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Molecular Characterization and Expression Analysis of *Cyp19a* Gene in *Schizothorax waltoni*

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LW, SW, QT, ZG, JX, and XZ conceived and designed the research. JC, RZ carried out the experiment. WS, LZ, HS, XL, XW wrote the manuscript with contributions. WW, KC and LS analysed the data.

Key words
***Schizothorax waltoni, Schizothorax O'connori, Cyp19a*, Sex differentiation, 3D molecular modelting, *SwCyp19a* protein, Expression analysis of protein**

ABSTRACT

Schizothorax waltoni has garnered significant interest due to its ecological and evolutionary significance. Despite its pivotal role in estrogen biosynthesis and sex differentiation, the *Cyp19a* gene has been underexplored in this species. This study showed that the *SwCyp19a* gene's full-length open reading frame was found to consist of 1554 nucleotides, encoding a protein with 517 amino acid residues. Phylogenetic analysis indicated high sequence identity with *Cyp19a* genes from other fish species, positioning *SwCyp19a* within a closely related clade. Tissue distribution analysis revealed predominant expression of Cyp19a in the ovaries, with sexually dimorphic expression patterns showing higher levels in females compared to males. Sexual dimorphic expression patterns refer to the phenomenon where the expression of certain genes or proteins varies significantly between males and females. This study provides the first comprehensive characterization of the *Cyp19a* gene in *S. waltoni*, highlighting its sexually dimorphic expression and potential role in sex differentiation. These findings contribute to the broader understanding of reproductive biology in high-altitude fish species and offer valuable insights for future conservation and genetic studies, and consider linking this to practical applications for fish conservation efforts.

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INTRODUCTION

Schizothorax waltoni, a species endemic to the Tibetan Plateau, is an important subject of ichthyological research due to its ecological and evolutionary significance. Recent studies have focused on its genetic makeup, reproductive biology, and adaptive mechanisms to its unique environment. The transcriptome of *S. waltoni* has been sequenced, revealing immune-related genes and potential microsatellite markers, which are essential for population genetic studies and conservation efforts (Ye *et al*., 2018). Phylogenetic analyses have placed *S. waltoni* within a distinct clade of the Cyprinidae family, offering insights into its evolutionary history and biogeographic patterns,s particularly in the Yunnan-Guizhou Plateau (Yang *et al*., 2012). Additionally, research has delved into the reproductive biology of *Schizothorax* species, shedding light on their spawning behaviors, fecundity, and environmental influences on gonad activity (Lam, 1983; Ma *et al*., 2012).

Studies on the reproductive biology of related species, such as *S. o'connori*, in the Yarlung Zangbo River provide valuable comparative data, highlighting similarities and differences in reproductive strategies among high-altitude cyprinids (Ma *et al*., 2012; Yao *et al*., 2009). The research on *Schizothorax o'connori* offers valuable insights into early re-diploidization in polyploid genomes and provides a genetic resource for environmental adaptation studies of endemic fish of the Qinghai-Tibet Plateau. This research can serve as a comparative model for studying *S. waltoni*, as it reveals genetic differentiation and adaptability mechanisms that are crucial for understanding the biology of high-altitude fish species. These studies emphasize the critical role of environmental factors, such as temperature and riverine conditions, in shaping reproductive cycles and success rates. Furthermore, investigations into the

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genetic diversity of *Schizothorax* species have utilized SNP markers, facilitating more robust population structure analyses and aiding in the management of these fish in their native habitats (Yu *et al*., 2015).

The *Cyp19a* gene, encoding the enzyme aromatase, is vital for estrogen biosynthesis and plays a significant role in the reproductive biology of fish. The *Cyp19a* gene's primary function is the conversion of androgens to estrogens, essential for female reproductive function. The gene's expression is sexually dimorphic and is crucial during gonadal differentiation. Studies on various fish species, such as Nile tilapia and pejerrey, have highlighted the gene's role in sex determination and its regulation by environmental factors like temperature (Ijiri *et al*., 2008; Karube *et al*., 2007; Kitano *et al*., 1999). Research in *Schizothorax* has focused on the expression and regulation of the *Cyp19a* gene. DNA methylation of the *Cyp19a* promoter has been linked to sex differentiation, with temperature influencing these epigenetic modifications (Navarro-Martín *et al*., 2011; Ospina-Álvarez and Piferrer, 2008). Additionally, gonadotropins and SF-1 have been found to regulate *Cyp19a1a* during oocyte development, providing further insights into reproductive biology (Shen and Wang, 2014).

In this study, we identified the *Cyp19a* gene in *S. waltoni* and analyzed its mRNA expression patterns across various somatic tissues in both female and male individuals. The aim of this research is to enhance our fundamental understanding of the *Cyp19a* gene in *S. waltoni*.

MATERIALS AND METHODS

All fish handling and experimental procedures conducted in this study received approval from the. This research adhered to the ARRIVE guidelines, and all methods were executed in strict compliance with relevant guidelines and regulations. The authors affirm that the study was carried out ethically and responsibly.

Sampling

The *S. waltoni* individuals used in this study were procured from local fisheries. We selected three healthy 4-year-old *S. waltoni* (male and female) after a 24-h fasting period. Deep anesthesia was induced with a 0.05% solution of ethyl 3-aminobenzoate mesylate (MS-222, Sigma). Tissues including the pituitary, brain, hypothalamus, heart, liver, stomach, intestine, muscle, testicles, and ovaries were carefully dissected and rapidly frozen in liquid nitrogen for gene cloning and tissue distribution studies.

RNA extraction and cDNA preparation

Using Trizol lysate buffer, total RNA was extracted and then purified using the RNA simple kit (Takara, Dalian, China) in accordance with the manufacturer's guidelines. The quality and integrity of the RNA were evaluated using agarose gel electrophoresis and spectrophotometric measurements at 260 nm. The SMART™ cDNA kit (Clonetech, Shanghai, China) was employed for cDNA synthesis, and qPCR analysis was performed with the PrimeScript real-time quantitative PCR kit (Takara, Dalian, China). The resulting cDNA was stored at -80°C as a template for subsequent qPCR analysis.

Molecular cloning of Cyp19a *and bioinformatic analysis*

Primers for the open reading frame of *Cyp19a* were designed based on expressed sequence tag (EST) sequences from transcriptome data obtained in our laboratory, as detailed in Table I. PCR amplification was used to obtain cDNA fragments of *Cyp19a* from *S. waltoni*. The 50 µL PCR reaction included 2 µL cDNA, 4 µL 10 mmol/L dNTP mixture, 5 µL reaction buffer, 1 µL of each primer solution (Table I), 0.4 µL Taq polymerase (Takara), and 36.6 µL nuclease-free water. The protocol began with initial denaturation at 94°C for 3 min, followed by 35 cycles (94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec), and a final extension at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Cloning of the putative gene fragments into the PMD18-T vector (Takara, Japan) was followed by purification and sequencing on an ABI3730XL sequencer (Applied Biosystems, Foster City, CA).

Table I. Primers used in this study.

Primer name	Sequence 5`→3`
For fragment PCR *Cyp19a*-P	ATCCAAAGCACTGACTACTAAA
	CCCAGACTTGAAGATGGC
For relative real-time PCR *Cyp19a*-RT-PCR	GCACAGGAAGCACAAGAGAG
	ACACACACTGCTTGACGTTC
β-actin-RT-PCR	TAGCCTCTCTCGGTCAGGAT
	ACACTGTGCCCATCTACGAG

In our study, DNAMAN version 7 was employed to convert the DNA sequence. The compute pI/Mw tool on the ExPASy platform (http://web.expasy.org/compute_pi/) provided molecular weight (MW) and theoretical isoelectric point (pI) data for the inferred Sw*Cyp19a* protein. SignalP-5.0 Server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) predicted signal peptide cleavage sites in largemouth bass lamps. NetNGlyc-1.0 (https://services.healthtech.dtu.dk/service.

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php?NetNGlyc-1.0) predicted N-linked sites, respectively. CELLO (cello.life.nctu.edu.tw) was used for subcellular localization predictions. PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) and SWISS-MODEL (http://swissmodel.expasy.org/) were utilized for secondary structure and 3D molecular modeling predictions of the Sw*Cyp19a* protein. Sequence and phylogenetic analyses of *Cyp19a* involved BLAST (http://www.ncbi.nlm.nih.gov/BLAST) for sequence analysis and MEGA5.0 software for amino acid alignments and phylogenetic analysis using the bootstrapped neighbor-joining method. Additionally, multiple sequence alignment among Sw*Cyp19a* and other orthologs was performed.

Quantitative real-time PCR

PCR reactions were carried out in a 96-well ABI PRISM 7500 Real-Time PCR System with a total volume of 20 μL per reaction. Each reaction mixture consisted of 0.5 μL of 5-fold diluted cDNA template, 10 μL of 2×SYBR Green Real-Time PCR Master Mix (ABI, cat no. 4367659), 0.2 μL of each primer (10 μM), and 9.1 μL of H2O. The thermal cycling conditions were: An initial hold at 50°C for 2 min, followed by 5 min at 95°C, then 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, concluding with a dissociation curve analysis. Sterile deionized water was used as a negative control in place of the cDNA template. Each experiment was performed in triplicate to ensure technical replication. Results from different assay plates for the same gene were normalized using a consistent threshold before applying external calibration curves, within the acceptable error range of amplification efficiency (R).

Statistical analyses

Analyses of statistical data were carried out using SPSS Statistics version 22.0. The concentrations of sex steroids and the real-time PCR results were expressed as mean ± standard error of the mean (SEM). Initially, the Shapiro-Wilk test and Levene's test were used to assess the normality and homogeneity of variance of the data. Following the verification of these assumptions, one-way analysis of variance (ANOVA) and Tukey's post hoc test were utilized for further analysis. Data that did not meet the assumptions underwent transformation (e.g., log10, sine, or positive square root) and were re-tested for normality and homogeneity of variance. For data that failed to meet these assumptions even after transformation, the non-parametric Kruskal-Wallis test was performed for all pairwise comparisons. Statistical significance was defined as $p < 0.05$.

RESULTS

Characteristics of Cyp19a

The Sw*Cyp19a* gene's full-length open reading frame was acquired through PCR amplification. Sequence analysis demonstrated that the coding sequence consists of 1554 nucleotides, encoding a protein with 517 amino acid residues (Fig. 1). The calculated molecular weight of the resultant protein is 58.4 kDa, with an isoelectric point of 7.66. Moreover, the Sw*Cyp19a* protein includes 3 potential N-glycosylation sites. The three potential N-glycosylation sites are shown in Figure 1. CELLO, an online subcellular localization predictor, suggested that Sw*Cyp19a* is likely localized in the endoplasmic reticulum.

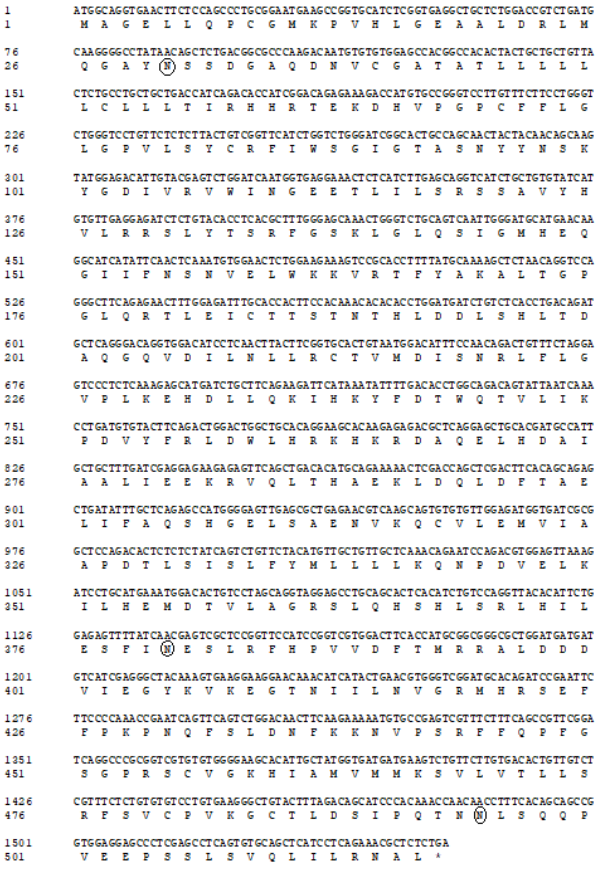

Fig. 1. CDS sequence and deduced protein sequence of *SwCyp19a*. The nucleotide and amino acid positions are indicated on the left. The stop codon is represented by a black asterisk (*). Potential N-glycosylation sites are marked with circles.

The Sw*Cyp19a* protein's secondary structure was analyzed, revealing the presence of 34 strands, as predicted by online protein structure prediction software (Fig. 2A).

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Using Swiss-Model, a 3D molecular model of Sw*Cyp19a* was developed. The analysis showed an 91.68% sequence identity with the template protein, O73686.1. A Ovarian aromatase (AlphaFold DB model of CP192_CARAU), indicating that the 3D model of Sw*Cyp19a* is accurate and consistent with its classification in the *Cyp19a* family (Fig. 2B).

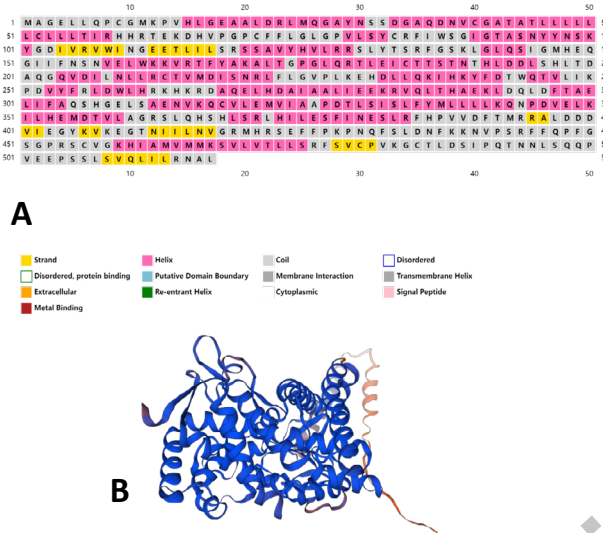

Fig. 2. Secondary structure (A) and of 3D structure (B) of the SwCyp19a protein.

To elucidate sequence similarities, the *Cyp19a* gene of the *S. waltoni* was compared with those from other vertebrates using NCBI resources. The *Cyp19a* sequence showed higher similarity to those of fish species such as *Schizothorax*. Multiple sequence alignments revealed that the *S. waltoni Cyp19a* has high sequence identity with *Cyp19a* from other species (Fig. 3). To explore the evolutionary relationships between the *S. waltoni Cyp19a* and its counterparts in other species, a phylogenetic tree was constructed (Fig. 4). The analysis suggested that Sw*Cyp19a* is most closely related to that of *Schizothorax*, consistent with the species' classification and evolutionary relationships.

Expression patterns of Cyp19a *in somatic tissues of* S. waltoni

To determine the tissue-specific distribution of *Cyp19a*, RT-PCR was performed on ten different tissues: Pituitary, brain, hypothalamus, heart, liver, stomach, intestine, muscle, testes, and ovaries (Fig. 5). Transcripts of the *Cyp19a* gene were present in both males and females. The gene showed predominant expression in the ovaries of 4-years old *S. waltoni*, with comparatively lower levels

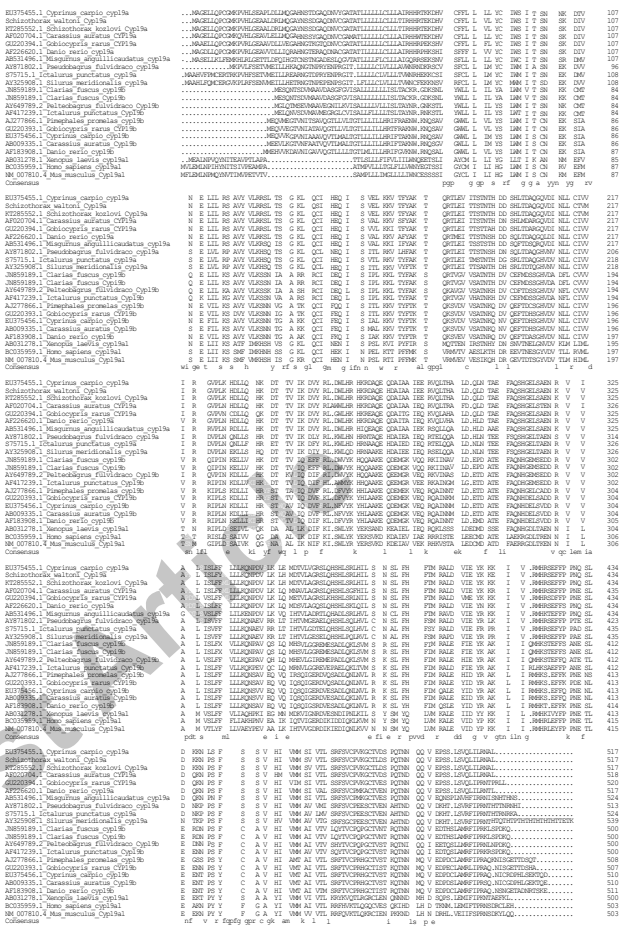

Fig. 3. Multiple sequence alignments of Sw*Cyp19a* amino acid sequences with that in other species. The putative conserved domain is indicated.

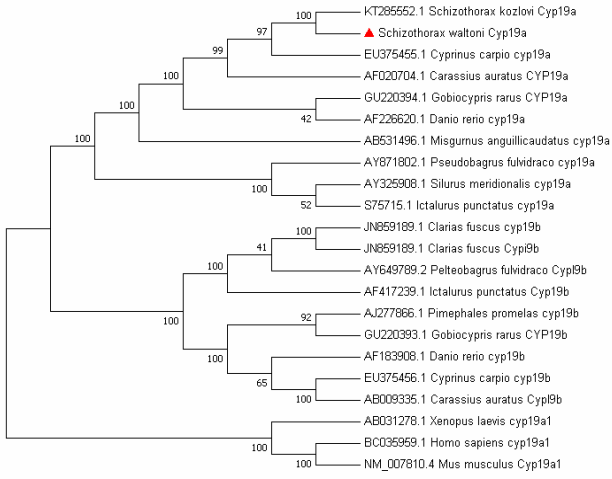

Fig. 4. Phylogenetic tree of *Cyp19a* constructed by the neighbor-joining method.

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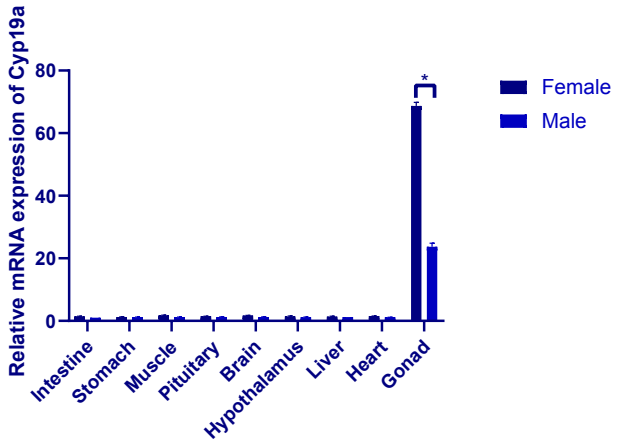

Fig. 5. Expression pattern of Sw*Cyp19a* determined by qPCR analysis. Data with black asterisk was significantly different ($p < 0.05$).

in various somatic tissues. Sw*Cyp19a* expression displayed sexual dimorphism in the differentiated gonads, with high expression in the ovary and moderate levels in the pituitary and hypothalamus. A β-actin fragment was amplified in all samples, confirming the integrity of the RNA. The study revealed significantly higher *Cyp19a* mRNA levels in females compared to males ($P < 0.05$).

DISCUSSION

During sex determination and differentiation, a highly conserved factor-estrogens and their synthesizing enzyme-plays a central role in ovarian differentiation among nearly all teleost fishes. Gonadal aromatase (*Cyp19a*), a microsomal enzyme situated in the smooth endoplasmic reticulum of steroidogenic cells, is essential for catalyzing the conversion of androgens to estrogens. *Cyp19a* mRNA is primarily expressed in the gonads, but its tissue-specific expression is not consistent among different fish species (Piferrer and Blázquez 2005). In *S. kozlovi*, *Cyp19a* expression is confined to the gonads and heart, with the highest levels in the ovary, indicating significant tissue specificity. Comparing *S. kozlovi* to other teleost fishes, the expression of *Cyp19a* shows both differences and similarities. For example, in Zebra fish (*Danio rerio*), *Cyp19a* is expressed only in the ovary, brain, and eye (Sawyer *et al*., 2006); in *Gobiocypris rarus*, it is found only in the ovary and testis (Cao *et al*., 2012), and in sablefish (*Anoplopoma fimbria*), it is expressed only in the ovary, testis, and pituitary (Smith *et al*. 2013). Furthermore, in different ploidy cyprinid fishes, *Cyp19a* is expressed in the ovary, testis, brain, and spleen (Tao *et al*., 2014). Overall, *Cyp19a* expression varies across different tissues, between sexes, during breeding and non-breeding seasons, and across developmental stages (Hong and Fang, 2000; Piferrer and Blázquez, 2005). In this study, *S. waltoni* showed a sex-specific expression pattern of *Cyp19a* in the gonads. These findings are consistent with observations in zebrafish (Sawyer *et al*., 2006), Nile tilapia (*Oreochromis niloticus*) (Chang *et al*., 2005), European sea bass (*Dicentrarchus labrax*) (Blázquez *et al*., 2008), pufferfish (*Takifugu rubripes*) (Rashid *et al*., 2007), and *Gobiocypris rarus* (Cao *et al*., 2012). The sexually dimorphic expression in *S. waltoni* may reflect adaptive strategies to high-altitude environments, where differential gene expression could influence physiological responses to hypoxia and temperature fluctuations, potentially affecting reproductive success and survival. The ecological significance of such dimorphism in *S. waltoni* could be linked to the allocation of resources and energy under harsh environmental conditions, with gene expression patterns indicating how these fish adapt to the unique challenges of the Qinghai-Tibet Plateau. The study of *Cyp19a* gene expression in *S. waltoni* under high-altitude conditions provides insights into the molecular mechanisms of sex steroid regulation, which could be crucial for understanding the adaptation and reproductive strategies in these species. Utilizing targeted methodologies such as CRISPR/Cas9 can provide a precise genetic approach to elucidate the role of the *Cyp19a* gene in sex differentiation, as demonstrated by the knockout of zebrafish ovarian aromatase gene (*Cyp19a1a*) leading to all-male offspring due to failed ovarian differentiation. RNA interference offers another potent tool for gene-specific silencing, which can further dissect the functional contributions of *Cyp19a* to sexual dimorphism and reproductive biology.

Our study marks the first successful cloning of the full-length cDNA of *Cyp19a* in *S. waltoni*, establishing a robust basis for examining the gene's function in sex determination and differentiation. The suppression of the *Cyp19a* gene exhibited a sex-specific expression pattern in the gonads. These findings indicate that *Cyp19a* in *S. waltoni* is a sex-related gene exhibiting sexually dimorphic expression, with epigenetic modifications potentially playing a significant role in gonad differentiation.

DECLARATIONS

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Data availability

Data are available from the corresponding author upon reasonable request.

Additional information

Correspondence and requests for materials should be addressed to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interests.

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