www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 14: 785-793 (2014) DOI: 10.4194/1303-2712-v14_3_21

The Genomic Structure and Expression Patterns of Cyp19a1a and Cyp19a1b Genes in the Ayu *Plecoglossus altivelis*

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Received 25 April 2014
Accepted 5 September 2014

Abstract

Here, we report the genomic structure and expression pattern of the *cyp19a1a* and *cyp19a1b* of ayu. The cDNAs of *cyp19a1a* and *cyp19a1b* coded 529 and 540 amino acid residues with a similarity of 76% and 77% with *Salmo trutta* (*cyp19a1a*) and *Oncorhynchus mykiss* (*cyp19a1b*), respectively. Genomic structure analysis revealed that ayu *cyp19a1a* and *cyp19a1b* genes contained six and seven introns, respectively. The insert sites of *cyp19a1b* agreed with other fish species, while that of *cyp19a1a* varied slightly. Sequence analysis of the 5′-flank region showed that the coding start sites were 71 bp and 72 bp from the translation start sites (TSS) of *cyp19a1a* and *cyp19a1b*, respectively. The expression patterns of *cyp19a1a* and *cyp19a1b* in adult ayu tissues were same as most teleostomi. *Cyp19a1a* was predominantly expressed in the ovary and female brain. Unexpectedly, *Cyp19a1a* also expressed strongly in the muscle. However, *cyp19a1b* was predominantly expressed in the brain. The expression patterns of ayu embryos and juveniles revealed that *cyp19a1a* expression fluctuated significantly during ovarian differentiation, while *cyp19a1b* showed no significant variations during this stage. Based on our data, *cyp19a1a* might play an important role in gonad differentiation and *cyp19a1b* might involve in the development of ayu nervous system.

Keywords: Cytochrome P450 aromatase, promoter, real time PCR.

Introduction

Cytochrome P450 aromatase (P450arom) is a member of the cytochrome P450 superfamily and is the terminal enzyme in the pathway responsible for generating sex steroids, which plays an important role in maintaining the physiological balance between the sex steroid hormones(Yu *et al*., 2008). *Cyp19* gene, which has already been identified in many different animal phyla, encodes an enzyme controlling the synthesis of estrogens(Uno *et al*., 2012). In most vertebrates, P450arom is encoded by a single copy of the *cyp19* gene; however, two forms of the P450arom protein have sometimes been reported: P450aromA, which is encoded by *cyp19a1a* and mainly expressed in the ovary, and P450aromB, which is encoded by *cyp19a1b* and primarily expressed in the brain(Goto-Kazeto *et al*., 2004). *Cyp19a1a* and *cyp19a1b* have different tissue distribution and expression patterns. To date, these two genes have been detected in many fishes, including *Danio rerio* (Chiang *et al*., 2001), *Carassius auratus* (Callard and Tchoudakova, 1997), *Oreochromis niloticus* (Chang *et al*., 1997), *Oncorhynchus mykiss* (Tanaka *et al*., 1992), *Cyprinus carpio* (Barney *et al*., 2008), *Trichogaster trichopterus* (Ezagouri *et al*., 2008), *Intalurus punctatus* (Trant, 1994), *Monoperus albus* (Yu *et al*., 2008), and *Melanotaenia fluviatilis* (Shanthanagouda *et al*., 2012). However, the P450arom (A/B) coding genes of ayu are still unknown.

Ayu, which belongs to the class Osteichthyes, suborder Salmonoidei and family Plecoglossidae, is a popular commercial fish throughout China and Japan. Ayu is widely consumed in these countries due to its bitter taste of the gut contents. However, this species exhibits sexual dimorphism following maturation. The price of pure females is sold for twice than a mixture of males and females. So how to regulate gender and to develop unisexual ayu breeding populations had become a key issue. Reported researches were mainly focused on ayu breeding, cultivation, disease prevention etc. (Awata *et al*., 2011; Lü *et al*., 2012). So far, the sex-determination mechanism of ayu is still untouched and genes involved in ayu gender regulation are unreported. Here, we firstly report the isolation and characterization of the complete *cyp19a1a* and *cyp19a1b* genes from the transcriptome of ayu gonads

and detected their expression patterns in the embryo, the larval and the adult tissues.

Materials and Methods

Experimental Materials

The ayu were sampled from the Zhejiang Mariculture Research Institute, Qingjiang Station, in December 2012. The fish were immediately dissected after anesthesia by 0.03% benzocaine solution and the different tissues were isolated, frozen, and stored in liquid nitrogen. The fertilized eggs and embryos at different stages of development were gathered and stored in liquid nitrogen.

DNA, RNA Extraction, and cDNA Synthesis

Genomic DNA was isolated from ayu muscle using a Genome DNA Isolation Kit (Sangon; no. SK8252). The total RNA from different tissues was isolated using the RNAiso Plus (Takara; no. 9108) according to the manufacturer's instructions. All the RNAs were treated by Rnase-free DNase I (Takara; no. D2215) and then purified. The reverse transcription reaction was performed in a 10 μl volume using the PrimeScript RT reagent Kit (Takara; no. RR037A) at 37°C for 15 min, followed by 85°C for 5 s, and then stored at -20°C.

cDNA Cloning and Intron Analysis

The internal fragments of *cyp19a1a* and *cyp19a1b* were amplified from the single-stranded cDNA library of the ayu gonad by gene-specific primers (Table 1). The PCR conditions were as follows: 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and a final 72°C for 5 min. PCR products were observed on a 2% agarose gel, then cloned into the pMD®19-T vector and sequenced.

Rapid amplification of cDNA ends (RACE) was performed to generate the 3′- and 5′-ends of *cyp19a1a* and *cyp19a1b* by the 3′-Full RACE Core Set Ver.2.0 (Takara; no. 6106) and 5′-Full RACE kits (Takara; no. D315) respectively, according to the manufacturer's instructions. Specific primers (Table 1) were designed according to the internal sequences. The reaction products were purified by DNA extraction Kit (Sangon; no. SK8252), then ligated into the pMD®19-T vector and sequenced.

Cyp19a1a and *cyp19a1b* genomic fragments were amplified from a genomic DNA template by specific primers (Table 1). The products were observed on a 2% agarose gel, and fragments with the desired length were purified, then ligated into pMD®19-T vector and sequenced.

Isolation of Ayu cyp19a1a and cyp19a1b 5′-Flank Region

The 5′-flank regions of *cyp19a1a* and *cyp19a1b* were isolated using the genome walking method and nested primers (Table 1) specific to the cDNA sequences of the two genes. The experimental procedure was performed using the Genome walking kit (Takara; no. D316) according to the manufacturer's instructions. PCR products with the

Table1. The primers used in this investigation

Purpose	Name	Sequence (5→3)
Degenerate primers for isolation of cyp19a1a and genomic amplification	cyp19a1a-F	TCTGGGTTCTGCTTGAGG
	cyp19a1a-R	TCTGCTCGTGGCTATCTG
	cyp19a1a-2F	GGTTGTTGGTATGTGGGATG
	cyp19a1a-2R	GACCAGGTTGATGTTCTCAGTT
3′-RACE of cyp19a1a	cyp19a1a-3-I	TGAGCCCCCAGAAGAGTGTG
	cyp19a1a-3-O	CCCACATACCAACAACCTTTCAC
5′-RACE of cyp19a1a	cyp19a1a-5-I	CTGACGATGTCCCCGTACTTGTTAT
	cyp19a1a-5-O	CAGACGAGGATACGACGAGGC
RT-qPCR for cyp19a1a	a-F	TCCAGCCCAGTCGGAAGTAG
	a-R	GACTCTTGTAGTTGGACCAGACG
Degenerate primers for isolation of 5′-flank region of cyp19a1a	a-5-I	TCGCATAGAACGAACGCTCAC
	a-5-M	GTTCCCAGACGAGGATACGACG
	a-5-O	CTCCAGATAGCCACGAGCAGA
Degenerate primers for isolation of cyp19a1b and genomic amplification	cyp19a1b-F	TCTCCTCCTGACGACTGC
	cyp19a1b-R	GCTCCACCTTTGGGTTCT
	cyp19a1b-2F	TGTCATCTTTGCCCAGAATC
	cyp19a1b-2R	CGGTGTAGCGGCTCAGTA
3′-RACE of cyp19a1b	cyp19a1b-3-I	TCGCTTCTTCCAGCCGTTC
	cyp19a1b-3-O	GTCACCTTACTGAGCCGCTACAC
5′-RACE of cyp19a1b	cyp19a1b-5-I	GACTCCAGGGTTGGTGTTCGT
	cyp19a1b-5-O	CCAGCCCAAAGCAATAGGAAG
Degenerate primers for isolation of 5′-flank region of cyp19a1b	b-5-I	AATGACGAGGTGTATCTGGTGTGA
	b-5-M	GACTCCAGGGTTGGTGTTCGT
	b-5-O	AGCCCAAAGCAATAGGAAGGA
RT-qPCR for cyp19a1b	b-F	TTTGACCTTCGCACCCAC
	b-R	TCACATAACCTTACCTACCCTC
RT-qPCR for β-actin	β-actin(+)	TCGTGCGTGACATCAAGGAG
	β-actin(-)	CGCACTTCATGATGCTGTTG

desired length were purified, ligated into the pMD®19-T vector, and sequenced.

Fluorescent Real Time RT-qPCR

Real time PCR was used to quantify *cyp19a1a* and *cyp19a1b* expression, according to the manufacturer's instructions (SYBR® Premix Ex Taq™, Takara; no. DRR081A) using the realplex^2 thermocycler (Eppendorf). RT-qPCR was performed using specific primers (Table 1) in a 20μL volume under the following cycling conditions: 40 cycles of 95°C for 30 s; 60°C for 30 s; and 72°C for 30 s. *β-Actin* was used as a housekeeping control. Samples of adult were collected from three different individuals and quantified respectively. Samples of embryo and larvae were randomly collected 50 individuals at the same development stage as a pond and three ponds were quantified. Every sample was repeated for three times. Pure water was used as template in blank control and repeated for three times. Genes was considered unexpressed when samples' mean 2^{-Ct} value wasn't larger than that of blank control. RT-qPCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method. The samples with the lowest expression level were used as the control test. Data were expressed as the mean ± SD and analyzed by one-way analysis of variance (ANOVA) with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P<0.05$.

Data Analysis

Sequences were compared against those in the National Center for Biotechnology Information (NCBI) database by using the Basic Local Alignment Search Tool (BLAST) tool to ensure their accuracy. All *cyp19a1a* and *cyp19a1b* putative amino acid sequences from different species were aligned using ClustalX2.0 (Larkin *et al*., 2007), and phylogenetic trees were constructed by the Maximum Evolution (ME) method by using MEGA6.0 (Tamura *et al*., 2013). An unrooted consensus tree was bootstrapped (1000 replications) to ensure statistical validity of each node. The putative protein-binding site at the promoter of *cyp19a1a* and *cyp19a1b* were examined using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). RT-qPCR analysis indicated differential expression of *cyp19a1a* and *cyp19a1b* in the different stages of embryo and larval and in different adult tissues.

Results

Cloning and cDNA sequence analysis of Ayu cyp19a1a and cyp19a1b

Two genes encoding P450arom were cloned from ayu and whole cDNA sequences were obtained. The *cyp19a1a* cDNA (1849 bp) had a 1590-bp open reading frame (ORF) which encoded a protein of 529 amino acid residues, a 72-bp 5′-untranslated region (UTR), and a 187-bp 3′-UTR. The *cyp19a1b* cDNA (2174 bp) has a 1623-bp ORF, which encoded a protein of 540 amino acid residues, a 71-bp 5′-UTR, and a 480-bp 3′-UTR. The I-helix region, Ozol's peptide region, aromatase-specific conserved region, and heme-binding region were all detected. The GenBank accession number of ayu *cyp19a1a* and *cyp19a1b* was KF246582 and KF296361 respectively.

The NCBI BLAST search results showed that the species with the highest homology to ayu *cyp19a1a* and *cyp19a1b* were *Salmo trutta cyp19a1a* (AAR04775) and *Oncorhynchus mykiss cyp19a1b-1* (CAC84574), and the similarities were 76% and 77%, respectively. In contrast, the ayu *cyp19a1a* and *cyp19a1b* only showed a similarity of 49%. The amino acid sequences of ayu *cyp19a1a* and *cyp19a1b* were compared against their homologs in other vertebrates, including *Danio reio cyp19a1a* (AF004521) and *cyp19a1b* (AF226619), *Carassius auratus cyp19a1a* (AB009336) and *cyp19a1b* (B009335), *Cynoglossus semilaevis cyp19a1a* (ABL74474) and *cyp19a1b* (ABM90641), *Hippoglossus cyp19a1a* (CAC36394) and *cyp19a1b* (AAY26901), *Homo sapiens cyp19* (NP_112503), and *Mus musculus cyp19* (NP_031836) by using ClustalX2. The results showed that the I-helix region, Ozol's peptide region, aromatase-specific conserved region, and heme-binding region of ayu *cyp19a1a* and *cyp19a1b* were the most conserved across species. The phylogenetic tree constructed from the above analysis revealed that the *cyp19a1a* and *cyp19a1b* genes clustered into two distinct clades along with their respective teleostean variants (Figure 1).

Genomic Organization of Ayu cyp19a1a and cyp19a1b

The exon/intron organization of the ayu *cyp19a1a* and *cyp19a1b* genes was determined by comparing their genomic sequences with the cDNA sequences. The GenBank accession number of ayu *cyp19a1a* and *cyp19a1b* was KF296362 and KF296363 respectively. The results showed that the ayu *cyp19a1a* gene consisted of seven exons and six introns, and the ayu *cyp19a1b* gene consisted of eight exons and seven introns. The insert sites of ayu *cyp19a1b* were same as those found in *Homo sapiens*, *Oryzias latipes*, *Dicentrarchus labrax*, and *Monopterus albus*, but lacked the second intron of the 5′-end in the corresponding *Dicentrarchus labrax* L. gene(Dalla Valle *et al*., 2002). The first insert site of ayu *cyp19a1a* was located at the position of the introns that were missing from the ayu *cyp19a1b* and lacked the first and third introns of the 5′-end of the corresponding gene in *Dicentrarchus labrax* L. The insert sites of introns 3 and 4 of ayu *cyp19a1a* were located at the same position as the introns of ayu *cyp19a1b*, and the others were located just near the

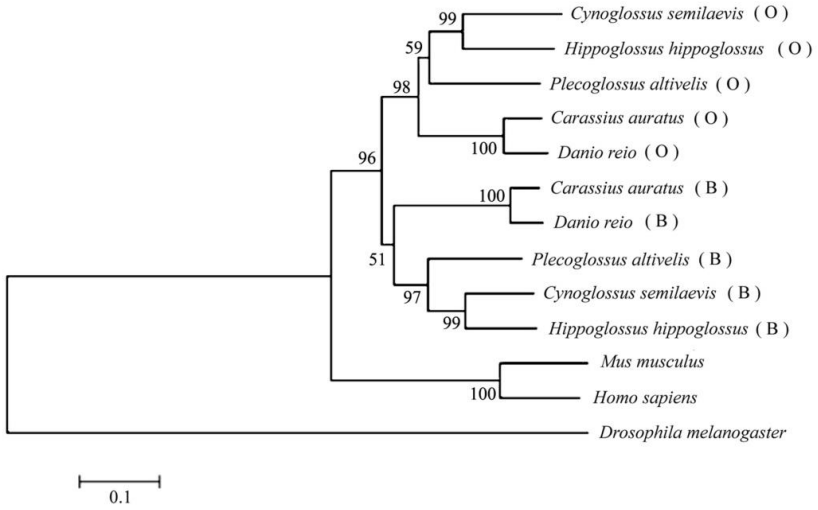

Figure 1. Phylogenetic tree of cyp19a1a and cyp19a1b based on the putative amino acid sequences, constructed by the ME (Maximin Evolution) method with the bootstrap 1000 times. The O indicates ovarian cyp19 (cyp19a1a) and B indicates brain cyp19 (cyp19a1b). The *Drosophila melanogaster* CYP19 (AAB05550) was used as outgroup.

corresponding insert sites. In addition, the null intron of ayu *cyp19a1a* and *cyp19a1b* was detected in the conserved region (Figure 2). The sizes of introns in the ayu *cyp19a1a* and *cyp19a1b* genes were smaller than those in humans, their total length being 1162 bp and 857 bp, respectively. Intron 3 of *cyp19a1a* and intron 4 of *cyp19a1b* were interrupted in their reading frame in the codon triplet (Figure 3).

Analysis of the 5′-Flank Region of Ayu cyp19a1a and cyp19a1b

Genome walking analysis revealed 1028 and 728 bp fragments for *cyp19a1a* and *cyp19a1b*, respectively. Sequence analysis showed that the translation start sites of *cyp19a1a* and *cyp19a1b* were 71 and 72 bp long, respectively, which was confirmed by 5′-RACE. Within the *cyp19a1a* 5′-flank region, five caudal type homeobox A (CdxA) sites, three copies of the sex-determining region Y (SRY) binding motif, two GATA-2 sites, a myeloid zinc finger1 site (MZF-1), an NK homeobox gene (Nkx2) binding site, and an acute myeloid leukemia 1a (AML-1a) site were identified as putative protein-binding sites. Within the *cyp19a1b* 5′-flank region, six SRY binding sites, four CdxA sites, two GATA-1, a GATA-2, pre-B transcription factor 1 (Pbx-1), E2 promoter binding factor (E2F), NK homeobox gene 2 (Nkx-2), CAAT-enhancer binding protein (C/EBP), RAR-related orphan receptor alpha1 (RORa1p), octamer-binding factor 1 (Oct-1), S8 homeobox gene, and Sry-related high mobility group box5 (Sox5) site were identified (Figure 4).

Expression of Ayu cyp19a1a and cyp19a1b in Adult Tissues

The expression of ayu *cyp19a1a* and *cyp19a1b* was examined in the adult gonad, brain, heart, liver, intestine, muscle, spleen and kidney. Results of the experiments performed are presented in Figure 5. The results showed that *cyp19a1a* was mainly expressed in the female ayu gonad and brain and in muscle of both sexes. *Cyp19a1b* was primarily expressed in the gonad and brain, and to a lesser extent also in the spleen, kidney, heart, and muscle of both sexes. The results also indicated significant differences in the expression of *cyp19a1a* and *cyp19a1b* in the ovary and brain. *Cyp19a1a* was highly expressed in the ovary and only weakly expressed in the testis, whereas *cyp19a1b* was weakly expressed in both gonads. Both *cyp19a1a* and *cyp19a1b* were strongly expressed in the female brain; while only *cyp19a1b* was strongly expressed in the male brain.

Expression of Ayu cyp19a1a and cyp19a1b in Embryo and Larvae

RT-qPCR analysis of *cyp19a1a* and *cyp19a1b* was performed to detect their expression patterns in the embryo and larval stages of development. The results showed that in the embryo, expression of *cyp19a1a* was restricted to the oosperm, morula, neurula, and tail bud stages, and it reached its peak in the tail bud ($P<0.05$). The expression of *cyp19a1b* was mainly restricted to the oosperm, morula, blastula, and neurula stages, and it reached its peak in the neurula ($P<0.05$) (Figure 6). In the larvae, expression of both *cyp19a1a* and *cyp19a1b* can be detected from day 1 to 70 post hatching (dph), but with obvious differences in their temporal expression patterns. *Cyp19a1a* had a higher level of expression between 22–64 dph and reached its peak at 28 dph ($P<0.05$), two obvious fluctuations had been detected as well. The expression of *cyp19a1b* was mainly detected between 46–54 dph and reached its peak on day 46 ($P<0.05$) (Figure 7).

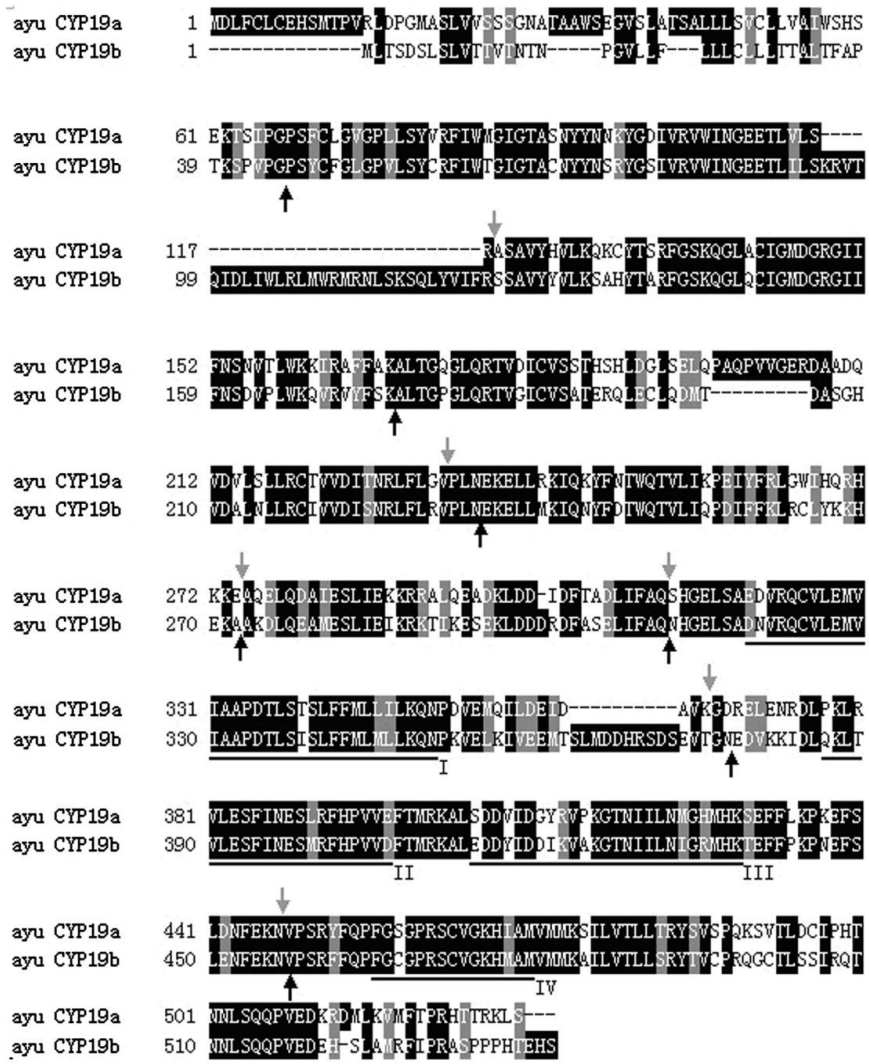

Figure 2. Comparison of ayu cyp19a1a and cyp19a1b amino acid sequences (the numbers from I to IV indicate the I-helix region, Ozol's peptide region, aromatase-specific conserved region and the heme-binding region. The black arrow indicates the insert sites of ayu cyp19a1b introns and the gray arrow indicates the insert sites of ayu cyp19a1a introns).

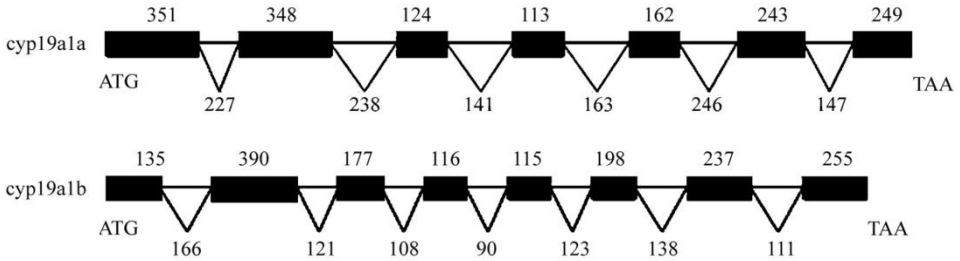

Figure 3. The exon/intron organization of ayu cyp19a1a and cyp19a1b.

Discussion

Several recent studies in fishes have shown that P450arom has two structurally and functionally different variants, commonly termed P450aromA and P450aromB, which are encoded by *cyp19a1a* and *cyp19a1b*, respectively. The conserved domains of the cytochrome CYP family are the I-helix region, the Ozol's peptide region, the aromatase-specific region, and the heme-binding region. In this study, we cloned the complete ORFs of ayu *cyp19a1a* and *cyp19a1b*, sequence analysis showed that these conserved sequences were highly homologous with those of other fishes. In addition, we found the genomic organization of ayu *cyp19a1a* and *cyp19a1b* to be highly conserved with respect to their insert sites.

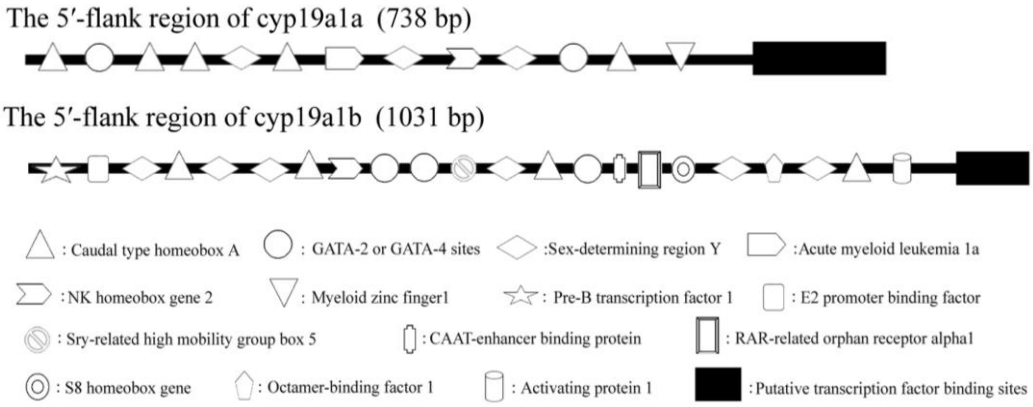

Figure 4. The 5′-flank region of ayu cyp19a1a gene and cyp19a1b gene.

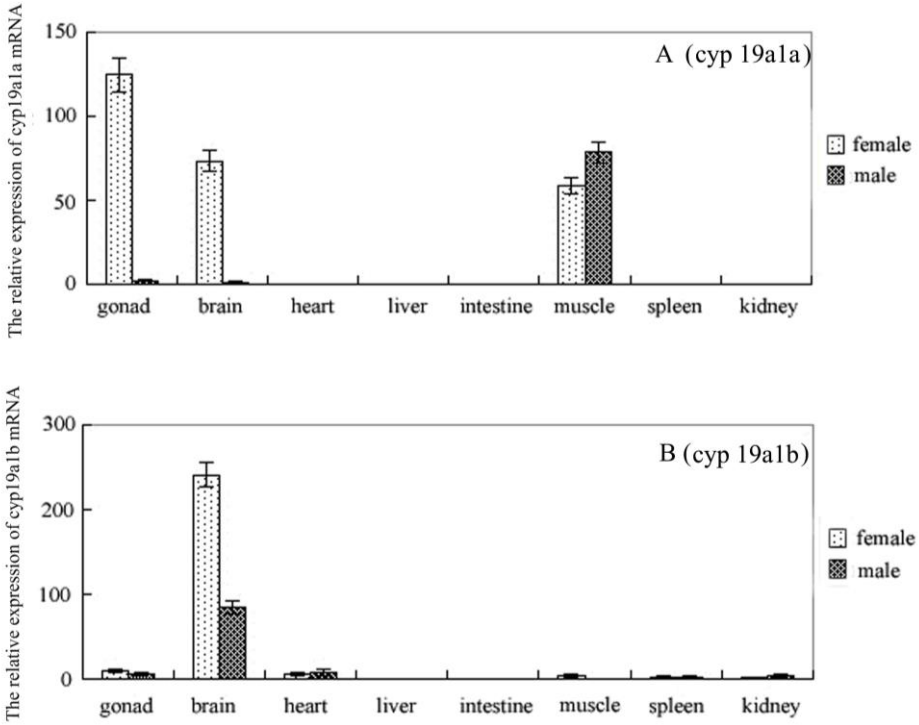

Figure 5. Expression of cyp19a1a and cyp19a1b in ayu different adult tissues. Relative expression was normalized against β-actin. Data are expressed as the mean ± SD. The expression of cyp19a1a was significant differences between the male and the female in gonad, brain and muscle ($P<0.05$). The expression of cyp19a1b was significant differences between the male and the female in brain and muscle ($P<0.05$). Y-axis values in Figure 5 A indicate the folds of expressions in the tissues relative to the average expression in male brain. Y-axis values in Figure 5 B indicate the folds of expressions in the tissues relative to the average expression in female kidney.

Some insert sites of ayu *cyp19a1a* had changed, which neared the relative conserved insert sites of *cyp19a1a* of other fishes. This shows that although the catalytic activity and structure of *cyp19a1a* and *cyp19a1b* are conserved among different fish species, the differential evolution still exists.

The results of the 5′-flank region analysis showed that ayu *cyp19a1a* and *cyp19a1b* had greatly diverged from zebrafish (Tong and Chung, 2003), goldfish(Tchoudakova *et al*., 2001), and *Monopterus albus* (Yu *et al*., 2008) *cyp19*. Many of the common binding sites found in other fishes were not found in ayu. The estrogen response element (ERE) is thought to directly upregulate *cyp19a1b* mRNA levels in response to estrogen in zebrafish and goldfish (Callard *et al*., 2001). However, EREs are neither found in the human, mouse or zebra finch nor in ayu promoter regions of *cyp19* (Honda *et al*., 1994; Honda *et al*., 1999; Ramachandran *et al*., 1999). Therefore, the effects of estrogen on *cyp19a1b* expression in birds and mammals must be exerted through other transcription factors or response elements (Callard *et al*., 2001). Finally, ayu *cyp19a1a* and *cyp19a1b* displayed differed considerably from each other. The

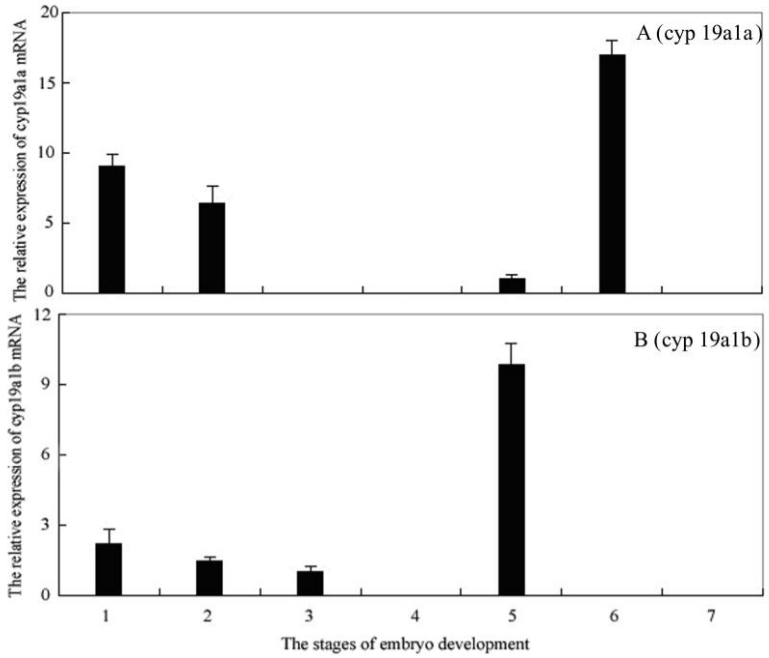

Figure 6. Expression of cyp19a1a and cyp19a1b in the different embryo development stages of ayu (the numbers from 1 to 7 indicate oosperm, morula, blastula, gastrula, neurula, tail bud, and hatch stages, respectively). Relative expression was normalized against β-actin. Data are expressed as the mean ± SD. The expression of cyp19a1a was significant differences between tail bud stage and the other stage ($P<0.05$). And cyp19a1b was significant differences between neurula stage and the other stage ($P<0.05$). Y-axis values in Figure 6 A indicate the folds of expressions in the tissues relative to the average expression in neurula. Y-axis values in Figure 6 B indicate the folds of expressions in the tissues relative to the average expression in blastula.

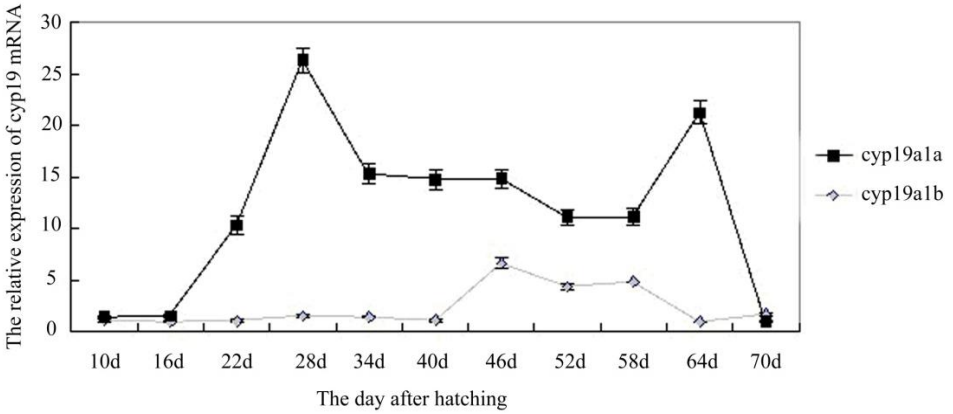

Figure 7. Expression of cyp19a1a and cyp19a1b in the larval stage of ayu. Relative expression was normalized against β-actin. Data are expressed as the mean ± SD. Y-axis values indicate the folds of expressions in the stages relative to the average expressions in 70d fry for cyp19a1a and to the average expressions in 22d fry for cyp19a1b respectively.

putative protein-binding sites of the *cyp19a1b* promoter were associated with more factors involved in embryogenesis and ontogenesis than did the *cyp19a1a* promoter. Taken together, these results indicate that the ayu *cyp19a1a* and *cyp19a1b* genes may have distinct regulatory mechanisms and that the *cyp19a1b* gene may be controlled more accurately of the two.

The expression patterns of *cyp19a1a* and *cyp19a1b* have been reported in many fishes. For example, zebrafish *cyp19a1a* is mainly expressed in the mature ovary and has a feeble expression in the brain and heart; zebrafish *cyp19a1b* is only expressed in the brain(Chiang *et al*., 2001). In the blue gourami (Ezagouri *et al*., 2008), *cyp19a1a* is mainly expressed in the gonad, pituitary gland and liver and is weakly expressed in the spleen and testis, whereas *cyp19a1b* is expressed in the eyes, pituitary gland, gall bladder, liver, and brain. The main sites of *Monopterus albus cyp19* expression were in the female brain, ovary, liver, and male pituitary gland(Yu *et al*., 2008). These results indicate that the highest expression of *cyp19* occur in the brain and gonad. In this study, we show that both ayu *cyp19a1a* and *cyp19a1b* are expressed

in the brain, gonad, and liver and that these genes display some sex-specific expression. The expression patterns were found to be similar to most other fishes, except for the presence of *cyp19a1a* in muscles.

The expression of *Danio rerio cyp19a1b* but not *cyp19a1a* was estrogen-inducible (Sawyer *et al*., 2006). In the embryo, increased expression level of *cyp19a1b* was detected in the oosperm, blastula, and seeding stage, whereas decreased expression level was detected in the multicellular and tail bubble period. The expression of *cyp19a1b* was in a state of flux (Kishida and Callard, 2001). Our data showed that ayu *cyp19a1a* and *cyp19a1b* were significant difference in expression in the embryo. The expression of *cyp19a1a* was mainly restricted to the oosperm, morula, neurula, and tail bud stage and reached its peak in the tail bud stage. The expression of *cyp19a1b* was mainly restricted to the oosperm, morula, blastula, and neurula and reached its peak in the neurula. These differences might due to the different regulations mechanism of *cyp19a1a* and *cyp19a1b* in ayu. The aromatizing enzyme P450arom can convert androgen to estrogen via the brain-pituitary gland-gonad, and the neurula is the most important stage in the development of the central nervous system. The high expression of *cyp19a1b* in the neurula indicates that this gene might play an important role in the development of ayu nervous system.

Using paraffin-mounted larval sections not shown, we inferred the key time points of sex differentiation (20-45 dph for ovary differentiation; 50-70 dph for testis differentiation) in ayu (unpublished data). The expression patterns of *cyp19a1a* and *cyp19a1b* in the juvenile showed that *cyp19a1a* mRNA levels fluctuated significantly during ovary differentiation and testis differentiation. This indicates that *cyp19a1a* is likely to play an important role in gonad-differentiation. The expression of *cyp19a1a* gene can be used as a marker to detect the gonad differentiation and also might be used as the target gene for ayu gender regulation.

Acknowledgments

This study was supported by funding from the Special Preliminary Study of 973 (2008CB117015), Changjiang Scholars and Innovative Research Team Projects (IRT0734), Priority themes of Major Science and Technology in Zhejiang Province (2009C12077), and the K.C. Wong Magna Fund of Ningbo University.

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