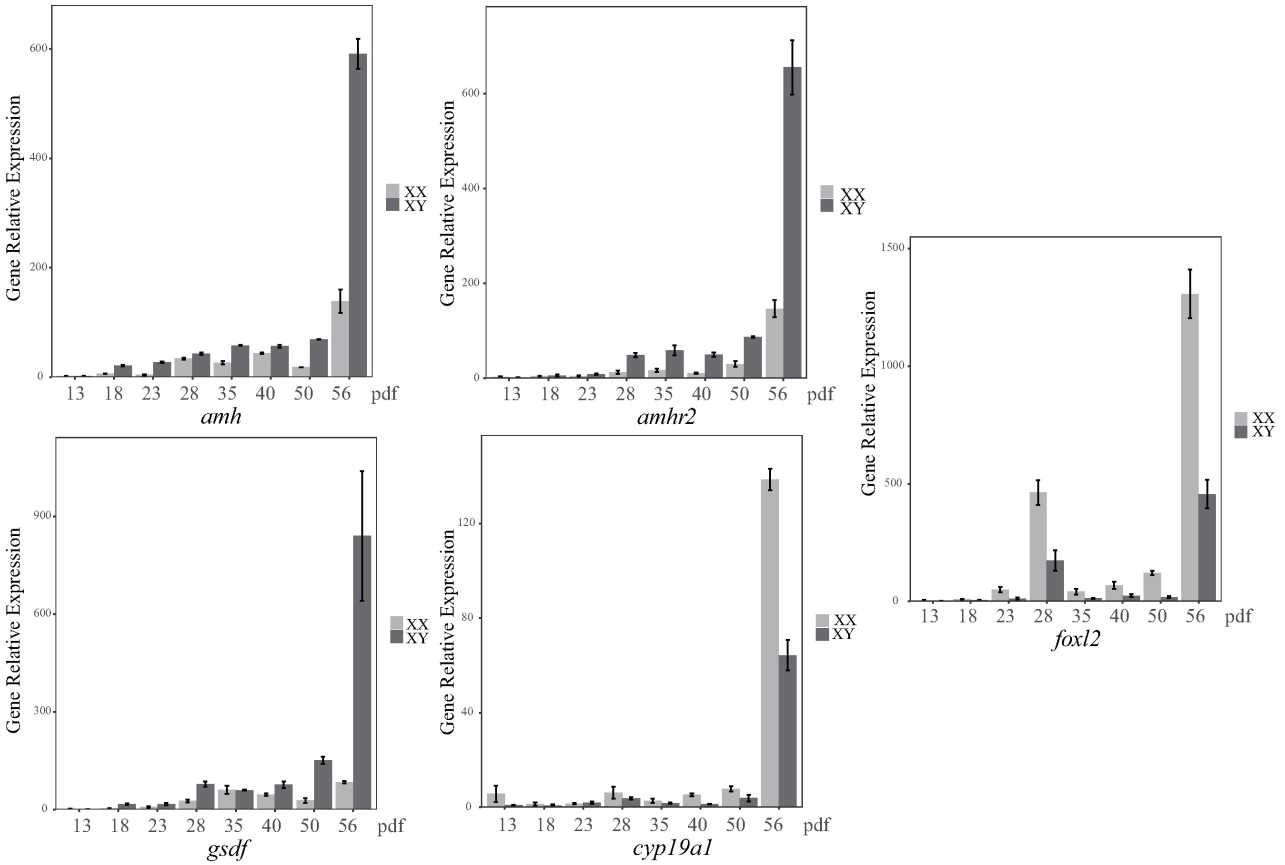

Fig. 5. Relative transcription levels of the five sex-related genes at eight different developmental stages in the gonads of largemouth bass. Data were normalized to *ß-actin*. Values are expressed as relative gene expression (mean ± SD).

rockfish (*Sebastes schlegelii*) [65–67]. *gsdf* for sablefish [68], and *bmpr1bby* for Atlantic herring (*Clupea harengus*) [69]. TGF-beta signaling pathway may also be able to influence sex determination by inhibiting aromatase activity [70]. Knockdown of the sex determination gene *amhy* in male Patagonian silverside can activate the expression of downstream *foxl2* and *cyp19a1a*. These are important genes in the female signaling pathway and can lead to increased estrogen synthesis, thereby causing sexual reversal from male to female [39]. However, the detailed mechanisms involved in sex determination, sex differentiation, and gonadal development in largemouth bass have thus far remained unclear.

In our current study, evolutionary and collinear analyses revealed that the 5 sex-related genes (*amh*, *anhr2*, *cyp19a1*, *gsdf* and *foxl2*) were generally conserved in 11 representative teleost fishes. However, some genes were found to be duplicated (*amh*, *amhr2* and *gsdf*), mutated (*gsdf*) or lost (*amhr2*) in several fish genomes (Figs. 1,2,3). In certain Perciformes species, some of these genes (*amh*, *amhr2* and *gsdf*) were reported to be replicated or altered to become the master sex-determining genes [67]. Therefore, most sex-determining genes may be derived from their orthologous genes (usually sex-related genes) by altering the structures of encoded proteins.

In male Nile tilapia, for example, an extra *amhΔy* gene is inserted into the Y-specific sequence (Fig. 1B). Knockout of this gene results in male-to-female sex reversal [62], suggesting that it is a master sex-determining gene. Sablefish have different X and Y chromosome copies of *gsdf*, with *gsdfY* possessing a spatio-temporal expression profile that is characteristic of a male master sex determination gene [68]. The present study also found differences in the *gsdf* gene coding sequence between male and female Nile tilapia. However, their roles and regulatory mechanisms in sex determination need further investigation. *amhr2* is an *amh* receptor involved in the sex determination and sex differentiation of various fishes [34]. In yellow perch, a male-specific duplicate of *amhr2*, considered to be a candidate sex determining gene, was inserted at the proximal end of the Y chromosome (NC_041339.1) [33]. *amhr2* SNPs in tiger pufferfish are sex-linked, with heterozygous males and homozygous females [5]. These sex-specific SNPs are also identified in Panther puffer (*Takifugu pardalis*) and spotted puffer (*Takifugu poecilonotus*). However, cyprinid species such as zebrafish, common carp, grass carp and golden-line barbels appear to have lost *amhr2* during evolution. The *amh* receptor role might be replaced by another type II receptor, most likely bone morphogenetic protein receptor type 2 (*bmpr2*) [26]. Of

Table 4. Representative DEGs involved in sex-related KEGG pathways.

KEGG pathway	Annotation	Gene name	log_2FC (XY/XX)	Sex biased
TGF-beta signaling pathway	bone morphogenetic protein 7	*bmp7*	–9.01	ovary
	bone morphogenetic protein receptor type-1A	*bmpr1a*	–1.85	ovary
	bone morphogenetic protein 8A	*bmp8a*	–12.6	ovary
	bone morphogenetic protein 15	*bmp15*	–6.67	ovary
	anti-Mullerian hormone	*amh*	4.51	testis
	follistatin related protein	*fsrp*	4.35	testis
	follistatin-related protein 3	*fsrp3*	5.56	testis
	transforming growth factor beta-2	*tgfb2*	5.53	testis
FoxO signaling pathway	insulin receptor	*ir*	2.77	testis
	growth differentiation factor 9	*gdf9*	–6.51	ovary
	epidermal growth factor-like protein 7	*egfl7*	3.8	testis
	transcriptional regulator Myc-B-like	*mycbl*	–4.07	ovary
	transcription factor Sp1	*sp1*	–1.68	ovary
Estrogen signaling pathway	heat shock cognate 71 kDa protein	*hsc71*	–1.64	ovary
	SHC-transforming protein 2	*shc1*	–11.4	ovary
Ovarian steroidogenesis	scavenger receptor class B member 1	*scarb1*	–6.05	ovary
	cytochrome P450 2K1	*cyp2k1*	–8.89	ovary
	estradiol 17-beta-dehydrogenase 1	*hsd17b1*	–5.67	ovary
	cytochrome P450 1A1	*cyp1a1*	–3.5	ovary
	cytochrome P450 2U1	*cyp2u1*	–7.25	ovary
	cytochrome P450 2J2	*cyp2j2*	–2.89	ovary
	cytochrome P450 2F2	*cyp2f2*	–4.0	ovary
	steroidogenic acute regulatory protein	*star*	5.67	testis
	insulin-like growth factor 1	*igf1*	6.79	testis
	cytochrome P450 20A1	*cyp20a1*	1.5	testis
	cytochrome P450 27C1	*cyp27c1*	4.33	testis
Steroid hormone biosynthesis	steroid 11-beta-hydroxylase	*cyp11b*	13.29	testis
	steroid 17-alpha-hydroxylase/17,20	*cyp17a1*	7.42	testis
	estradiol 17-beta-dehydrogenase 8	*17β-hsd8*	2.17	testis
Wnt signaling pathway	frizzled-3	*fzd3*	4.3	testis
	frizzled-6	*fzd6*	6.5	testis
	frizzled-7	*fzd7*	3.1	testis
	transcription factor 7	*tcf7*	15.3	testis

KEGG, Kyoto Encyclopedia of Genes and Genomes.

note, zebrafish have a different sex-determination mechanism compared to other teleost fishes, partially due to the absence of *amhr2* [32]. In contrast to cyprinid fishes, results from the present study show the *amhr2* gene was present in all Perciformes, and even duplicated in some other species (Fig. 3A). It appears therefore that the *amhr2* gene may have gradually evolved during the fish evolutionary process. These results also confirm the diversity and complexity of sex-determining genes in fish. This complexity poses a great challenge to the practical identification of sex determination genes.

Compared with other fishes, largemouth bass and smallmouth bass are conservative in that they contain all five sex-related genes studied here, without duplication, loss, or differences in gene features between males and females. Nevertheless, having no differences at the gene level does not rule out that they are sex-related, since many studies have reported that differences at the expression level can also determine a fish's sex. For example, the sex-determining gene in channel catfish, breast cancer anti-resistance 1 (*bcar1*), shows similar male and female genome sequences. However, a comparative transcriptome analysis revealed that sex-specific isoform expression through alternative splicing may underlie the sex determination processes [71]. Based on our comparative transcriptome data of gonads from adult largemouth bass, we found no new alternative splicing transcripts for the five sex-related genes, suggesting that their transcript sequences were also conserved in the mature gonads. Interestingly, the sex-determining region (~1.7 Mb) in largemouth bass was previously reported on Chr7. However, this large non-recombinant region of the Chr7 does not contain any obvi-

ously potential master genes for sex determination [43]. In our current study, the 5 sex-related genes were distributed on different chromosomes of the largemouth bass genome (i.e., not just on the Chr7). This finding suggests that the identification of sex-determining genes in largemouth bass remains difficult.

To further investigate the 5 sex-related genes, we conducted expression analyses of adult tissues and gonads at different developmental stages using transcriptome sequencing and qRT-PCR. The 5 sex-related genes displayed significant differences in transcription between the ovary and testis at different developmental stages and in adult gonads (Figs. 4,5). The *gsdf* gene in particular exhibited the highest transcription level in males (XY) (Table 2 and Fig. 4), consistent with those findings reported in Nile tilapia [32]. Overexpression of *gsdf* in the developing gonads of medaka and Nile tilapia converted XX individuals into functional males [72,73]. Furthermore, knockout of *gsdf* in XY fish resulted in male-to-female sex reversal [37,74], suggesting that *gsdf* could be involved in male sex differentiation, sex maintenance, and testis development in medaka and Nile tilapia. Both *amh* and *amhr2* exhibited higher transcription levels in the testis than the ovary, implying that these genes may play a role in testis development in largemouth bass.

During the natural sex reversal process of ricefield eel (*Monopterus albus*), increased expression of *amh* and decreased expression of dosage-sensitive sex reversal (*dax1*) in the ovaries are important for the activation of testis development. High expression of *amh* and low expression of *dax1* are necessary for the maintenance of testis functions [75]. In contrast, *cyp19a1* and *foxl2* are expressed at higher levels in the ovary than in the testis. In olive flounder (*Paralichthys olivaceus*), *foxl2* may play an important role in ovarian differentiation by maintaining *cyp19a1* expression and antagonizing the expression of *dmrt1* [76]. These results indicate that *foxl2* and *cyp19a1* promote ovarian development and male-related gene expression. In largemouth bass, the expression of the 5 sex-related genes was significantly different between adult male and female gonads, and during gonadal development. *amh*, *amhr2* and *gsdf* were predominantly expressed in the testis, whereas *cyp19a1* and *foxl2* were mainly transcribed in the ovary. These differences indicate the genes may have important roles in the process of sex differentiation and the sex stability of largemouth bass. At 86 dpf in Senegalese sole (*Solea senegalensis*), the follicle stimulating hormone receptor (*fshr*) can activate other gonadal marker genes (such as *amh* and *cyp19a1*) to promote gonad differentiation [77]. In the present study, *amh*, *amhr2*, *gsdf*, *cyp19a1* and *foxl2* were significantly expressed in the testes and ovaries on 56 dpf. We therefore speculate that differentiation between males and females in largemouth bass starts at about the 50th day after fertilization.

In the present study, we also performed comprehensive transcriptomic analyses of the testis and ovary in largemouth bass. Differential expression analysis allowed us to identify multiple sex-biased genes that may be associated with steroidogenic hormones, ovarian steroidogenesis, and sex differentiation. In particular, several additional members of the TGF-beta signaling pathway were identified that showed significantly different expression between the testis and ovary. These include *bmp7*, *bmpr1a*, *bmp8a*, *fsrp*, *fsrp3*, *tgfb2*, *ir*, *gdf9*, SMAD family member 1 (*smad1*), *smad2*, *smad6b*, *smad7*, inhibitor of DNA binding 3 (*id3*), repulsive guidance molecule BMP co-receptor a (*rgma*), and activin A receptor type 1 (*acvr1*). A comparative transcriptome analysis of yellowfin bream (*Acanthopagrus latus*) identified several genes in the TGF-beta signaling pathway that were differentially expressed, including *bmp1-8*, *smad1-8*, follistatin and transcription factor e2f4 [78]. *id2* and *id3* are members of the dominant-negative, basic helix-loop-helix transcription factor family. The *id2bbY* gene is a duplicated copy of the autosome *id2b* gene on the Y chromosome, which has been reported as a sex-determining gene in arapaima [4]. Gonadal transcriptome analyses of *Channa argus* and *C. maculate* showed that the transcription level of *id2* in the testis was significantly lower than that in the ovary [79]. The *id3* expression level was also significantly different between the male and female gonads of largemouth bass, suggesting that it may be critical for gonad development in this fish.

Dmrt and the transcription factor Sox (*sox*) are well-known sex-related gene families in various animals. *Dmrt1* plays an important role in sex determination, male germ cell differentiation, and sex maintenance in diverse mammals, birds, and fishes [42,80–82]. Moreover, *dmrt2-5* are important for testis development in many fish species [83–85]. Our results showed that *dmrt1*, *dmrtb1*, *dmrta1*, *dmrt2* and *dmrt3* were highly expressed in largemouth bass testis (Table 4). The *sox* family has multiple roles in several biological processes, including gonad development and sex determination. *sox3* is a sex-determining gene in Indian ricefish (*Oryzias dancena*) [72], and *sox9* cooperates with *sox8* to protect the adult testis from male-to-female genetic reprogramming and complete degeneration [86,87]. High expression levels of *sox2*, *sox5*, *sox7*, *sox9a* and *sox10* in the testis, and of *sox3*, *sox11* and *sox19b* in the ovary suggests that *sox* genes may have complex roles in the sex differentiation of largemouth bass. Interestingly, similar results were reported in yellowfin bream [88].

5. Conclusions

This study characterized 5 sex-related genes (including *amh*, *amhr2*, *cyp19a1*, *foxl2* and *gsdf*) in largemouth bass and in 11 other teleost representatives, including their transcription profiles. Phylogenetic and comparative genomics analyses provided additional evidence for validating the orthologs of these genes. Transcriptome sequencing

and qRT-PCR revealed distinct transcription levels for these sex-related genes in various tissues of largemouth bass and at different stages of development. In summary, this study provides a comprehensive overview of these sex-related genes in largemouth bass. These interesting data establishes a basic foundation for in-depth functional analyses of these genes in relation to sex determination and sex differentiation.

Availability of Data and Materials

The transcriptome raw reads were deposited in NCBI BioProject database under accession number PRJNA973811.

Author Contributions

QS and XYe conceived and designed the research. XZ, CS, ZR, YH, XL, HL and XYou performed data analyses. XZ, ZR, CH, JX, XW and CS prepared samples and data. XZ and CS wrote the manuscript. QS, XYe, XL, HL, YH and XYou revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All the experiments on fishes were conducted in accordance with the specific guidelines on the care and use of animals for scientific purposes as outlined by the Institutional Animal Care and Use Committee (IACUC) of the Pearl River Fisheries Institute, Chinese Academy of Fishery Sciences (CAFS), China. The IACUC approved this study under the CAFS project "Breeding of LMB-2019".

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2902063.

References

[1] Schartl M, Schories S, Wakamatsu Y, Nagao Y, Hashimoto H, Bertin C, *et al*. Sox5 is involved in germ-cell regulation and sex determination in medaka following co-option of nested transposable elements. BMC Biology. 2018; 16: 16.

[2] Chen J, Zhu Z, Hu W. Progress in research on fish sex determining genes. Water Biology and Security. 2022; 1: 100008.

[3] Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, *et al*. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99: 11778–11783.

[4] Adolfi MC, Du K, Kneitz S, Cabau C, Zahm M, Klopp C, *et al*. A duplicated copy of id2b is an unusual sex-determining candidate gene on the Y chromosome of arapaima (Arapaima gigas). Scientific Reports. 2021; 11: 21544.

[5] Kamiya T, Kai W, Tasumi S, Oka A, Matsunaga T, Mizuno N, *et al*. A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, Takifugu rubripes (fugu). PLoS Genetics. 2012; 8: e1002798.

[6] Koyama T, Nakamoto M, Morishima K, Yamashita R, Yamashita T, Sasaki K, *et al*. A SNP in a Steroidogenic Enzyme Is Associated with Phenotypic Sex in Seriola Fishes. Current Biology: CB. 2019; 29: 1901–1909.e8.

[7] Liao X, Xu G, Chen SL. Molecular method for sex identification of half-smooth tongue sole (Cynoglossus semilaevis) using a novel sex-linked microsatellite marker. International Journal of Molecular Sciences. 2014; 15: 12952–12958.

[8] Xiao Y, Xiao Z, Ma D, Zhao C, Liu L, Wu H, *et al*. Chromosome-Level Genome Reveals the Origin of Neo-Y Chromosome in the Male Barred Knifejaw Oplegnathus fasciatus. IScience. 2020; 23: 101039.

[9] Baroiller JF, D'Cotta H, Saillant E. Environmental effects on fish sex determination and differentiation. Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation. 2009; 3: 118–135.

[10] Díaz N, Piferrer F. Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. BMC Genomics. 2015; 16: 679.

[11] Yamamoto Y, Zhang Y, Sarida M, Hattori RS, Strüssmann CA. Coexistence of genotypic and temperature-dependent sex determination in pejerrey Odontesthes bonariensis. PloS one. 2014; 9: e102574.

[12] Chen J, Hu W, Zhu Z. Progress in studies of fish reproductive development regulation. Chinese Science Bulletin. 2012; 58: 7–16.

[13] Mei J, Gui JF. Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish. Science China. Life Sciences. 2015; 58: 124–136.

[14] Peichel CL, McCann SR, Ross JA, Naftaly AFS, Urton JR, Cech JN, *et al*. Assembly of the threespine stickleback Y chromosome reveals convergent signatures of sex chromosome evolution. Genome Biology. 2020; 21: 177.

[15] Takehana Y, Naruse K, Hamaguchi S, Sakaizumi M. Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, Oryzias hubbsi and O. dancena. Chromosoma. 2007; 116: 463–470.

[16] Xiong Y, Wang S, Gui JF, Mei J. Artificially induced sex-reversal leads to transition from genetic to temperature-dependent sex determination in fish species. Science China. Life Sciences. 2020; 63: 157–159.

[17] Smith C, Wootton RJ. The remarkable reproductive diversity of teleost fishes. Fish and Fisheries. 2016; 17: 1208–1215.

[18] Nagahama Y. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiology and Biochemistry. 2005; 31: 105–109.
[19] Nagahama Y, Chakraborty T, Paul-Prasanth B, Ohta K, Nakamura M. Sex determination, gonadal sex differentiation, and plasticity in vertebrate species. Physiological Reviews. 2021; 101: 1237–1308.
[20] Forconi M, Canapa A, Barucca M, Biscotti MA, Capriglione T, Buonocore F, *et al*. Characterization of sex determination and sex differentiation genes in Latimeria. PloS one. 2013; 8: e56006.
[21] Shen ZG, Wang HP. Molecular players involved in temperature-dependent sex determination and sex differentiation in Teleost fish. Genetics, Selection, Evolution: GSE. 2014; 46: 26.
[22] Wu M, Chen G, Li YP. TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Research. 2016; 4: 16009.
[23] Massagué J. TGFβ signalling in context. Nature Reviews. Molecular Cell Biology. 2012; 13: 616–630.
[24] Mullen AC, Wrana JL. TGF-β Family Signaling in Embryonic and Somatic Stem-Cell Renewal and Differentiation. Cold Spring Harbor Perspectives in Biology. 2017; 9: a022186.
[25] Long J, Zheng SQ, Wang XS, Zhang S, Wang DS. Role of TGF-β signaling pathway in sex determination and differentiation in fish. Journal of Fisheries of China. 2020; 44: 166–177. (In Chinese)
[26] Yan YL, Batzel P, Titus T, Sydes J, Desvignes T, BreMiller R, *et al*. A Hormone That Lost Its Receptor: Anti-Müllerian Hormone (AMH) in Zebrafish Gonad Development and Sex Determination. Genetics. 2019; 213: 529–553.
[27] Klüver N, Pfennig F, Pala I, Storch K, Schlieder M, Froschauer A, *et al*. Differential expression of anti-Müllerian hormone (amh) and anti-Müllerian hormone receptor type II (amhrII) in the teleost medaka. Developmental Dynamics: an Official Publication of the American Association of Anatomists. 2007; 236: 271–281.
[28] Halm S, Rocha A, Miura T, Prat F, Zanuy S. Anti-Müllerian hormone (AMH/AMH) in the European sea bass: its gene structure, regulatory elements, and the expression of alternatively-spliced isoforms. Gene. 2007; 388: 148–158.
[29] Cáceres G, López ME, Cádiz MI, Yoshida GM, Jedlicki A, Palma-Véjares R, *et al*. Fine Mapping Using Whole-Genome Sequencing Confirms Anti-Müllerian Hormone as a Major Gene for Sex Determination in Farmed Nile Tilapia (*Oreochromis niloticus* L.). G3 (Bethesda, Md.). 2019; 9: 3213–3223.
[30] Pan Q, Feron R, Yano A, Guyomard R, Jouanno E, Vigouroux E, *et al*. Identification of the master sex determining gene in Northern pike (Esox lucius) reveals restricted sex chromosome differentiation. PLoS Genetics. 2019; 15: e1008013.
[31] Wu GC, Chiu PC, Lyu YS, Chang CF. The expression of amh and amhr2 is associated with the development of gonadal tissue and sex change in the protandrous black porgy, Acanthopagrus schlegeli. Biology of Reproduction. 2010; 83: 443–453.
[32] Zheng S, Long J, Liu Z, Tao W, Wang D. Identification and Evolution of TGF-β Signaling Pathway Members in Twenty-Four Animal Species and Expression in Tilapia. International Journal of Molecular Sciences. 2018; 19: 1154.
[33] Feron R, Zahm M, Cabau C, Klopp C, Roques C, Bouchez O, *et al*. 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. Molecular Ecology Resources. 2020; 20: 531–543.
[34] Zheng S, Tao W, Yang H, Kocher TD, Wang Z, Peng Z, *et al*. Identification of sex chromosome and sex-determining gene of southern catfish (*Silurus meridionalis*) based on XX, XY and YY genome sequencing. Proceedings. Biological Sciences. 2022; 289: 20212645.
[35] Nakamoto M, Uchino T, Koshimizu E, Kuchiishi Y, Sekiguchi R, Wang L, *et al*. A Y-linked anti-Müllerian hormone type-II receptor is the sex-determining gene in ayu, Plecoglossus altivelis. PLoS Genetics. 2021; 17: e1009705.
[36] Gautier A, Le Gac F, Lareyre JJ. The gsdf gene locus harbors evolutionary conserved and clustered genes preferentially expressed in fish previtellogenic oocytes. Gene. 2011; 472: 7–17.
[37] Zhang X, Guan G, Li M, Zhu F, Liu Q, Naruse K, *et al*. Autosomal gsdf acts as a male sex initiator in the fish medaka. Scientific Reports. 2016; 6: 19738.
[38] Wang DS, Zhou LY, Kobayashi T, Matsuda M, Shibata Y, Sakai F, *et al*. Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. Endocrinology. 2010; 151: 1331–1340.
[39] Hattori RS, Murai Y, Oura M, Masuda S, Majhi SK, Sakamoto T, *et al*. A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109: 2955–2959.
[40] Zhang C, He Q, Cheng H, Li L, Ruan X, Duan X, *et al*. Transcription factors foxl2 and foxl3 regulate cyp19a1a and cyp11b in orange-spotted grouper (Epinephelus coioides). Aquaculture Reports. 2022; 25: 101243.
[41] Zhang X, Li M, Ma H, Liu X, Shi H, Li M, *et al*. Mutation of foxl2 or cyp19a1a Results in Female to Male Sex Reversal in XX Nile Tilapia. Endocrinology. 2017; 158: 2634–2647.
[42] Li MH, Yang HH, Li MR, Sun YL, Jiang XL, Xie QP, *et al*. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. Endocrinology. 2013; 154: 4814–4825.
[43] Wen M, Zhang Y, Wang S, Hu F, Tang C, Li Q, *et al*. Sex locus and sex markers identification using whole genome pool-sequencing approach in the largemouth bass (Micropterus Salmoides L.). Aquaculture. 2022; 559: 738375.
[44] Sun C, Li J, Dong J, Niu Y, Hu J, Lian J, *et al*. Chromosome-level genome assembly for the largemouth bass Micropterus salmoides provides insights into adaptation to fresh and brackish water. Molecular Ecology Resources. 2021; 21: 301–315.
[45] Sun CF, Zhang XH, Dong JJ, You XX, Tian YY, Gao FY, *et al*. Whole-genome resequencing reveals recent signatures of selection in five populations of largemouth bass (*Micropterus-salmoides*). Zoological Research. 2023; 44: 78–89.
[46] He Q, Ye K, Han W, Yekefenhazi D, Sun S, Xu X, *et al*. Mapping sex-determination region and screening DNA markers for genetic sex identification in largemouth bass (Micropterus salmoides). Aquaculture. 2022; 559: 738450.
[47] Du J, Zhou J, Li S, Shao J, Jiang P, Dong C, *et al*. A PCR-based method for genetic sex identification and evidence of the XX/XY sex determination system in largemouth bass (Micropterus salmoides). Aquaculture. 2021; 545: 737220.
[48] Sun F, Liu S, Gao X, Jiang Y, Perera D, Wang X, *et al*. Male-biased genes in catfish as revealed by RNA-Seq analysis of the testis transcriptome. PloS one. 2013; 8: e68452.
[49] Zhang Z, Wang Y, Wang S, Liu J, Warren W, Mitreva M, *et al*. Transcriptome analysis of female and male Xiphophorus maculatus Jp 163 A. PloS one. 2011; 6: e18379.
[50] Yano A, Guyomard R, Nicol B, Jouanno E, Quillet E, Klopp C, *et al*. An immune-related gene evolved into the master sex-determining gene in rainbow trout, Oncorhynchus mykiss. Current Biology: CB. 2012; 22: 1423–1428.
[51] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research. 2004; 32: 1792–1797.
[52] Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-

TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution. 2015; 32: 268–274.
[53] Hussein GHG, Chen M, Qi PP, Cui QK, Yu Y, Hu WH, *et al*. Aquaculture industry development, annual price analysis and out-of-season spawning in largemouth bass Micropterus salmoides. Aquaculture. 2020; 519: 734901.
[54] Chen Y, Chen Y, Shi C, Huang Z, Zhang Y, Li S, *et al*. SOAPnuke: a MapReduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. GigaScience. 2018; 7: 1–6.
[55] Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nature Biotechnology. 2019; 37: 907–915.
[56] Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011; 12: 323.
[57] Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods. 2008; 5: 621–628.
[58] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics (Oxford, England). 2010; 26: 139–140.
[59] Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols. 2009; 4: 44–57.
[60] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.). 2001; 25: 402–408.
[61] Mascoli A, Zapater C, Pizarro J, Crespo B, Gómez Peris A. Sex-specific subfunctionalization of gsdf duplicates in early gametogenesis of sea bass. 2021. Available at: https://digital.csic.es/handle/10261/267674 (Accessed: 16 September 2021).
[62] Li M, Sun Y, Zhao J, Shi H, Zeng S, Ye K, *et al*. 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 Genetics. 2015; 11: e1005678.
[63] Tao W, Cao J, Xiao H, Zhu X, Dong J, Kocher TD, *et al*. A Chromosome-Level Genome Assembly of Mozambique Tilapia (*Oreochromis mossambicus*) Reveals the Structure of Sex Determining Regions. Frontiers in Genetics. 2021; 12: 796211.
[64] Rajendiran P, Jaafar F, Kar S, Sudhakumari C, Senthilkumaran B, Parhar IS. Sex Determination and Differentiation in Teleost: Roles of Genetics, Environment, and Brain. Biology. 2021; 10: 973.
[65] Song W, Xie Y, Sun M, Li X, Fitzpatrick CK, Vaux F, *et al*. A duplicated *amh* is the master sex-determining gene for *Sebastes* rockfish in the Northwest Pacific. Open Biology. 2021; 11: 210063.
[66] Xing TF, Li YL, Liu JX. Female-specific genomic regions and molecular sex identification of the clearhead icefish (Protosalanx hyalocranius). BMC Genomics. 2021; 22: 495.
[67] Han C, Zhu Q, Lu H, Wang C, Zhou X, Peng C, *et al*. Screening and characterization of sex-specific markers developed by a simple NGS method in mandarin fish (Siniperca chuatsi). Aquaculture. 2020; 527: 735495.
[68] Herpin A, Schartl M, Depincé A, Guiguen Y, Bobe J, Hua-Van A, *et al*. Allelic diversification after transposable element exaptation promoted *gsdf* as the master sex determining gene of sablefish. Genome Research. 2021; 31: 1366–1380.
[69] Rafati N, Chen J, Herpin A, Pettersson ME, Han F, Feng C, *et al*. Reconstruction of the birth of a male sex chromosome present in Atlantic herring. Proceedings of the National Academy of Sciences of the United States of America. 2020; 117: 24359–24368.
[70] Oliveira MAD, Silva Filho AS, Araújo FE. TGF-β Superfamily: an overview of amh signaling into sex determination and differentiation in fish. Brazilian Archives of Biology and Technology. 2023; 66: e23220371.
[71] Bao L, Tian C, Liu S, Zhang Y, Elaswad A, Yuan Z, *et al*. The Y chromosome sequence of the channel catfish suggests novel sex determination mechanisms in teleost fish. BMC Biology. 2019; 17: 6.
[72] Takehana Y, Matsuda M, Myosho T, Suster ML, Kawakami K, Shin-I T, *et al*. Co-option of Sox3 as the male-determining factor on the Y chromosome in the fish Oryzias dancena. Nature Communications. 2014; 5: 4157.
[73] Kaneko H, Ijiri S, Kobayashi T, Izumi H, Kuramochi Y, Wang DS, *et al*. Gonadal soma-derived factor (gsdf), a TGF-beta superfamily gene, induces testis differentiation in the teleost fish Oreochromis niloticus. Molecular and Cellular Endocrinology. 2015; 415: 87–99.
[74] Jiang DN, Yang HH, Li MH, Shi HJ, Zhang XB, Wang DS. gsdf is a downstream gene of dmrt1 that functions in the male sex determination pathway of the Nile tilapia. Molecular Reproduction and Development. 2016; 83: 497–508.
[75] Hu Q, Guo W, Gao Y, Tang R, Li D. Molecular cloning and characterization of amh and dax1 genes and their expression during sex inversion in rice-field eel Monopterus albus. Scientific Reports. 2015; 5: 16667.
[76] Fan Z, Zou Y, Liang D, Tan X, Jiao S, Wu Z, *et al*. Roles of forkhead box protein L2 (foxl2) during gonad differentiation and maintenance in a fish, the olive flounder (Paralichthys olivaceus). Reproduction, Fertility, and Development. 2019; 31: 1742–1752.
[77] de la Herrán R, Hermida M, Rubiolo JA, Gómez-Garrido J, Cruz F, Robles F, *et al*. A chromosome-level genome assembly enables the identification of the follicule stimulating hormone receptor as the master sex-determining gene in the flatfish Solea senegalensis. Molecular Ecology Resources. 2023; 23: 886–904.
[78] Zhu KC, Zhang N, Liu BS, Guo L, Guo HY, Jiang SG, *et al*. A chromosome-level genome assembly of the yellowfin seabream (Acanthopagrus latus; Hottuyn, 1782) provides insights into its osmoregulation and sex reversal. Genomics. 2021; 113: 1617–1627.
[79] Sun D, Wen H, Qi X, Li C, Sun C, Wang L, *et al*. Comparative study of candidate sex determination regions in snakeheads (Channa argus and C. maculata) and development of novel sex markers. Aquaculture. 2023; 575: 739771.
[80] Lin Q, Mei J, Li Z, Zhang X, Zhou L, Gui JF. Distinct and Cooperative Roles of *amh* and *dmrt1* in Self-Renewal and Differentiation of Male Germ Cells in Zebrafish. Genetics. 2017; 207: 1007–1022.
[81] Webster KA, Schach U, Ordaz A, Steinfeld JS, Draper BW, Siegfried KR. Dmrt1 is necessary for male sexual development in zebrafish. Developmental Biology. 2017; 422: 33–46.
[82] Schulz RW, Bogerd J, Male R, Ball J, Fenske M, Olsen LC, *et al*. Estrogen-induced alterations in amh and dmrt1 expression signal for disruption in male sexual development in the zebrafish. Environmental Science & Technology. 2007; 41: 6305–6310.
[83] Lyu Q, Hu J, Yang X, Liu X, Chen Y, Xiao L, *et al*. Expression profiles of dmrts and foxls during gonadal development and sex reversal induced by 17α-methyltestosterone in the orange-spotted grouper. General and Comparative Endocrinology. 2019; 274: 26–36.
[84] Sheng Y, Chen B, Zhang L, Luo M, Cheng H, Zhou R. Identification of Dmrt genes and their up-regulation during gonad transformation in the swamp eel (Monopterus albus). Molecular Biology Reports. 2014; 41: 1237–1245.
[85] Zhu Y, Cui Z, Yang Y, Xu W, Shao C, Fu X, *et al*. Expression

analysis and characterization of dmrt2 in Chinese tongue sole (Cynoglossus semilaevis). Theriogenology. 2019; 138: 1–8.

[86] Vidal VP, Chaboissier MC, de Rooij DG, Schedl A. Sox9 induces testis development in XX transgenic mice. Nature Genetics. 2001; 28: 216–217.

[87] Barrionuevo FJ, Hurtado A, Kim GJ, Real FM, Bakkali M, Kopp JL, *et al*. Sox9 and Sox8 protect the adult testis from male-to-female genetic reprogramming and complete degeneration. ELife. 2016; 5: e15635.

[88] Li S, Lin G, Fang W, Huang P, Gao D, Huang J, *et al*. Gonadal Transcriptome Analysis of Sex-Related Genes in the Protandrous Yellowfin Seabream (*Acanthopagrus latus*). Frontiers in Genetics. 2020; 11: 709.