# Cloning, characterization and expression of estrogen receptor beta in the male half-smooth tongue sole, *Cynoglossus semilaevis*

Wenge Li · Jiaren Zhang · Weijie Mu · Haishen Wen

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Abstract A full-length sequence encoding the estrogen receptor beta was isolated from half-smooth tongue sole, Cynoglossus semilaevis (hstsER $\beta$ ) using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends procedures. The hstsER $\beta$  cDNA clone was found to contain 1,791 nucleotides including an open reading frame that encodes 578 amino acids. The deduced hstsER $\beta$ protein consisted of six nuclear receptor-characteristic domains. Based on a phylogenetic analysis, the hstsER $\beta$  C and E domains are highly conserved compared to other fishes. The potential phosphorylation sites for PKC, CK-2 and PTK are also found in this protein. Highest amino acid identities were found for hstsER $\beta$  with common carp (*Cyprinus carpio*) ER $\beta$  (76 %) and Japanese flounder (Paralichthys olivaceus) ER $\beta$  (76 %). Tissue expression analysis confirmed that the hstsER $\beta$  was widely distributed and predominantly expressed in testis, brain and liver. Seasonal changes in the testis, brain and liver expression profiles of hstsER $\beta$  were examined by RT-PCR; the present results suggest that level of  $hstsER\beta$  in brain increased to the highest then decreases with gonadal growth; whereas in the testis and liver, the *hstsER* $\beta$  mRNA level dropped to lowest then slightly increased.

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**Keywords** Estrogen receptor · *Cynoglossus* semilaevis · Teleost fish · Clone · Expression

#### Introduction

It has been revealed that estrogen takes an important part in gonadal differentiation, development and fertilization processes for both female and male vertebrates (Nakamura et al. 1998). All of these activities of estrogen are mainly separately supported by classical nuclear estrogen receptor (ER) and G protein-coupled receptor family (Levin 2005). It is well known that the proteins of this superfamily have the common features and can be divided into six distinct domains (Weinberger et al. 1985; Krust et al. 1986; Kumar et al. 1987). The C domain (DNAbinding domain; DBD) has two zinc-finger structures that are essential for recognition and specific binding of the receptor to DNA, and the E domain (ligandbinding domain; LBD) whose role is the liganddependent transactivation of specific gene transcription are highly conserved among species (Xia et al. 1999). There are also variable regions at the N and C termini and between the DBD and LBD (A/B, F and D domains, respectively) (Choi and Habibi 2003).

Two isoforms of ER, which designated ER $\alpha$  and ER $\beta$ , have been described in vertebrates. ER $\alpha$  has been cloned from several vertebrate species (Greene

W. Li · J. Zhang · W. Mu · H. Wen (⊠) Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao 266003, China e-mail: wenhaishen@ouc.edu.cn

et al. 1986; Koike et al. 1987; White et al. 1987), and ER $\beta$  was found to be present in rat which expressed widely in the male reproductive tract (Kuiper et al. 1996) and human (Mosselman et al. 1996; Saunders et al. 1997). A cDNA coding ER was first isolated in rainbow trout (Pakdel et al. 1989), then was cloned in *Oreochromis aureus, Oreochromis niloticus, Ictalurus punctatus* and other species. (Tan et al. 1996; Chang et al. 1999; Xia et al. 1999; Patino et al. 2000; Wu et al. 2001). Interestingly, in teleost fish, a third estrogen receptor subtype which is genetically distinct from the other two types has been found, it has been named as ER $\beta$ 2 (Tchoudakova et al. 1999; Ma et al. 2000).

The estrogen receptors (ERs) are members of a large ligand-activated nuclear receptors superfamily containing receptors for other steroid hormones, thyroid hormone (Mangelsdorf et al. 1995; O'donnell et al. 2001). From mammals and non-mammalian vertebrates, various ER subtypes have been isolated, and the function studies of them have suggested the complexity by which estrogen receptors are activated and transformed (Pennie et al. 1998; Price et al. 2001; Menuet et al. 2002). There are many studies related to ER gene suggested that it is expressed very early during embryonic development and gonadal differentiation in fishes, showing an important role for estrogens in sexual differentiation (Guiguen et al. 1999; Lassiter et al. 2002). Cavaco et al. (1998) demonstrated estrogen receptors have affect on main events during sexual development, such as puberty, as sexual steroids represent the key elements for these processes.

The half-smooth tongue sole (*Cynoglossus semilaevis*) is a native commercially important marine fish in China. Interestingly, the females of the species grow faster than their male counterparts (Chen et al. 2012). Because of the weak reproductive capacity in male could not establish a fish hearing, typically, it was not seen as a fishing target in the wild. Therefore, study of male fish has important economic significance. Due to the commercial values, the half-smooth tongue sole was considered as one of the main artificial seeding fish in China. At present, there are some reports about the artificial propagation and breeding technology research of the half-smooth tongue sole (Liu et al. 2005), and there are no reports about ER $\beta$  in half-smooth tongue sole.

In the half-smooth tongue sole,  $ER\alpha$  gene was previously isolated, and we analyze it in another

coming work (unpublished yet). In our present study, we have cloned and characterized the ER $\beta$  in the male half-smooth tongue sole, described the tissue distribution and expression profiles of ER $\beta$  isoforms using male fish, combined with serum levels of E<sub>2</sub>. Knowledge about the expression and function of these genes is a step toward understanding the molecular mechanisms of ER $\beta$  action.

# Materials and methods

# Experimental fish

The experimental male fish half-smooth tongue sole reared in a pond at a commercial fish farm (Laizhou, Shandong, PRC). Fishes were maintained for 3-4 days in indoor culture tanks with natural seawater under controlled conditions (20  $\pm$  0.5 °C;  $\geq$ 4 mg/l O2; 14:10 h light; dark cycle). All fishes were anesthetized in 100 mg/l tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO) prior to sampling tissue. The blood was also collected from caudal vein, clot at 4 °C for 4-6 h; serum was then separated by centrifugation at  $16,000 \times g$  for 10 min and stored at -40 °C until processed for steroid assay. Organs collected from fish were immediately snap-frozen into liquid nitrogen and stored at -80 °C until RNA extraction. Moreover, parts of the gonads were stored in Bouin's solution for hematoxylin and eosin (HE) staining in order to identify the developmental stages of gonad. Body weight, viscera weight and gonad weight were recorded in each sample fish to calculate gonadosomatic index (GSI = [gonad weight/(body weightviscera weight)]  $\times$  100).

Total RNA extraction and reverse transcription (RT)

Total RNA was extracted using RNAiso reagent (Takara, Japan) following the manufacturer's protocols. RNA concentration of each sample was quantified in UV spectrophotometer (Ultrospec-2100Pro, Amersham), and an agarose gel was applied to check RNA integrity. Then, first-strand cDNA was synthesized, respectively, with 1  $\mu$ g total RNA from each sample using random primers and Reverse Transcriptase M-MLV (Takara, Japan) in a 10  $\mu$ l reaction. Isolation and PCR amplification of  $\text{ER}\beta$  cDNA fragments

In order to clone  $\text{ER}\beta$  half-smooth tongue sole cDNA fragment, a pairs of degenerated primers (ER $\beta$ F1/  $ER\beta R1$ ) were designed by a web-based primer design program named CodeHop (Chen et al. 2009) (Table 1). PCR was carried out in a final volume of 25 µl containing 2 µl of cDNA from ovarian tissue, 2.5  $\mu$ l of 10× reaction buffer, 2  $\mu$ l of a 10-mM dNTP mix, 0.5 µl of 25 µM solution of each primer, 0.2 µl of Taq polymerase (Takara, Japan) using touchdown PCR program as follows: 5 min denaturing step at 94 °C, 13 cycles of 35 s at 94 °C, 35 s at a range of annealing temperature from 68 °C to 56 °C, decreasing 1 °C each cycle and 35 s at 72 °C, then followed by additional 25 cycles of 35 s at 94 °C, 35 s at 55 °C and 35 s at 72 °C, finally ended with 10 min at 72 °C for extension. PCR product was then electrophoresed on a 1.5 % agarose gel showing the predicted molecular weight. The target fragments were purified using TIAN gel midi Purification Kit (TIAGEN, China), then cloned into pGEM-T vector (TIAGEN, China), followed by propagation in E. coli DH5 $\alpha$ , and subsequently sequenced using the ABI3730XL sequencer to give at least threefold coverage.

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

In order to achieve full-length cDNA of  $\text{ER}\beta$ , SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech, USA) was used to 3' and 5' ends RACE-PCR. The genespecific primers and nested primers for amplification of 5' and 3' cDNA ends were listed in Table 1. The PCR products which have corresponding predicted length were excised, purified and cloned into vector, then sequenced as described above. BLASTN (Altschul et al. 1997) searches were used to verify gene identity and determine similarities with other vertebrates.

Phylogenetic analysis and sequence analysis

Amino acid sequences were got from GenBank (Altschul et al. 1990), and the sequences of  $\text{ER}\beta$  of the halfsmooth tongue sole were aligned with other homologous fish estrogens receptors. Multiple protein sequence alignments were aligned by the ClustalX version 1.81 (Thompson et al. 1997). Phylogenetic analyses, of fulllength amino acid sequences, were conducted using MEGA version 2.0 (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 1,000 bootstrapping replicates. Protein sequence analysis was preformed 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 black rockfish and other species were calculated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index. html). Sequences used for comparison and their Gen-Bank accession numbers are as follows: Solea solea  $\text{ER}\beta$  (csER $\beta$ , CAL09961.1), *Danio rerio* ER $\beta$  (zfER $\beta$ 1, AAK16742.1; zfERβ2, CAC93849.1), Oncorhynchus mykiss ER $\beta$  (rtER $\beta$ 1, NP\_001118225.1), Oncorhynchus mykiss ER $\beta$ 2 (rtER $\beta$ 2, NP\_001118042.1), Para*lichthys olivaceus* ER $\beta$  (JfER $\beta$ , BAB85623.1), Sebastes

 Table 1
 Primer sequences for cloning and mRNA expression analysis

Primer	Nucleotide sequence $(5' \text{ to } 3')$	Usage	
$ER\beta F1$	AGCGGTCCATCCAGGG(A/C/G/T)CA(C/T)AA(C/T)GA	Degenerate primer	
$ER\beta R1$	GCCAGATCAGGCCGATCAT(A/C/G/T)A(A/G)(A/C/G/T)AC	ERaR1	
ER-C-5-1	AGCAGGAACTGAGGATGGGGGGAGAGC	5'-RACE primer	
ER-C-5-2	GGGGCGGTGGTCATCGTTACTACAGG	Nested 5'-RACE primer	
ER-C-3-1	ACTGACCCGTCTGTCCACGCAGAGCA	3'-RACE primer	
ER-C-3-2	GTGGTATGAGGAAGGAACACGGAAGC	Nested 3'-RACE primer	
ER-e-f	CTGCGAGGTTGGAATGAC	Expression primer	
ER-e-r	ACCCTGGGATCTTCTTGG	Expression primer	
18SF	CCTGAGAAACGGCTACCACATC	Control primer	
18SR	CCAATTACAGGGCCTCGAAAG	Control primer	

schlegelii ER $\beta$  (brfER $\beta$ 1, ACN38898.3; brfER $\beta$ 2, ADR73047.1), Sparus aurata ER $\beta$  (sbER $\beta$ 1, CAD 33851.1; sbER $\beta$ 2, CAE30470), Oreochromis aureus ER $\beta$  (tER $\beta$ 1, ACF75102.1; tER $\beta$ 2, ACF75103.1), Carassius auratus ER $\beta$  (gfER $\beta$ 1, AAD26921.1; gfER  $\beta$ 2, AAF35170.1), Micropogonias undulates ER $\beta$  (acER $\beta$ 1, AAG16711.1; acER $\beta$ 2 AAG16712.1), C. semilaevis ER $\beta$  (hstsER $\beta$ ).

# **RT-PCR** analysis

Semi-quantitative RT-PCR assays were performed to evaluate the level of  $ER\beta$ -mRNA expression in different tissues, including liver, spleen, head kidney, kidney, brain, intestine, gill, heart, stomach, testis, muscle of male adult half-smooth tongue sole. Total RNA of those organs were extracted and reversetranscribed as described above. RNA was used for studying the temporal expression pattern of  $ER\beta$ during the annual reproductive cycle, total RNA of whose gonads were also prepared for analysis. Specific primers developed based on the sequences generated were listed in Table 1 (ER-e-f and ER-er). PCR amplification of 18S ribosomal RNA was applied to ascertain reverse transcription efficiency and as internal control to normalize the concentration of templates for semi-quantitative RT-PCR. The expression of housekeeping gene (18S) did not fluctuate throughout the reproductive cycle.

Annealing temperatures and cycle number were optimized as follows: 5 min denaturing step at 94  $^{\circ}$ C, 22 cycles of 35 s at 94  $^{\circ}$ C, 30 s at 57  $^{\circ}$ C and 35 s at 72  $^{\circ}$ C, followed by final 10 min elongation period at 72  $^{\circ}$ C. Then, the PCR products were electrophoresed in 1.5 % agarose gel and visualized using ethidium bromide staining. Electrophoretic images and the optical densities of amplified bands were analyzed using the software of Gel Image System Ver3.60 (Tanon, China).

# Steroid radioimmunoassay

Serum levels of estradiol-17  $\beta$  in male were measured using Iodine [<sup>125</sup>I] Radioimmunoassay Kits (Tianjin Nine Tripods Medical and Bioengineering Co., Ltd., Sino-US joint-venture enterprise), according to the manufacturer's protocol. The binding rate is highly specific with an extremely low cross-reactivity to other naturally occurring steroids, which was less than 0.1 % to most circulating steroids. The coefficients of intra-assay and inter-assay variations were 7.4–9.8 %. Any samples with coefficient of variation higher than 10 % were not included in the analyses. The assay sensitivity reached to 2.1 pg/ml for  $E_2$  by the kit protocol, respectively.

#### Statistics

The relevant values in this study were analyzed through ANOVA followed by Duncan's multiple range tests. Statistical significance was considered as P < 0.05.

# Results

Isolation and characterization of ER $\beta$  cDNA

Phylogenetic analysis implicated that this hstsER belonged to ER $\beta$  subtype, closely related to *S. solea* ER $\beta$  and *P. olivaceus* ER $\beta$  (Fig. 2). The full-length cDNA of half-smooth tongue sole ER $\beta$ (GenBank accession number ACN39246.2)consisted of 1,791 bp, including an open reading frame (ORF) that is predicted to encode a protein of 578 amino acid residues.

The comparison result of deduced amino acid sequences between hstsER $\beta$  and other similar ER $\beta$ , such as  $csER\beta$ ,  $JfER\beta$ ,  $KrER\beta$ ,  $gsER\beta$ ,  $hmER\beta$  and hER $\beta$  (Fig. 3), showed that the hstsER $\beta$  exhibited high identity with other teleosts. The hstsER $\beta$  was also divided into six nuclear receptor-characteristic domains: variable A/B domain at N terminal, highly conserved C domain (DNA-binding domain, DBD), E domain (ligand-binding domain, LBD) as well as the hinge region D domain between the DBD and LBD and F domain at C terminal. The A/B domain possesses a mitogen-activated protein kinase phosphorylation site which was considered to make up for ligand-independent transactivation function motif (AF-1). In the C domain, eight cysteine residues of the two zinc-finger motifs, as well as the D-box (EGCKAFF) and P-box (PATNQ) were completely conserved. In the E domain, a protein kinase C phosphorylation site, a tyrosine kinase phosphorylation site and a ligand-depended transactivation function motif (AF-2) were also presented (Figs. 1, 2).

As it is shown in Table 2, identities compared hstsER $\beta$ -deduced amino acid sequence with other teleosts' ER $\beta$  including csER $\beta$ , JfER $\beta$ , KrER $\beta$ , rtER $\beta$ 1, rtER $\beta$ 2, ccER $\beta$ 1, ccER $\beta$ 2, zbER $\beta$ 1, zbER $\beta$ 2, gsER $\beta$ 2,

$ \begin{array}{c} \textbf{M} \ T \ T \ A \ P \ L \ E \ K \ E \ Q \ P \ L \ L \ Q \ L \ Q \ E \ V \ G \ S \ S \ R \ V \ R \ G \ 25 \\ \hline 121 \\ ATGCATGCTTGCCCCATCCTCAGTTGCTCCTCGTCCTCTCTCGGGGAGGACCTTGGACCCAGCATCCCATCTGCATCGCCCCTCGCCCCTGGGCCAGGACTTGCACCAGACTTGCACCCAGCACTTGCCCCCCCC$	1	TGATGATGTAGGAGCTGCGTCGACACCCAGGCTGCCTGTAGTAACGATGACCACCGCCCCTCTGGAGAAGGAGCAGCCCCCTCTTCAGCTGCAGGAGGTGGGCTCCAGCCGCGTTAGAGG	20
$ \begin{array}{c} 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 $	1	M T T A P L E K E Q P L L Q L Q E V G S S R V R G 2	25
$\begin{array}{c} 241 \\$	121	ATGCATGCTCTCCCCCATCCTCAGTTCCTGCTCCTCCTCCTCCCGGGGATGACCTTGGACCCCAGCCATCCCATCTGCATCCCCTTACACAGATCTGGGCCACGACTTCAC	10
A S L P F Y S P T I F T Y P S P S V V D G S S G R Q S L S P S V F W A G H G R V 105 GGGCTCAACCGTTCCCCTGCATCACCCCACAGGGTGGACTCGCCCGACCTGCCACAGGACGTGGGTGAGTAACCCCACGGGAGAGGGGGGGG	41	CMLSPILSSCSSSSPGMTLDPSHPICIPSPYTDLGHDFT68	5
$\begin{array}{rcccccccccccccccccccccccccccccccccccc$	241	CGCCAGCCTTCCCTTCTACAGCCCCACCATCTTCACCTACCCCAGTCCCAGCGTCGTCGACGGCTCCTCTGGGCGTCAGTCCCTCAGCCCGTCCGT	50
121GSTVPQGRPQGRPQRTLQRTVVELTPRESVLSSS<	81	A S L P F Y S P T I F T Y P S P S V V D G S S G R Q S L S P S V F W A G H G R V 10	)5
481GAGAGGCTCCAGGAGAAGGAGGAGGGGGTGCATGTGAGCGGAGAAGAGGAGCAGCGATCATCATTCTGCGCGCGTGTGCAGCAGGGTTTTGACTACGGGCTGGTGCTCCGGG600161RRSQEKEGVVSCDRKTDHHFCAVCHYGVNSCIIIS5601AGGATGTAAGGCGTTCTTCTAAGGAGGGACCAGACAACAGGAACACGAATACATCTGTCGGGCCACATCATCGTGCGCACTACGGACAAAAAATCGCAGGGGGGGG	361	GEGETCAACCGTTCCGCTGCATCACCCACAGGGTCGACCCCAGCATGCTCCGACCCTGCAGAGGACGTGGGTGG	30
161R R S Q E K E E G V V S C D R K T D H H F C A V C H D F A S G Y H Y G V W S C 1185601AGGATETAAGGCCTTETTCAAGAGGAGCATCCAGGGGACAAAGGACTACATCTGTCCGGGCCACAATCAGTCCATATCGACAAAATCGCCTAAGAGCGCGGTGTCCCCTCTG720201 $\mathbf{C}$ K A F F K R S I Q G H N D Y I $\mathbf{D}$ P A T N Q C T I D K N R R K S C Q A C R L R 225721AAAGTCTGCGAGGTTGGAATGACCAAGTGTGGTATGAGGAAGGA	121	G S T V P L H H P Q G R P Q H A P T L Q R T W V E L T P R E S V L S S S K S T R 14	15
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	481	GAGACGCTCCCAGGAGAAGGAGGAGGGCGTGGTGTCATGTGACCGGAAGACGGATCATCACTTCTGCGCCGTGTGTCACGACTTTGCCTCGGGTTATCACTACGGCGTGTGGTCCTGCGA 60	00
201 <b>C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R R K S C Q A C R L R</b> 225 721 AAAGTGCTGCGAGGTTGGAATGACCAAGTGTGGTATGAGGAAGGA	161	R R S Q E K E E G V V S C D R K T D H H F <mark>C A V C H D F A S G Y H Y G V W S C E</mark> 18	35
721AAAGTGCTGCGAGGTTGGAATGACCAAGTGTGGTATGAGGAAGAACACGGAAGCTACCGGAGCCCACAGTGAGCGAGGGGCGACTGACCCGGAGGGCAAACTCAACGGAACCAAA840241K C C E V G M T K C G M R K E H G S Y R T P K S R R L T R L S T Q S K L N G P K 265841GGCGTCAGCGACGCGGAGGGGGGGGGGGGCGCGCGCGCGGGGGGG	601	AGGATGTAAGGCCTTCTTCAAGAGGAGCATCCAGGGACACAACGACTACATCTGTCCGGCCACCAATCAGTGCACTATCGACAAAAATCGCCGTAAGAGCTGCCAGGCGTGTCGCCTTCG 72	20
241KCCKCGMRKEHGSYRTPKSRLTRLSTQSKLNGPK265841GGCGTCAGCGGCACGCGCAGCGGCAGGGGGGGGGGGGGG	201	G C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R R K S C Q A C R L R 22	25
841GGCGTCAGCTGCAGCGGAGAGTTTGCTCAAGGAGCCGCAGCTCCCGGGGGGGG	721	AAAGTGCTGCCAAGGTTGGAATGACCAAGTGTGGTATGAGGAAGGA	10
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	241	K C C E V G M T K C G M R K E H G S Y R T P K S R R L T R L S T Q S K L N G P K 26	55
961CATGAGCGGCCCATGACGGAGGCCACCGTCATGATGTCACTACCTAC	841	GGCGTCAGCTGCACCAGCGGAGAGTTTGCTCAAGGAGCCGCAGCTCCCGGTGCTGACACCGGAGGCGCTGATCGCGAGGATCATGGAGGCGGAGCCGCCCGACATCTACCTCATGAGGGA	50
321       M S G P M T E A T V M M S L T N L A D K E L V H M I T W A K K I P G F V D L N L 345         1081       CCTGGACCAGGTGCACCTGCTGGAGGTGCTGCTGGAGGTGCTGATGAGGGGGGGG	281	A S A A P A E S L L K E P Q L P V L T P E A L I A R I M E A E P P D I Y L M R D 30	)5
1081 CCTGGACCAGGTGCACCTGCTGGAGTGCTGCTGGAGGTGCTGAAGGTGCTGATGAGGGGCTGATGTGGCGGTCAGTGGACCATCCTGGGAAAACTCATCTTCTCCCCTGACCTCAGGCCTGAGCAGGAGGAGGAGTACGTGGTGCTGAGGGGGGAGGTGCGAGGAGGGGGGGG	961	CATGAGCGGGCCCATGACGGAGGCCACCGTCATGATGTCACTCAC	30
361 L D Q V H L L E C C W L E V L M M G L M W R S V D H P G K L I F S P D L S L S R 385 1201 AGAAGAGGGGAGCTGCTCGTGGAGATCTATGACATGCTGATAGCTGCCAGTGCGAGAGGAGGTGGAGAGGGGGAGGAGGAGGAGGAGGAGGAG	321	M S G P M T E A T V M M S L T N L A D K E L V H M I T W A K K I P G F V D L N L 34	15
1201AGAAGAGGGGAGCTGCGTCCAGGGCTTCGTGGAGATCTATGACATGCTGATAGCTGCCACGTCCAGGGTGAGAGAGCTGCAGGAGAGGAGGAGGAGGAGGAGGAGGAGCATGAT1320401 $E E G S C V Q G F V E I Y D M L I A A T S R V R E L K L Q R E E Y V C L K A M I 4251321CCTGCTCAACTCCAACATGTGCCTGAGCTCCTCAGAGGGGGAGGAGCTACAGAGTCGTTCCAGGCTGCTGCGTCTCCTGGACGCCATGACCGACGCTCTGGTGGGGCCATGCCCCAA441L L N S N M C L S S S D G G E E L Q S R S R L L R L L D A M T D A L V W A I A K 4651441GAGCGGCCTGTCGTTCCGTCAGCAGTACACCCGCCTCCCTC$	1081	CCTGGACCAGGTGCACCTGCTGGAGTGCTGCTGGCTGGAGGTGCTGATGATGGGGGCTGATGTGGGCGGTCAGTGGACCATCCTGGGAAACTCATCTTCTCCCCTGACCTCAGCCTGAGCGCGGAGTGCTGGGCGGTCAGTGGGCCGGTCAGTGGACCATCCTGGGAAACTCATCTTCTCCCCTGACCTCAGCCGGAGGGCGGCGGCGGCGGCGGTCAGTGGGCGGTCAGTGGACCATCCTGGGAAACTCATCTTCTCCCCTGACCTCAGCCGGAGGGCGGCGGCGGCGGCGGCGGTCAGTGGGCGGTCAGTGGACCATCCTGGGAAACTCATCTTCTCCCCTGACCTCAGCCGGCGGCGGCGGCGGCGGTCAGTGGGCGGTCAGTGGACCATCCTGGGAAACTCATCTTCTCCCCTGACCTCAGCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG	)()
401 E E G S C V Q G F V E I Y D M L I A A T S R V R E L K L Q R E E Y V C L K A M I 425 1321 CCTGCTCAACTCCAACATGTGCCTGAGCGGGGGGGGGGG	361	L D Q V H L L E C C W L E V L M M G L M W R S V D H P G K L I F S P D L S L S R 38	35
1321CCTGCTCAACTCCAACATGTGCCTGAGGCTGCTGAGGGGGGGG	1201	AGAAGAGGGGAGCTGCGTCCAGGGCTTCGTGGAGATCTATGACATGCTGATAGCTGCCACGTCCAGGGTGAGAGAGCAGCTGCAGAGAGAG	20
441       L       L       N       S       N       M       C       L       S       S       D       G       G       E       E       L       Q       S       R       S       R       L       L       L       L       L       L       L       N       M       T       D       A       L       V       W       A       I       A       K       465         1441       GAGCGGCCTGTCGTCCGTCAGCAGCAGCACCCCCCCCCC	401	EEGSCVQGFVEIYDMLIAATSRVRELKLQREEYVCLKAMI42	25
1441       GAGCGGCCTGTCGTCGTCGCAGCAGTACACCCGCCTCGCTCACCTGCTGCTGCTGCTGCTGCGACATCCGACATGCAGCAACAAAGGCATGGACCACCTTCACTGCATGAAGAA 1560         481       S       G       L       S       F       R       Q       Y       T       R       L       L       L       S       H       I       H       V       S       N       K       G       M       D       H       L       H       L       S       S       S       S       G       M       N       K       M       K       N       505         1561       CATGGTGCCTCTGTATGACCTGCTGGGGAGGTGCTGGGACGCTCACATCATGCACGCTCCCGGTCGGGCGCGCGC	1321	CCTGCTCAACTCCAACATGTGCCTGAGCTCCTCAGACGGAGGGGGGGG	10
481       S G L S F R Q Q Y T R L A H L L M L L S H I R H V S N K G M D H L H C M K M K N 505         1561       CATGGTGCCTCTGTATGACCTGCTGGAGATGCTGGACGCTCACATCATGCACAGCTCCCGCTCGGGCCGCGCGCG	441	LLNSNMCLSSSDGGEELQSRSRLLRLLDAMTDALVWAIAK46	55
1561       CATGGTGCCTCTGTATGACCTGCTGGAGATGCTGGACGCTCACATCATGCACAGCTCCCGTCGGGCCGCCGTGCTGCTCCCCTCCCCCCCC	1441	GAGCGGCCTGTCGTCAGCAGCAGCACCACCCGCCTCGCTCACCTGCTCATGCTGCTGCCGCACATCCGACATGTCAGCAAAAGGCATGGACCACCTTCACTGCATGAAAAAAA 156	50
521       M V P L Y D L L L E M L D A H I M H S S R L G R R A A P S P H P P S R A C D G Q 545         1681       GGGTGTCACGGACCAGAAGGAGGACGTACTCGCAGACTCTGGGAAGGAGCTCCTCACACACA	481	SGLSFRQQYTRLAHLLMLLSHIRHVSNKGMDHLHCMKMKN50	)5
1681 GGGTGTCACGGACCAGAAGGAGACGTACTCGCAGTCTGCAGACTCTGGGAAGAGCTCCTCACACACA	1561	CATGGTGCCTCTGTATGACCTGCTGCTGGAGATGCTGGACGCTCACATCATGCACAGCTCCCGTCTGGGCCGCCGTGCTGCTCCCCTCCCCCCCC	30
	521	M V P L Y D L L E M L D A H I M H S S R L G R R A A P S P H P P S R A C D G Q 54	15
561 GVTDQKETYSQSADSGKSSSHTWTPGSPRVDGH* 578	1681	GGGTGTCACGGACCAGAAGGAGACGTACTCGCAGTCTGCAGACTCTGGGAAGAGCTCCTCACACACA	0
	561	G V T D Q K E T Y S Q S A D S G K S S S H T W T P G S P R V D G H *	18

Fig. 1 Nucleotide and deduced amino acid sequence of  $hstsER\beta$ . Two zinc-finger motifs in DNA-binding domain were identified by gray open boxes, and eight cysteines in the same domain were also *underlined*. The initiation codon and termination codon were boxed

hER $\beta$ , hmER $\beta$  were 76, 76, 72, 64, 56, 64, 63, 64, 63 %, respectively, and lower identities (46, 49 %) were found with hER $\beta$  and cER $\beta$ . However, the identities between hstsER $\beta$  and gsER $\beta$  are low which both are teleosts. The highest identity in the C domain and E domain, suggested that both ERs had the similar function, but derived from distinct ancestral genes.

# Phylogenetic analysis

The phylogenetic analysis, based on deduced amino acids, shows that the ERs are divided into two groups in the phylogenetic tree (Fig. 2), the ER $\beta$ 1 group and ER $\beta$ 2 group. The sequence homology implicates that our hstsER is apparently belonging to ER $\beta$ 1 cluster. Furthermore, hstsER $\beta$  was more closely related to *Solea* solea ER $\beta$  and *Paralichthys olivaceus* ER $\beta$  (Fig. 3).

# Expression of $\text{ER}\beta$ in different adult organs

The tissue expression pattern of the hstsER $\beta$  was analyzed by RT-PCR (Figs. 3, 4). It is revealed that the ER $\beta$ -mRNA was detected in all the organs, and abundant in liver, gill and kidney, and, at lowest levels, in the head kidney. Primers ER $\beta$ F and ER $\beta$ R were listed in Table 1. Primers 18SF and 18SR were used to normalize the PCR products of ER $\beta$ -mRNA to obtain semi-quantitative results.

 $ER\beta$ -mRNA expression in gonad during the male reproductive cycle

According to Chen et al., we divided the testis into four stages HE staining (Chen and Wen 2012). The four stages are as following: stage of spermatogonia (February to

	MAPK (AF-1)						
tsER β	-MTTAPL-EKE-QPLLQLQEVGSSRVRGCMLSPILSSCSSSSSPGMTLDPSHPICIPSPYTDLGHDFTASLPFYSPTIFTYPSPS						
CSER β	-MAAISA-EKE-QPLLHRQEVDSSRVR-SCVLSPILGSSSPALSIDTGQPICIPSPYTDLGHDFPTIPFYSPTIYSYASPG						
JfER B1	-MAAASA-EKD-QPLLQLQEVDSSRVR-SCVLSPILSTSSPGLSLDGSQPICIPSPYTELGHDFATIPFYGPTIFSYAAPS						
KrER β	MAVASPP-EKD-QPLLQLHEVDSSRVG-SRVLTSSSPALSMETSQPICIPSPYTDLGHEFTTIPFVSPTIFTYAGPG						
gser β2	-MAASPELDSRSLLQLQEVDSSKPSERPSSPRQLPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQPPAAYSPPLGMDS-HTVCIPSPYADSSHEYNHGHGPLNFYSQSVLSYARQP						
hmer β	-MSICASSHKDFSQLRPTQDMEIKNSPSSLTSPASYNCSQSILPLEH-GPIYIPSSYVESRHEYSAMTFYSPAVMNYSVPSSTGN						
hser β	MNYSIPSNVTN						
	A/B -						
tsER β	VVDGSS-GRQSLSPSVFWAGHGRVGSTVPLHH-PQGRPQHAPTLQRTWVELTPRESVLSSSKSTRRRSQEKEEGVVSCDRKTDH						
CSER β	IPDCPS-VHQSLSPSLFWPGHGHMGPTVPLHR-SQARPQHSQPIQSQWVELTPRDSVLMSSKSVRRRSQESDEGAVSSAVKADH						
JfER B1	IPDCPS-VHQSLSPSLFWPSHGHMGPPMTLHR-SQGRSQQGQPIQSPWGELTPRDGVLANSKGVRRRSQESEDGVVSSGGKSDL						
KrER β	ISDCPS-VHQSLNPSLFWPSHGHVGPSIPLHP-SQARPQHGQPIQSPWMELSQRDSVLATSKNVRRRSQESEEAVVSSGGKADL						
gser β2	VTDSPSYLCPSISPSAFWPSHNHPSMPSLTLQCPQPHVYNEPSPHAPWLEPKAHAVTTSSAVISCNKLPGKRSDERGEGANSSSCSSAVEKADM						
hmer B	LEGGPVRQTASPNVLWPTSGHLS-PLATHCQSSLLYAEPQKSPWCEARSLEHTLPVNRETLKRKLGGSGCASPVTSPSAKRDA						
hser β	LEGGPGRQTTSPNVLWPTPGHLS-PLAIHCQPSVLYAEPQKSPWRETRSLEHTLPVNRETLKRKVSGNRCASPVTGPGSKRDA						
	$\begin{array}{cccccccccccccccccccccccccccccccccccc$						
tsER β	HFCAVCHDFASGYHYGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCCEVGMTKCGMRKEHGSYRTPKSRRLTRLS						
CSER B	HYCAVCHDYASGYHYGVWSCEGCKAFFKRS1QGHNDY1CPATV4CT1DKNRRKSCQACRLRKCCEVGMTKCGMRKEHGSYRNPKTRRLTRLS						
JfER B1	HYCAVCHDYASGYHYGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMTKCGMRKDHGSYRNPKTRRLTRLS						
KrER β	HYCAVCHDYASGYHYGVWSCEGCKAFFKRSIQGHNDYICPAINQCTIDKNRRKSCQACRLRKCCEVGMTKCGMRKERGNY-RNPQARRVTRLS						
gser \$2	HFCAVCHDYASGYHYGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMMKCGVRRERCSYRGARHRRGGLQA						
hmer β	HFCAVCSDYASGYHYGVWSCEGCKAFFKRS1QGHNDY1CPATNQCT1DKNRRKSCQACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLRKCYEVGMVKCGSRRERCGYRIVRQRSASEQCACRLFACACRL						
hser β	HFCAVCSDYASGYHYGVWSCEGCKAFFKRS1QGHNDY1CPATYQCT1DKNRRKSCQACRLRKCYEVGMVKCGSRRERCGYRLVRRQRSADEQ						
	*:**** *:******************************						
tsER β							
CSER B	TQSKLNGPKASA-APAESLLKEPQLP-VLTPEALIARIMEAEPPDIYLMRDMSGPMTEATVMMSLTNLADKELVHMITW TQGRVNGPKVLT-GPAAGLTSEPQPPAALTPEQLIERIMEAEPPDIYLMKDTSGPLTEANVMMSLTNLADKELVHMISW						
JfER B1	SQGRASGPKALT-GPVALMNELQPP-ALTPEQLIERIMEAEPPDITLMKD/SGPLTEANVMMSLTNLADKELVMMITW						
KrER B	SQGRANGPKALT-RPAEGSFNAPNPP-ALTPEQLIGRIMEAEPPEIYLMNDMRRPLTEANVMMSLTNLADKELVHMISW						
gser \$2	RDPTGRGLVRVGLGSRGQRHLHLEAPLTPLPQAKRVHHSAMSPEEFISRIMEAEPPEIYLMEDMNKPFTESSMMMSLTNLADKELVLMISW						
hmer β	VHCLNKAKRTSGHTPRVKELLLNSLSPEQLVLTLLEAEPPNVLVSRPS-MPFTEASMMMSLTKLADKELVHMIGW						
hser β	LHCAGKAKRSGGHAPRVRELLLDALSPEQLVLTLLEAEPPHVLISRPS-APFTEASMMMSLTKLADKELVHMISW						
	: .* :: :: **** : : . :**:.:* ****:********						
	CK-2 PKC						
tsER β csER β	AKKIPGFVDLNLLDQVHLLECCWLEVLMMGLMWRSVDHPGKLIFSPDLSLSREEGSCVQGFVEIYDMLIAATSRVRELKLQREEYVCLKAMILL AKKIPGFVELSLLDQVHLLECCWLEVLMMGLMWRSVDHPGKLIFSPDLSLSREEGNCVQGFVEIFDMLIAATSRVRELKLQREEYVCLKAMILL						
JfER B1	ARATPOPVELSILDQVHLLECCWLEVLMMGLMWRSVDHPGKLTFSPDLSLSREEGSCVQGFSETFDMLTAATSRVRELKLQREETVCLKAMTLL AKKTPGFVELGLLDQVHLLECCWLEVLMMGLMWRSVDHPGKLTFSPDLSLSREEGSCVQGFSETFDMLTAATSRVRELKLQREEYVCLKAMTLL						
KrER B	AKKIPGFIELGLLDQVHLLECCWLEVLMIGLWRSVDHPGKLIFSPDLSLSREEGNCVQGFSEIFDMLIAATSRVRELKLQREEYVCLKAMILL						
gser \$2	AKKIPGFVELSLADQIHLLKCCWLEILMLGLMWRSVDHPGKLIFSPDFKLNREEGQCVEGIMEIFDMLLAATSRFRELKLQREEYVCLKAMILL						
hmer B	AKKIPGFVELSLLDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTARFRELKLQHKEYLCVKAMILL						
hser β	AKKIPGFVELSLFDQVRLLESCWMEVLMMGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILL						
	*******::* * **::**:.**:**:**:**::***::***:**:**:**:*						
tsER β	CK-2 PKC AF-2 E NSNMCLSSSDGGEELQSRSRLLRLLDAMTDALVWAIAKSGLSFRQQYTRLAHLLMLLSHIRHVSNKGMDHLHCMKMKNNVPLVDLLLEMLDAHI						
CSER B	NSNWCLSSSF00EEQSSSRLRLRLDAWTAALWATAAS0CSPRQTTRLAHLLMLLSHTPHVSNK0MDHLRCMKMKNWVFET0LLEWLDAHT NSNWCLSSADGSEELQSRSKLLHLLDAVTDALVWATAKTGLTFRQQYTRLAHLLMLLSHTPHVSNK0MDHLRCMKMKNWVFLYDLLEWLDAHT						
JfER β1	NSNICLSSRIDSEEUSSISKLLILLDAVTDALVWATAKTOLTFRQYTTLAHLLILLSHTIFTYS NKONDELIKMISIKKOVYT ETPELLEMEDATT NSNICLSSSEGSEELHSRSKLLLLDAVTDALVWATAKTGLTFRQYTTLAHLLMLLSHTIFTYSNKGMDHLHKMKNKVPLYDLLEMEDATT						
KrER β	NSNMCLSSSEGSEELQSRSKLLRLLDAVTDALVWATAKTGLTFRQQYTRLAHLLMLLSYTRHASNKGMDHLHGMKMKNMVPLYDLLLEMLDAHT						
gser <sup>β2</sup>	NSYLCTNSPETAEELESRNKLLRLLDSVIDALVWAISKLGLTTQQQTLRLGHLTMLLSHIRHVSNKGMDHLSTMKRKNVVLVYDLLLEMLDANT						
hmerβ	NSSMYPLAT-ASQEAESSRKLTHLLNAVTDALVWVISKSGISSQQQSVRLANLLMLLSHVRHISNKGMEHLLSMKCKNVVPVYDLLLEMLNAHT						
hser <b>b</b>	NSSMYPLVT-ATQDADSSRKLAHLLNAVTDALVWVTAKSGTSSQQQSMRLANLLMLLSHVRHASNKGMEHLLNMKCKNVVPVyDLLLEMLNAHV						
	** : . :: .* :* :*::: *****.*:* *:: :*: **.:* *****: * ***** * ** *::* :*******:*:						
torn 0	F MHSSRLGRRAAPS PHPPSRACDGQGVTDQKETYSQSADSGKSSSHTWTPGSPRVDGH						
tsER β csER β	MISSRLPRCATASTTTTTTTTAPPPPPPPHHHQQEFTEQRATATRPPTCGKSSPNTWTP-GSNRDAGEAQ						
JfER β1	MISSRER REALASTITITITIAL TITITITIAL MURANI AND THE AND						
KrER β	MHGSRLPHRPPQQEPGDQTVVPAQPHSSDSGPSNTWTP-SSTGDGGEPQ						
gser \$2	TTSGSQASSSPTSETFPDQHQYPQAPSHLQPGSDQAAADHTAVPPRGPAEAP1LDGHLQALTLQSSPHFQSLEMTHMDSNQY1HPEQWSLETRD						
hmer B	LRGYKSSISGSECCSTEDSKSKEGSQNLQSQ						
hser β	LRGCKSS1TGSECSPAEDSKSKEGSQNPQSQ						
	Description of the second seco						
tsER <b>B</b>							
CSER β							
JfER <b>β</b> 1							
KrER β							
gser <sup>β2</sup>	AALSVDGSVDYMSPDPTVMDTDLVNGL						
hmer <b>b</b>							
hser <b>b</b>							

Fig. 2 Amino acid alignment of hstsER $\beta$  with  $ccER\beta$ , JfER $\beta$ , KrER $\beta$ , gsER $\beta$ , hER $\beta$  and hsER $\beta$ (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 [DNAbinding domain], D, E [ligand-binding domain] and F) and the P- and D-boxes in C domain were indicated. The activation domains (AF-1 and AF-2) in the A/B and D domain as well as potential phosphorylation sites for MAPK, PKA, PKC, CK-2 and PTK were indicated with open gray boxes, respectively. MAPK mitogen-activated protein kinase, PKA protein kinase A, PKC protein kinase C, CK-2 casein kinase II, PTK protein tyrosine kinase

Species	hstsER $\beta$	A/B domain	C domain	D domain	E domain	F domain
csERβ	76 (589/578)	63 (159/163)	95 (84/85)	60 (38/37)	92 (238/293)	30 (70/55)
$JfER\beta 1$	76 (565/578)	60 (159/163)	93 (84/85)	54 (37/37)	92 (238/293)	35 (47/55)
$KrER\beta 2$	72 (561/578)	55 (156/163)	92 (84/85)	54 (37/37)	89 (238/293)	26 (46/55)
rtERβ1	64 (594/578)	41 (177/163)	89 (85/85)	38 (37/37)	88 (238/293)	21 (57/55)
rtER $\beta 2$	56 (604/578)	34 (175/163)	87 (85/85)	29 (42/37)	78 (240/293)	20 (62/55)
$ccER\beta 1$	64 (559/578)	49 (156/163)	84 (83/85)	20 (44/37)	86 (238/293)	18 (38/55)
$ccER\beta 2$	63 (544/578)	48 (156/163)	82 (87/85)	22 (40/37)	87 (238/293)	22 (23/55)
zbERβ2	64 (553/578)	48 (155/163)	80 (87/85)	23 (39/37)	86 (238/293)	20 (33/55)
zbERβ1	63 (553/578)	44 (155/163)	80 (87/85)	23 (40/37)	85 (238/293)	20 (33/55)
gsERβ2	49 (668/578)	32 (176/163)	87 (84/85)	16 (51/37)	74 (238/293)	8 (119/55)
$hER\beta$	46 (477/578)	13 (93/163)	84 (84/85)	13 (33/37)	66 (236/293)	13 (29/55)
hmER $\beta$	49 (549/578)	26 (165/163)	85 (84/85)	24 (35/37)	66 (236/293)	7 (29/55)

**Table 2** Amino acid identities between hstsER $\beta$  and ERs in fish and mammals

See "Materials and methods" for sequence references

The total score of amino acids and the number of residues per domain are marked in brackets



Fig. 3 Phylogenetic tree based on amino acid sequences for  $\text{ER}\beta$ s in teleosts. Bootstrap values are indicated (1,000 replicates) (see "Materials and methods" for sequence references and abbreviations)

October, stage III); immature sperm (May to July, stage IV); mature testes (August to October, stage V); testes after spermiation (November to January, stage VI). The

temporal expression profiles of hstsER $\beta$  in testis, spleen, brain and kidney during the reproductive cycle were shown in Fig. 5. It indicated that hstsER $\beta$  was expressed



**Fig. 4** RT-PCR expression analysis of  $hstsER\beta$  in organs of adult male half-smooth tongue sole. Various tissue-specific expressions of the hstsER $\beta$  gene were determined by RT-PCR; 18S ribosomal RNA was used as an internal control for relative

throughout the reproductive cycle of these organs in male. The expression levels of hstsER $\beta$  in testis and liver were decreased from February to October (stage III), then increased gently from August to October (stage V) (P < 0.05). In the stages of February to April (stage III), the expression level was the highest, and in the August to October (stage V), the expression level was the lowest. The expression level of hstsER $\beta$  in the brain was gently increased from February to July, decreased from July and sustained at the same level (P < 0.05).

Serum steroid hormone level of  $E_2$  during the reproductive cycle

Levels of serum  $E_2$  over the testis development cycle were detected (Fig. 6). The average level of serum  $E_2$  in male black rockfish between February and April was  $5.00 \pm 1.50 \text{ pg ml}^{-1}$ , then remarkably elevated to  $21.97 \pm 2.11 \text{ pg ml}^{-1}$  between May and July, and then reached to  $25.94 \pm 2.13 \text{ pg ml}^{-1}$  between August and October, finally fell to  $10.93 \pm 1.62 \text{ pg ml}^{-1}$  between November and January.

#### Discussions

In this study, we cloned and characterized the  $\text{ER}\beta$  from half-smooth tongue sole, *C. semilaevis*. The

quantity of cDNA (*lower panel*). *H* heart, *L* liver, *S* spleen, *He* head kidney, *C* caeca, *T* testis, *St* stomach, *K* kidney gill, *G* gill, *B* brain, *I* intestine, *M* muscle

hstsER $\beta$  possessed common domains (domains A–F) typical for ERs with other ER $\beta$ s, the highly conserved zinc-finger motif, including the P- and D-boxes and the eight cysteine residues, being indispensable for DNA binding (Schwabe et al. 1993). The N-terminal region (domain A/B) was variable (Table 2; Fig. 2) in hstsER $\beta$ ; it had a cell-type and promoter-specific transactivation function (AF-1) (Tora et al. 1989, Tzukerman et al. 1994). In these listed species, a potential MAPK phosphorylation site was found in this domain; it is suggested that  $ER\beta$ s transcriptional activity may activated by MAPK pathway in ligandindependent manner (Lannigan 2003). The C domain (DNA-binding domain, DBD) was highly conserved among species (Halm et al. 2004; Xia et al. 1999), and it was considered to responsible for DNA binding (Kumar et al. 1987). Furthermore, there were highly conserved potential phosphorylation sites for PKC, CK-2 and PTK in this domain and the important role of them required further testing. The two sequence motifs: EGCKAF and CPATNQC, which were supposed to essential for species binding to estrogen response element (ERE) on target genes and receptor dimerization, respectively, were also conserved in both the teleostean and the tetrapods. The E domain (or ligand-binding domain, LBD) which was in the C-terminal domain, was required for ligand binding (Kumar et al. 1987) and included a ligand-dependent Fig. 5 The mRNA expression of  $ER\beta$  in testis (a), brain (b) liver (c) in half-smooth tongue sole during the male annual reproductive cycle. Values are expressed as mean  $\pm$  SE of mean. *Different letters* indicate significant difference (P < 0.05, oneway ANOVA, followed by Duncan's test)



transactivation function (AF-2) (Danielian et al. 1992). We also located a PKC phosphorylation site that was completely conserved in this domain in all fish ER $\beta$  but not the tetrapods; it may illustrate the different function of PKC site in fish and mammals. Lastly, identity scores of F domain in these species were lowest, and Montano et al. (1995) thought that the function of the F domain was proposed to play a modulatory role in affecting agonist/antagonist effectiveness of anti-estrogens and the transcriptional activity of the ligand–receptor complex in cells.

Analysis based on amino acids sequence identity revealed that the isolated cDNA, hstsER $\beta$ , was closely related to the ER $\beta$  subtype (Table 2) in teleost rather than to in the tetrapods. The hstsER $\beta$  showed a high degree of conservation in the DNA-binding (95– 80 %) and ligand-binding domains (92–85 %) revealed the conservation of these domains. The high degree of homology in DNA-binding domain between hstsER $\beta$  and others may indicate that all ERs bind to the same type of ERE (Ma et al. 2000). In contrast to the DBD and LBD, the A/B, D and F domains of



**Fig. 6** Serum 17b-Estradiol (E<sub>2</sub>) and Gonadosomatic (GSI) levels in male half-smooth tongue sole. Values are expressed as mean  $\pm$  SE of mean. *Different letters* indicate significant difference (P < 0.05, one-way ANOVA, followed by Duncan's multiple test)

hstsER $\beta$  were poorly conserved in aa sequence compared with other ER $\beta$  of fish and of tetrapods. The identity range of A/B was from 13 to 63 %, the D domain was from 13 to 60 % and the F domain was from 13 to 60 %. Curiously, the residue A456 in LBD of human ER $\beta$  which was conserved in all the ERs has been shown to form the dimer interface in both ER $\alpha$ and ER $\beta$  homodimers (Pike et al. 1999).

Tissue distribution of hstsER $\beta$  mRNA analyzed through RT-PCR (Fig. 4) showed this gene was widely distributed in the organs of male; this result was similar with the expression files of goldfish (Carassius auratus) (Choi and Habibi 2003) and fathead minnow (Pimephales promelas) (Filby and Tyler 2005). In our research, the ER $\beta$  showed highest expression level in the liver; this result was confirmed with rainbow trout (Nagler et al. 2007) and fathead minnow (Filby and Tyler 2005). Some research showed the liver is the site of synthesis of the yolk protein precursor, VTG, which is stimulated by E2 and mediated through the hepatic ER (Maitre et al. 1985). The expression level of testis was lower than liver, gill and kidney, possessed the same result with study on goldfish and gilthead sea bream. In the goldfish, the  $ER\beta1$  showed higher expression pattern in gonads than other non-gonads organs in both of the male and female suggesting it played the key role in the regulation of ovarian and testicular function (Choi and Habibi 2003). Socorro found that the ER $\beta$ revealed a higher expression level in gonads than intestine, brain and heart (Socorro et al. 2000). Interestingly, in our study, the expression level in gill was also high; it suggested that expression in gill might be connected with a role for estrogen. The result of osmoregulation RT-PCR showed that the hstsER $\beta$ expression levels varied with reproductive stages in the testis, brain and liver of males.

The expression of hstsER $\beta$  in testis in our study showed that the level was decreased with the sexual development and increased in the stage of August to October, which result was similar to the pattern in liver. The research on European sea bass suggested that the ER $\beta$  may had an important function during gonadal development and/or maturation (Halma et al. 2004), and studies by Rodríguez et al. (2001) illustrated that  $ER\beta$  have initiated spermatogenesis and even spermiate. Variation of  $ER\beta$  expression of testis during reproductive cycle showed little related to the level of  $E_2$  and GSI. During the stage III to stage V, as the testis develop (GSI tended to increase), the mRNA expression of ER $\beta$  decreased, simultaneously the serum of  $E_2$  appeared to increase. However, during the stage VI, the level of  $ER\beta$  increased but the serum level of E2 down to low level. These findings showed that estrogen played an important role in the early spermatogenetic cycle, which was also suggested by Miura (Miura et al. 1999) and Gomez (Gomez et al. 1999). In addition, our study showed that  $ER\beta$  might not the predominant receptor for estrogen binding during the spermatogenesis in male.

By RT-PCR, the expression files of hstsER $\beta$  in brain were detected, and result showed that the  $ER\beta$ was expressed in all stages during the gonadal development in half-smooth tongue sole. Some studies illustrated that the expression of  $ER\beta$ -mRNA was found in many regions of brain, suggesting the important role of ER $\beta$  on regulation the reproductive behavior and pituitary gonadotropin secretion (Bernard et al. 2001; Patisaul et al. 1985). Our result showed the highest expression level of  $ER\beta$  in brain was founded in the stage IV, and the serum level of E<sub>2</sub> was increased at that stage, then the expression level of  $ER\beta$  tended to decrease, but the serum level of  $E_2$  still increased until highest. It was seemed that the  $ER\beta$ plays an important role during the early spermatogenesis in brain.

It is established that VTG was also induced on exposure to estrogens in male fish (Sumpter and Jobling 1995), but due to the negligible circulating  $E_2$ level in male fish, the variable expression in liver during gonad development was difficult to interpret. The high expression level in the liver in spermatocytes stage suggested ER $\beta$  may have the key role in the beginning of the spermatogenesis, and further analysis is required in protein to confirm these. In addition, it is said that the ER $\beta$  expression in liver was not significantly affected by E<sub>2</sub> of fathead minnow, but the ER $\alpha$  was (Filby and Tyler 2005), the observation may suggest the ER $\beta$  and ER $\alpha$  play a different role in the liver. In order to demonstrate these claims, more additional work is required to elucidate the mechanisms in protein levels and receptor binding assay.

In summary, cDNAs for half-smooth tongue sole  $\text{ER}\beta$  were cloned and characterized in this study, and it was classified phylogenetically. The expression of hsts $\text{ER}\beta$  in gonadal and somatic organs of adult male was also analyzed, demonstrating the widely distribution of this gene in organs. RT-PCR was used to found the expression pattern in testis, liver and brain in male. The cDNA encoding  $\text{ER}\beta$  and expression profiles are a prerequisite for investigating the regulation of estrogen action of half-smooth tongue in more details.

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