Identification, Expression and Evolutional Analysis of Two cyp19-Like Genes in Amphioxus

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Article

Identification, Expression and Evolutional Analysis of Two *cyp19*-like Genes in Amphioxus

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Simple Summary: The mechanism of sex determination and differentiation in animals has remained a central focus of reproductive and developmental biology research. Among these, the study of sex differentiation mechanisms in amphioxus, an ancient and unique organism, holds significant value for understanding the evolution and origin of sex determination mechanisms in vertebrates. However, the current understanding of the sex differentiation regulatory mechanisms in amphioxus remains limited. This study aims to enrich and deepen our comprehension of the sex differentiation mechanisms in amphioxus through the identification of two *cyp19* homologous genes and the analysis of their expression patterns in male and female gonads. Additionally, this research provides valuable insights into the formation and evolutionary pathways of sex determination mechanisms in vertebrates.

Abstract: The mechanism of sex determination and differentiation in animals remains a central focus of reproductive and developmental biology research, and the regulation of sex differentiation in amphioxus remains poorly understood. *Cytochrome P450 Family 19 Subfamily A member 1 (CYP19A1)* is a crucial sex differentiation gene that catalyzes the conversion of androgens into estrogens. In this study, we identified two aromatase-like genes in amphioxus: *cyp19-like1* and *cyp19-like2*. *Cyp19-like1* is more primitive and may represent the ancestral form of *cyp19* in zebrafish and other vertebrates. *Cyp19-like2*, on the other hand, is likely the result of gene duplication within amphioxus suggesting its potential role as a key gene in sex differentiation. To gain further insights into the expression patterns of these two *aromatase-like*, we examined their expression in different gonad tissues and during different stages of gonad development. While the expression patterns of the two genes differ in gonad tissues, both are highly expressed in the gonad primordium and are primarily localized to microsomal membrane systems. However, as development proceeds, their expression levels decrease significantly. This study enhances our understanding of sex differentiation mechanisms in amphioxus and provides valuable insights into the formation and evolution of sex determination mechanisms in vertebrates.

Keywords: amphioxus; invertebrates; sex differentiation; Cyp19

1. Introduction

Sex determination and differentiation, crucial for species continuation, is a complex process influenced by various factors within and outside cells. It involves genetic gonad differentiation shaped by the environment, with multiple cells and organs participating. Gender differentiation builds on this to determine an organism's female or male phenotype. Despite diverse sex determination mechanisms among animals, they all rely on gene expression products. Understanding these molecular mechanisms is crucial. Vertebrates show diverse and uncertain sex determination mechanisms, yet conserved pathways and genes exist across species. For instance, *Sry* is a switch gene in mammals [1], and other genes related to female and male sex formation have been discovered,such as *Cyp19a1*, *Wnt4*, *Foxl2*, *Amh*, *Sf1*, *Dmrt1*, and *Dax1* [2–6]. Signaling molecules like

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BMP and WNT also regulate sex differentiation [7–10]. Invertebrates may have sex chromosomes or alternative systems like haploidy. The functions of conserved genes and pathways in genetic sex determination remain unclear, warranting further amphioxus research.

As a Protochordata, the amphioxus bridges invertebrates and vertebrates, playing a key role in evolution [11]. Since its discovery in 1774, it has been a valuable model for vertebrate studies [12,13]. With fewer lineage-specific changes than *Urochordate* [14], the amphioxus offers a simpler body structure for studying vertebrate origins and invertebrate evolution [15,16]. It exhibits sexual dimorphism with a 1:1 sex ratio [17,18]. The gonads, attached to the inner walls of the peribranchial cavity, are composed of 26 pairs of rectangular sacs with thick walls and its gonads can be clearly observed due to skin transparency. Research on amphioxus gonadal development is fragmented, but studies suggest involvement of estrogen, androgen, and other hormones [19,20]. Chromosome analysis hints at sex chromosome involvement [21] , and genome comparisons reveal species-specific sex-determining regions and genes [22]. Amphioxus lacks LH and FSH but has a thyrostimulin [23], suggesting a primitive pituitary-gonadal axis [24–26]. However, the sex determination mechanism in amphioxus remains largely unknown.

The CYP superfamily is extensive, with over 13,000 genes across 400 gene families [27]. The *Cytochrome P450 Family 19 Subfamily A member 1* (*CYP19A1*) is crucial for sex differentiation. This enzyme converts androgens to estrogens [28], regulating vertebrate sex development [29–32]. In most vertebrates, there is only one aromatase gene. However, pigs have three aromatase genes, and the *Actinopterygii* of *Osteichthyes* have two aromatase genes. In *Actinopterygii*, there are two types of aromatase: ovarian and brain aromatase, encoded by the genes *cyp19a1a* and *cyp19a1b*, respectively. The ovarian aromatase plays a crucial role in sex differentiation. The Cyp19a1a is primarily expressed in the ovaries, and its knockout in zebrafish leads to female-to-male sex reversal and delayed testis development [33–37]. Cyp19a1b is primarily expressed in the brain and has significant impacts on brain development and function. It is involved in neuroendocrine metabolism during gonad development and also maintains the testis [38,39]. Cyp19a1a and Cyp19a1b differ structurally and functionally, possibly due to genome duplication under selective pressure during evolution [38,40]. Over the course of evolution, vertebrate *cyp19a1* have exhibited high sequence conservation and good synteny [41].

Mizuta cloned amphioxus cytochrome P450 members, which encode proteins involved in catalyzing key reactions in the synthesis of progesterone, androgen, and estrogen from cholesterol, such as CYP11A, CYP17, and CYP19 [42,43]. We searched the amphioxus genome and found two *cyp19-like* on separate chromosomes. These genes don't cluster with zebrafish *cyp19a1a* or *cyp19a1b* but sit at the evolutionary tree's base. We cloned these aromatase-like sequences from amphioxus *Branchiostoma japonicum* and used bioinformatics and molecular techniques to understand their functions, structures, and evolutionary relationships. We also studied their expression in amphioxus tissues and gonads using various methods. This comprehensive approach allowed us to determine the expression pattern of *cyp19* in the male and female gonads of amphioxus, providing valuable insights into its role in gonadal differentiation and development.

2. Materials and Methods

2.1. Experimental Materials

The Amphioxus (*Branchiostoma japonicum*) can be collected near the Shazikou sea area of Qingdao in China in early May every year. Immediately after collection, preliminary processing such as cleaning, weighing, and recording should be performed. When sexually mature, amphioxus can be distinguished by the color of its gonads: the testes appear white, while the ovaries appear pale yellow. Depending on the experimental requirements, the amphioxus can be used fresh or preserved appropriately. The experimental cells are HEK 293T human embryonic kidney cell line, purchased from the American Type Culture Collection (ATCC). Upon receiving the cells, they should be revived, passaged, and cryopreserved according to the operating guidelines provided by ATCC. It is essential to ensure that the cells maintain good growth under suitable conditions. Six-week-old ICR female mice were used for antibody preparation. Before antibody preparation, the mice should

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undergo adaptive breeding to ensure they are in good health. During the immunization process, strict adherence to experimental animal handling guidelines is crucial to ensure reliable experimental results and animal welfare.

2.2. RNA Extraction and cDNA Synthesis

Prepare and quickly process Amphioxus samples. Use RNA extraction reagents for tissue lysis and RNA isolation, followed by washing, drying, and dissolving the RNA. Quality check the RNA. Then, remove genomic DNA from the RNA and reverse transcribe it into cDNA using reverse transcriptase and primers. Purify the cDNA if needed, and detect its quality by PCR. All steps should be performed in an RNase-free environment, adhering to laboratory safety protocols.

2.3. Cloning of the cyp19

Initially, a comprehensive BLAST search was executed on the NCBI (ncbi.nlm.nih.gov), utilizing the zebrafish *cyp19a1a* sequence as the query. This meticulous exploration yielded two promising gene sequences from the Florida amphioxus database, designated as *aromatase-like* (XP_035672840.1) and *aromatase-like* (XP_035669280.1). Leveraging the transcriptome and genome data from the amphioxus from Qingdao, meticulous sequence alignments were then performed. These alignments facilitated the identification of two homologous sequences, namely *cyp19-like1* (corresponding to XP_035672840.1) and *cyp19-like2* (corresponding to XP_035669280.1). Subsequently, primers were carefully designed for the cloning of the ORF sequences specific to the *cyp19-like1* and *cyp19-like2*, paving the way for further downstream analyses.

Table 1. The sequences of primers and peptides used in this study.

Experiment	Description	Sequences (5′–3′)
5′RACE	P1(*cyp19-like1* F)	ATGTACGGAGTGATCTCTCTCCTTA
	P2(*cyp19-like1* R)	CTAGTTTCTCTCTTCAAAGTACATG
	P3(*cyp19-like2* F)	ATGGACCTAGGCGAAGGCTGGGACG
	P4(*cyp19-like2* R)	TCAGCTGTTGTCCACCCTTGGGTAC
Real-time PCR	P5(*actin* F)	TGCTGATTGTGGCTGCTGGTACTG
	P6(*actin* F)	GGTGTAGGCCAGCAGGGCGTG
	P7(*cyp19-like1* F)	GCTCAGGAGGACGACAGGATTG
	P8(*cyp19-like1* R)	GCAGCAGCGTACACATGATGG
	P9(*cyp19-like2* F)	TTCGCCGCTGCTCTCATCCA
	P10(*cyp19-like2* R)	CGGTCTCCGACGACTTCTCTGA
In situ hybridization	P11(*cyp19-like1* F)	GCGTGGTCGCCGTTGTCGTT
	P12(*cyp19-like1* R)	CGCCGCAAGAAATCCAGAGCT
	P13(*cyp19-like2* F)	GTGTATCCGCCATTGCTACC
	P14(*cyp19-like2* R)	TCTCCGACGACTTCTCTGATT
Subcellular Localization	P15(*cyp19-like1* F)	gcacagtggcggccgctcgagATGTACGGAGT GATCTCTCTCCTTACC
	P16(*cyp19-like1* R)	gctcaccattctagactcgagGTTTCTCTCTTCAA AGTACATGTAACTAGC
	P17(*cyp19-like2* F)	gcacagtggcggccgctcgagATGGACCTAGG CGAAGGCTG
	P18(*cyp19-like2* R)	gctcaccattctagactcgagGCTGTTGTCCACC CTTGGG
Polyclonal antibody preparation (mouse)	Cyp19-like1	N'-CREELKTAPPSDKPD-C'
	Cyp19-like2	N'-CPSRDHKSLDVSRNL-C'

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2.4. Bioinformatics Analysis

Commence by acquiring the genome sequences of both organisms from the NCBI website. Make use of TBtools-, a versatile bioinformatics toolbox, to extract the exon and intron sequences pertaining specifically to the cyp19. These sequences can then be uploaded to the GSDS2.0 (Gene Structure Display Server 2.0 (gao-lab.org)) to generate illustrative gene structure diagrams that elucidate the intricacies of the gene's organization. In parallel, clone the *cyp19-like1* and *cyp19-like2* cDNA sequences from the amphioxus and translate them into protein sequences with the aid of EditSeq. Subsequently, align these protein sequences with the zebrafish *Cyp19a1a* and *Cyp19a1b* amino acid sequences retrieved from NCBI using Megalign7.1.0, enabling a comparative assessment of sequence similarity and an analysis of molecular parameters such as molecular weight. Additionally, harness the capabilities of SMART7.1.0 (SMART: Main page (embl.de)) and PHYRE2.0 (PHYRE2) to predict signal peptides, transmembrane regions, functional domains, and three-dimensional structures of the Cyp19 in both organisms, providing valuable insights into their structural and functional attributes. Lastly, embark on a phylogenetic analysis using MEGA10.1.6 to elucidate the evolutionary relationships within the Cyp superfamily. Gather amino acid sequences for Cyp19 from a diverse array of species and steroid biosynthesis-related Cyp subfamilies (Cyp3a, Cyp17a, Cyp21) from NCBI. Align these sequences using Clustal W and construct a phylogenetic tree employing the Maximum Likelihood method, revealing the evolutionary history and domain architecture of the Cyp19.

2.5. Real-Time PCR

Real-Time PCR is a technique for quantifying nucleic acid sequences in real-time during PCR amplification. It involves sample preparation, primer design, PCR setup with fluorescent probes or dyes, and real-time detection using a dedicated instrument. Data analysis provides quantitative results of the target nucleic acid in the sample. This method offers sensitivity, specificity, and direct quantification, making it useful in genetics, molecular biology, and diagnostics.

2.6. Paraffin Section In Situ Hybridization

The paraffin section in situ hybridization (ISH) technique involves several steps to localize specific mRNA sequences within histological specimens. Firstly, the paraffin-embedded sections undergo preprocessing, including dewaxing, hydration, and protease treatment to enhance probe accessibility. Next, the probe, which is a labeled oligonucleotide complementary to the target mRNA, is prepared and denatured. The denatured probe is then applied to the sections and incubated under controlled temperature conditions, allowing for hybridization between the probe and the target mRNA. After hybridization, unbound probe is removed, and the bound probe is detected using appropriate methods such as immunohistochemical staining. This technique enables the direct visualization of gene expression patterns within cells or tissues, providing valuable insights into gene regulation and function.

2.7. Hematoxylin and Eosin staining

Begin with tissue sample preparation, including dehydration, clearing, and embedding in paraffin. Slice the paraffin-embedded tissue into thin sections using a microtome. Then, remove the paraffin with xylene and hydrate the sections through graded alcohol concentrations. Next, stain the nuclear DNA blue with hematoxylin and the cytoplasm red with eosin. Post-processing involves dewatering, clearing, and mounting the sections on slides（0.4μm~0.8μm）. Finally, observe the stained sections under a microscope, where the nuclei should appear blue and the cytoplasm red, providing a clear view of the tissue's morphology.

2.8. Protocol for Generating Mouse Polyclonal Antibodies

Select and prepare the antigen, mix it with an immune adjuvant, and emulsify. Immunize mice via intraperitoneal injection. Monitor antibody production periodically, collect mouse sera, and

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purify the antibodies. Identify the specificity and purity of the antibodies before storing them for future experiments.

2.9. Western Blot

Prepare protein samples, determine their concentration, and load them onto an SDS-PAGE gel for electrophoresis. After electrophoresis, transfer the proteins to a nitrocellulose membrane. Incubate the membrane with specific antibodies, wash to remove unbound antibodies, and visualize the target protein using a suitable substrate. Analyze the results, observing band positions and intensities to assess protein expression.

2.10. Immunohistochemical Staining

Obtain the tissue sample, perform dehydration, defatting, and fixation. Slice the sample into thin sections and place them on slides. Bind specific antibodies to the tissue sample, followed by washing to remove unbound antibodies. Then, bind specific secondary antibodies to the bound primary antibodies and wash again. Subsequently, conjugate the marker to the secondary antibodies and add a chromogen for color development, revealing the specific protein. Finally, mount the slides and observe the results under a microscope.

2.11. Subcellular Localization

Cultivate target cells and transfect them with plasmids labeled for the protein of interest. Fix and permeabilize the cells to allow antibody access. Add specific antibodies to recognize the target protein, followed by washing to remove unbound antibodies. Then, conjugate fluorescently labeled secondary antibodies to the primary antibodies. Observe the cells under a fluorescence microscope to localize the protein of interest within subcellular structures.

2.12. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9, with all assays conducted in triplicate technical and three times biologic replicates. Data were analyzed using One-way ANOVA or two-tailed Student's t-test and presented as means ± SD. Significance levels were set at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, with 'ns' indicating non-significance.

3. Results

3.1. Cloning of the cyp19

Using female amphioxus cDNA as a template, the complete ORF sequence of the *cyp19-like* gene in amphioxus *B. japonicum* was cloned through 5'RACE technology. The *cyp19-like1* has a total length of 1491bp, encoding 496 amino acids (Figure A). The *cyp19-like2* has a total length of 1482bp, encoding 494 amino acids (Figure B). Sequencing results confirmed the accuracy of the sequences.

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Figure 1. Complete ORF nucleotide sequences of two *cyp19-like* fragments in amphioxus. Figure A shows the *cyp19-like1* sequence fragment in amphioxus, with a size of 1491bp. Figure B displays the *cyp19-like2* sequence fragment in amphioxus, with a size of 1482bp.

3.2. Bioinformatic Analysis of the cyp19

3.2.1. Comparison of *cyp19* Structures between Zebrafish and Amphioxus

The zebrafish *cyp19a1a* gene has 9 exons located on chromosome 18, while *cyp19a1b* has 9 exons on chromosome 25. In amphioxus, *cyp19-like1* has 10 exons on chromosome 4, and *cyp19-like2* has 9 exons on chromosome 3. Since the zebrafish *cyp19a1a* and *cyp19a1b* genes are located on different chromosomes and have different functions, we hypothesize that the two *cyp19-like* in amphioxus, located on different chromosomes, may also exhibit functional differences.

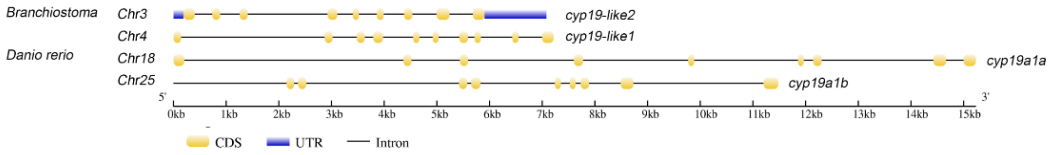

Figure 2. Comparison of the gene structures of *cyp19a1a*, *cyp19a1b*, and *aromatase-like* genes in zebrafish and Florida amphioxus.

3.2.2. Comparison of Sequence Similarity between Zebrafish and Amphioxus Cyp19

The amino acid sequence similarity between zebrafish *cyp19a1a*, *cyp19a1b*, and amphioxus *cyp19-like1*, *cyp19-like2* is low (Figure A). Sequence alignment revealed that the similarity between the two amphioxus *cyp19-like* is only 39.4%. The sequence similarity between *cyp19-like1* and *cyp19a1a*, *cyp19a1b* is 35.2% and 35.5%, respectively. The sequence similarity between *cyp19-like2* and *cyp19a1a*, *cyp19a1b* is 37.1% and 37.8%, respectively (Figure B).

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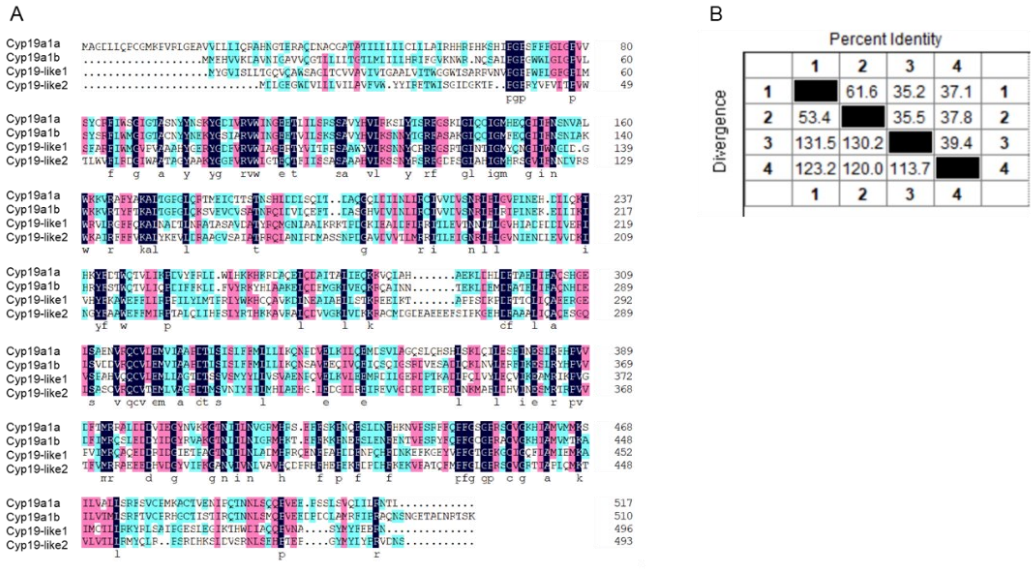

Figure 3. Alignment of amino acid sequences of *cyp19* in zebrafish and amphioxus. A: Amino acid sequence alignment; B: Sequence similarity comparison. 1 represents *cyp19a1a*, 2 represents *cyp19a1b*, 3 represents *cyp19-like1*, and 4 represents *cyp19-like2*.

3.2.3. Prediction and Comparison of Cyp19 Structures between Zebrafish and Amphioxus

SMART website was used to predict signal peptides, transmembrane regions, and domains of Cyp19 in zebrafish and amphioxus. The results showed that both amphioxus and zebrafish possess a conserved cytochrome p450 domain. Zebrafish Cyp19a1a does not have a transmembrane region, while Cyp19a1b, Cyp19-like1, and Cyp19-like2 have a transmembrane region at the N-terminus (Figure A). Three-dimensional structure prediction of Cyp19 encoded by zebrafish and amphioxus revealed a high degree of similarity in their three-dimensional structures (Figure B), indicating potential similar functions.

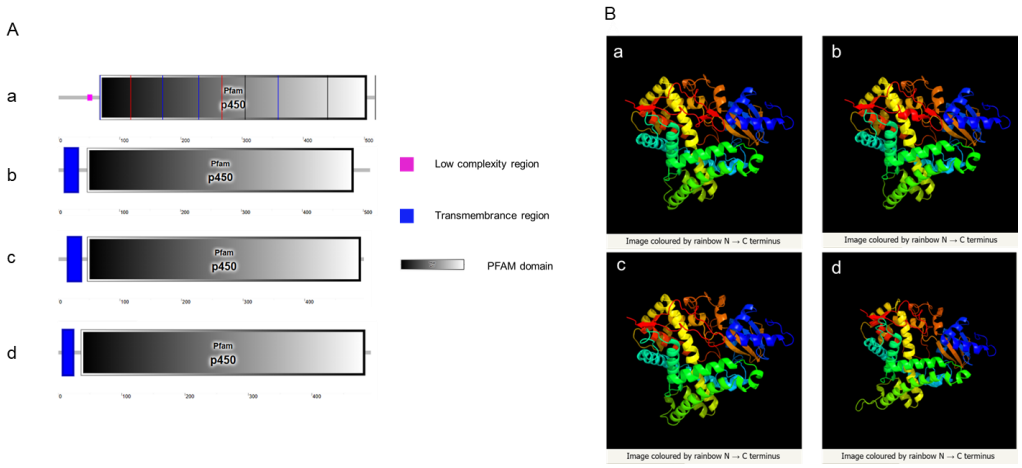

Figure 4. Prediction of Cyp19 structures in zebrafish and amphioxus. A: SMART prediction of domains in zebrafish Cyp19a1a (a), Cyp19a1b (b), and amphioxus Cyp19-like1 (c), Cyp19-like2 (d); B: PHYRE2 prediction of three-dimensional structures in zebrafish Cyp19a1a (a), Cyp19a1b (b), and amphioxus Cyp19-like1 (c), Cyp19-like2 (d).

3.2.4. Synteny and Evolutionary Analysis of Cyp19

Through Cyp19 synteny analysis, it was found that the protein has synteny in humans, mice, zebrafish, and amphioxus. *Cyp19-like1* and *cyp19-like2* exhibit synteny, but *cyp19-like2* has stronger

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synteny with humans, mice, and zebrafish, suggesting it may play a more important role in sex determination (Figure A). The main function of Cyp19 is to participate in steroid synthesis. Among the Cyp superfamily, other subfamilies related to steroid biosynthesis include Cyp3a, Cyp17a, and Cyp21. The results showed that the three amphioxus Cyp19-like sequences belong to the Cyp19 subfamily. Amphioxus Cyp19-like1 clusters with invertebrates such as hydroids and sea lilies, indicating a more primitive evolutionary status. In contrast, Cyp19-like2 clusters with vertebrates, suggesting it may be a key gene in sex determination (Figure B). Analysis revealed that the two *cyp19-like* sequences in amphioxus do not have a one-to-one correspondence with *cyp19a1a* and *cyp19a1b*. Amphioxus *cyp19-like1* may be the primitive form before the evolution of other vertebrates, while *cyp19-like2* may be the result of amphioxus's own genome

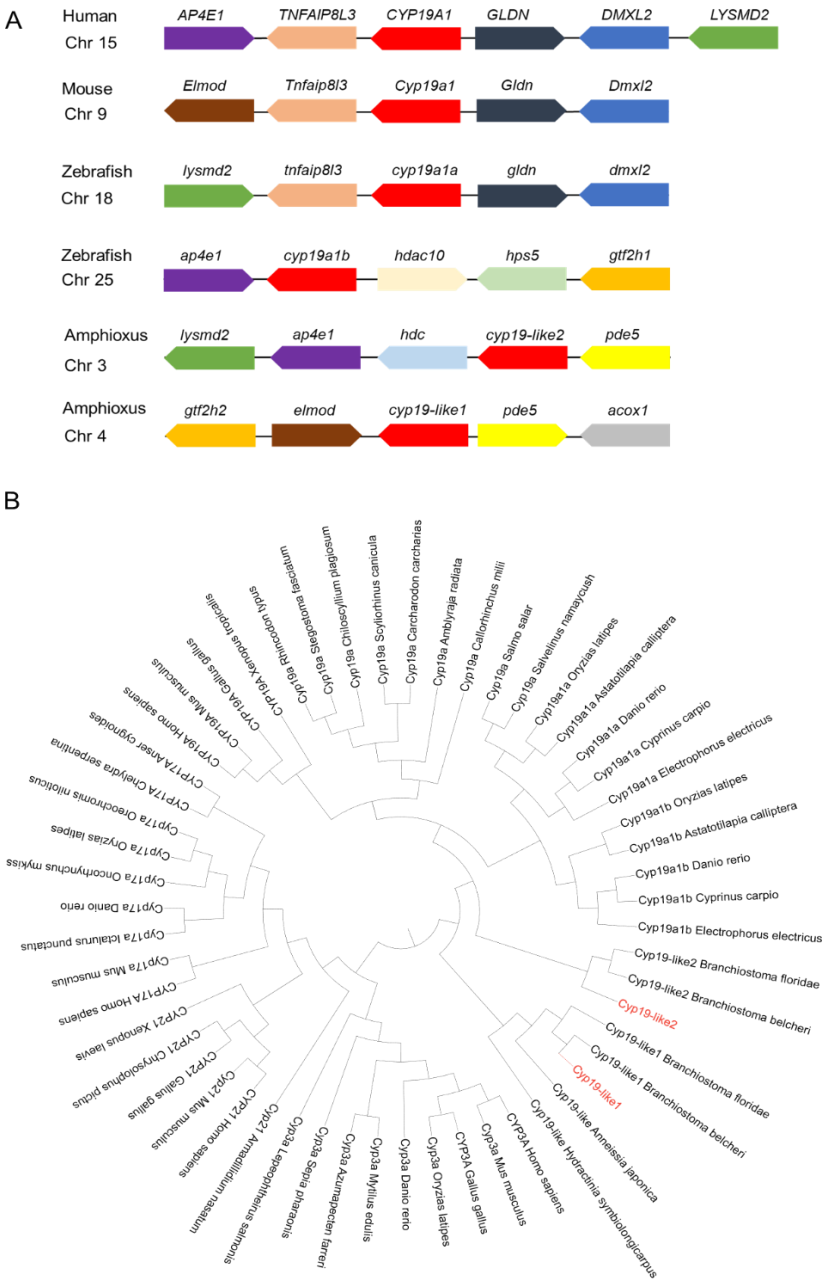

Figure 5. Analysis of synteny and phylogeny of Cyp superfamily across different species. A: Location maps of Cyp19 on amphioxus and other vertebrate chromosomes. Boxes represent genes, and the direction of the boxes indicates the orientation of the genes. B: Phylogenetic analysis of Cyp superfamily across different species. Sequence sources: see Supplemental Table S1.

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3.3. Expression patterns of two cyp19-like genes in the gonads of male and female amphioxus

3.3.1. Differential tissue expression of cyp19

We have demonstrated through semi-quantitative, Real-time PCR, and in situ hybridization techniques that there are differences in the expression of *cyp19-like1* and *cyp19-like2* in different tissues of adult amphioxus. Semi-quantitative and Real-time PCR results show that *cyp19-like1* is highest expressed in the gills, followed by the ovaries in amphioxus. *Cyp19-like2* is highly expressed in the ovaries but is also present in tissues such as the gills (Figures A and B). In situ hybridization results show that both *cyp19-like1* and *cyp19-like2* have significant positive signals in the gills and ovaries (Figure C). The Real-time PCR and in situ hybridization results are similar to the characteristic of high expression of *cyp19a1* in the ovaries of vertebrates. However, unlike vertebrates, *cyp19-like* are also highly expressed in the gills of amphioxus. To further investigate the expression of *cyp19-like* in the gills of male and female amphioxus, we conducted Real-time PCR using male and female gill templates. The results showed no significant difference in the expression of *cyp19-like1* and *cyp19-like2* in the gills (Figure D).

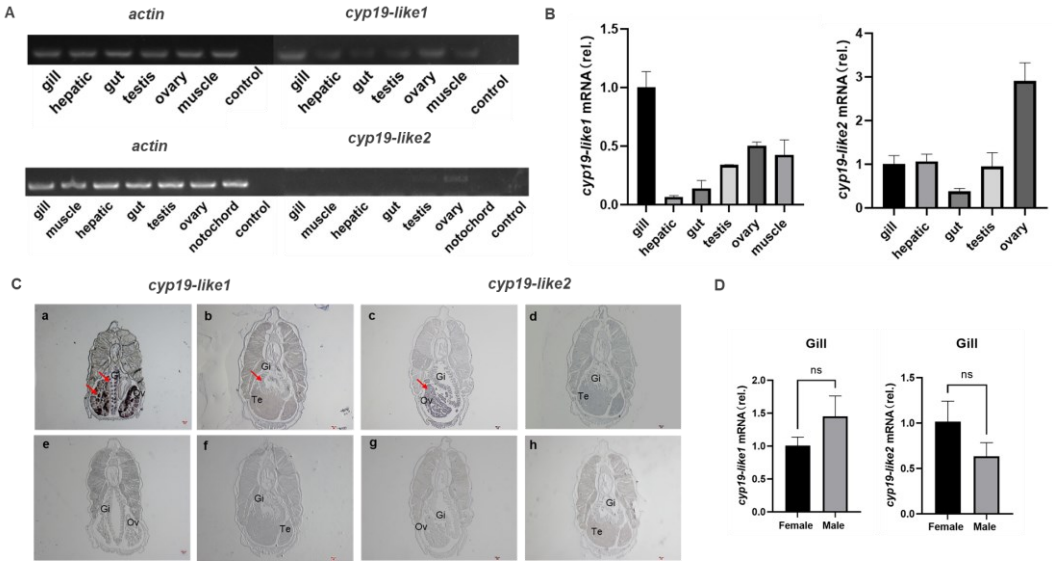

Figure 6. Tissue expression and localization of *cyp19-like* in amphioxus. A: Semi-quantitative PCR detection of cyp19-like gene expression in different tissues of amphioxus. B: Real-time PCR detection of cyp19-like gene expression in different tissues of amphioxus, including gill, hepatic, gut, testis, ovary, muscle and notochord; C: In situ hybridization results of *cyp19-like* on paraffin sections of amphioxus. a-d show antisense probe hybridization results; e-h show sense probe hybridization results (negative control). Gi: Gill; Ov: Ovary; Te: Testis. Arrows indicate positive signals. Scale bar: 100μm. D: Expression of *cyp19-like* in the gills of male and female amphioxus. ns indicates no significant difference.

3.3.2. Expression of cyp19 at different stages

During our laboratory breeding period, we observed the gonadal development of amphioxus and found that the gonads begin to develop in April, with the appearance of small black particles (gonadal primordia) on both sides of the amphioxus body. The development time of male and female gonads and both sides of the gonads is relatively consistent. In May, the gonads begin to mature, and at this time, they are transparent vesicular structures, and the black particles become larger and lighter with development. June to July is the peak period of maturity, with large and full gonads, rectangular shape, white testes in males, and yellow ovaries in females. The spawning peak occurs in July.

Based on observations of gonadal morphology and histology, we divided the gonadal development cycle of amphioxus into four stages: undifferentiated gonad (T0), gonadal primordium (T1), gonadal development (T2), and gonadal maturity (T3) (Figure A). We used Real-time PCR to

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detect the expression patterns of the two *cyp19-like* at different stages of gonadal development in amphioxus. The results showed that both *cyp19-like1* and *cyp19-like2* are highly expressed at the beginning of gonadal development, i.e., the gonadal primordium stage, and their expression decreases with the maturation of the gonads (Figure B).

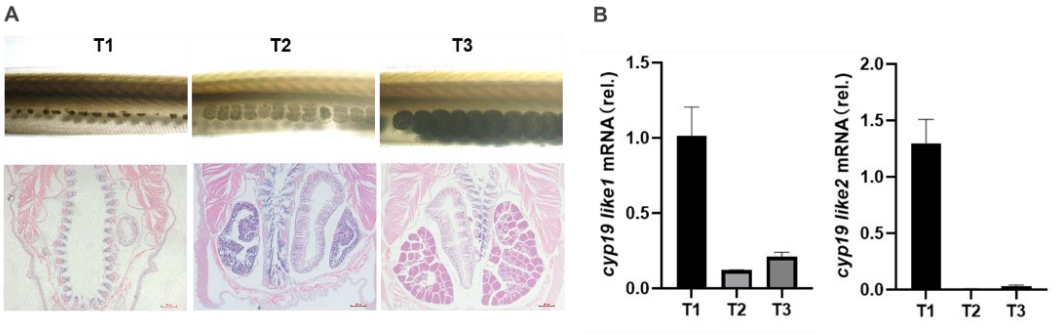

Figure 7. Expression of *cyp19-like* in amphioxus at different developmental stages. A: Tissue morphology and HE staining results at different stages of gonad development in amphioxus. B: Real-time PCR detection of *cyp19-like* expression at different stages of gonad development in amphioxus. T1, T2, and T3 represent the primordial, developing, and mature gonad stages, respectively.

3.3.3. Temporal expression and cellular localization of Cyp19

The immunogenic peptide sequences of Cyp19-like1 and Cyp19-like2 are N'-CREELKTAPPSDKPD-C' and N'-CPSRDHKSLDVSRNL-C', respectively. These immunogenic peptide sequences are located on the outer side of the predicted Cyp19 protein three-dimensional structure (Figure A). The antibody preparation effect was enhanced by KLH coupling. We used WB to detect the specificity of the prepared Cyp19 polyclonal antibody and the size of the Cyp19. The results showed good specificity of the Cyp19 antibody (Figure B).

We selected amphioxus individuals at three different stages and detected the protein expression levels of the two Cyp19-like at different stages of gonadal development using immunohistochemical staining. The immunohistochemical staining results were consistent with the Real-time PCR results, showing that Cyp19-like1 and Cyp19-like2 are highly expressed at the beginning of gonadal development, i.e., the gonadal primordium stage, and their expression gradually decreases with the maturation of the gonads. Additionally, the expression level of Cyp19-like2 was higher (Figure C). Furthermore, we examined the localization of Cyp19 in cells and found that the protein is a membrane protein mainly localized to mitochondrial membranes, endoplasmic reticulum membranes, and other microsomal membrane systems (Figure D).

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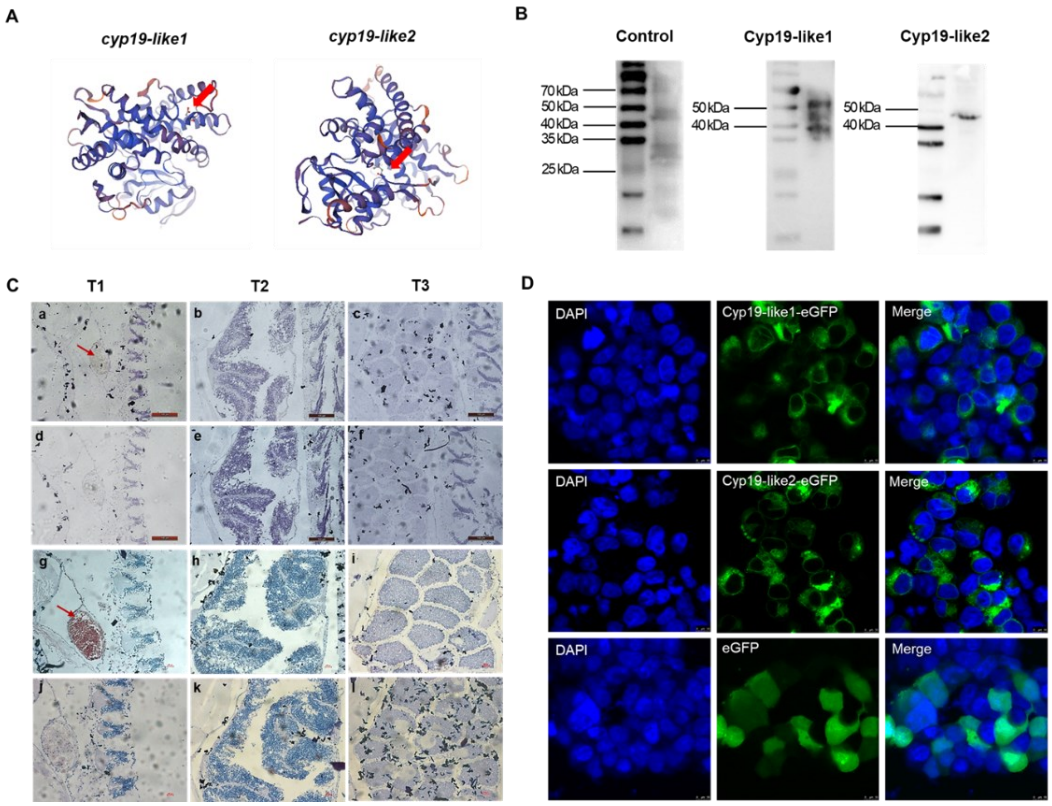

Figure 8. Antibody preparation and protein expression of amphioxus Cyp19-like. A: Designated sites for immunogenic peptides which were indicated with arrows. B: Western blot results of Cyp19-like in Amphioxus. C: Immunohistochemical results of Cyp19-like in Amphioxus. T1, T2, and T3 represent the primordial, developing, and mature gonad stages, respectively. a-f show Cyp19-like1 immunohistochemical results, while g-l show Cyp19-like2 immunohistochemical results. a-c, g-i are experimental groups, while d-f, j-l are control groups. Scale bar: 100μm. D: Subcellular localization of amphioxus Cyp19-like in HEK cellls using LSCM. Scale bar: 10μm.

4. Discussion

The aromatase, encoded by the *cyp19a1*, plays a crucial role in vertebrate reproduction by catalyzing the conversion of androgens to estrogens. While aromatase is known to be critical in sex determination and differentiation in animals, the origin of the *cyp19a1* remains enigmatic. In vertebrates, the majority of animals possess a single aromatase gene. However, fish belonging to the *Actinopterygii* subclass of *Osteichthyes* exhibit a unique feature: they possess two aromatase genes, ovarian aromatase and brain aromatase. These genes are expressed in distinct locations and exhibit structural and functional differences. Through sequence alignment, we discovered the existence of two *aromatase-like* in Florida amphioxus, situated on chromosomes 4 and 3, respectively. Utilizing amphioxus *B. japonicum* as our material, we successfully cloned these two homologous sequences, naming them *cyp19-like1* and *cyp19-like2*.

Given the presence of amphioxus *cyp19-like1* and *cyp19-like2*, which are also situated on different chromosomes and display structural and sequence variations, it raises intriguing questions regarding their specific functions in amphioxus and whether both genes play a role in sex differentiation. *cyp19-like1* has 10 exons, while the other sequences possess 9 exons, which aligns with the basic structure of vertebrate *cyp19* having 9 exons [42,43]. Amphioxus and zebrafish share a conserved cytochrome p450 domain, indicating a common ancestral origin. Notably, zebrafish Cyp19a1a lacks a transmembrane region, whereas zebrafish Cyp19a1b, amphioxus Cyp19-like1, and amphioxus Cyp19-like2 all possess a transmembrane region at their N-termini. This feature suggests potential differences in their subcellular localization and function.

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The question at hand concerns is the functional relationship between the two *cyp19-like* in amphioxus and the *cyp19a1a* and *cyp19a1b* in fish, particularly in the context of their evolutionary history. Zhang and colleagues' observations about the strong synteny and sequence conservation of vertebrate *cyp19a1* during evolution provide a valuable baseline for comparison. Despite this conservation, amphioxus Cyp19 (ABA47317.1) does not share synteny with vertebrate *cyp19a1*, suggesting that the direct evolutionary link between these two genes is tenuous. In their opinion, it appears that the *cyp19a1* in bony fish may have originated from an ancestor that evolved alongside amphioxus, rather than directly descending from it. Alternatively, significant chromosomal rearrangements in the region surrounding the *cyp19a1* locus could have occurred in the basal vertebrate ancestor closely related to amphioxus[40]. Our findings, however, introduce a new element to this discussion. Our analysis indicates that amphioxus *cyp19-like* exhibit some synteny with vertebrate *cyp19a1*. Yet, *cyp19-like2* exhibits stronger synteny with humans, mice, and zebrafish. This suggests that *cyp19-like2* may have a closer evolutionary affinity with these vertebrate genes. In addition, phylogenetic analysis provides valuable insights into the evolutionary history and potential functions of genes. In the context of *cyp19*, the finding that *cyp19-like1* represents a more primitive and ancestral form suggests that it played a crucial role in the early stages of vertebrate evolution. The proposed origin of *cyp19-like2* from genome duplication within amphioxus itself highlights the dynamic nature of genome evolution and the potential for novel functions to arise from such duplications.

In bony fish, the observed differences in tissue expression patterns [44,45], affinities for substrates [46,47], and inducibility by estrogens and xenoestrogens [45,48–50] between the *cyp19a1a* and *cyp19a1b* are consistent with the idea that duplicated genes can diverge in function. Similar observations in pigs further support this notion [51,52]. Given these findings, it is intriguing to explore whether the two amphioxus *cyp19-like* exhibit differences in expression patterns, particularly in male and female gonads. Our study using real-time PCR and in situ hybridization techniques has revealed interesting patterns of *cyp19-like* expression in amphioxus. The high expression of *cyp19-like1* in the gills, followed by the ovaries, suggests a role for this gene in reproductive and other functions. In contrast, the predominant expression of *cyp19-like2* in the ovaries is consistent with its putative role in sex differentiation. We investigated whether there are differences in *cyp19-like* expression in the gills of males and females using Real-time PCR and found no sex-specific differences in expression in the gills. The absence of sex-specific differences in *cyp19-like1* expression in the gills is noteworthy, indicating that this gene may play a more general role in amphioxus physiology. However, the expression pattern of *cyp19-like* in the heads of adult amphioxus remains enigmatic and deserves further investigation.

The examination of *cyp19* expression during gonad development in amphioxus provides further insights into the potential roles of these genes in sex differentiation and reproduction. The high expression of both *cyp19-like* at the onset of gonad development suggests their involvement in the initial stages of gonad formation. However, the subsequent downregulation of expression as development progresses might indicate that these genes are not essential for maintaining gonad function but rather play a crucial role in initiating gonad development. Immunohistochemical staining also confirmed that the proteins encoded by these two genes were most abundant in the early stages of gonad development, with subsequent downregulation. Overall, the expression of *cyp19-like2* and its encoded protein was more prominent in the gonad primordium. Aromatase proteins are monomeric and anchored within the endoplasmic reticulum via a transmembrane domain at their amino-terminal end [53,54]. We investigated the subcellular localization of Cyp19 and found that the protein is likely localized primarily to microsomal membrane systems such as mitochondrial and endoplasmic reticulum membranes, consistent with its expression pattern in vertebrate cells.

Future studies should aim to further investigate the molecular mechanisms underlying the observed differences in gene expression levels during gonad development and elucidate the precise functions of *cyp19-like1* and *cyp19-like2* in amphioxus, including their potential roles in sex differentiation, reproduction, and other physiological processes.

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5. Conclusions

We comprehensively examined the expression of two *cyp19-like* genes in amphioxus using techniques such as Real-time PCR, ISH, and IHC. This study clarifies Cyp19 expression patterns during amphioxus gonad development and highlights its importance in vertebrate reproduction evolution. Differences in gene expression and protein localization of *cyp19-like1* and *cyp19-like2* offer insights into sex determination and gonad development mechanisms. However, further research is needed to fully understand these complex mechanisms.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: The GenBank accession numbers.

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