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Cloning and expression analysis of *Foxl2* during the reproductive cycle in Korean rockfish, *Sebastes schlegeli*

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Abstract Foxl2 is a member of the winged helix/forkhead family of transcription factors and is known to regulate ovarian aromatase, which plays a crucial role in ovarian differentiation. To address the role of *Foxl2* in gonads and brain during gonadal development, we isolated the full-length cDNA of *Foxl2* and analyzed its spatiotemporal expression patterns in the viviparous teleost Korean rockfish, *Sebastes schlegeli*. Tissue distribution pattern revealed that the *Foxl2* was detected in the liver, fat, gill, brain, and ovary, but could hardly be found in the testis. Reverse transcriptase PCR suggested that *Foxl2* in Korean rockfish may involve in ovary development in the study of expression level during gonads development. It also revealed that the stage of highest expression level for *Foxl2* was almost much earlier than *cyp19a1a* and *cyp19a1b* during the gonadal development stage in gonads and brain except for *cyp19a1a* in brain. Furthermore, the expression pattern of *Foxl2* as well as aromatases may imply the role of *Foxl2* in the up-regulation of aromatases not only in the female fish but also in male.

Keywords *Sebastes schlegeli* · Foxl2 cDNA clone · mRNA expression · P450 aromatases

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Introduction

In the sex determination, a primary signal initiates the onset of a cascade of transcriptional or mRNA splicing factors, allowing the final differentiation of the gonads into testis or ovary (Naimi et al. 2009). It has been considered that estrogen plays critical roles in sex differentiation, and the effects of environmental factors such as high temperature on sex differentiation have been demonstrated in Korean rockfish (Devlin and Nagahama 2002; Omoto et al. 2010). It is well established that the FOX (forkhead box) family of transcription factors with a 100-amino-acid domain was conserved and played a central role in ovarian differentiation and the regulation of cellular differentiation and proliferation (Carlsson and Mahlapuu 2002). One of the FOX family members is *Foxl2*, a putative winged helix/forkhead transcription factor gene involved in ovarian development and function. In mammals, its mutation leads to the blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), involving eyelid malformations and premature ovarian failure (POF) (BPES type I) or occurring without premature ovarian failure (type II) (Zlotogora et al. 1983; Crisponi et al. 2001; De Baere et al. 2003; Cocquet et al. 2002). A study demonstrated the *Foxl2* was capable of interacting at GnRH receptor–activating sequence (GRAS), which mediated the activin responsiveness of murine GnRH receptor gene promoter (Ellsworth et al. 2003). In mice, *Foxl2* suppressed the testicular differentiation that promoted

testis-specific expression of Sox9 mainly through the repression of Sox9 regulatory element, which may lead to gonadal sex reversal (Uhlenhaut et al. 2009).

Foxl2 cDNA was isolated in several teleost species, including mammals and non-mammalian species, such as medaka (*Oryzias latipes*) and Nile tilapia (*Orechromis niloticus*) (Wang et al. 2004). The expression of *Foxl2* in some vertebrate species ovarian is specifically initiated before morphological sex differentiation in gonads and maintained throughout the ovarian development (Pailhoux et al. 2001; Cocquet et al. 2002; Loffler et al. 2003). In addition, a report of mouse demonstrated that *Foxl2* mRNA was detected in both granulosa cells and oocytes of fetal and adult individuals (Loffler et al. 2003). However, the follicular cells displayed a strong protein expression of *Foxl2* while the stroma showed more diffused (Cocquet et al. 2002), and no protein signal was detected in the oocytes of mammals (Pannetier et al. 2003). The existing data denote the cytochrome P450 aromatase restricted the proportion of sex steroids, specifically androgens and estrogens which were very important for sexual/gonadal differentiation (Govoroun et al. 2004). And *Foxl2* binds to the sequence ACAAATA in the promoter region of the *cyp19a1a* gene directly through its forkhead domain and activates the transcription of *cyp19a1a* with its C terminus (Wang et al. 2007). It was suggested that *Foxl2* may play an important role in the ovarian differentiation by the entire steroidogenic pathway (Yamaguchi et al. 2007).

Korean rockfish (*Sebastes schlegeli*), a widely distributed marine ovoviviparous fish, is of a great requirement in sea fishing these years. It is a typical ovoviviparous species which mainly inhabits the coast waters of Korea, Japan and China. In addition, there is less reported information about reproductive physiology in ovoviviparous species. So it is meaningful to get more information about this kind of fish.

In our study, it would be intriguing to explore the spatiotemporal expression pattern of *Foxl2*, *cyp19a1a*, and *cyp19a1b* in gonads and brains during gonadal reproductive cycle. This analysis might further provide valuable evidences for the significance of *Foxl2* in the regulation of aromatases. To accomplish this, we cloned full-length cDNA of *Foxl2* from the ovary of Korean rockfish, and then the sequence information was employed to characterize the expression at molecular level.

Materials and methods

Experimental fish and sampling

About twenty samples including mature male and female Korean rockfish were obtained from Shandong coastal area every 2 months. All fish were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO). After excising the gonads, sexual maturity was determined by the presence of mature ova and sperm. In order to perform seasonal cycle studies, adult male fishes ($n \geq 3$) were collected in mainly at stage II (spermatogonia stage), stage III (immature sperm stage), stage IV (mature testes stage), and stage V (spermiation stage); and female fishes were mainly at stage II (perinucleolar oocyte stage), stage III (primary yolk stage), stage IV (secondary yolk stage), stage V (tertiary yolk stage), and stage VI (gestational ovary stage) (according to Shi et al. 2011). Tissues including brain, heart, ceca, liver, gill, head kidney, bowel, stomach, spleen, gonad and pituitary samples at various stages from Korean rockfish were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until some of them were selected for the extraction of total RNA.

Total RNA extraction and reverse transcription (RT)

Total RNA was prepared using RNAiso reagent (Takara, Japan) following the manufacturer's instructions. The RNA concentration of each sample was quantified by UV spectrophotometer (Ultrospec-2100Pro, Amersham), and a 1.5 % agarose gel was applied to check RNA integrity. Then, first-strand cDNA was synthesized respectively with total RNA (1 μg) in a total volume of 10 μl, using random primers and M-MLV reverse transcriptase (Takara, Japan).

Isolation and PCR amplification of *Foxl2* cDNA fragments

For amplification of the full-length sequence of Korean rockfish, a pair of degenerated primers (FOXL2F/FOXL2R, listed in Table 1) were designed by a Web-based primer design program, CodeHop (Chen et al. 2009). PCR was carried out in a final volume of 50 μl containing cDNA of ovary using Taq polymerase (Takara, Japan) following the

manufacturer's instructions. The touchdown PCR cycling conditions were as follows: 5-min denaturation step at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at a range of annealing temperature from 72 to 62 °C, decreasing 1 °C each cycle, and 30 s at 72 °C, then followed by additional 30 cycles of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C, finally ended with 10-min extension phase at 72 °C. The PCR products were resolved on a 1.5 % agarose gel, and target DNA fragments were purified using the gel extraction kit (Tiangen, China). Then, target amplicons were cloned into the PGM-T vector (Tiangen, China), propagated in *E. coli* Trans5α (Transgen, China). Clones were sequenced using the ABI3730XL sequencer (ABI, Life Technology, Carlsbad, CA, USA).

Rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

To obtain the 5′ and 3′ cDNA ends, the 5′ and 3′ cDNA RACE was carried out using the SMART™ RACE cDNA Amplification Kit (Clontech, USA); gene-specific primers and nested primers are shown in Table 1. For first-strand cDNA synthesis, RT-PCR required 1 μg of total RNA and 1 μM of primers. We designed two pairs of gene-specific primers from each gene based on the fragments. Subsequently, a nested PCR was conducted for cloning the full-length cDNA. PCR was performed using the following PCR cycling conditions: 5-min denaturation step at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 63 °C for Foxl2-5-1, or 64 °C for Foxl2-5-2, or 30 s at 69 °C for Foxl2-3-1 and Foxl2-3-2, then followed by additional step at 72 °C for 1 min, finally ended with 10-min extension phase at 72 °C. The PCR products were resolved on a 1.5 % agarose gel, and target DNA fragments were purified using the gel extraction kit (Tiangen, China). Then, target amplicons were cloned into the PGM-T vector (Tiangen, China), propagated in *E. coli* Trans5α (Transgen, China). Clones were sequenced using the ABI3730XL sequencer (ABI, Life Technology, Carlsbad, CA, USA).

Phylogenetic analysis and sequence analysis

Multiple protein sequences of *Foxl2* cDNA were obtained from GenBank (Altschul et al. 1990) and aligned with ClustalX 1.81 (Thompson et al. 1997). Phylogenetic analyses of full-length amino acid sequences were conducted using MEGA 4.0 (Tamura et al. 2007). Phylogenetic trees were constructed using the maximum likelihood method (1,000 bootstrapping replicates, indicating the credibility of each branch) using the neighbor-joining method (Saitou and Nei 1987). All the Foxl2 amino acid sequences used in the

Table 1 Primers and probes used for cloning, RT-PCR, and qPCR of Foxl2 gene

Primers	Sequence (5′–3′)	Position	Usage
RT-PCR			
FOXL2F	GAGAAGMGBCTYACGCTGTCCGGCTTGTACTT	399–421	Degenerate primer
FOXL2R	CCCARTAWGAGCARTGCATCAT	1,057–1,078	Degenerate primer
3′, 5′ RACE			
FOXL2-5-R1	TCCCTCACGAGGAACTTTGATGAAACACTC	514–543	5′-RACE primer
FOXL2-5-R2	GATGAAACACTCGTTGAGACTCAGGTTGTG	975–1,002	Nested 5′-RACE primer
FOXL2-3-R1	AGTCTTTATTCGGAGGAGACGGCTATGGTT	677–706	3′-RACE primer
FOXL2-3-R2	GAACTCTTACAACGGCATGAGTCACCATCA	894–923	Nested 3′-RACE primer
RT-qPCR			
FOXL2-e-F	CGACCAAGGAGAAAGAGCGA	269–288	RT-PCR and qPCR primer
FOXL2-e-R	GCGATGAGAGCCACATAGGA	358–377	RT-PCR and qPCR primer
cyp19a-e-F	GATACGCACCTACTTCACCAA	553–573	RT-PCR and qPCR primer
cyp19a-e-R	GACATCCACATGAGCCAAACT	653–673	RT-PCR and qPCR primer
cyp19b-e-F	AGACGGAGAAGTTGGACGAT	571–590	RT-PCR and qPCR primer
cyp19b-e-R	CAGCATGAAGAAGAGGCTGA	725–706	RT-PCR and qPCR primer
18S-e-F	CCTGAGAAACGGCTACCATC	–	Reference primer
18S-e-R	CCAATTACAGGGCCTCGAAAG	–	Reference primer

phylogenetic analyses were obtained from GenBank (http://www.ncbi.nlm.nih.gov/), except the Korean rockfish Foxl2 sequences. The GenBank accession numbers are as follows: *Clarias gariepinus* North African catfish, AEM63537; *Oreochromis niloticus* Nile tilapia, XP_003459563; *Paralichthys olivaceus* Japanese flounder, BAF69017; *Oncorhynchus mykiss* rainbow trout, ND_001117957; *Denio rerio* zebrafish, NP_001038717; *Cynoglossus semilaevis* tongue sole, ACY05959; *Halichoeres trimaculatus* threespot wrasse, BAJ15129; *Misgurnus anguillicaudatus* oriental weatherfish, BAJ19137; *Epinephelus merra* honeycomb grouper, ACD62374; *Xenopus laevis* African clawed frog, BAH22852; *Sus scrofa* pig, NP_001231594; *Homo sapiens* human, AF301906; *Mus musculus* house mouse (NM_012020), *Gallus gallus* chicken, AEE80502.

Tissue distribution pattern of *Foxl2* transcripts

Expression pattern of *Foxl2* was examined in various tissues by RT-PCR assays. Total RNA was extracted from ovary, liver, kidney, head kidney, brain, heart, spleen, ceca, stomach, fat, gills, intestine, and pituitary of a female fish at late-vitellogenic stage and the testis of male fish at sperminated stage using RNAiso reagent (Takara, Japan). Extracted RNA was treated with RNase-free DNase I (Takara, Japan) for 30 min at 37 °C and inactivated at 75 °C for 10 min before proceeding for the first-strand synthesis. Absence of DNA in total RNA was reverse-transcribed using M-MLV RT (Promega, USA) following the manufacturer's instructions, and the primers used for examination of tissue expression pattern are listed in Table 1. Primers for 18S (Mu et al. 2012) used as a reference gene were designed from 18S rRNA sequence obtained from Korean rockfish. The sense and the antisense primers were listed in the Table 1. PCR was carried out in a final volume of 25 µl with 1 µl cDNA; the reaction conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. Six microliters of each reaction products was electrophoresed by a Gel system (Tanon, China) on a 1.5 % agarose gel containing ethidium bromide (EB).

Quantitative real-time PCR (qPCR)

The relative expression of *Foxl2*, *cyp19a1a* and *cyp19a1b* mRNA during different phases of reproductive cycle was determined by qRT-PCR using total RNA absence of DNA prepared from gonads and brains of Korean rockfish. Real-time PCR assays (25 µl), each individual sample of which was run in triplicate wells, were carried out using iQ^{TM} SYBR Green Supermix (Takara, Japan) performing on Multicolor Real-Time PCR Detection System (Roche Lightcycler480, Germany). The sequences of primer of *Foxl2* (FOXL2-e-F and FOXL2-e-R) are listed in Table 1. To remove trace genomic DNA from the samples and prevent potential genomic DNA amplification, the mRNA was treated with DNase I (Takara, Japan) and Ribonuclease Inhibitor (Takara, Japan). The *Foxl2* qPCR conditions were as follows: 1 cycle of denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C (for *Foxl2*), or 59.6 °C (for *cyp19a1a*) or 57.8 °C (for *cyp19a1b*) of 30 s, and extension at 72 °C for 30 s. As an internal control, 18S rRNA was amplified under the same conditions using Korean rockfish–specific primers (Table 1, see "Tissue distribution pattern of *Foxl2* transcripts"), and no significant changes were observed in the 18S rRNA expression level during gonadal development. Melting curve analysis for each amplicon was performed to check for single amplification. The threshold cycle (Ct) values according to the manufacturer's protocol (Roche) were obtained from the exponential phase of qPCR amplification. Samples in the initial stage were used as calibrator for comparative relative qPCR. The relative expression of target gene/18S rRNA was analyzed according to the expression $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). ΔCt = target gene Ct− reference gene Ct.

Statistical analysis

The relevant values in this study were analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range tests. Statistical significance was considered as $P < 0.05$.

Results

Isolation and characterization of Korean rockfish *Foxl2* cDNAs

Korean rockfish *Foxl2* cDNA (JN998083) was isolated by employing RT-PCR, 5′- and 3′-RACE. It consists of a 924-nucleotide open reading frame

(ORF)-encoded 307-amino-acid polypeptide, and the ATG codon starts at nucleotides 199–201. Its sequence contained a 201-bp 5′-untranslated regions (UTR) and 927-bp 3′-untranslated regions (UTR), but no poly (A) was attached in its 3′-UTR.

Homology and phylogenetic analysis of putative amino acid sequence of *Foxl2*

The Korean rockfish Foxl2 containing a conserved 110-amino-acid sequence named forkhead box shares high identity (98–100 %) with human and mouse Foxl2s. Aligning by BLASTP search (http://www.ncbi.nlm.nih.gov/blast/) with other kinds of fish, Korean rockfish Foxl2 was 76, 76, and 77 % identical to *Human sapiens*, *Mus musculus* and *Sus scrofa* Foxl2s at the amino acid level, respectively. Foxl2s in teleost and *Gallus gallus* lack polyalanine tract (A), glycine-rich (G), and proline–alanine–rich domain, which are conserved in mammalian Foxl2s (Fig. 1). Comparing the fish, chicken, and mammalian Foxl2 sequences showed that Foxl2's forkhead domain and the C-terminal region were rather conserved, and the C-terminal region was more conserved than the N-terminal region (Fig. 2). Based on a complete alignment of other teleost fish, chicken, and mammals Foxl2 sequences, a phylogenetic tree was constructed by using a neighbor-joining method. The tree showed that Korean rockfish Foxl2 fitted within the subgroup of fish Foxl2, and it was most related to that of threespot wrasse (Fig. 3).

Tissue distribution of *Foxl2* mRNA

RT-PCR analysis was employed to analyze the *Foxl2* expression in various tissues distribution. *Foxl2* mRNA was detected in the liver, fat, gill, brain, and ovary, with the highest level in the stomach, whereas it was found at lower levels in the testis (Fig. 4).

Expression pattern of *Foxl2*, ovarian and brain type of aromatase at gonad and brain during development gonadal stages during the reproductive cycle by qPCR

During the sampled periods, expression of *Foxl2* mRNA was observed in gonads and brains of both male and female Korean rockfish throughout the reproductive cycle (Figs. 5, 6).

The *Foxl2* expression level in testis increased from stage II (mean ± SEM: 1.28 ± 0.43) to the highest at the stage III (mean ± SEM: 2.69 ± 0.84) and then substantially decreased from stage II to the lowest at stage V (mean ± SEM: 0.28 ± 0.02) ($P < 0.05$). Expression of *cyp19a1a* gene increased from stage II (mean ± SEM: 1.10 ± 0.38) to the highest level at stage IV (mean ± SEM: 5.17 ± 2.14). The expression of *cyp19a1b* decreased from stage II (mean ± SEM: 1.02 ± 0.20) to stage III (mean ± SEM: 0.24 ± 0.08), then sharply increased in the stage IV, with the highest level (mean ± SEM: 1.37 ± 0.60), and finally dropped in stage V (mean ± SEM: 0.18 ± 0.05). The expression pattern in ovary of female was quite different from that in testis of male; that is, the *Foxl2* expression level continuously increased from the stage II (mean ± SEM: 0.9 ± 0.34) to the highest level in the stages IV–V (mean ± SEM: 6.76 ± 1.69) and then sharply decreased to the lowest in stage VI (mean ± SEM: 0.53 ± 0.22). The expression level of *cyp19a1a* gene went up from stage II (mean ± SEM: 0.92 ± 0.30) to the highest at stage III (mean ± SEM: 5.97 ± 1.7) and then went down to the lowest at stage VI (mean ± SEM: 0.19 ± 0.06). And the expression level of *cyp19a1b* decreased from the stage II (mean ± SEM: 1.02 ± 0.13) to the lowest at stages IV–V (mean ± SEM: 0.4 ± 0.14) and finally greatly increased at stage VI (mean ± SEM: 4.07 ± 1.26) ($P < 0.05$).

In male brain, from stage II to IV, the expression levels of *Foxl2* increased (mean ± SEM: 1.00 ± 0.05; 1.53 ± 0.08). However, the *cyp19a1a* expression increased from stage II to III (mean ± SEM: 1.95 ± 0.54) and dropped to the lowest level at stage V (mean ± SEM: 0.65 ± 0.11). At stage II, *cyp19a1b* decreased to the lowest level at stage III (mean ± SEM: 0.31 ± 0.09) and increased to the highest level until stage IV (mean ± SEM: 2.09 ± 0.77). In females, expression of *foxl2* continuously decreased until stages IV–V (mean ± SEM: 0.27 ± 0.05) and then increased at stage VI. At stage II, the expression of *cyp19a1a* increased and peaked at stage III (mean ± SEM: 1.50 ± 0.10) and then dropped to the lowest level at stage VI (mean ± SEM: 1.15 ± 0.35). However, the *cyp19a1b* decreased to lowest at stage III (mean ± SEM: 0.67 ± 0.11) and increased from stages IV–V to the highest at stage VI (mean ± SEM: 1.89 ± 0.49).

Discussion

Our study focuses on the role of *Foxl2* in ovary and brain, so we cloned the full length of *Foxl2* cDNA in Korean rockfish (GenBank accession number: JN998083). To begin with, the full-length *Foxl2* cDNA was isolated from ovarian tissue of Korean rockfish using RT-PCR and RACE amplification strategies. Compared with other fish and mammalian amino acid in Foxl2s, the amino acid of Korean rockfish was well conserved in the forkhead domain and the C-terminal region. Cocquet et al. suggested that the forkhead domain and C-terminal region may have conserved functions through evolution, whereas the N-terminal region would evolve under weaker constraints (Cocquet et al. 2002). The proline–alanine-rich domain is considerably reduced in Korean rockfish and other teleosts, but was found in mammalian Foxl2. Govorou et al. indicated that Foxl2 lengthened considerably during evolution in species, because of the accumulation of proline, alanine, and glycine residues in the region after the DNA-binding domain (Govoroun et al. 2004). In addition, this kind of homopolymeric repeat resulted from a "replication slippage" mechanism and was considered to be

Fig. 2 Alignment of predicted amino acid sequence of Korean ▶ rockfish Foxl2 with that of other teleosts Foxl2s. Alignments were generated using ClustalX, and all the GenBank accession numbers are provided in "Materials and methods". Amino acids are presented in conventional single-letter code and numbered on the left side of the sequence. Identical amino acids among species are shown in *white letters* on a *black background*. The missing amino acids are represented by *dash*. The *bar* indicates the forkhead domain. The putative nuclear localization signal (NLS) sequence is shown with *double underline*. The glycine-rich repeats (G), polyalanine tract (A), and proline repeats of mammals are *boxed*

Fig. 1 Nucleotide and amino acid sequences of Korean rockfish Foxl2 cDNA (GenBank accession no. JN998083). The translated amino acid sequence is shown in standard one-letter code below the nucleotide sequence. The forkhead domain is *boxed* by *gray box*. *Asterisk* indicates the stop codon

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Korean rockfish      1   MVATYQNPEDDAMTLMIHDTNTTTKEKERPKE-----EPVH-QDKVPEKPDPSQKPPYSY
Nile tilapia         1   MVATYQNPEDDAVALMIHDTN-TTKEKERPKE-----EPV--QDKVSEKPDPSQKPPYSY
threespot wrasse     1   MVATYPNPEDDAVALMIHDTN-TTKEKERPKE-----EPV--QEKVPEKPDPTQKPPYSY
honeycomb grouper    1   MVATYQNPEDDAVALMIHDTN-TTKEKERPKE-----EPV--QEKVPEKPDPSQKPPYSY
Japanese flounder    1   MVATYQNSEDDAVALMIHDTN-ASKEKERPKE-----EPV--QEKVSEKPDPSQKPPYSY
rainbow trout        1   MMDTYQNPEDDAVALMVHDTN-MAKDKERPKE-----EPV--QEKVSEKTDPSQKPPYSY
tongue sole          1   MVAAYRSAEGDEVALLIHDSNANNKEKERPKE-----EPV--QEKVCEKPDPTQKPPYSY
chicken              1   MVSGYADGEEDAVAMLAHDGG-GSKEPERGKE-----ELS--AEKGPEKPDPSQKPPYSY
sus scrofa           1   MVASYPEPEDAAGALLAPETGRTAKEPEAPPPLSPGKGGGGGASTAPEKPDPAQKPPYSY
human                1   MVASYPEPEDAAGALLAPETGRTVKEPEGPPP-SPGKGGGGGGTAPEKPDPAQKPPYSY
house rat            1   MVASYPEPEDTAGTLLAPESGRAVKEAEASPP-SPGKGGG---TTPEKPDPAQKPPYSY

Korean rockfish      55  VALIAMAIRESTEKRLTLSGIYQYIITKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
Nile tilapia         53  VALIAMAIRESSEKRLTLSGIYQYIITKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
threespot wrasse     53  VALIAMAIRESTEKRLTLSGIYQYIITKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
honeycomb grouper    53  VALIAMAIRESSEKRLTLSGIYQYIISKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
Japanese flounder    53  VALIAMAIRESSEKRLTLSGIYQYIISKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
rainbow trout        53  VALIAMAIRESTEKRLTLSGIYQYIISKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
tongue sole          54  VALIAMAIRESSEKRLTLSGIYQYIISKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
chicken              53  VALIAMAIRESAEKRLTLSGIYQYIISKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
sus scrofa           61  VALIAMAIRESAEKRLTLSGIYQYIIAKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
human                60  VALIAMAIRESAEKRLTLSGIYQYIIAKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG
house rat            56  VALIAMAIRESAEKRLTLSGIYQYIIAKFPFYEKNKKGWQNSIRHNLSLNECFIKVPREG

Korean rockfish      115 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
Nile tilapia         113 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKALFG-----------
threespot wrasse     113 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
honeycomb grouper    113 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
Japanese flounder    113 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
rainbow trout        113 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
tongue sole          114 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
chicken              113 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPTHFQPGKSLFG-----------
sus scrofa           121 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPAHFQPGKGLFGAGGAAGGCGVA
human                120 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPAHFQPGKGLFGAGGAAGGCGVA
house rat            116 GGERKGNYWTLDPACEDMFEKGNYRRRRRMKRPFRPPPAHFQPGKGLFGSGGAAGGCGVP

Korean rockfish      164 ---GDGYG-YLSPPKYLQSSFMNNSWS----PTPMSYTSCQMSSGNVSPVN---------
Nile tilapia         162 ---GDSYG-YLSPPKYLQSSFMNNSWSLGQPPTPMSYTSCQMASGNVSPVN---------
threespot wrasse     162 ---GDGYG-YLTPPKYLQSSFMNNSWSLGQPPTPMSYTSCQMASGNVSPVN---------
honeycomb grouper    162 ---GDGYG-YLSPPKYLQSSFMNNSWSLGQPPTPMSYTSCQMASGNVSPVN---------
Japanese flounder    162 ---GDGYG-YLSPPKYLQSSFMNNSWSLGQPPTPMSYTSCQMASGNVSPVN---------
rainbow trout        162 ---GDGYG-YLSPPKYLQSSFMNNSWSLGQPPTPMSYTSCQMASGNVSPVN---------
tongue sole          163 ---TDSYG-YLSPPKYLQSSFMNNSWSLSQPPAPLSYTSCQMASGNVSPVN---------
chicken              162 ---PDGYG-YLSPPKYLQSTFMNNSWPLPQPPAPVPYASCQMSGGSVSPVN---------
sus scrofa           181 GAGADGYG-YLAPPKYLQSGFLNNSWPLPQPPSPMPYASCQMAAAAAAAAAAAAAAGPGS
human                180 GAGADGYG-YLAPPKYLQSGFLNNSWPLPQPPSPMPYASCQMAAAAAAAAAAAAAGPGS
house rat            176 GAGADGYG-YLAPPKYLQSGFLNNSWPLPQPPSPMPYASCQMAAAAAAAAAAAAGPGS

Korean rockfish      208 ------VKGLSAPS-SYNPYSRVQSMALP-SMVNSYNGMS-------------------H
Nile tilapia         210 ------VKGLSAPS-SYNPYSRVQSMALP-SMVNSYNGMS-------------------H
threespot wrasse     210 ------VKALSAPS-SYNPYSRVQSMALP-SMVNSYNGMG-------------------H
honeycomb grouper    210 ------MKGLSAPS-SYNPYSRVQSMALP-SMVNSYNGMS-------------------H
Japanese flounder    210 ------VKGLSPPS-SYNPYSRVQSMALP-SMVNSYNGMG-------------------H
rainbow trout        210 ------VKGLSAPS-SYNPYSRVQSMGLP-SMVNSYNGMS-------------------H
tongue sole          211 ------MKGLSPPS-SYNPYSRVQSMALP-GMVNSYNGMS-------------------H
chicken              210 ------VKGLSGPA-SYGPYSRVQSVALP-GMVNSYNGVA-------------------H
sus scrofa           241 PGAAAVVKGLAGPAASYGPYSRVQSMALPPGVVNSYNGLGGPPAAPPPPP---HPHSHPH
human                240 PGAAAVVKGLAGPAASYGPYTRVQSMALPPGVVNSYNGLGGPPAAPPPPP---HPHPHPH
house rat            236 PGAAAVVKGLAGPAASYGPYSRVQSMALPPGVVNSYNGLGGPPAAPPPPPPPPHPHPHPH

Korean rockfish      242 HHHPVHPH-------------HHAQQLSPATA-AAPPPVPSGNGAAGLQFACSRQPAELS
Nile tilapia         244 HHHP-----------------HHTQQLSPAT--AAPPPVSSSNG-AGLQFACSRQPAELS
threespot wrasse     244 HHHPAHP--------------HHAQQLSPAT--AAPPPVSSSNG-AGLQFACSRQPAELS
honeycomb grouper    244 HHHPAHP--------------HHSQQLSPAT--AAPPPVSSSNG-AGLQFACSRQPAELS
Japanese flounder    244 HHHPAHP--------------HHAQQLSPAT--AAPPPVSSSNG-AGLQFACSRQPAELS
rainbow trout        244 HHHP-HA--------------HHAQQLNPAT--VAPPPVSSSNG-AGLQFACSRQPTELS
tongue sole          244 HHHPVHP--------------HPGQQLSSAA--AGPPSVSSSNG-AGLQFACSRQPAELS
chicken              244 PHHPHAH--------------HPQQLGPAS--PAPPAAPAANG-AGLQFACARQPAELS
sus scrofa           299 AHHLHAAAAPPPAPPHHGAAAPPPGQLSPASPATAAPPAPAPTNAPGLQFACARQ-PELA
human                298 AHHLHAAAAPPPAPPHHGAAAPPPGQLSPASPATAAPPAPAPTSAPGLQFACARQ-PELA
house rat            297 AHHLHAAAAPPPAPPHHGAAAPPPGQLSPASPATAAPPAPAPTSAPGLQFACARQ-PELA

Korean rockfish      288 MVHCSYWEHETKHSALHTRIDI
Nile tilapia         285 MVHCSYWEHETKHSALHTRIDI
threespot wrasse     288 MVHCSYWEHETKHSALHTRIDI
honeycomb grouper    288 MVHCSYWEHETKHSALHTRIDI
Japanese flounder    288 MVHCSYWEHETKHSALHTRIDI
rainbow trout        288 MVHCSYWDHESKHSALHARIDI
tongue sole          288 MVHCSYWDHESKHSALHTRIDI
chicken              288 MVHCSYWDHESKHSALHTRIDI
sus scrofa           359 VMHCSYWEHDSKHGALHSRIDI
human                358 MVHCSYWDHDSKTGALHSRLDL
house rat            357 MVHCSYWDHDSKTGALHSRLDL
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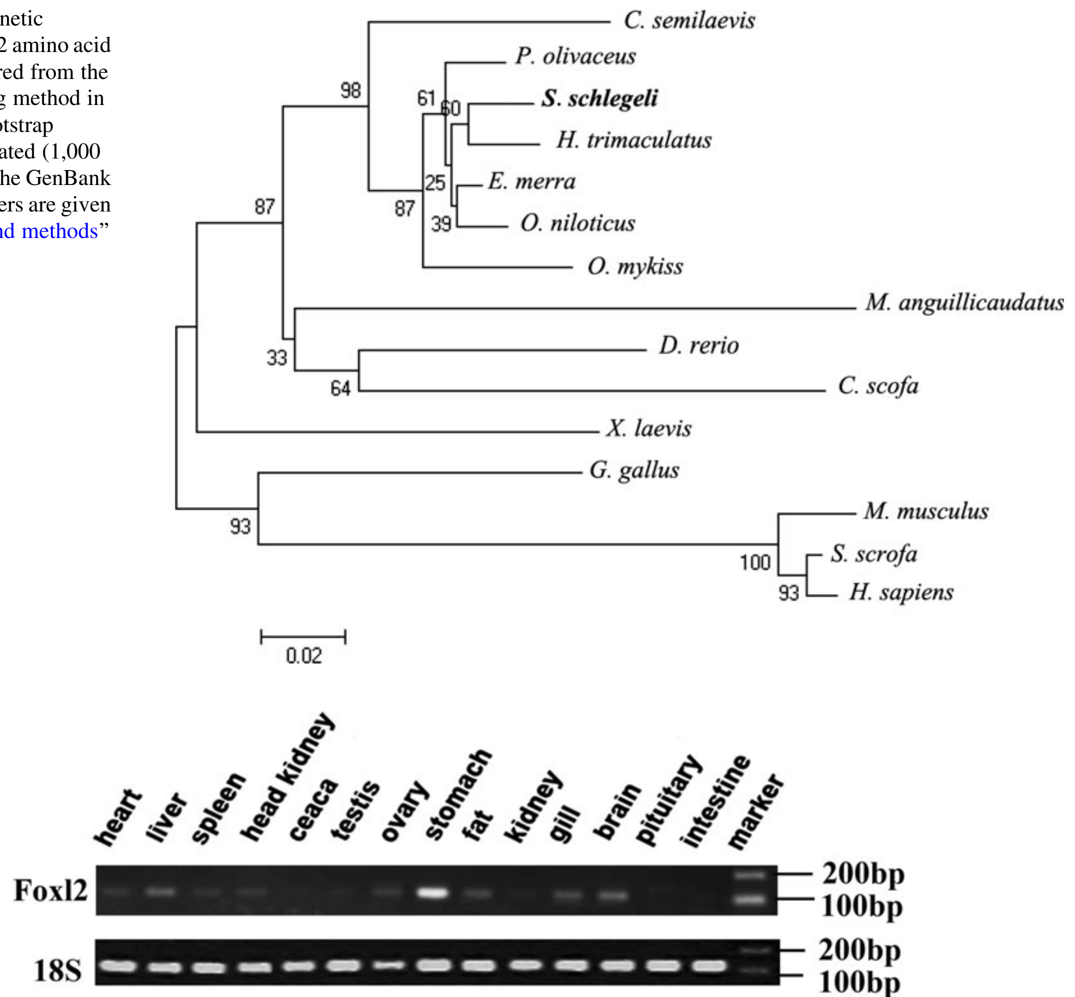

Fig. 3 Phylogenetic analysis of Foxl2 amino acid sequences inferred from the neighbor-joining method in vertebrates. Bootstrap values are indicated (1,000 replicates). All the GenBank accession numbers are given in "Materials and methods"

Fig. 4 Tissue distribution of *Foxl2* gene in adult Korean rockfish by (reverse transcription) RT-PCR analysis. The integrity of the RNA from the each tissue was ensured by uniform amplification of 18S rRNA transcripts (*lower panel*)

subsequently followed by point mutations (Mortlock et al. 2000). In mammals, homopolymeric amino acid such as polyalanine tract (A), glycine (G), and proline (P) were contained in Foxl2 gene, whereas they were also absent in non-mammalian vertebrates including fish. All the present study further confirmed the conservation and differences in Foxl2 between mammals and fish.

In the present study, we did not get any multiple forms of Foxl2 indicating the existence of single form, but two isoforms, Foxl2a and Foxl2b, were isolated from the rainbow trout (Baron et al. 2005). The phylogenetic analysis of Foxl2 revealed high homology of Korean rockfish Foxl2 with threespot wrasse, showing that Korean rockfish Foxl2 is closer to marine fish than freshwater fish.

Tissue distribution pattern revealed predominant expression of *Foxl2* in stomach of adult Korean rockfish, while it was moderate expressed in liver, fat, gill, brain and ovary. However, the expression of *Foxl2* was hardly found in testis of Korean rockfish. The *Foxl2* expressed in ovary but was hardly detected in testis of our data corroborates with studies of many other kinds of teleost fish, such as medaka (Nakamoto et al. 2007). By Northern blotting, studies in tilapia found there is no band of *Foxl2* in testis, but a single transcript was detected in the ovary; meanwhile, in situ hybridization also gave strong signals in the adult ovary, whereas no signal was detected in the testis (Wang et al. 2004). These results indicated that there is no involvement of *Foxl2* during testicular development at least with reference to many kinds of

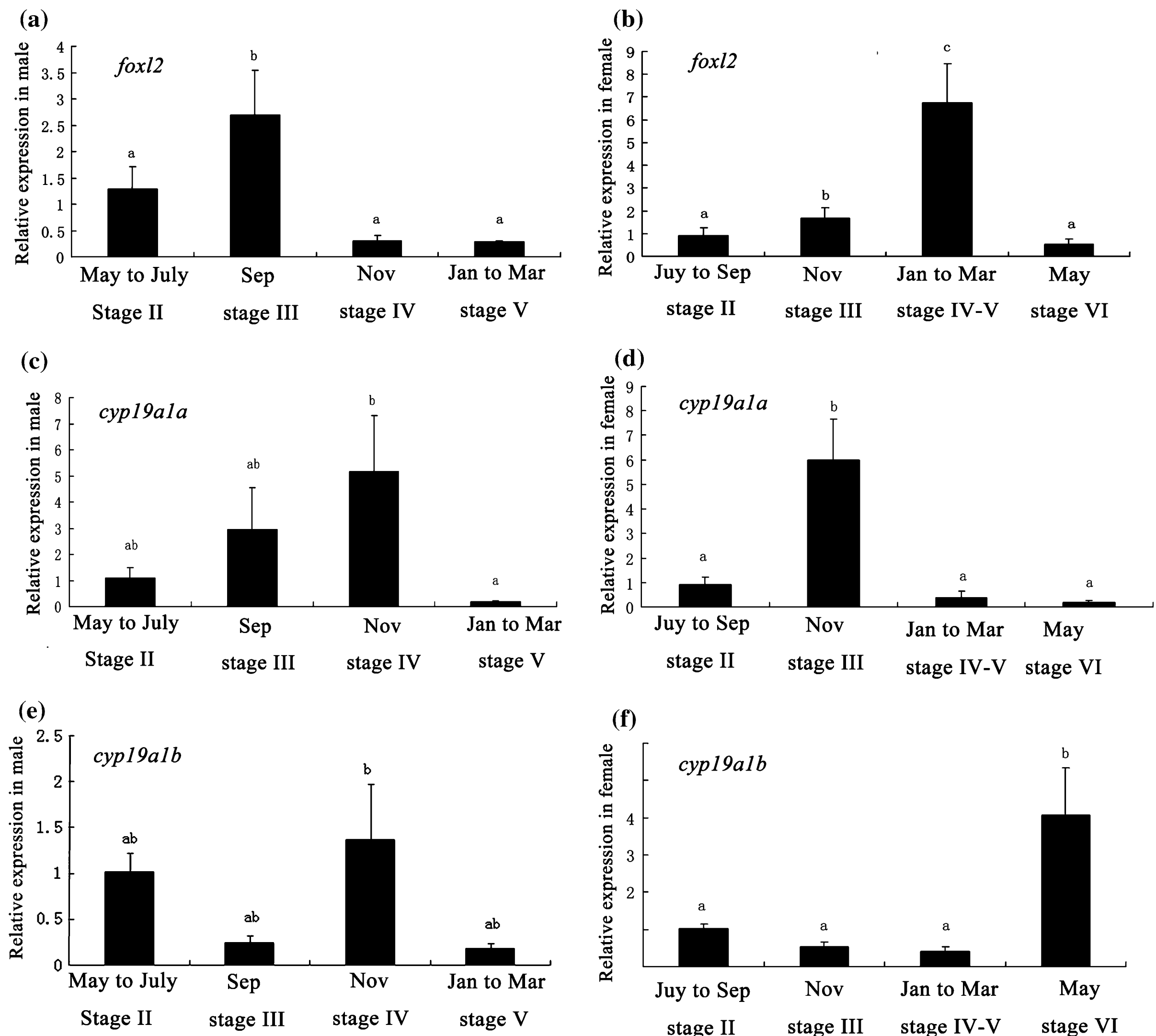

Fig. 5 *Foxl2* mRNA expressions during the reproductive cycle in gonads. Relative mRNA expression levels of Foxl2 (**a**, **b**), cyp19a1a (**c**, **d**), and cyp19a1b (**e**, **f**) are measured during the reproductive cycle by real-time PCR. Results are expressed as normalized fold expression with respect to 18S rRNA levels for the same sample. *Vertical bars* represent mean ± SEM ($n \geqq 3$); groups with *different letters* are significantly different ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

teleosts. Many studies showed *Foxl2* was highly expressed in the ovary and is present even before morphological differentiation in mammals and birds (Cocquet et al. 2002; Govoroun et al. 2004; Loffler et al. 2003; Pannetier et al. 2003) and was expressed in ovary-specific manner in mouse embryos, chickens, and turtle (Loffler et al. 2003). It is also interesting that *Foxl2* was highly expressed in stomach of Korean rockfish, which was never mentioned in other fish species. This result may indicate that Foxl2 also plays a role in the non-gonadal-related tissues. However, further studies should be conducted to investigate the function of *Foxl2* in stomach. In addition, we did not find any expression of *Foxl2* in Korean rockfish pituitary, which was found in honeycomb grouper (Alam et al. 2008) and catfish (Sridevi and Senthilkumaran 2011). The study of rare minnow suggested *Foxl2* probably plays a new function in eye tissue (Wang et al. 2012).

We studied the expression patterns of the *Foxl2* gene during the gonads development in Korean rockfish as well as ovarian and brain type of

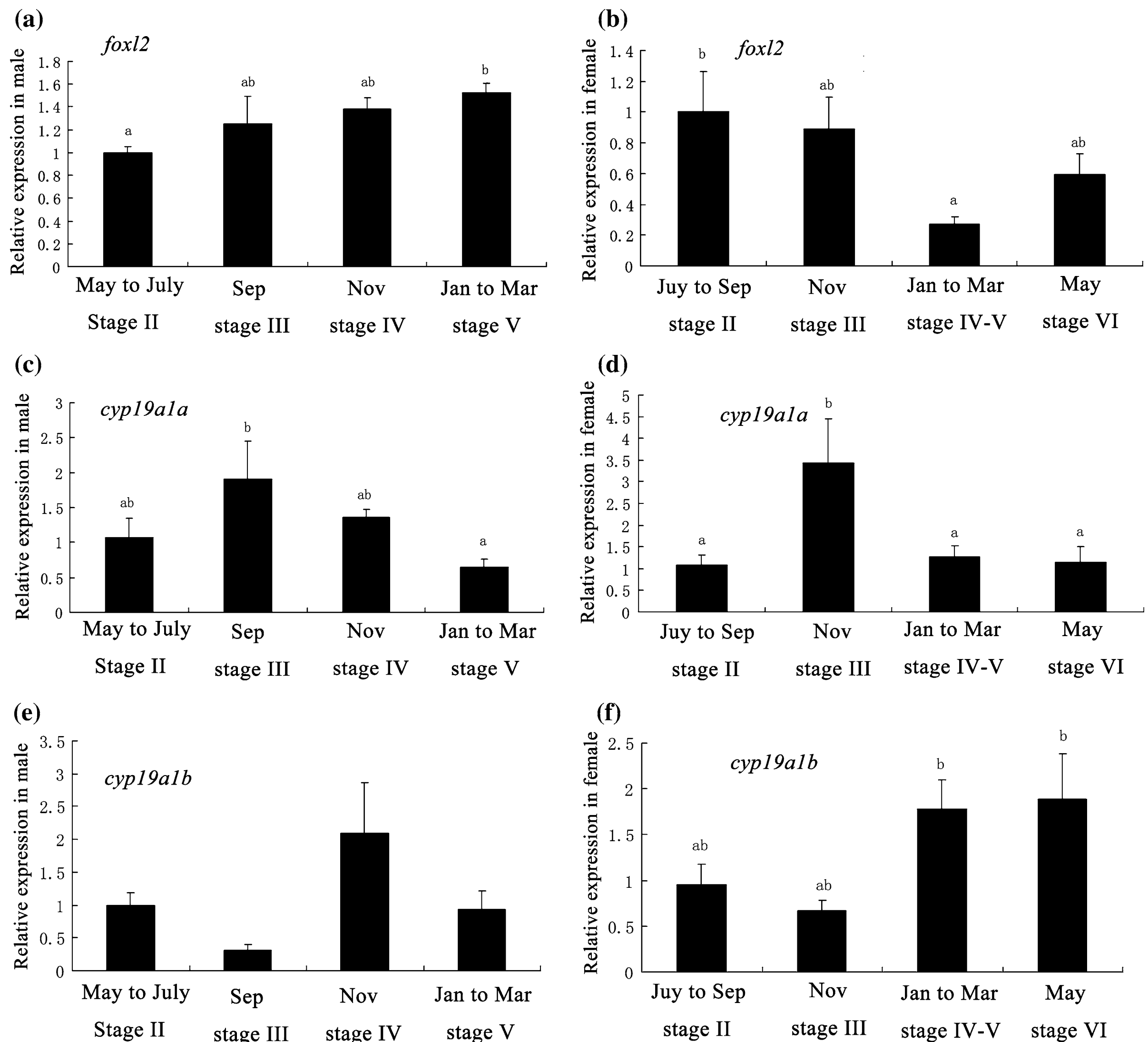

Fig. 6 *Foxl2* mRNA expressions during the reproductive cycle in brains. Relative mRNA expression levels of Foxl2 (**a**, **b**), cyp19a1a (**c**, **d**) and cyp19a1b (**e**, **f**) are measured during the reproductive cycle by real-time PCR. Results are expressed as normalized fold expression with respect to 18S rRNA levels for the same sample. *Vertical bars* represent mean ± SEM ($n \geqq 3$); groups with *different letters* are significantly different ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

aromatase. Of *Foxl2* gene, a sharp increase from immature gonadal stage following by a peak at stage III in testis depicts an important role for this correlate in testis differentiation as the critical window of gonadal differentiation in catfish (Raghuveer and Senthilkumaran 2009, 2010; Raghuveer et al. 2011). In the female fish, the expression level of *Foxl2* gene increased from immature stage and peaked at late yolk stage, which was similar to the result about chicken (Govoroun et al. 2004). Whether this may suggest the possible role of *Foxl2* in oocyte maturation needs to be confirmed by further studies (Govoroun et al. 2004). In the female catfish, *Foxl2* was highly expressed in pre-spawning and spawning stage (Sridevi and Senthilkumaran 2011). By the immunolocalization studies of previtellogenic and vitellogenic ovary catfish, results demonstrated the localization of *Foxl2* in the follicular layer with an extended immunoreactivity toward cytoplasm. In addition, the studies on the localization pattern of *Foxl2* in tilapia and medaka also denote its localization in follicular layer (Nakamoto et al. 2009; Wang et al. 2004). All of these results may imply that

Foxl2 may be an important transcription factor for gonads development in mammals as well as teleosts.

It is well known that the *Foxl2* expression correlated with the level of ovarian and brain type of aromatase (Baron et al. 2005; Sridevi and Senthilkumaran 2011). In our study, *Foxl2* expression with highest level in testis and ovary commonly appeared earlier than that of *cyp19a1a* and *cyp19a1b*, except for *cyp19a1a* gene in ovary. By reverse transcriptase PCR, *Foxl2* and *cyp19a1b* were highly expressed in prespawn testis in rainbow trout (von Schalburg 2010), which was similar with our result of Korean rockfish. There are many reports demonstrating that *Foxl2* promotes the transcription of *cyp19a1a* in fish and mammals, such as medaka, Japanese flounder (Yamaguchi et al. 2007; Nakamoto et al. 2007), and goat (Galay-Burgos et al. 2006). In medaka, it suggested that the onset of *Foxl2* expression was earlier than that of aromatase by in situ hybridization (Nakamoto et al. 2007). Studies in tilapia found that *Foxl2* binds to the sequence ACAAATA in the promoter region of the *cyp19* gene, resulting in the activation of *cyp19* expression (Wang et al. 2007). In rainbow trout, the *cyp19* expression is activated by Foxl2 protein (Baron et al. 2005). In addition, *Foxl2* may be involved in activating the *cyp19* transcription in somatic cells, which was demonstrated by Oshima et al. in the study of frog (Oshima et al. 2008). All of these may imply that *Foxl2* was involved in the initiation of aromatase transcription in testis and ovary differentiation.

There are a few studies that have addressed *Foxl2* and its regulation to ovarian and brain type of aromatase. In the study of catfish, it is a strong correlation between *cyp19a1bcyp19a1b*, FTZ-F1 and *Foxl2* expression during the brain development in catfish, investigating the involvement of FTZ-F1 and *Foxl2* in the regulation of expression of these two types of aromatase in brain (Sridevi and Senthilkumaran 2011). In the Korean rockfish, the abundant *Foxl2* expression level in early gonadal development stage in female brain may imply its transcriptional activation of *cyp19a1a*. However, we found that the *cyp19a1a* and *cyp19a1b* had no correlation with *Foxl2* in male brain. This indicated that expression of aromatase might be regulated by many other specific factors besides *Foxl2*.

In conclusion, we described the cloning and expression analysis of *Foxl2* gene in gonads and brains during the reproductive cycle, finding its expression relationship with *cyp19a1a* and *cyp19a1b* in typical ovoviviparous fish, Korean rockfish. These results may help in further understanding the endocrinological and reproductive development mechanism in the teleost.

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References

Alam MA, Kobayashi Y, Horiguchi R, Hirai T, Nakamura M (2008) Molecular cloning and quantitative expression of sexually dimorphic markers Dmrt1 and Foxl2 during female to male sex change in *Epinephelus merra*. Gen Comp Endocrinol 157:75–85

Altschul SF, Gish W, Miller W, Myers EW, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Baron D, Houlgatte R, Fostier A, Guiguen Y (2005) Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73:959–966

Carlsson P, Mahlapuu M (2002) Forkhead transcription factors: key players in development and metabolism. Dev Biol 250:1–23

Chen CF, Wen HS, He F, Dong SL (2009) Programming design of degenerate primers and cloning half-smooth tongue sole *Cynoglossus semilaevis* CYP17 gene. J Ocean Univ China 39:1213–1218 (Chinese with English abstract)

Cocquet J, Pailhoux E, Jaubert F, Servel N, Xia X, Pannetier M, De Baere E, Messiaen L, Cotinot C, Fellous M, Veitia RA (2002) Evolution and expression of FOXL2. J Med Genet 39:916–921

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G (2001) The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27:159–166

De Baere E, Beysen D, Oley C, Lorenz B, Cocquet J, De Sutter P, Devriendt K, Dixon M, Fellous M, Fryns JP, Garza A, Jonsrud C, Koivisto PA, Krause A, Leroy BP, Meire F, Plomp A, Van Maldergem L, De Paepe A, Veitia R, Messiaen L (2003) FOXL2 and BPES: mutational hotspots, phenotypic variability, and revision of the genotype–phenotype correlation. Am J Hum Genet 72:478–487

Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191–364

Ellsworth BS, Burns AT, Escudero KW, Duval DL, Nelson SE, Clay CM (2003) The gonadotropin releasing hormone (GnRH) receptor activating sequence (GRAS) is a composite regulatory element that interacts with multiple

classes of transcription factors including Smads, AP-1 and a forkhead DNA binding protein. Mol Cell Endocrinol 206:93–111

Galay-Burgos M, Gealy C, Navarro-Martin L, Piferrer F, Zanuy S, Sweeney GE (2006) Cloning of the promoter from the gonadal aromatase gene of the European sea bass and identification of single nucleotide polymorphisms. Comp Biochem Physiol A 145:47–53

Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, Couty I, Batellier F, Cotinot C (2004) Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev Dyn 231:859–870

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-(\triangle\triangle CT)}$ method. Methods 25:402–408

Loffler KA, Zarkower D, Koopman P (2003) Etiology of ovarian failure in blepharophimosis- ptosis-epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 144:3237–3243

Mortlock DP, Sateesh P, Innis JW (2000) Evolution of N-terminal sequences of the vertebrate HOXA13 protein. Mamm Genome 11:151–158

Mu WJ, Wen HS, He F, Li JF, Liu M, Zhang YQ, Hu J (2012) Cloning and expression analysis of follicle-stimulating hormone and luteinizing hormone receptor during the reproductive cycle in Korean rockfish (*Sebastes schlegeli*). Fish Physiol Biochem. doi:10.1007/s10695-012-9699-9

Naimi A, Martinez AS, Specq ML, Diss B, Mathieu M, Sourdaine P (2009) Molecular cloning and gene expression of *Cg-Foxl2* during the development and the adult gametogenetic cycle in the oyster *Crassostrea gigas*. Comp Biochem Physsiol B Biochem Mol Biol 154:134–142

Nakamoto M, Wang DS, Suzuki A, Matsuda M, Nagahama Y, Shibata N (2007) Dax1 suppresses P450arom expression in medaka ovarian follicles. Mol Reprod Dev 74:1239–1246

Nakamoto M, Muramatsu S, Yoshida S, Matsuda M, Nagahama Y, Shibata N (2009) Gonadal sex differentiation and expression of Sox9a2, Dmrt1, and Foxl2 in *Oryzias luzonensis*. Genesis 47:289–299

Omoto N, Koya Y, Chin B, Yamashita Y, Nakagawa M, Noda T (2010) Gonadal sex differentiation and effect of rearing temperature on sex ratio in black rockfish (*Sebastes schlegeli*). Ichthyol Res 57:133–138

Oshima Y, Uno Y, Matsuda Y, Kobayashi T, Nakamura M (2008) Molecular cloning and gene expression of Foxl2 in the frog *Rana rugosa*. Gen Comp Endocrinol 159:170–177

Pailhoux E, Vigier B, Chaffaux S, Servel N, Taourit S, Furet JP, Fellous M, Grosclaude F, Cribiu EP, Cotinot C, Vaiman D (2001) A 11.7-kb deletion triggers intersexuality and polledness in goats. Nat Genet 29:453–458

Pannetier M, Servel N, Cocquet J, Besnard N, Cotinot C, Pailhoux E (2003) Expression studies of the PIS regulated genes suggest different mechanisms of sex determination within mammals. Cytogenet Genome Res 101:199–205

Raghuveer K, Senthilkumaran B (2009) Identification of multiple dmrt1s in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. J Mol Endocrinol 42:437–448

Raghuveer K, Senthilkumaran B (2010) Isolation of sox9 duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up-regulation of sox9 in testicular slices. Reproduction 140:477–487

Raghuveer K, Senthilkumaran B, Sudhakumari CC, Sridevi P, Rajakumar A, Singh R, Murugananthkumar R, Majumdar KC (2011) Dimorphic expression of various transcription factor and steroidogenic enzyme genes during gonadal ontogeny in the air-breathing catfish, *Clarias gariepinus*. Sex Dev 5:213–223

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Shi D, Wen HS, He F, Li JF, Yang YP, Chen CF, Zhang JR, Chen XY, Jin GX, Shi B, Qi BX, Li N (2011) The physiology functions of estrogen receptor a (ERa) in reproduction cycle of ovoviviparous black rockfish, *Sebastes schlegeli* Hilgendorf. Steroids 14:1597–1608

Sridevi P, Senthilkumaran B (2011) Cloning and differential expression of *FOXL2* during ovarian development and recrudescence of the catfish, *Clarias gariepinus*. Gen Comp Endocrinol 174:259–268

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier AC, Klugmann C, Klasen C, Holter NI, Riethmacher D, Schutz G, Cooney AJ, Lovell-Badge R, Treier M (2009) Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. Cell 139:1130–1142

von Schalburg KR, Yasuike M, Davidson WS, Koop BF (2010) Regulation, expression and characterization of aromatase (cyp19b1) transcripts in ovary and testis of rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol B Biochem Mol Biol 155:118–125

Wang D, Kobayashi T, Zhou L, Nagahama Y (2004) Molecular cloning and gene expression of *Foxl2* in the Nile tilapia, *Oreochromis niloticus*. Biochem Biophys Res Commun 320:83–89

Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi K, Nagahama Y (2007) Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. Mol Endocrinol 21:712–725

Wang H, Wu T, Qin F, Wang LH, Wang ZZ (2012) Molecular cloning of *Foxl2* gene and the effects of endocrine-disrupting chemicals on its mRNA level in rare minnow, *Gobiocypris rarus*. Fish Physiol Biochem 38:653–664

Yamaguchi T, Yamaguchi S, Hirai T, Kitano T (2007) Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun 359:935–940

Zlotogora J, Sagi M, Cohen T (1983) The blepharophimosis, ptosis, and epicanthus inversus syndrome: delineation of two types. Am J Hum Genet 35:1020–1027