



# Molecular cloning and expression analysis of estrogen receptor betas (ER $\beta$ 1 and ER $\beta$ 2) during gonad development in the Korean rockfish, *Sebastes schlegeli*

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## ABSTRACT

Estrogen receptors (ER) play a crucial role in mediation of estrogen activities. Here we report the isolation and expression analysis of ER $\beta$ 1 and ER $\beta$ 2 from ovary Korean rockfish (*Sebastes schlegeli*). were isolated using reverse transcription-polymerase chain reaction (PCR) and rapid amplification of cDNA ends procedures. The cDNA of this study, ER $\beta$ 1 (588 amino acids) and ER $\beta$ 2 (659 amino acids) were identified using reverse-transcriptase PCR (RT-PCR) and rapid amplification of cDNA ends procedures. Structural analysis showed both ER $\beta$ s contain six typical nuclear receptor-characteristic domains. Phylogenetic analysis indicated that Korean rockfish ER $\beta$ s were highly conserved among teleost. RT-PCR confirmed that the ER $\beta$ s were widely distributed in both gonads and extra gonadal tissues. Further, we analyzed the expression patterns of male and female *S. schlegeli* during the reproductive cycle using quantitative real-time PCR (qRT-PCR). The results showed that the highest expression levels were observed in testis at immature sperm stage for both of KrER $\beta$ 1 and KrER $\beta$ 2. For female, the expressions of KrER $\beta$ 1 and KrER $\beta$ 2 were significantly higher in the ovary at the early-oocyte stage. Cloning these two ER $\beta$  subtypes in the Korean rockfish, together with the information on expression levels in adult fish has given us the foundation to investigate their possible role in brain-pituitary-gonad neuroendocrine axis in future studies.

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## 1. Introduction

It is well known that estrogen is one of the most important hormones in vertebrates, and plays an important role in growth, differentiation, reproductive behavior and pituitary gonadotropin secretion (Cavaco et al., 1998; Krisfalusi and Nagler, 2000; Lange et al., 2002). Increasing evidences demonstrate that most estrogens actions are mediated by the member of the steroid receptor super-family which is called estrogen receptors (ERs) (Fairbrother, 2000; Filby and Tyler, 2005; Lange et al., 2002). The protein members of this family have been well recognized that they contain six distinct domains labeled from A to F (Krust et al., 1986; Kumar et al., 1987). Among these, C domain (DNA-binding domain; DBD) and E domain (ligand-binding domain; LBD) are responsible for ligand binding, nuclear localization, and transcriptional activation (AF-2), and they are highly conserved among species (Choi and Habibi, 2003; Ma et al., 2000; Sumida and Saito, 2008). In contrast, A/B domain in the N-terminal and F domain

in the C-terminal are less conserved (Filby and Tyler, 2005). In addition, the D domain (hinge region) is necessary for the maintenance of ER three-dimensional structure (Hu and Lazar, 1999; Zilliacus et al., 1995).

Two subtypes of ER have been described in vertebrates so far, called ER $\alpha$  and ER $\beta$  (Hawkins et al. 2000), which are found in the cell or tissue specific context (Choi and Habibi, 2003; Enmark and Gustafsson, 1999). They exhibit distinct differences in ligand binding affinities, transcriptional activities and knockout phenotypes (Nilsson et al., 2001). A current hypothesis suggests that ER $\alpha$  and ER $\beta$  resulted from a whole genome duplication event in ray-finned fishes after they diverged from the lobe-finned fishes (Amores et al., 1998). In addition, many studies in zebrafish (Bardet et al., 2002; Menuet et al., 2002), Atlantic croaker (Hawkins et al., 2000), goldfish (Ma et al., 2000; Tchoudakova et al., 1999) suggested that two forms of ER $\beta$  existed in teleost. Bardet et al. and Robinson-Rechavi et al. revealed that ER $\beta$ 1 and ER $\beta$ 2 were generated by duplication of an ancestral ER $\beta$  subtype (Bardet et al., 2002; Robinson-Rechavi et al., 2001).

Based on the research in yellow perch, gilthead sea bream, common eelpout, and largemouth bass, ER $\beta$ s have been detected in a wide range of tissues including kidney, muscle, heart, liver, ovary, testis, gill, pituitary, brain, etc. (An et al., 2008; Andreassen et al., 2003; Lynn et al., 2008; Pinto et al., 2006; Sabo-Attwood et al., 2004). The study of variation of genes expression and serum steroid hormone during gonad development in the ER $\beta$  knockout mouse indicated

Abbreviations: ER, Estrogen receptor; HE, Hematoxylin and eosin; PCR, Polymerase chain reaction; UTR, Untranslated terminal region; E<sub>2</sub>, Estradiol-17 $\beta$ ; RT-PCR, Reverse transcription polymerase chain reaction.

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that ER $\beta$  might regulate expressions of androgen receptor and other ovulation-related factor genes to modulate GnRH release thus further affecting ovulation (Cheng et al., 2002). In teleost, the study of zebrafish ER showed it can be transcriptionally activated by estradiol (E<sub>2</sub>) (Bardet et al., 2002). ERs expressed early during embryonic development and gonadal differentiation in teleost, suggesting the important role for estrogens in sexual differentiation (Guiguen et al., 1999; Lassiter et al., 2002). Cavaco et al. also suggested that ERs was paramount important for main events during sexual development, such as puberty (Cavaco et al., 1998).

The Korean rockfish (*Sebastes schlegeli*) is a widely distributed fish, occurs in the coastal areas of the northwestern Pacific Ocean, especially in the East China Sea, Yellow Sea, and the coastal areas of the South Sea of Korea and Japan (Kang and Hwang, 2003). As a typical ovoviviparous fish with the high economic value, it is therefore of strong interest to know more about its molecular mechanisms of endocrine regulatory during the reproductive cycle. In this study, we focused on the two forms of ER $\beta$  in Korean rockfish since the ER $\alpha$  has been described in our previous work (Shi et al., 2011). We aimed to characterize two subtypes of ER $\beta$ , for the first time, investigated its tissue distribution and temporal expression in Korean rockfish as a step to further understanding the molecular mechanisms of ER action.

## 2. Materials and methods

### 2.1. Experimental fish

Around twenty individuals of adult mature male and female Korean rockfish samples were obtained from Shandong coastal area every 2 months. They were maintained for 3–4 days in indoor culture tanks with natural seawater under controlled conditions (20  $\pm$  0.5  $^{\circ}$ C; C4 mg/l O<sub>2</sub>; 14:10 h light; dark cycle). Sexual maturity was determined after excising the gonads defined by the presence of mature ova and sperm, according to Mu et al. (Mu et al., 2013). All fish were anesthetized in 100 mg/L tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). Tissues of stomach, intestine, gill, heart, spleen, kidneys, head kidneys, brain, muscle, pituitary and liver were collected from each fish, and sectioned in two parts, one fixed in Bouin's solution for hematoxylin and eosin (HE) staining in order to identify the development stages of gonads, the other one was snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}$  C until RNA extraction.

### 2.2. Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from Korean rockfish tissue samples using RNAiso reagent (Takara, Japan) following the manufacturer's instructions. RNA concentration and purity of each sample was quantified in UV spectrophotometer (Ultrospec-2100Pro, Amersham; A260 nm/A280 nm ratios > 1.8). The electrophoresis on ethidium bromide-stained 1.5% agarose gels was applied to check RNA integrity. The first-strand cDNA was synthesized, respectively, with 1  $\mu$ g total RNA from each sample and d(T)<sub>18</sub> primers in 10  $\mu$ l reactions at 70  $^{\circ}$  C for 5 min and Reverse Transcriptase M-MLV (Takara, Japan) following the manufacturer's protocol.

### 2.3. Isolation and PCR amplification of ER $\beta$ s cDNA fragments

To obtain core partial-length fragments KrER $\beta$ 1 and KrER $\beta$ 2 cDNAs, two pairs of degenerated primers were designed by a web-based primer design program-codehop (Chen et al., 2009) from highly conserved amino acid sequences among fish species (Table 1). PCR reaction was carried out in a final volume of 50  $\mu$ l containing 2  $\mu$ l of cDNA from ovarian tissue following the manufacturer's instructions (Takara, Japan). PCR cycling conditions were as follows: 5 min denaturing step at 94  $^{\circ}$  C, 10 cycles of 30 s at 94  $^{\circ}$  C, 30 s at a range of annealing temperature from 70  $^{\circ}$  C to 60  $^{\circ}$  C (both ER $\beta$ 1 and ER $\beta$ 2), decreasing 1  $^{\circ}$  C each

cycle and 30 s at 72  $^{\circ}$  C, then followed by additional 30 cycles of 30 s at 94  $^{\circ}$  C, 30 s at 60  $^{\circ}$  C (both ER $\beta$ 1 and ER $\beta$ 2) and 30 s at 72  $^{\circ}$  C, finally ended with 10 min at 72  $^{\circ}$  C for extension. The PCR reactions were separated by a 1.5% agarose gel and purified using TIAN gel midi Purification Kit (TIAGEN, China), then cloned into pGM-T vector (Tiagen, China), followed by propagation in *Escherichia coli* DH5 $\alpha$ , clones were subsequently sequenced on the ABI3730XL sequencer (ABI, USA).

### 2.4. 3' and 5' RACE-PCR

The 3' and 5' ends RACE PCR were applied using the SMART<sup>TM</sup> RACE cDNA amplification Kit (Clontech, USA). Specific primers and nested primers for amplification of 5' and 3' ER $\beta$  cDNA ends were listed in Table 1. The first-strand cDNA synthesis and RT-PCR were used 1  $\mu$ g of total RNA and 1  $\mu$ mol L<sup>-1</sup> each primers. PCR was performed using the cycling conditions: 5 min denaturing step at 94  $^{\circ}$  C, 40 cycles of 30 s at 94  $^{\circ}$  C, 30 s at 68  $^{\circ}$  C (ER $\beta$ 1-C-3-2), or at 69  $^{\circ}$  C (ER $\beta$ 2-C-5-1; ER $\beta$ 2-C-5-2), or at 70  $^{\circ}$  C (ER $\beta$ 1-C-5-1; ER $\beta$ 1-C-3-1; ER $\beta$ 2-C-3-1), then followed by additional step at 72  $^{\circ}$  C for 1 min, finally ended with 10 min at 72  $^{\circ}$  C for extension. The PCR reactions were separated by a 1.5% agarose gel and purified using TIAN gel midi Purification Kit (TIAGEN, China), then cloned into pGM-T vector (Tiagen, China), followed by propagation in *Escherichia coli* DH5 $\alpha$ , selected clones were sequenced on the ABI3730XL sequencer (ABI, USA).

### 2.5. Phylogenetic analysis and sequence analysis

Multiple protein sequence of ER $\beta$ s cDNA were obtained from Genbank, and alignments were aligned by the ClustalX version 1.81 (Thompson et al. 1997). Phylogenetic analyses, of full length amino acid sequences, were conducted using MEGA version 4.0 (Tamura et al. 2007). A rooted phylogenetic tree was constructed by means of the Neighbor-Joining algorithm (Saitou and Nei, 1987), and the data were re-sampled via 1000 bootstrapping replicates (Felsenstein, 1985). Protein sequence analysis was performed with the ExPASy Molecular Biology Server (<http://www.expasy.ch/>) scanning all known PROSITE motifs based on PROSITE database (Bairoch et al. 1997). Percent identities of proteins motifs between Korean rockfish and other species were calculated using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

### 2.6. Tissue distribution analysis by reverse transcription (RT)-PCR

The expression profiles of ER $\beta$ 1 and ER $\beta$ 2 mRNA in different tissues were examined using RT-PCR (reverse transcriptase-polymerase chain reaction) assays. Total RNA was extracted from tissues of ovary, liver, kidney, head kidney, brain, heart, spleen, caeca, stomach, fat, gill, intestinal and pituitary of one female fish in late-vitellogenic stage and testis from one male fish at spermiated stage. To avoid genomic contamination, extracted RNA was treated with RNA DNase I before reverse transcription. Total RNA of those organs were extracted and reverse-transcribed as described above (see 2.2). Specific primers developed based on the sequences generated were listed in Table 1 (ER $\beta$ 1-e-f and ER $\beta$ 1-e-r; ER $\beta$ 2-e-f and ER $\beta$ 2-e-r). Tissue expression was normalized using 18S rRNA (Table 1) as an internal control. Semi-quantitative RT-PCR was performed using a Biometra TPersonal Thermal Cycler (Biometra, Germany), the PCR cycling conditions were as follows: 94  $^{\circ}$  C for 5 min, followed by 40 cycles of 94  $^{\circ}$  C for 5 s, 58  $^{\circ}$  C for 30 s (both for ER $\beta$ 1 and ER $\beta$ 2), 72  $^{\circ}$  C for 30 s, finally 72  $^{\circ}$  C for 10 min. Reaction product was checked by 1.5% agarose gel (containing ethidium bromide) electrophoresis and visualized on a Gel system (Tanon, China).

**Table 1**  
Primer sequences used for cloning and mRNA expression analysis.

Primers	Sequence (5'–3')	Usage	Position
ERβ1RF-1	CACTTCTGCCCGTGTGYCANGAYTAYG	Degenerate primer	710–735
ERβ1RR-1	TCA AAGATCTCCGAGAAGCCYTSNACRCA	Degenerate primer	1740–1759
ERβ1RF-2	GAGCTGGGCTGTGGAYCARGTNC	Degenerate primer	1586–1608
ERβ1RR-2	TGCATGATGTGGGCGTCNARCATYTC	Degenerate primer	2105–2130
ERβ2RF-1	TGCGCCGTGTGCCAYGAYYGC	Degenerate primer	716–738
ERβ2RR-1	GGTAGATCTCCGGGGCTCNGCYTCCAT	Degenerate primer	1145–1172
ERβ2RF-2	ACCTGCTGA AGTGCTGYTGGYTNGA	Degenerate primer	1313–1336
ERβ2RR-2	CGTCCAGCATCTCCAGCARNARRTCRTA	Degenerate primer	2092–2120
ERβ1-C-5-1	ACGACACCACGGCTTCTCGCTCT	5'-RACE primer	1012–1053
ERβ1-C-3-1	GCCTCACCTTCCGCCAACAGTACAC	3'-RACE primer	1963–1987
ERβ1-C-3-2	AGGTGATGGACCATCCAGGGAAAC	Nested 3'-RACE primer	1662–1686
ERβ2-C-5-1	CACTTCGTAGCATTACGTAGGCGGC	5'-RACE primer	882–907
ERβ2-C-5-2	GCCTCGGTGAAGTCTTCTCATGTC	Nested 5'-RACE primer	1181–1206
ERβ2-C-3-1	TCCGCCACGTAGTAAACAAAGGCAT	3'-RACE primer	1728–1752
ERβ1-e-f	CTCCGGCTCGTAATCTTTGTC	PCR primer	523–542
ERβ1-e-r	AAGGTATGGTGGTGAAGTCTG	PCR primer	752–771
ERβ2-e-f	GATGATCGACGCTCTGGTCT	PCR primer	1630–1649
ERβ2-e-r	CCGCCGTAAGTCTGAAAT	PCR primer	2008–2027
18SF	CCTGAGAAACGGCTACCATC	Reference primer	–
18SR	CCAATTACAGGGCTCGAAAG	Reference primer	–

### 2.7. Quantitative real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR (qRT-PCR) was conducted to determine the relative expression of ERβs mRNA using the RNA extracted from gonads of Korean rockfish. PCR analyses were performed using Eppendorf iCycler iQ multicolor real-time PCR detection system (Eppendorf, Hamburg, USA) and the iQ™ SYBR Green Supermix (Takara, Japan) according to the manufacturer's protocol. The primer sequences for ERβ1 (ERβ1-e-f and ERβ1-e-r) and ERβ2 (ERβ2-e-f and ERβ2-e-r) are listed in Table 1. As an internal control, 18S rRNA was amplified under the same conditions using Korean rockfish-specific primers (Table 1), and no significant changes were observed in the 18S rRNA expression level during gonadal development. The mRNA was treated using DNase I (Takara, Japan) and Ribonuclease Inhibitor (Takara, Japan) to remove trace genomic DNA and prevent potential genomic DNA amplification. The ERβs qPCR conditions were as follows: 1 cycle of denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 20 s, annealing at 59 °C for 20 s, and extension at 72 °C for 20 s. Three PCR reactions were performed for each sample and then averaged. Relative expression levels (ERβ; reference gene) were determined using a development of the arithmetic comparative method,  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). The samples from one stage with serial dilutions of total cDNA were used as calibrators in this experiment.  $\Delta\Delta CT = (\text{Avg. } Ct_{\text{target}} - \text{Avg. } Ct_{\text{reference gene}}) - (\text{Avg. } Ct_{\text{calibrator gene}} - \text{Avg. } Ct_{\text{reference gene}})$ .

## 3. Results

### 3.1. Isolation and characterization of ERβ cDNA

The full-length KrERβ1 cDNA (2451 bp, FJ646610.3) was found to have an open reading frame (ORF) of 1683 bp which began with the first ATG codon at position 588 bp and ended with a TGA stop codon at position 2273 bp. It encoded 561 amino acids (Fig. 1). The cDNA of KrERβ2 (2338 bp, HQ452829.1), was found to have an open reading frame (ORF) of 1977 bp which began with the first ATG codon at position 182 bp and ended with a TGA stop codon at position 2161 bp. It encoded 659 amino acids (Fig. 2). Both of them were lacking a typical polyadenylation signal in the 3'-UTR but containing a poly (A) tail.

The KrERβ1 and KrERβ2 sequences can be classified into six domains (A/B, C, D, E and F) based on its sequence identity to other species' ERβs (Krust et al., 1986) (Fig. 3). In A/B domain of ERβ2, there was a PCK (protein kinase C) phosphorylation site which was considered to make up for ligand-independent transactivation function motif (AF-1), and

there was lack of typical MAPK kinase phosphorylation sites in this domain. In the C domain, motifs of D-box (EGCKAFF), P-box (PATNQ), PKA (protein kinase A) and eight cysteine residues of ERβs were completely conserved compared with *O. mykiss* ERβs and *Sparus aurata* ERβs. A conserved motif of CK-2 as well as a protein kinase C phosphorylation site was found in ERβ2, another CK-2 motif was in ERβ1. In the E domain, two PKC sites and a ligand-dependent transactivation function motif (AF-2) were conserved in both of ERβ1 and ERβ2, and a motif of CK-2 was found in ERβ2.

Amino acid sequences of KrERβ1 and KrERβ2 showed an overall identity of 58%. The two ERβs share 38% identity in the A/B domain, 89% in the C domain (the DNA-binding domain), 17% in the D domain, 73% in the E domain (the ligand-binding domain), and 17% in the F domain (Tables 2 and 3). In comparison of Korean rockfish ERβs with other species, KrERβs shared 79–21, 96–87, 71–7, 93–66, and 75–8% identities in the A/B, C, D, E and F domains, respectively (Tables 2 and 3), showing the high conserved features of C domain.

### 3.2. Phylogenetic analysis

The phylogenetic analysis was conducted using the amino acid sequences for detection of the evolutionary relationship among ERβs genes (Fig. 4). All ERβs proteins appeared to be clustered in two distinct clades-ERαs and ERβs. Three subclades including fish ERβ1, fish ERβ2 and tetrapod ERβ were classified into ERβs clades. In the teleost ERβ1 clade, KrERβ1 had the highest similarity with *Perca flavescens* ERβa (86% similarity). Inside the teleost ERβ2 clade, KrERβ2 had the highest similarities with *Micropterus salmoides* ERβ (88% similarity).

### 3.3. Expression of ERβs in different adult tissues

Primers (ERβ1eF and ERβ1eR; ERβ2eF and ERβ2eR, listed in Table 1), were applied to determine tissue expression of KrERβs. In order to avoid cross amplification, the primers were constructed from stretches of sequence that exhibit significant differences between ERs. The length of generated PCR products of ERβ1 and ERβ2 were 249 bp and 389 bp respectively (Fig. 5). Both of ERβ1 and ERβ2 displayed a widely distribution. The results showed that ERβ1 and ERβ2 were found in pituitary, brain, kidney, gonads, head-kidney and spleen. However, ERβ1 was expressed in fat and heart, but ERβ2 were not detected in these tissues. The expression of ERβ1 was not found in intestine, caeca as well as liver which was common found the expression in all kind of fish, but ERβ2 was detectable in these tissues.





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1      GGTAACAGGAGGCTGGAAMACCAGACAACAAGTCCAAAAATCATCAGCTGATCTTCCCTCCGCCCACTGGACTCAGTGACAGACAGGACTCGTCTTACCAGAACATGCAATAGA 120
121    GGAAGCAGCTGCATCATATATTATCACCAGTGTGATCAGTACTCCCCCCCCCGAATGGCCCTCTCCCCGGGCTGGATGCTGACCCGTTACCTCTGATCCAGCTCCAGGAGGT 240
1      M A S S P G L D A D P L P L I Q L Q E V 20
241    GGACTCCAGAAACCGGCAGAGAGGCCGAGTCCCGGGACTCCGTCGCCGTGTACAGCCCTCCTGTGGCATGGACGGCCACACCGTCTGCATCCCTCTCCGTACATGGACAGTAG 360
81     D S S K P A E R P S S P G L R P A V Y S P P V G M D G H T V C I P S P Y M D S S 60
361    CCACGAGTACAACCGGCCAGCGACCTCTAACCTTCTACAGCCGCTGTGTGCTGAGCTACGCCAGGCCCCATCACGACAGCCGCTCGTCTCTGTGCTGCCCTCAGCCGCTCGGC 480
121    H E Y N H G H G P L T F Y S P S V L S Y A R P P I T D S P S S L C S S L S P S A 100
481    CTTCTGGCGTCCACGGCCACCCCAACATGCCCTCACTGACTCTGGGCTGCCCTCAGCCTCTCGTCTACAATGAGCCAGCCACATGCACCCCTGGATGGAGGCCAAAACCCACTGTAT 600
161    F W P S H G H P N M P S L T L R C P Q P L V Y N E P S P H A P W M E A K T H C I 140
601    CAACAGCAGCAGCTCTATCATGGGCTGTAACAAGCGCTGGGGAAGAGGTGAGAGGAGGAAGTCTGAACTCCTCTTGTGCTGCTGCGGGCGGCAAGCCGACATGCACCTCTCGGC 720
201    N S S S S I M G C N K P L G K R S E E E V V N S S L C S S A A G K A D M H F C A 180
721    CGTGTGCCAGACTACGCTCGGGTACCCTACGGCGTGTGCTGCGAGGGTGAAGGCTTTTTCAGAGGAGTATCCAAGGACACAACGACTACATCTGCCCGCCACCAATCA 840
241    V C H D Y A S G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y I C P A T N Q 220
841    GTGCGCCATCGACAAGAACCGACGTAAGAGTCCAGGCGCTGCCGCCTACGTAATGCTACGAAGTGGGCATGATGAAGTGGGTGAAGGCCGCAACGCTGCAGCTATCGAGGAGCCCG 960
281    C A I D K N R R K S C Q A C R L R K C Y E V G M M K C G V R R E R C S Y R G A R 260
961    GCACCGCCGCGTGGACTCAGCCTCGGGATCCACAGCAGGGGTTTGGTCAGGGTGGGGCTGGTCTCGGGCCAGCGCACCTCCACCTGGAGGCTCCCTCGCACCGCTCGCCCC 1080
321    H R R G G L Q P R D P T G R G L V R V G L G P R A Q R H L H L E A P L A P L A P 300
1081   CCTCCCTCAGGCCAACCCGCGCACCCTCGGCATGAGGCCGAGGAGTTCATCTCCCGCATATGGAGGCGGAGCCTCCGAGATCTACCTCATGGAGGACATGAAGAAGCAGTTCAC 1200
361    L P Q A N H A H H S A M R P E E F I S R I M E A E P P E I Y L M E D M K K Q F T 340
1201   CGAGGCCAGATGATGATGTCCTCACCAACCTGGCCGACAAAGGAGTGGCTCCTATGATCAGCTGGGCTAAAAGATCCCGGTTTGTAGAGCTGAGTCTAGCTGACAGATCCACCT 1320
401    E A S M M M S L T N L A D K E L V L M I S W A K K I P G F V E L S L A D Q I H L 380
1321   GCTGAAGTCTGCTGGCTGGAGTCTGATGTTGGCCCTGATGTGGAGTCCGTGGATATCCCGAAAATCATCTCTCTCCAGACTCAAACCAAGGAGGAGGGCCAGTGTGT 1440
441    L K C C W L E I L M L G L M W R S V D H P G K L I F S P D F K L N R E E G Q C V 420
1441   GGAGGCCATCATGGAGATTTTCGACATGCTGCTGCAGCCATTTCTCGTTCGTGAGCTGAAGCTTCAGAGAGAGGAGTACGCTGTCTGAAGGCCATGATCTCTCACTCACTCAATCT 1560
481    E G I M E I F D M L L A A T S R F R E L K L Q R E E Y V C L K A M I L L N S N L 460
1561   GCGTACGAGCTCCCTCAGACAGCCGAGGAGCTGGAGAGCAGGAGCAAGCTGCTGCATCTGCTGGACTCGATGATCGACGCTCTGGTCTGGGCCATTTCAAGATGGGCGCTGTCGCCCA 1650
521    R T S S P Q T A E E L E S R S K L L H L L D S M I D A L V W A I S K M G L S T Q 500
1681   GCAGCAGACTCTGCTGTTGGACACCTCACCATGCTCTCTCCACATCCGCCAGTCAAGTAAACAAAGGCATGGCCACCTGTCCAGCATGAAGAGGAAGAACGTTGCTGGTGTACGA 1800
561    Q Q T L R L G H L T M L L S H I R H V S N K G M A H L S S M K R K N V V L V Y D 540
1801   CCTCCTCTGGAGATGCTGGACGCAACACGTCACGACGAGCAAGCGTCTCGCCGAGTTCGAGCTCGTACTCCGACAGCAGCAGTACCCCAAACTCCGTCGGCCGCGCA 1920
601    L L L E M L D A N T S S S S S Q A S S S P S S D S Y S D Q H Q Y P Q T P S A A D 580
1921   CCACACCCCGTGCCTCGGATGGACCAATGAGGCCCGATCCTGGACAGACACTGCAGGCTCTGCCCTCCGGTCTCAGCGCCATTTAGAGTTTTCAGCGCGGCTCAGATGGTTCAG 2040
641    H T A V P P H G P N E A P I L D R H L Q A L P L R S S A P F Q S L A A A H M V S 620
2041   CAACGATTACATCCATCAGGACGAGTGTGCTGATGAGGAGACGAGTCCATCGTGGAAACCGAGCGGCTACATCATCGCTGATCGGGTTGTATGGAGACGGCCATCGAGGGTTG 2160
681    N D Y I H Q E Q W S L D A G D D G P S V E P T G Y I I A D R V V M E T A I E G * 659
2161   ACGGACTGGAAGAGTCTAACTGAGACTGAACTTTTAATGGCTTTTCAACCCCTCCCCCAAGATCGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2258

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Initiation codon: ATG Termination codon: TGA

Fig. 2. Nucleotide and deduced amino acid sequence of KrERβ2 isolated from ovary. Two zinc-finger motifs in DNA binding domain were underlined, and eight cysteines in the same domain were also shaded. The initiation codon and termination codon were boxed.

during May to July (stage II), then peaked to  $1.33 \pm 0.12$  during September, dropped to  $1.05 \pm 0.21$  during January to March (stage V), and finally slightly increased to  $1.02 \pm 0.14$ .

In the female, the expression pattern of ERβ1 and ERβ2 was similar. During July to September (stage II), the expression level of ERβ1

and ERβ2 was  $0.92 \pm 0.35$  and  $2.49 \pm 0.49$  respectively. The highest level of ERβ1 was  $3.73 \pm 1.48$  during November (stage III), and ERβ2 was  $5.99 \pm 1.78$ . Finally, the level of ERβ1 and ERβ2 dropped to the lowest level of  $0.18 \pm 0.04$  and  $1.05 \pm 0.21$  during May (stage V).





**Table 2**

Amino acid identities between ER $\beta$ 1 in Korean rockfish and ERs in fish and mammals (see Section 2.5 for sequence references. The total score of amino acids and the number of residues per domain are marked in brackets).

Species/domain	<i>S.schlegelii</i> , $\beta$ 1	A/B	C	D	E	F
<i>P. flavescens</i> , $\beta$ a	86(555/561)	78(156/152)	96(87/85)	71(38/40)	93(238/238)	75(36/46)
<i>D. labrax</i> , $\beta$ 1	86(517/561)	79(106/152)	95(85/85)	67(41/40)	94(228/238)	69(47/46)
<i>S. aurata</i> , $\beta$ 1	83(559/561)	70(149/152)	92(85/85)	65(40/40)	95(238/238)	65(47/46)
<i>O. mykiss</i> , $\beta$ 1	70(594/561)	53(174/152)	91(85/85)	32(40/40)	99(238/238)	26(57/46)
<i>O. mykiss</i> , $\beta$ 2	60(604/561)	37(172/152)	90(85/85)	17(45/40)	78(238/238)	10(64/46)
<i>M. salmoides</i> , $\beta$	59(670/561)	40(174/152)	90(85/85)	22(57/40)	75(228/238)	23(116/46)
<i>S. schlegelii</i> , $\beta$ 2	58(659/561)	38(172/152)	89(85/85)	17(57/40)	73(238/238)	17(106/46)
<i>M. undulatus</i> , $\beta$	57(673/561)	81(175/152)	90(85/85)	17(57/40)	75(228/238)	13(118/46)
<i>S. aurata</i> , $\beta$ 2	56(559/561)	70(149/152)	90(85/85)	15(54/40)	75(238/238)	13(119/46)
<i>H. sapiens</i> , $\beta$	51(530/561)	21(142/152)	88(85/85)	7(38/40)	66(226/238)	17(29/46)
<i>R. norvegicus</i> , $\beta$	50(549/561)	24(161/152)	87(85/85)	13(38/40)	66(226/238)	10(29/46)
<i>P. olivaceus</i> , $\beta$	41(553/561)	73(155/152)	94(85/85)	67(40/40)	93(228/238)	58(48/46)
<i>H. sapiens</i> , $\alpha$	49(444/561)	14(28/152)	83(83/85)	12(49/40)	57(239/238)	11(45/46)
<i>R. norvegicus</i> , $\alpha$	42(600/561)	6(184/152)	83(83/85)	15(49/40)	57(239/238)	15(45/46)
<i>S. aurata</i> , $\alpha$	42(581/561)	13(145/152)	82(83/85)	15(52/40)	57(239/238)	17(69/46)
<i>S. schlegelii</i> , $\alpha$	41(624/561)	18(185/152)	80(86/85)	12(33/40)	56(253/238)	8(67/46)
<i>P. olivaceus</i> , $\alpha$	41(578/561)	12(135/152)	81(83/85)	15(49/40)	55(239/238)	19(73/46)

**Table 3**

Amino acid identities between ER $\beta$ 2 in Korean rockfish and ERs in fish and mammals (see Section 2.5 for sequence references. The total score of amino acids and the number of residues per domain are marked in brackets).

Species/domain	<i>S. schlegelii</i> , $\beta$ 2	A/B	C	D	E	F
<i>M. salmoides</i> , $\beta$	89(670/659)	87(174/172)	98(85/85)	87(57/57)	95(228/238)	75(116/106)
<i>M. undulatus</i> , $\beta$	86(673/659)	81(175/172)	98(85/85)	85(57/57)	94(228/238)	67(118/106)
<i>S. aurata</i> , $\beta$ 2	82(668/659)	69(172/172)	98(85/85)	85(54/57)	93(238/238)	62(119/106)
<i>O. mykiss</i> , $\beta$ 2	69(604/659)	56(172/172)	98(85/85)	31(45/57)	85(238/238)	14(64/106)
<i>D. labrax</i> , $\beta$ 1	59(517/659)	33(106/172)	87(85/85)	9(41/57)	75(228/238)	17(47/106)
<i>P. flavescens</i> , $\beta$	58(555/659)	33(156/172)	85(87/85)	23(38/57)	74(238/238)	10(36/106)
<i>S. schlegelii</i> , $\beta$ 1	58(561/659)	38(152/172)	89(85/85)	17(57/40)	73(238/238)	17(46/106)
<i>P. olivaceus</i> , $\beta$	58(553/659)	37(155/172)	88(85/85)	22(40/57)	74(228/238)	12(48/106)
<i>O. mykiss</i> , $\beta$ 1	56(594/659)	34(174/172)	91(85/85)	20(40/57)	75(238/238)	10(57/106)
<i>S. aurata</i> , $\beta$ 1	55(559/659)	31(149/172)	87(85/85)	22(40/57)	74(238/238)	8(47/106)
<i>H. sapiens</i> , $\beta$	52(530/659)	24(142/172)	91(85/85)	15(38/57)	66(226/238)	17(29/106)
<i>R. norvegicus</i> , $\beta$	52(549/659)	23(161/172)	90(85/85)	13(38/57)	67(226/238)	10(29/106)
<i>H. sapiens</i> , $\alpha$	49(444/659)	14(28/172)	81(83/85)	12(49/57)	57(239/238)	6(45/106)
<i>R. norvegicus</i> , $\alpha$	40(600/659)	11(184/172)	81(83/85)	8(12/49/57)	56(239/238)	11(45/106)
<i>S. aurata</i> , $\alpha$	40(581/659)	17(145/172)	85(83/85)	17(52/57)	54(239/238)	11(69/106)
<i>P. olivaceus</i> , $\alpha$	40(578/659)	11(135/172)	84(83/85)	10(49/57)	53(239/238)	10(73/106)
<i>S. schlegelii</i> , $\alpha$	38(624/659)	18(185/172)	82(86/85)	21(33/57)	54(253/238)	5(67/106)

#### 4. Discussion

Two full-length cDNA of ER $\beta$ s in the Korean rockfish were cloned in this study. The KrER $\beta$ 1 cDNA was found to contain an ORF of 1683 nucleotides encoding the protein of 588 amino acids, and the ER $\beta$ 2 cDNA was 1977 bp, encoded the protein of 659 amino acids. The feature that ER $\beta$ 2 protein of Korean rockfish contained more amino acids than ER $\beta$ 1 was found in many teleosts. The two sequences possess the domain structure (A, B, C, D, E domains) which are typical for ER $\beta$ s, and the highly-conserved zinc-finger motif, including the P- and D-boxes, are indispensable for DNA-binding (Schwabe et al., 1993). It is a common feature that ER $\alpha$  has a mitogen-activated protein kinase (MAPK kinase site) in the A/B domain (Halm et al., 2004; Shi et al., 2011). However, the lack of typical MAPK kinase phosphorylation site in both of KrER $\beta$ s in the A/B domain was similar with the *S. aurata* (Pinto et al., 2006), showing the different species may have different signal transduction pathways according to their evolutionary status. Furthermore, it is supposed that the lacking of MAPK (AF-1) in A/B domain in ER $\beta$ s may be a reason that Korean rockfish ER $\alpha$  and ER $\beta$ s have different functions. The two zinc-finger motifs

in the C domain as well as the estrogen-dependent activation domain AF-2 (DLLLEMLD) in the E domain were completely conserved, the latter one was related to ligand dimerization, ligand binding and ligand-dependent transcription activation function (Pinto et al., 2006). The function of F domain was unknown, however, it was considered to control gene transcription interacting with nuclear cofactors by affected ER/Sp1 action (Kim et al., 2003; Montano et al., 1995).

In human ER $\alpha$ , 64 amino acids residues (M342-L354, W383-R394, L402-L410, V418-L428, M517-M528 and L539-H547) in the E domain formed the 17 $\alpha$ -estradiol binding cavity (Brzozowski et al., 1997). In KrER $\beta$ 1 and ER $\beta$ 2, 48 and 50 amino acids residues of these 64 were conserved, respectively. Furthermore, 15 amino acids residues (L346, A350, L387, L391, R394, S395, E397, L402, L403, F404, A405, P406, L408, L410 and L525) also in this domain were essential for ligand contact within binding pocket of human ER- $\alpha$ . There were 12 of them could be identified in KrER $\beta$ 1 (except E397, L403 and A405), and 11 of them were found in KrER $\beta$ 2 (except E397, L403, A405 and L408). In addition, four residues (Leu<sup>349</sup>, Met<sup>421</sup>, Tyr<sup>526</sup>, and Cys530) changes which existed in KrERs were at positions surrounding the human ER $\alpha$  pocket, in accordance with the study by Hawkins and Thomas

**Fig. 3.** Amino acid alignment of KrER $\beta$ 1 and KrER $\beta$ 2 with sbER $\beta$ , sbER $\beta$ 2, rtER $\beta$ 1 and rtER $\beta$ 2 (see Materials and methods for sequence references and abbreviations). Asterisks (\*) and dots (.) marked for completely conserved and conserved amino acids, respectively. The functional domains (A/B, C [DNA-binding domain], D, E [ligand-binding domain], and F) are showed by boxes. And the P- and D-box in C domain, as well as the activation domains (AF-1 and AF-2) in the A/B and D domain, respectively, are indicated by gray boxes. Motifs for ligand interaction, receptor dimerisation and transactivation are marked with triangle, square and double underlines respectively. Potential phosphorylation sites for PKA, PKC and CK-2 are also boxed in gray. PKA: protein kinase A; PKC: protein kinase C; CK-2: casein-kinase II.

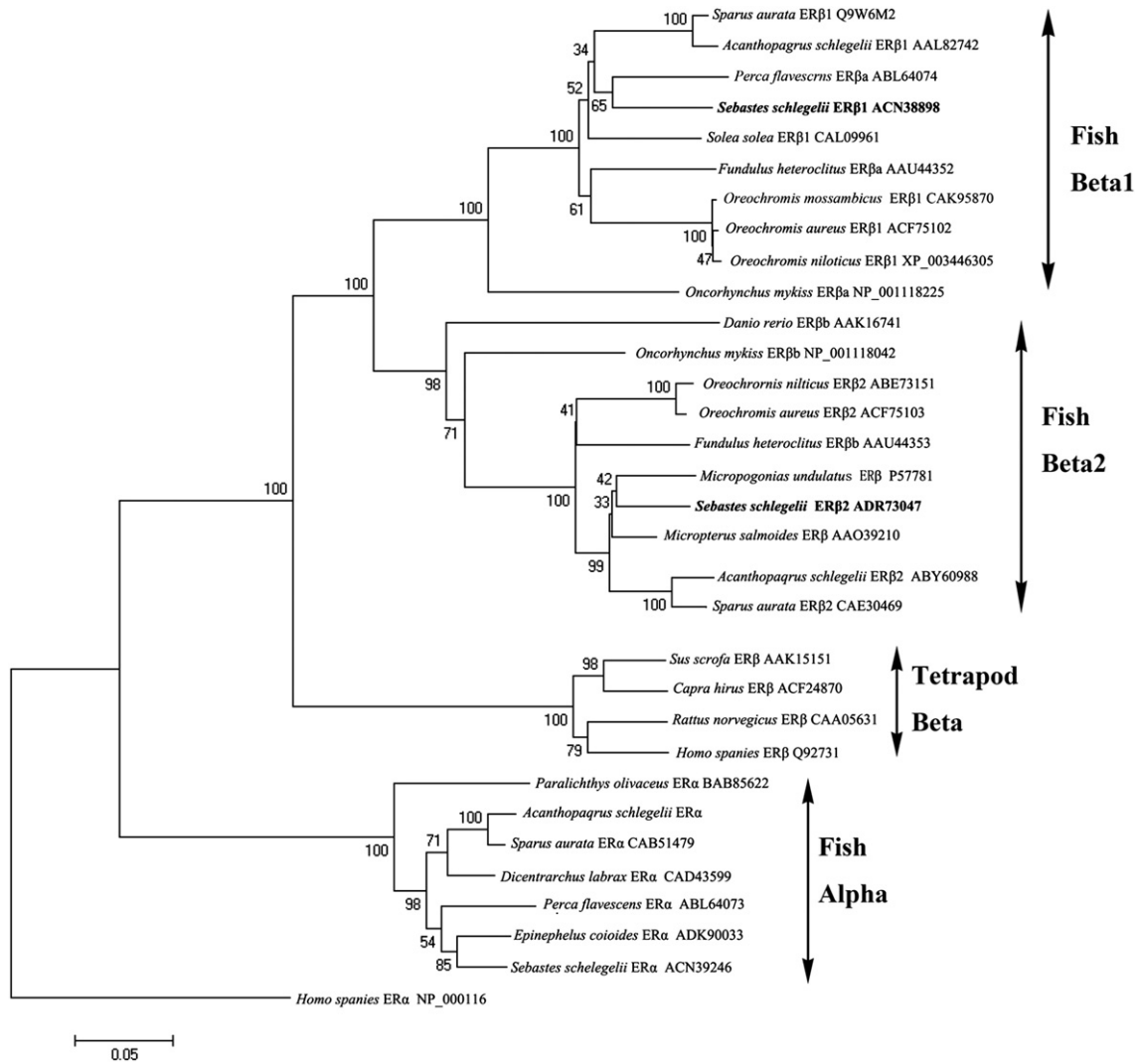
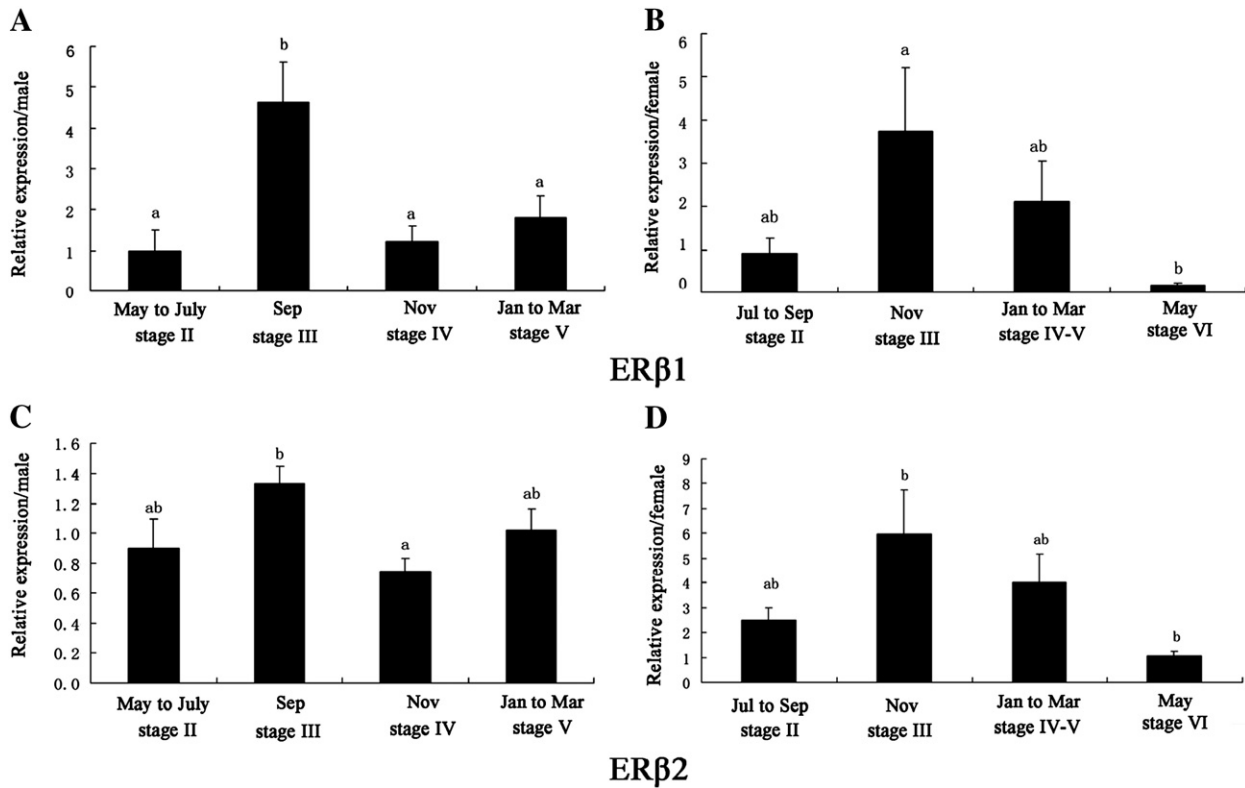


Fig. 4. Phylogenetic tree based on amino acid sequences for ERβs in tetrapods and teleosts. The human estrogen receptor α is used as an outgroup. Bootstrap values are indicated (1000 replicates) (see Materials and methods for sequence references and abbreviations).



Fig. 5. RT-PCR expression analysis of KrERβs in tissues. The integrity of the RNA from the each tissue was ensured by uniform amplification of 18S transcripts (lower panel).





**Fig. 6.** Expression of ERβ1 in testis (A) and ovary (B) and ERβ2 in testis (C) and ovary (D) mRNA of Korean rockfish during the annual reproductive cycle. Values are expressed as mean  $\pm$  standard error of mean. Different letters indicate significant difference ( $P < 0.05$ , one-way ANOVA, followed by Duncan's test) ( $n \geq 3$ ) In male fish: stage II spermatogonia stage; stage III testes full of immature sperm; stage IV mature testes; stage V post-spermiation. In female: stage II perinucleolus stage oocyte; stage III early-oocyte; stage IV-V post-oocyte; stage VI gestational ovary.

(2004). All of these significant conserved residues showed the proposed function for determining species and subtype-specific ligand binding characteristics in ERβs. By comparing the functional domains, we found that ERβs in various species were highly conserved. The motifs of EFCKAF and CPATNQC, highly conserved in all ERβs, were important for species binding to ERE (estrogen response element) on target genes and receptor dimerization respectively (Forman and Samuels, 1990; Ma et al., 2000). These results indicate there was repetition in combination of target DNA with ER, redundancy function of starting target genes transcription. It also suggested these two subtypes of ERβs were capable to combine with different ligands, thus exerting different effects on the biology activity.

The ER genes expressed in various tissues showed an important significance for estrogen action. Although the structures were similar, these ER subtypes had the different expression patterns. RT-PCR was used to measure the tissue distribution of ERβs mRNA in maturing Korean rockfish. As is shown in Fig. 3, RT-PCR analysis exhibited that both of KrERβs mRNA were highly detected in E<sub>2</sub> target tissues that were known related to the reproductive function, such as the pituitary, ovary and testis. And this result was similar with goldfish ERβ2 (Choi and Habibi, 2003) and sea bream ERβs (Socorro et al., 2000). Study in mammals showed that ERβ was expressed at relatively high levels in the reproductive system, including prostate, epididymis, testis, ovary and uterus, but level in the pituitary was low (Kuiper et al., 1996). The high levels of ERβs expression in the gonads and pituitary of teleost fish showed ERβs may play an important role in sexual differentiation and/or development (Halm et al., 2004). In addition, the high level of ERβ transcript levels in the adult zebrafish ovary indicated a special role for ERβ in reproductive tissue as well as ERβ in mammals (Byers et al., 1997). The high expression of ERβs is

also found in Korean rockfish brain, suggesting some relationship between ER and neuroendocrine functional control. Studies in other teleosts, such as pejerrey, goldfish and zebrafish, also detected the ERβ expressed in brain, suggesting the preoptic area and hypothalamus was the main target site for E<sub>2</sub>, which may involved in the regulation of neuroendocrine related gene expression (Marlatt et al., 2008; Menuet et al., 2002; Stroble-Mazzulla et al., 2008). The interesting thing is that the high level expression of KrERβ2 but undetectable expression of KrERβ1 was found in the liver. Most of teleosts showed the three subtypes of ER mainly expressed in liver, such as sea bream, goldfish and fathead minnow (Choi and Habibi, 2003; Filby and Tyler, 2005; Pinto et al., 2006). However, there was a high expression level of ERα and ERβ2 but low level of ERβ1 in liver of fathead minnow, which indicated the interspecific differences existing in the genes of ERs expression. Some studies showed that the high mRNA levels of ERα1 and ERβ2 were accordance with the effect of estrogen on liver to induce vitellogenesis (Campbell and Idler, 1980; Sumpter and Jobling, 1995). Leañes-Castañeda and Kraak found the vitellogenin production was mainly mediated through ERβ in male fish liver (Leañes-Castañeda and Kraak, 2007). Our research may suggest that KrERβ2 play the predominant role and that ERβ1 may have no function or major effect in this process. In this study, in contrast to ERβ1, expression of ERβ2 was more wide-spread in Korean rockfish tissues. Furthermore, the ERβ1 in Korean rockfish was expressed abundantly in kidney, moreover, the low level of mRNA expression was found in fat, head-kidney and heart. However, the KrERβ2 mRNA was almost highly expressed in intestine, kidney, caeca, head-kidney and spleen. The abundance transcripts of ERβ2 were reported in the sea bream intestine, suggested that it involved in the modulation of calcium transport, even though the mechanism was not yet well explained (Guerreiro et al., 2002). All of these results

above showed the potential complexity function of ER $\beta$  subtypes in these organs. Further studies were needed to elucidate any more biological effects of these subtypes in various tissues.

Using quantitative real-time PCR, high levels of ER $\beta$ s mRNA in Korean rockfish were observed in early development stage of testis, being different with that of trend of ER $\alpha$  (Shi et al., 2011), which suggested that those two kinds of ER played different roles during the testis development. In addition, the study in fathead minnow indicated different mechanisms of regulation for different ERs (Filby and Tyler, 2005), which may be another result for the different pattern of ERs. In the early stage of spermatogenesis in male Korean rockfish, the ERs mRNA level increased as well as the plasma E<sub>2</sub> level (Shi et al., 2011), which showed that the ERs was involved the regulation of estrogen-dependent spermatogenesis. The fact that some researches revealed the high level ER $\beta$  mRNA expression during early testis development stage suggests that ER $\beta$  was important for gonads development and maturation (Byers et al., 1997; Filby and Tyler, 2005; Halm et al., 2004). The study of European sea bass suggested that the ER had initiated spermatogenesis and even spermiate (Rodríguez et al., 2001). In the study of female, KrER $\beta$ 1 and KrER $\beta$ 2 exhibited a high expression level in the vitellogenic stage, which indicated that ER might play an important role in gonadal development. At the beginning of the ovary development, the expression of KrER $\beta$ 1 and KrER $\beta$ 2 mRNA level increased as well as plasma level of E<sub>2</sub> (Shi et al., 2011). An et al. (2008) pointed out that there was a co-action of ER and E<sub>2</sub> in promoting vitellogenesis and final maturation of oocyte. However, much more work are required to elucidate the mechanisms in protein levels and receptor binding assay.

In summary, in this study, full-length of sequences of ER $\beta$ 1 and ER $\beta$ 2 cDNAs are isolated from Korean rockfish for the first time, and specific tissue expression RT-PCR as well as the temporal expression during gonad development process are described. What is showed in our work is the expressions of ER $\beta$ 1 and ER $\beta$ 2 in mature Korean rockfish in related tissues of brain-pituitary-gonad neuroendocrine axis, except undetectable expression of ER $\beta$ 1 in liver, which indicates that ER $\beta$ s play a key role in this axis, and ER $\beta$ 1 may have the species specific pattern. For the first time, the expression patterns of ER $\beta$ s in this ovoviviparous Korean rockfish during gonadal development in male as well as female are studied. Furthermore, ER $\beta$ s highly expresses during the early stage of gonads in Korean rockfish, which indicates that ERs may play an important role in gonadal development and maturation. Studies above are indispensable for the research of genes related to brain-pituitary-gonad neuroendocrine axis. However, additional studies are needed to determine the roles of ER $\beta$ s in other issues during gonadal development cycle, in order to get further understanding of the role in regulation of reproductive endocrine system of teleost.

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