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Molecular physiology mechanism of cytochrome P450 aromatase-regulating gonad development in ovoviviparous black rockfish (*Sebastes schlegeli*)

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Abstract

Cytochrome P450 aromatase (P450arom) is an enzyme responsible for the conversion of androgen into oestrogen. To study the role of ovarian aromatase (Cyp19a1a) in sex differentiation, the novel cDNA clone encoding ovarian aromatase (Cyp19a1a) was identified from the ovary of the black rockfish (*Sebastes schlegeli*). The cloned cDNA is 1868 bp in length with 79 bp 5′U TR, 209 bp 3′UTR [excluding poly (A)] and contains 1550 bp ORF, encoding a protein of 516 amino acids. Alignment and phylogenetic analysis showed that the black rockfish ovarian aromatase (Cyp19a1a) shared 61–89% sequence identity with those of other seven fish species. The *cyp19a1a* transcript was found more highly abundant in ovary, but not detected in the testis, brain, liver, spleen, kidney, muscle, skin, heart, head kidney, gill, intestine and eyes by semi-quantitative reverse transcription (RT)-PCR. The *cyp19a1a* gene is not equivalent in tissue-specific expression in the female and male, showed the possibility of distinct promoters and regulatory mechanisms. The expression level of *cyp19a1a* in ovary declined with the ovary development, but was higher than that in testis in all phases. These results indicated that *cyp19a1a* might be involved in gonadal development in *S. schlegeli*.

Keywords: black rockfish, Cyp19a1a, mRNA expression, Steroid hormones

Introduction

In vertebrates, Cytochrome P450 aromatase is a terminal enzyme in the oestrogen biosynthetic pathway, which catalyses the formation of oestrogen (oestradiol-17β, E_2) and estrone from androgen (testosterone and androstenedione), and influences the physiological balance among the sex steroid hormones (Simpson, Mahendroo, Means, Kilgore, Hinshelwood, Graham-lorence, Amarneh, Ito, Fisher, Michael, Mendelson & Bulun 1994a; Conley & Hinshelwood 2001; Simpson & Davis 2001). It has been well established that the reproductive steroid hormones (oestrogens, androgens and progestins) play an important role in the development of gametes, expression of sexually dimorphic characteristics and initiation of reproductive behaviours in all vertebrates by many studies (Harmin, Crim & Weigand 1995; Li, Liu, Zhang & Lin 2006). Timely and appropriate changes in steroid hormone titres are fundamental to reproductive success as a direct result of alterations in the activity of one or more steroidogenic enzymes. The synthesis of oestrogens is dependent on the activity of the steroidogenic enzyme P450 aromatase. As aromatase transcriptional level and enzyme activity level are correlated (Fukada, Tanaka, Matsuyama, Kobayashi & Nagahama 1996; Gelinas, Pitoc & Callard 1998; Chang, Hung, Chiang & Lan 1999), aromatase mRNA levels have been studied as an indicator of oestrogen production and sexual differentiation in vertebrate.

A variety of reports revealed that P450arom was involved in sex differentiation and oocyte development of teleosts (Fukada *et al.* 1996; Gen, Okuzama, Kumakura, Yamaguchi & Kagawa 2001; Ijiri, Kazeto, Lokman, Adachi & Yamauchi 2003). A positive relationship between ovarian P450arom mRNA level and plasma reproductive steroid hormone levels during oocyte development

has been demonstrated in several species such as catfish (*Ictalurus punctatus*), red seabream (*Pagrus major*), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Kumar, Ijiri & Trant 2000; Gen *et al.* 2001; Nakamura, Jennifer, Evans, Kusakabe, Kusakabe, Nagahama & Young 2005; Rodrıguez-Marı, Yan, BreMiller, Wilson, Cañestro & Postlethwait 2005). The human aromatase gene is a single gene for which tissue-specific expression is controlled by alternate promoter splicing (Meinhardt and Mullis, 2002). Interestingly, teleost fish express two subtypes of aromatases cDNA, a predominantly ovarian form (*cyp19a1a*) and a predominantly brain form (*cyp19a1b*) (Piferrer *et al.*, 1994; Simpson *et al.*, 2002; Colbum, Walker & Berlinsky 2009). The cDNA encoding P450arom has been isolated from a number of vertebrates, including human (Corbin, Graham-Lorence, McPhaul, Mason, Mendelson & Simpson 1988; Harada 1988) and birds (McPhaul, Noble, Simpson, Mendelson & Wilson 1988). Among teleosts, the *cyp19a1a* cDNA has been cloned from many fish species, such as rainbow trout (Tanaka, Telecky, Fukada, Adachi, Chen & Nagahama 1992) Japanese eel (*Anguilla japonica*) (Ijiri *et al.* 2003), European eel (Tzchori, Degani, Hurvitz & Moav 2004), orange-spotted grouper (*Epinephelus coioides*) (Zhang, Zhang, Zhang, Zhu, Tian, Li & Lin 2004), killifish (*Fundulus heteroclitus*) (Greytak, Champlin & Callard 2005) and half-smooth tongue-sole (*Cynoglossuss semilaevis*) (Deng, Chen, Xu & Liu 2009). These studies suggested that aromatase was very important for understanding the physiological roles of steroid hormones, particularly oestrogens, in vertebrates.

Black rockfish (*Sebastes schlegeli*) is a typical marine ovoviviparous fish, whose yolk accumulated in oocytes during vitellogenesis as an energy source (Boehlert *et al.* 1991). The blackfish widely distributed in the coast of Japan, Korea and China (Mori, Nakagawa, Soyano & Koya 2003) accounts for a large percent of sea fishing these years. The present studies about black rockfish of reproductive physiology were limited, it is meaningful to research the endocrine regulatory mechanisms with this kind of fish to further understand the other kinds of ovoviviparous fish.

The purpose of this study was to isolate and characterize the full length cDNA encoding the ovarian P450arom in *S. schlegeli*, characterize its expression in different tissues and conduct correlation analysis between P450arom and serum steroid hormones such as oestradiol-17 (E2) and testosterone (T) in different gonad developmental stages. Our study indicated that the E_2 may induce the liver synthesis of vitellogenin and satisfy the reproductive activities, and T was synthesized as the precursor of E2, which was converted by P450arom.

Materials and methods

Animals and the sampling procedure

Ten cultivated black rockfish (*S. schlegeli*) were randomly captured from an aquatic farm (Qingdao City, Shandong, China) every 2 months, from September 2008 to July 2009. And fish were held for 3–4 days in tanks with natural sea water in our laboratory during holding. In this period, water in the aquaria was continuously filtered, aerated and the temperature in the aquaria was maintained between 20°C and 23°C by heating elements of the pumps or by air-conditioning. Sampled fish were anaesthetized in 100 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma, St Louis, MO, USA) and sacrificed after decapitation. The weights of the ovaries and livers were recorded for calculating the GSI and HSI respectively: GSI = [gonad weight/(body weight − viscera weight)] × 100, HSI = [liver weight/(body weight − viscera weight)] × 100, values are expressed as mean ± standard deviation of mean. The other tissues were also collected such as gonads, liver, spleen, kidney, brain, intestine, gill, heart, stomach, fat, pyloric caecum and head kidney. Then tissues immediately frozen in liquid nitrogen, and stored at −80°C until extraction of total RNA.

Histological analysis

Tissue sections were fixed in Bouin's solution, paraffin embedded, sectioned at 5–7 μm thick and were stained with HE (haematoxylin and eosin), then photographed by light microscopy (Nikon–E200; Tokyo's Chiyoda District, Japan). The classification of gonadal development stages was based on the previous work for Korean rockfish (Shi, Wen, He, Li, Yang, Chen, Zhang, Chen, Jin, Shi, Qi & Li 2011). The results are shown in Table 1.

Steroid radioimmunoassay (RIA)

Blood samples were collected from the caudal vein after anaesthetizing the fish with MS-222.

Table 1 Sample records and gonad stage of the black rockfish

Time	Stage of testis	Stage of ovarian
November	Later spermatogenesis period (Vstage)	Vitellogenic and ovulation period
January	Absorption period	(III-IV stage)
March	(VIstage)	Ovulation and embryonic period (IV-Vstage)
May	Spermatogonia	Embryonic (V stage)
July	proliferation period (IIIstage)	Immature period (II stage)
September	Early spermatogenesis period(IVstage)	

Blood was allowed to clot in 4°C for 4–6 hours and the serum was obtained by centrifuging at 16 000 g for 10 min. The serum was stored at −40°C for the steroid analysis. The E_2 and T levels were measured by 125I radioimmuno-assay (RIA) kits (Tianjin Nine Tripods Medical & Bioengineering, Sino–US joint–venture enterprise, China) following the procedure modified by He, Wen, Dong, Wang, Chen, Shi, Mu, Yao and Zhou (2008). The binding rate is highly specific with an extremely low cross reactivity to other naturally occurring steroids, less than 0.1% to most circulating steroids. The coefficients of intra-assay and inter-assay variation were 7.7–9.8% and 7.4–8.9% respectively, for the E_2 and T assay. Any samples with coefficients of variation higher than 10% were excluded in the analysis. The assay sensitivity reached 2.1 pg mL^{-1} and 1.9 ng mL^{-1} for E2 and P by the kit protocol respectively.

Total RNA isolation and first strand cDNA synthesis

Total RNA was extracted from black rockfish ovary using the commercial product RNAiso (Takara, Tokyo, Japan) according to the manufacturer's instructions. The concentration and purity of the total RNA were quantified and validated on a UV spectrophotometer (Ultrospec-2100Pro; Amersham, Baoshan district, Shanghai, China) and its integrity was confirmed by a 1.5% agarose-gel electrophoresis. DNaseI (Takara, Japan) treatment was performed to remove DNA contamination. First-strand cDNA was synthesized using oligo (dT) primer following the manuscript (Takara, Japan). The synthesized cDNA was stored at −20°C for further use.

Isolation and PCR amplification of P450aromA cDNA fragments

To clone the P450aromA cDNA core fragment of black rockfish, a pair of degenerate primers with the redundant positions (n = A, C, G, and T; r = A and G; y = C and T; h = A, C, and T; k = G and T) (AF1/AR1, Table 2) were designed by CODEHOP (Chen, Wen, He & Dong S 2009) of zebrafish (*D. rerio*), rainbow trout (*O. mykiss*), European seabass (*Dicentrarchus labrax*) and orange-spotted grouper (*E. coioides*) and used to amplify approximately 500 bp cDNA from ovary. The PCR amplification was carried out using touchdown PCR, with 94°C for 5 min, 10 cycles of 35 s at 94°C, 35 s at the annealing temperature and 35 s at 72°C with a range of annealing temperature from 63°C to 53°C, decreasing 1°C each cycle and followed by additional 31 cycles at the annealing temperature of 53°C, finally ended with an extension of 10 min at 72°C. The PCR products were separated through 1% agarose gel and purified using TIANGEL gel midi Purification Kit (TIAGEN, Beijing, China), then ligated into the PGM–18T plasmid vector (Takara, Japan). The recombinant was transformed into DH5α competent cells.

5′and 3′ RACE–PCR

To obtain 3′ and 5′ ends of P450aromA sequences, two gene-specific primers (GSPs), A1 for 3′ ends and A2 for 5′ ends (Table 2), were designed. SMART™ RACE cDNA Amplification Kit (Clontech, Washington, USA) was used to 5′ and 3′ ends RACE–PCR. Reverse transcription was carried out using 1 μg of total RNA from black rockfish ovary. The PCR amplifications were performed according

Table 2 Primers used for black rockfish CYP19A gene cloning and mRNA expression analysis. (n = A, C, G, and T; r = A and G; y = C and T; m = A and C)

Primer symbol	Primer sequence(5′-3′)	Amplicon size
AF1	TGAGCTGCATCGGCatgtaygarmg	500 bp
AR1	TCCAGCACGCACTGCacnacrttytc	
A1	CATCTGGACGACCTGGACAG-TTTGGCT	1868 bp
A2	GCCAAACTGTCCAGGTCGTC-CAGATGAG	
18sF	CCTGAGAAACGGCTACCACATC	119 bp
18sR	CCAATTACAGGGCCTCGAAAG	

to the manufacturer's instructions. The PCR product of the expected length was separated on 1.5% agarose gel and purified using TIAN gel midi Purification Kit (QIAGEN, Beijing, China). The purified fragments were then cloned into PGM-T18 vector (QIAGEN, China) followed by propagation in *E. coli* DH5α and subsequently sequenced on an ABI3730XL sequencer (Applied Biosystems, Forster city, CA, USA).

Semi-quantitative RT–PCR analysis

Semi-quantitative reverse transcription (RT)-PCR assays were performed to evaluate the expression pattern of *cyp19a1a* in 12 tissues from male and female black rockfish during different reproductive stages. Four fish were randomly chosen from male and female respectively for investigating the temporal expression of *cyp19a1a* in each gonad's developmental stage. The integrity of all RNA samples was verified by the successful amplification of 18s rRNA. The first-strand cDNAs were used as templates for PCR with specific primers (using primer 5.0 version) (AF2/AR2; Table 2), which amplified a PCR product of 204 nucleotides in the protein-encoding region. The forward and reverse primers for 18s rRNA were 18SF and 18SR. All sequences of primers were shown in Table 2.

Approximately 5 ng of total RNA was amplified by PCR. The cycling condition for 18s rRNA was as follows: 94°C, 5 min; followed by 21 cycles for 18s rRNA of 94°C, 35 s, 61°C, 35 s, 72°C, 35 s and a final extension at 72°C for 10 min. The cycling conditions for cyp19a1a (Primer AF2/AR2) was as follows: 94°C, 5 min; followed by 40 cycles of 94°C, 35 s, 60°C, 35 s, 72°C, 35 s and a final extension at 72°C for 10 min. The 18s rRNA are considered suitable internal control genes other than β-actin (Schmittgen and Zakrajesk 2000; Goidin *et al.* 2001). The RT-PCR products were separated on a 1% agarose gel and stained by ethidium bromide. Following densitometric measurements of amplified bands were analysed using the software, Gel Image System Ver. 3.60 (Tanon, Shanghai, China).

Sequence analysis and Statistics

Sequence alignments, comparisons and their similarity score of homology between various *cyp19a1a* cDNA sequences were performed using Clustal W and EBI clustalw online services provided by http://www.ebi.ac.uk/clustalw/. Cyp19a1a amino acid was carried out using the Clustalw programs and phylogenetic analysis was performed by multiple alignments of deduced amino acid sequences with the Neighbour–Joining method using MEGA 4.0 Software. Structure prediction analysis was carried out with the ExPASy Molecular Biology Server (http://www.expasy.ch/).

Relative values of *cyp19a1a* mRNA (*cyp19a1a* mRNA/18s rRNA) were subjected to one-way analysis of variance followed by Duncan's test. Differences were considered significant for $P < 0.05$. The statistical analyses of GSI and RIA were also processed by one-way analysis and Duncan's test. All the results are expressed as means ± SE.

Results

Relationship between GSI, HSI and the gonads developmental phase

Changes in GSI and HSI, according the result of HE-stained for black rockfish of female and male, are shown in Fig. 1a and Fig. 1b. The average GSI of female black rockfish increased significantly from the November to March ($P < 0.05$), and kept increasing significantly to May ($P < 0.05$), then dropped significantly during July–September ($P < 0.05$). The level of HSI remained high during November–March, and then dropped slightly from May.

In the male black rockfish, GSI changed steadily in the first three stages; from November to March, the level began to increase, and then dropped during May. In July–September, the level sharply increased to the highest. At the same time, the level of HSI rose from November to March, fell down from March to July to September, which had the lowest level.

Serum steroid levels

Significant titres of E_2 and T were detectable throughout the whole year. Fig. 2 shows the level changes of serum E_2 and T in female black rockfish. In female, serum E_2 levels steadily dropped from November to March, and dropped from March to May, and reached the bottom during September. T levels peaked in May and remained at low serum levels in other stages.

The changes in serum E_2 and T levels in male black rockfish are shown in Fig. 3. In male, serum

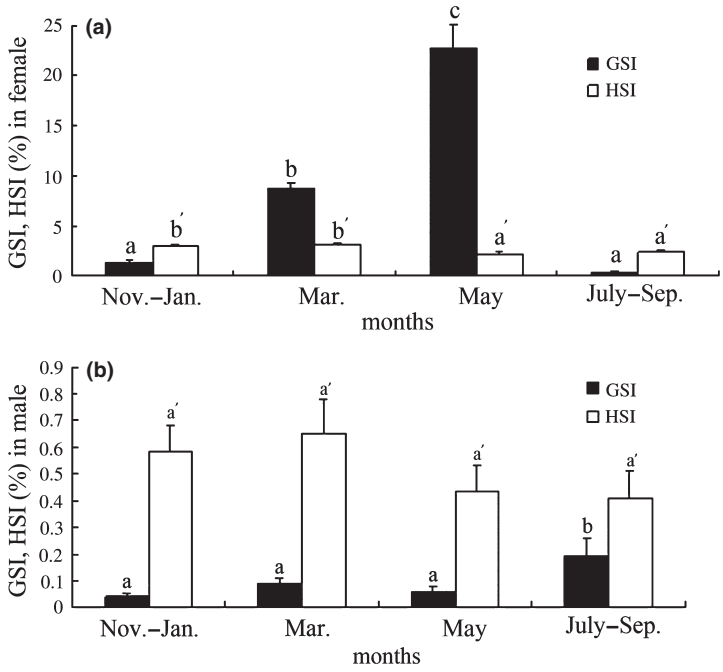


Figure 1 Gonadosomatic index (GSI) and Hepatosomatic (HSI) indices of female (a) and male (b) black rockfish. Values are expressed as mean ± standard deviation of mean. Different letters indicate significant difference ($P < 0.05$).

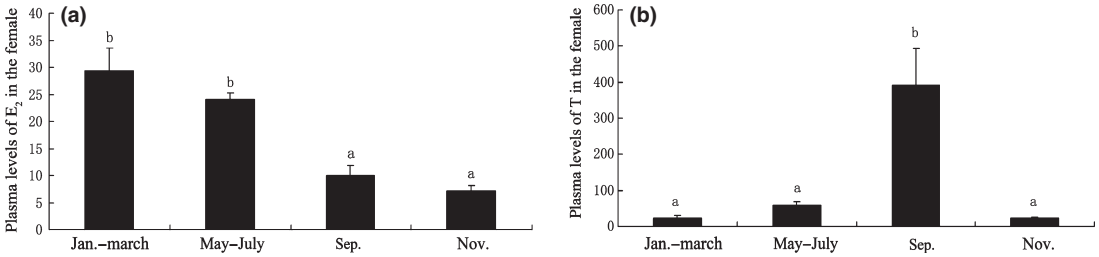


Figure 2 Serum 17-Oestradiol and testosterone levels in female black rockfish. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$).

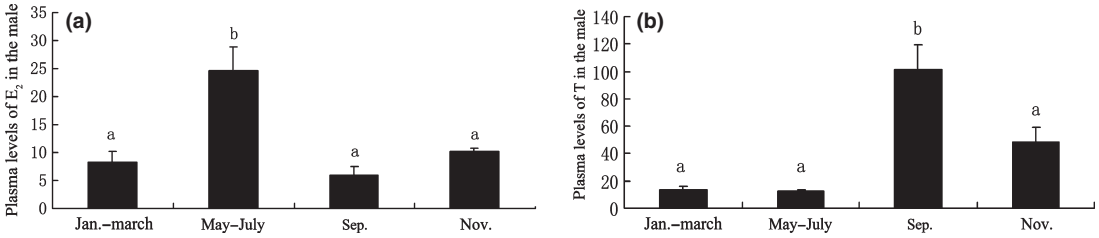


Figure 3 Serum 17-Oestradiol and testosterone levels in male black rockfish. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$).

E_2 levels increased from January to July, then significantly dropped in September, while, there is an increase during November. T levels decreased from January to July, then sharply increased to the highest level in September and constantly dropped to lower levels from this stage.

Isolation and characterization of Cyp19a1a cDNA from the ovary of black rockfish

Ovarian aromatase cDNA of 1868 nucleotides was obtained from the ovary of black rockfish (NCBI number: FJ594995). This full-length cDNA

included 1494 nucleotides open reading frame (ORF) encoding deduced proteins of 516 amino acid residues, a 79 nucleotides 5′-untranslated region (UTR), a 209 nucleotides 3′–UTR and 87 nucleotides poly (A) tail. Regions which are important to enzymatic function are highly conserved across species such as the membrane-spanning region, the I-helix, Ozol's peptide region, aromatic region, and haem-binding region (Fig. 4). Overall, the amino acid sequence identity between black rockfish Cyp19a1a and Cyp19a1b was 65%. Alignment and phylogenetic analysis showed that the ovarian P450arom of black rockfish shared 65% sequence identity with its brain aromatase and 61–89% identity with ovarian aromatases of *D. rerio* (AAG12243), *E. coioides* (AAR97601), *D. labrax* (AAM95455), *Mugil cephalus* (AAW72732), *Pelteobagrus fulvidraco* (AAW65999), *C. semilaevis* (ABM90641), *Kryptolebias marmoratus* (ABC68613), but only 58–65% with brain aromatases of the seven fish, 54%, 35% and 49% with *Xenopus tropicalis* (BAE93232), *Branchiostoma floridae* (ABA47317) and *Homo sapiens* (NP_112503) aromatases.

Phylogenetic analysis

The black rockfish Cyp19a1a amino acid sequence and several published Cyp19a1a and Cyp19a1b in various animals, including teleosts, cartilaginous fish, amphibian and mammals, were used to characterize phylogenetic relationships. The consensus tree shows two main branches (Fig. 5), the first main branch bifurcates into two clear branches. One of them clustered all *CYP19* of teleosts, and the other branch included *X. tropicalis*, *B. floridae* and *H. sapiens,* while the *CYP19* of teleosts group was divided into two distinct groups (Cyp19a1a and Cyp19a1b). The black rockfish Cyp19a1a was found to belong to the teleosts Cyp19a1a branch (Fig. 5) and most close to the *E. coioides* Cyp19a1a.

Expression of cyp19a1aCYP19A in different adult tissues

Semi-quantitative RT–PCR results in different tissues at stage III of male and stage II of female

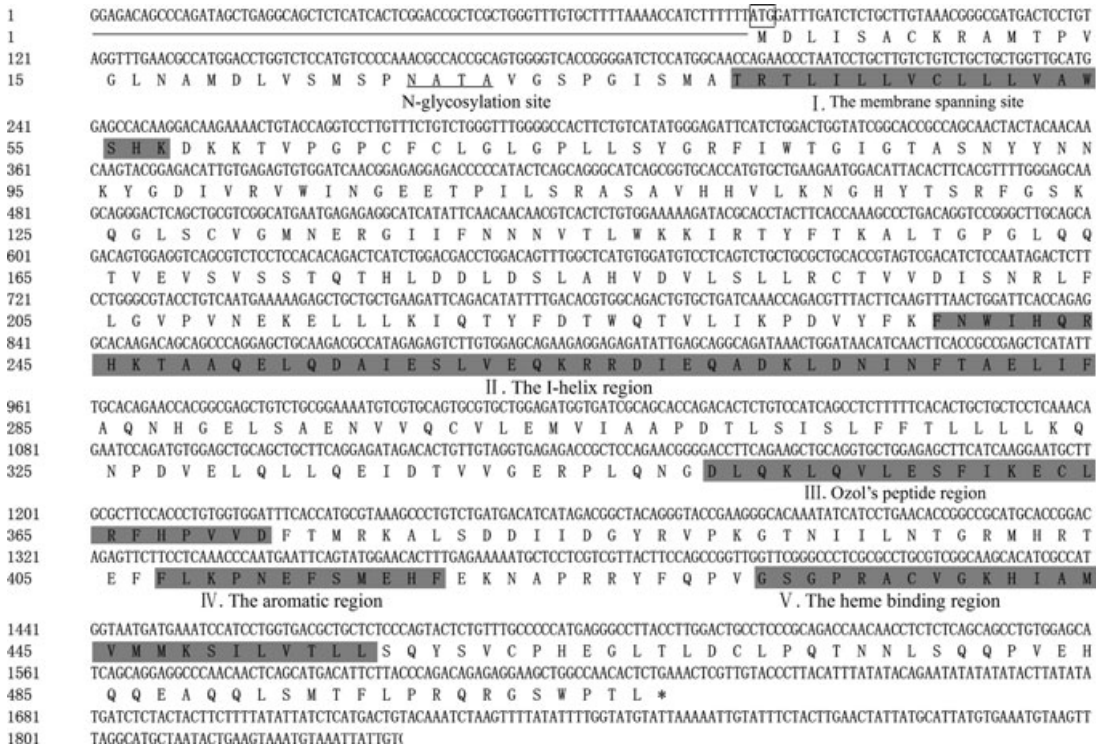


Figure 4 The 22 CYP1a1acDNA and the deduced amino acid sequence of Sebastes schlegeli. The membrane-spanning region, the I-helix, Ozol's peptide region, aromatic region, and haem-binding region signature are shaded. (*) is the meaning of termination codon. 5′UTR are underlined and 3′ are italics. An N-glycosylation site is marked with a dotted line. The start codon is marked with a box. GenBank accession no. NJ594995.

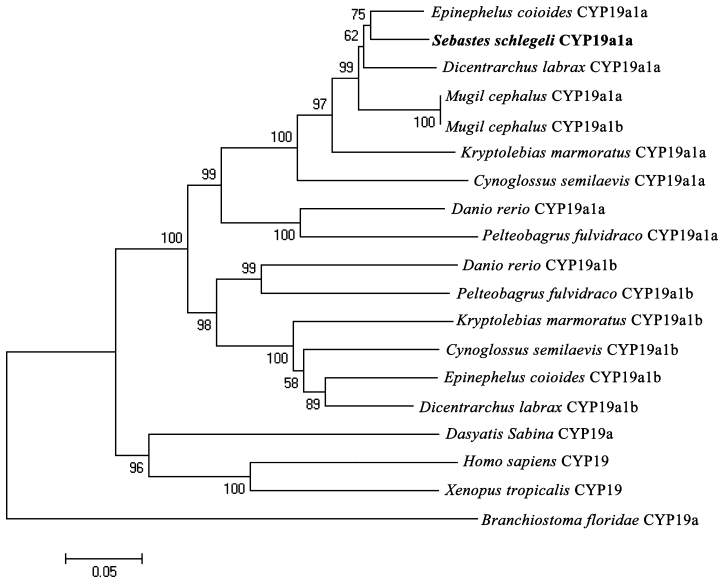


Figure 5 Phylogenetic tree for black rockfish P450arom proteins. All the proteins extracted from genbank, the accession numbers are: *Homo sapiens* CYP19a (NP_112503), *Xenopus tropicalis* CYP19a (BAE93232), *Dasyatis Sabina* CYP19a (AAF04617), *Branchiostoma floridae* CYP19a (ABA47317), *Danio rerio* CYP19a1b (AAG12245), *Epinephelus coioides* CYP19a1b (AAR97602), *Dicentrarchus labrax* CYP19a1b (CAC43178), *Mugil cephalus* CYP19a1b (AAW72732), *Pelteobagrus fulvidraco* CYP19a1b (AAU25806), *Cynoglossus semilaevis* CYP19a1b (ABL74474), *Kryptolebias marmoratus* CYP19a1b (BAF03500), *Sebastes schlegeli* CYP19a1a (ACN39247) *D. rerio* CYP19a1a (AAG12243) *E. coioides* CYP19a1a (AAR97601), *D. labrax* CYP19a1a (AAM95455), *M. cephalus* CYP19a1a (AAW72732), *P. fulvidraco* CYP19a1a (AAW65999), *C. semilaevis* CYP19a1a (ABM90641), *K. marmoratus* CYP19a1a (ABC68613).

(Fig. 6) demonstrated that the expression of ovarian aromatase was highly abundant in the ovary, but not present in the testis, liver, spleen, kidney, brain, intestine, gill, heart, stomach, fat, pyloric caecum, head kidney in both sexes.

P450aromA expression in gonads with annual changes

Fig. 7 and Fig. 8 show the variation in *cyp19a1a* in gonads of females and males during the different stages of gonadal reproductive cycle. The relative mRNA expression of *cyp19a1a* in female peaked from November to January,then dropped to the lowest level in May, and increased during July–September. In male, the expression was peaked during January–March, and less expressed in September; however, it was almost undetectable in May–July and November.

Discussion

Cytochrome P450aromatase, whose expression is driven by multiple tissue-specific promoter, is an important rate-limiting enzyme responsible for conversion of testosterone into oestrogens (Luckenbach, Lea, Ashlee, Borski, Daniels & Godwin 2005). In mammals, the P450arom protein is

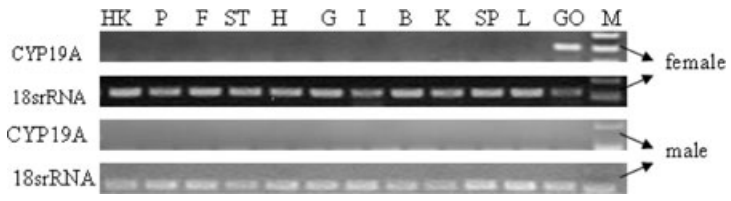


Figure 6 Expression pattern of P450arom in different adult tissues detected by RT–PCR. GO: gonad; L: liver; SP: spleen; K: kidney; B: brain; I: intestine; G: gill; H: heart; ST: stomach; HK: head kidney; F: fat; P: pyloric caecum; M: marker.

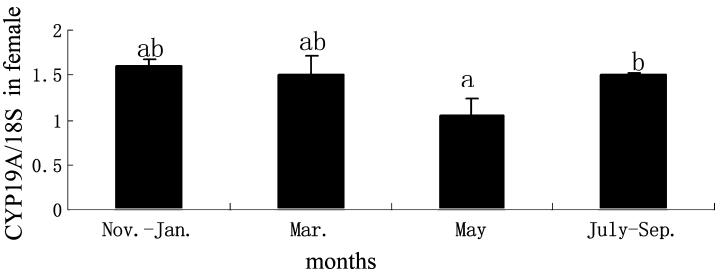
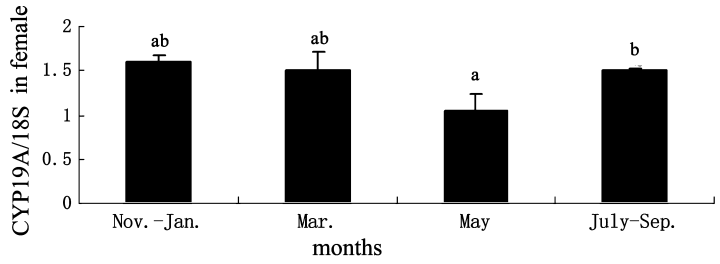


Figure 7 P450arom mRNA expression in ovarian with annual changes. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test).



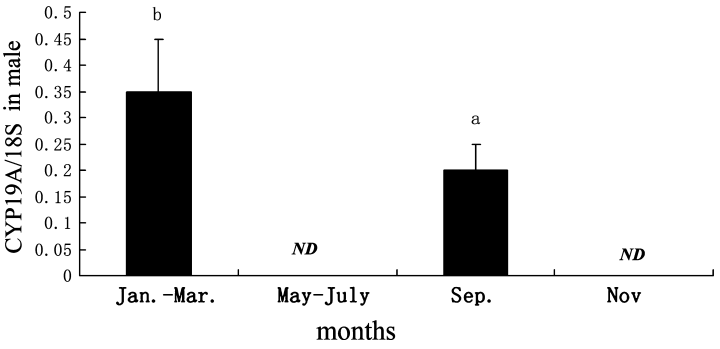


Figure 8 P450arom mRNA expression in testis with annual changes. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test).

encoded by a single P450arom gene (Simpson *et al.* 1994). However, fish have two forms of P450arom, termed cyp19a1a and cyp19a1b, which are encoded by separate gene loci (*CYP19A* and *-B*, or alternately *CYP19a1* and *-a2*) and predominantly express in ovary and brain respectively (Tchoudakova & Callard 1998; Zhao, Mak, Tchoudakova, Callard & Chen 2001). BLAST analysis result of the cDNA sequence isolated in this study indicates very high identity with other teleosts cyp19a1a sequences. When aligned with aromatase proteins from other vertebrate classes, the deduced amino acid sequence is highly conserved in the black rockfish protein, especially in several major functional domains, such as the membrane-spanning region, I-helix, Ozol's peptide region, aromatic region, and haem-binding region (Ijiri, Berard & Trant 2000; Deng *et al.* 2009). The sites of I133, E302, P308, D309, T310, R435, C437, which play an important role in the activity, are also exactly the same with other species (Kao, Cam, Laughton, Zhou & Chen 1996). Taken together, we provide substantial evidence that the isolated cDNA encoding black rockfish *cyp19a1a* belongs to gonad P450arom subfamily. In addition, this study indicates that the black rockfish *cyp19a1a* should be conserved among species in the catalytic activity, structure and function.

In our study, the GSI level of female black rockfish was increased to the top in the V stage, which is similar to the result with Senegalese sole (García-López, Couto, Canario, Sarasquete & Martínez-Rodríguez 2007). In amberjack and Korean spotted sea bass, the GSI level was also increased in the ovulation stage. (Micale & MaricchioloGenovese 1999; Lee & Yang 2002) . In addition, Johnson *et al.* suggested that the highest level during stage III in fish indicated the strong liver vitellogenin synthesis and secretion, and most of energy was used for protein synthesis and

transport in this stage (Johnson, Casillas, Myers, Rhodes & Olson 1991). In male black rockfish, the change in GSI level was not significant, and highest GSI was in the IV stage, showing mature testis in that period. However, the low level of HIS in stage VI may indicate the low level of liver vitellogenin synthesis and secretion.

Cyp19a1a is predominantly expressed in ovary (Tchoudakovaet & Callard 1998; Xu, Yu & Tang 2005; Greytak *et al.* 2005), while weakly in other tissues in many kinds of teleosts (Kitano, Takamune, Kobayashi, Nagahama & Abe 1999; Kobayashi, Nakamura, Sunobe, Morrey, Suzeki & Nagahama 2004; Luckenbach *et al.* 2005; Deng *et al.* 2009). In our study, *cyp19a1aCYP19A* was detected in testis and ovary by RT–PCR, and mRNA expression level was much higher in female ovary than in male testis. Expression analysis during reproductive cycle showed that the level was extremely low in vitellogenic stage (stage III), and that there is also no expression in testis in vitellogenic stage in tissue distribution research, all of the results demonstrating the low level of *cyp19a1a* expression in this stage. In addition, the expression was not detected in many fish species, which is similar to the research results in Atlantic halibut, tongue sole and zebrafish (Deng *et al.* 2009; Matsuoka, Nes, Andersen, Benfey & Reith 2006; Sherilyn *et al.,* 2006). Many studies showed that P450arom activity is responsible for endogenous E_2 biosynthesis in gonads, and thereby induced the process of oocyte development (Fukada *et al.* 1996; Gen *et al.* 2001; Ijiri *et al.* 2003; Deng *et al.* 2009). In our study, the higher expression level of P450aromA in ovary also demonstrated its correlation with oocyte development.

The *cyp19a1a* mRNA expression level correlated with plasma oestradiol levels was detected in many kinds of fish species (Kumar *et al.* 2000; Nakamura *et al.* 2005; Hong, Wu & Zhang 2009). In female gonad, we found that the transcription level of *cyp19a1a* dropped steadily from November to May in the following year when the ovary developed from vitellogenic stage (stage IIIto ovulation stage (stage VI). There were similar changes in the serum E_2 levels; however, the serum T levels were increased at the same time. From May to September, the amount of P450aromA transcripts and serum T levels increased, but serum E_2 levels in this period continued to decrease. This may be because E_2 was used to induce the liver synthesis of vitellogenin to satisfy the reproductive activities, and T was synthesized as the precursor of E_2. The hepatosomatic index, which reflects the production of vitellogenin by the liver (Nakamura *et al.* 2005), has a similar changing pattern with serum E_2 levels. All these results suggest that the activity of follicles to synthesize E_2, which are physiologically important during vitellogenesis, is at least partially regulated by P450arom gene. As we know, *cyp19a1a* can promote the conversion of T into E_2 (Roselli, Abdelgadir, Jorgensen & Resko 1996; Skolnessa, Durhan, Garcia-Reyero, Jensen, Kahl, Makynen, Martinovic-Weigelt, Perkins, Villeneuve & Ankley 2011;). Similar to *Bostrichthys sinensis*, peak serum T in black rockfish occurs later than the E_2. This may be due to the decrease in P450aromA activity at the end of vitellogenic stage (stage IV) phase of oocyte. However, the changes in *cyp19a1a* expression level were different with zebrafish (Goto-Kazeto, Kight, Zohar, Place & Trant 2004), in which the highest *cyp19a1a* expression level was found in the vitellogenic stage (stage II). But, the *cyp19a1a* expression level in black rockfish maintained at a high level in immature stage. Another kind of difference can be found in the expression pattern of *cyp19a1a* in black rockfish compared with killifish and rainbow trout (Valle, Ramina, Vianello, Belvedere & Colombo 2002; Dong & Willett, 2008), in which the expression of *cyp19a1a* cannot be detected in mature stage. This kind of difference may be explained by the special ovoviviparity reproduction mode of black rockfish, whose eggs hatched in the female body do not obtain nutrition from female, but rely on yolk proteins accumulating in oocytes as an energy source (Boehlert *et al.*, 1991). In addition, Guiguen *et al.* suggested that *cyp19a1a* down-regulation is the only necessary step for inducing a testicular differentiation pathway (Guiguen, Fostier, Piferrer & Chang 2010).

As an enzyme responsible for the conversion of androgen into oestrogen, P450arom also plays an important role in reproductive and gonad development in male teleosts (Robert & Yoshitaka 2002). Several studies have already demonstrated that several special physiological activities are required for testis differentiation such as the decrease in the aromatase expression level, inhibition of aromatase activity and dropping of E_2 level (Kitano *et al.* 1999; ;Kwon, McAndrew & Penman *2001*; Tzchoria *et al.,* 2004). We analysed the transcriptional profile of *cyp19a1a* in testis in the whole reproductive cycle and found that *cyp19a1a* had lower

expression level in early spermatogenesis period (stage IV and absorption period (stage VI) of the testis, and no expression was detected in other phases in male black rockfish. Some other studies showed that the aromatase remained undetectable in the developed testis of some species such as Atlantic halibut (Matsuoka *et al.* 2006), Japanese flounder (Kitano *et al.* 1999) and Nile tilapia (Kwon *et al.* 2001). Guiguen *et al.* suggested that the down-regulation is the necessary step for inducing a testicular differentiation pathway (Guiguen *et al.* 2010). Low level expression in testis of male black rockfish may also demonstrate the non-detection of aromatase in developed testis of Atlantic halibut (Matsuoka *et al.* 2006), Japanese flounder (Kitano *et al.* 1999) and Nile tilapia (Kwon *et al.* 2001). It is more likely that the regulation of P450arom plays an important role in males, rather than the presence of P450arom as it is the absence of this enzyme that keeps T levels high in developing male gonads. All these results suggested that P450arom and E_2 play an important role during the reproductive cycle of testis in black rockfish.

In this study, we cloned and characterized the full-length cDNA sequences of gonadal cytochrome P450 aromatase (*cyp19a1a*); and the mRNA levels in the testes and ovaries were analysed and correlated with E_2, T levels and HSI, GSI levels during reproductive cycle. The results demonstrated that *cyp19a1a* has a higher expression level in ovary, which indicated that the ovary of black rockfish was the main tissues of *cyp19a1a* expression. Our study also showed a kind of close relationship between E_2, T and *cyp19a1a* in endocrinological mechanism of black rockfish. All of these results would facilitate further advances in understanding the molecular physiology and endocrinological mechanism of fish.

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