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

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

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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 (DNA-binding domain; DBD) has two zinc-finger structures that are essential for recognition and specific binding of the receptor to DNA, and the E domain (ligand-binding domain; LBD) whose role is the ligand-dependent 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 ERα and ERβ, have been described in vertebrates. ERα has been cloned from several vertebrate species (Greene

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et al. 1986; Koike et al. 1987; White et al. 1987), and ERβ 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 ERβ2 (Tchoudakova et al. 1999; Ma et al. 2000; Menuet et al. 2002) or ERγ (Hawkins 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 ERβ in half-smooth tongue sole.

In the half-smooth tongue sole, ERα gene was previously isolated, and we analyze it in another coming work (unpublished yet). In our present study, we have cloned and characterized the ERβ in the male half-smooth tongue sole, described the tissue distribution and expression profiles of ERβ isoforms using male fish, combined with serum levels of E_2. Knowledge about the expression and function of these genes is a step toward understanding the molecular mechanisms of ERβ 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 ± 0.5 °C; ≥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×*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 weight-viscera weight)] × 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 μg total RNA from each sample using random primers and Reverse Transcriptase M-MLV (Takara, Japan) in a 10 μl reaction.

Isolation and PCR amplification of ERβ cDNA fragments

In order to clone ERβ half-smooth tongue sole cDNA fragment, a pairs of degenerated primers (ERβF1/ERβR1) 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 μl of 10× reaction buffer, 2 μl 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* DH5α, 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 ERβ, SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used to 3′ and 5′ ends RACE-PCR. The gene-specific 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 ERβ of the half-smooth 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 full-length 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 GenBank accession numbers are as follows: *Solea solea* ERβ (csERβ, CAL09961.1), *Danio rerio* ERβ (zfERβ1, AAK16742.1; zfERβ2, CAC93849.1), *Oncorhynchus mykiss* ERβ (rtERβ1, NP_001118225.1), *Oncorhynchus mykiss* ERβ2 (rtERβ2, NP_001118042.1), *Paralichthys olivaceus* ERβ (JfERβ, BAB85623.1), *Sebastes*

Table 1 Primer sequences for cloning and mRNA expression analysis

Primer	Nucleotide sequence(5′ to 3′)	Usage
ERβF1	AGCGGTCCATCCAGGG(A/C/G/T)CA(C/T)AA(C/T)GA	Degenerate primer
ERβR1	GCCAGATCAGGCCGATCAT(A/C/G/T)A(A/G)(A/C/G/T)AC	ERαR1
ER-C-5-1	AGCAGGAACTGAGGATGGGGGAGAGC	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 ERβ (brfERβ1, ACN38898.3; brfERβ2, ADR73047.1), *Sparus aurata* ERβ (sbERβ1, CAD 33851.1; sbERβ2, CAE30470), *Oreochromis aureus* ERβ (tERβ1, ACF75102.1; tERβ2, ACF75103.1), *Carassius auratus* ERβ (gfERβ1, AAD26921.1; gfERβ2, AAF35170.1), *Micropogonias undulates* ERβ (acERβ1, AAG16711.1; acERβ2 AAG16712.1), *C. semilaevis* ERβ (hstsERβ).

RT-PCR analysis

Semi-quantitative RT-PCR assays were performed to evaluate the level of ERβ-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 reverse-transcribed as described above. RNA was used for studying the temporal expression pattern of ERβ 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-e-r). 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 °C, 22 cycles of 35 s at 94 °C, 30 s at 57 °C and 35 s at 72 °C, followed by final 10 min elongation period at 72 °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 β in male were measured using Iodine [^{125}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 ERβ cDNA

Phylogenetic analysis implicated that this hstsER belonged to ERβ subtype, closely related to *S. solea* ERβ and *P. olivaceus* ERβ (Fig. 2). The full-length cDNA of half-smooth tongue sole ERβ(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 hstsERβ and other similar ERβ, such as csERβ, JfERβ, KrERβ, gsERβ, hmERβ and hERβ (Fig. 3), showed that the hstsERβ exhibited high identity with other teleosts. The hstsERβ 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 hstsERβ-deduced amino acid sequence with other teleosts' ERβ including csERβ, JfERβ, KrERβ, rtERβ1, rtERβ2, ccERβ1, ccERβ2, zbERβ1, zbERβ2, gsERβ2,

Fig. 1 Nucleotide and deduced amino acid sequence of *hstsERβ*. 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*

hERβ, hmERβ were 76, 76, 72, 64, 56, 64, 63, 64, 63 %, respectively, and lower identities (46, 49 %) were found with hERβ and cERβ. However, the identities between hstsERβ and gsERβ 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 ERβ1 group and ERβ2 group. The sequence homology implicates that our hstsER is apparently belonging to ERβ1 cluster. Furthermore, hstsERβ was more closely related to *Solea solea* ERβ and *Paralichthys olivaceus* ERβ (Fig. 3).

Expression of ERβ in different adult organs

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

ERβ-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

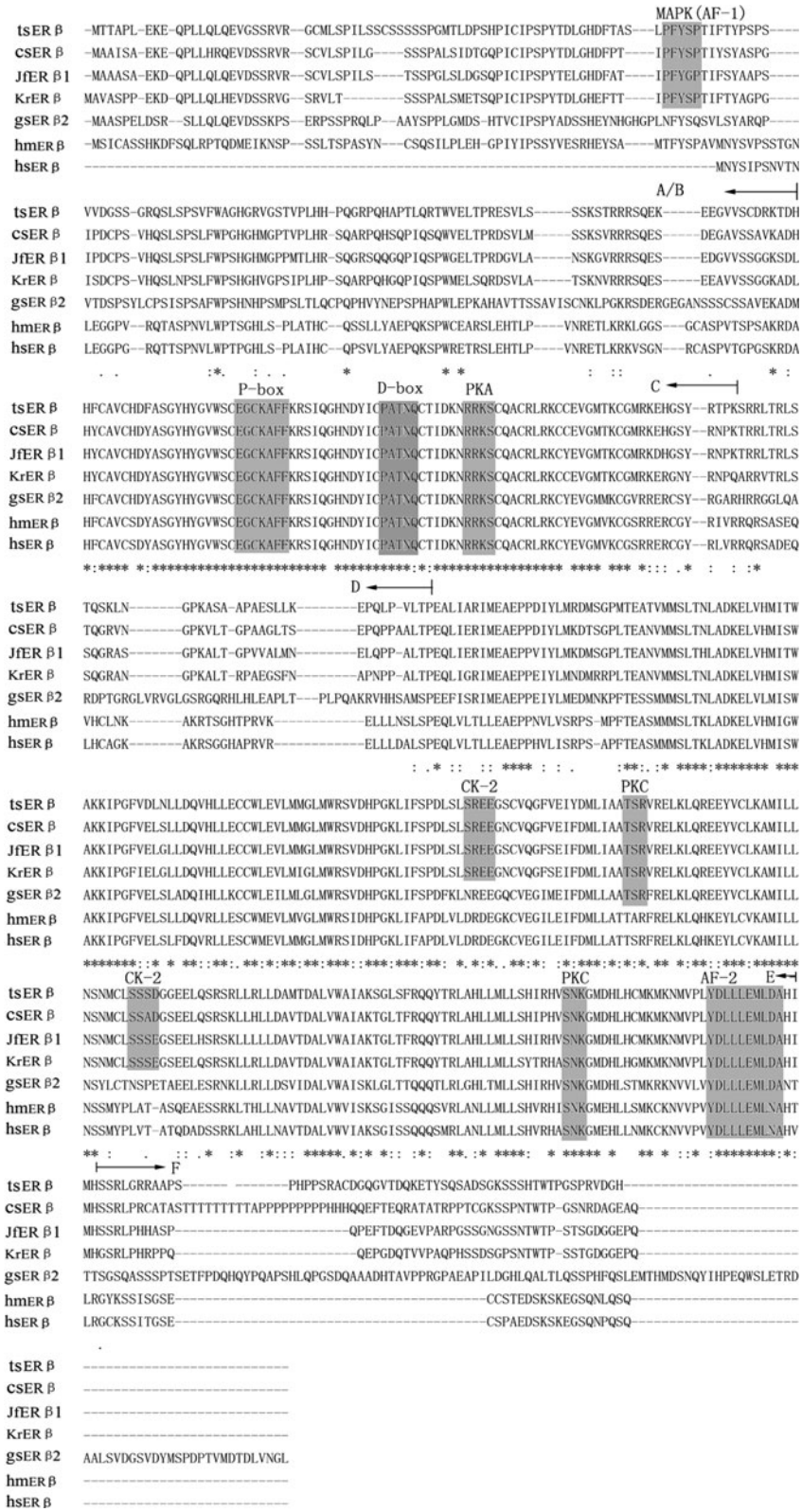

Fig. 2 Amino acid alignment of hstsERβ with ccERβ, JfERβ, KrERβ, gsERβ, hERβ and hsERβ (see "Materials and methods" for sequence references and abbreviations). *Asterisks* (*) and *dots* (:) marked for completely conserved and conserved amino acids, respectively. The functional domains (A/B, C [DNA-binding domain], D, E [ligand-binding domain] and F) 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

Table 2 Amino acid identities between hstsERβ and ERs in fish and mammals

Species	hstsERβ	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)
JfERβ1	76 (565/578)	60 (159/163)	93 (84/85)	54 (37/37)	92 (238/293)	35 (47/55)
KrERβ2	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)
rtERβ2	56 (604/578)	34 (175/163)	87 (85/85)	29 (42/37)	78 (240/293)	20 (62/55)
ccERβ1	64 (559/578)	49 (156/163)	84 (83/85)	20 (44/37)	86 (238/293)	18 (38/55)
ccERβ2	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)
hERβ	46 (477/578)	13 (93/163)	84 (84/85)	13 (33/37)	66 (236/293)	13 (29/55)
hmERβ	49 (549/578)	26 (165/163)	85 (84/85)	24 (35/37)	66 (236/293)	7 (29/55)

See "Materials and methods" for sequence references

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

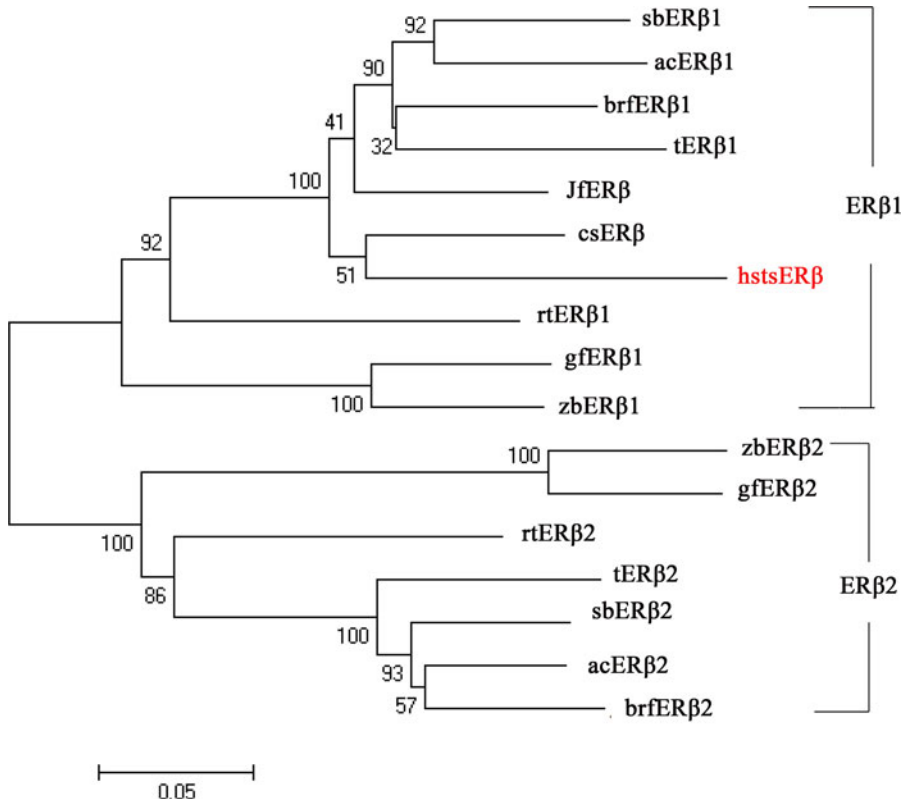

Fig. 3 Phylogenetic tree based on amino acid sequences for ERβs in teleosts. Bootstrap values are indicated (1,000 replicates) (see "Materials and methods" for sequence references and abbreviations)

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 hstsERβ in testis, spleen, brain and kidney during the reproductive cycle were shown in Fig. 5. It indicated that hstsERβ was expressed

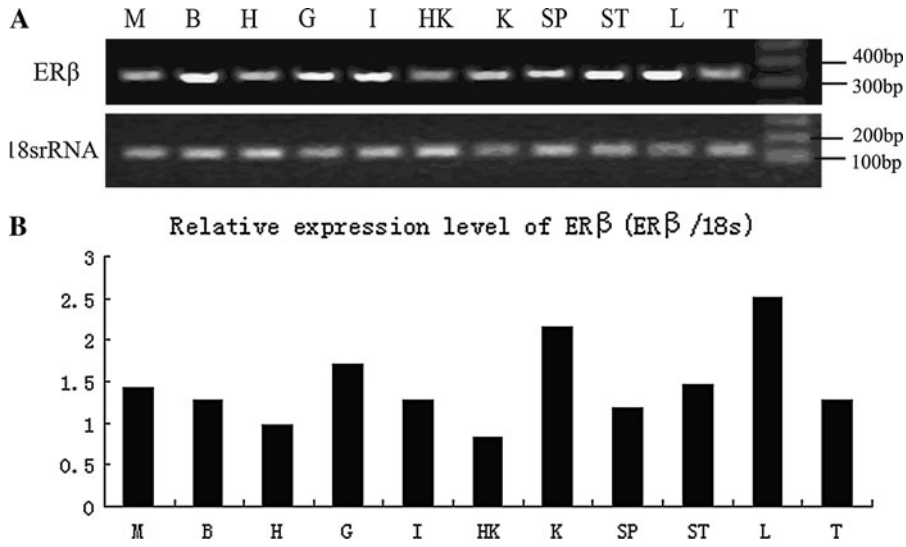

Fig. 4 RT-PCR expression analysis of *hstsERβ* in organs of adult male half-smooth tongue sole. Various tissue-specific expressions of the hstsERβ gene were determined by RT-PCR; 18S ribosomal RNA was used as an internal control for relative quantity of cDNA (*lower panel*). *H* heart, *L* liver, *S* spleen, *He* head kidney, *C* caeca, *T* testis, *St* stomach, *K* kidney gill, *G* gill, *B* brain, *I* intestine, *M* muscle

throughout the reproductive cycle of these organs in male. The expression levels of hstsERβ 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 hstsERβ 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 ± 1.50 pg ml^{-1}, then remarkably elevated to 21.97 ± 2.11 pg ml^{-1} between May and July, and then reached to 25.94 ± 2.13 pg ml^{-1} between August and October, finally fell to 10.93 ± 1.62 pg ml^{-1} between November and January.

Discussions

In this study, we cloned and characterized the ERβ from half-smooth tongue sole, *C. semilaevis*. The hstsERβ possessed common domains (domains A–F) typical for ERs with other ERβs, 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 hstsERβ; 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 ERβs transcriptional activity may activated by MAPK pathway in ligand-independent 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

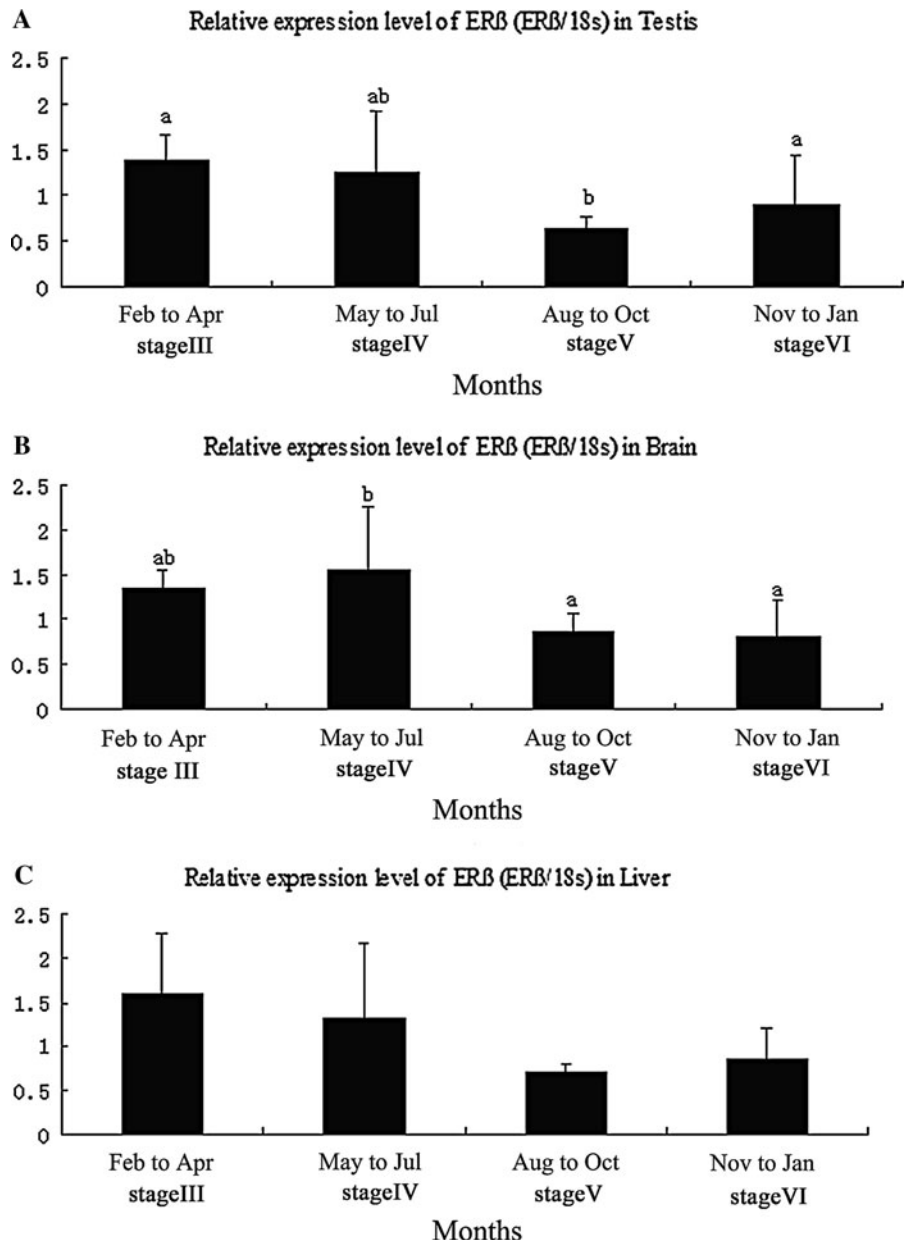

Fig. 5 The mRNA expression of *ERβ* in testis (**a**), brain (**b**) liver (**c**) in half-smooth tongue sole during the male annual reproductive cycle. Values are expressed as mean ± SE of mean. *Different letters* indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

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 ERβ 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, hstsERβ, was closely related to the ERβ subtype (Table 2) in teleost rather than to in the tetrapods. The hstsERβ 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 hstsERβ 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

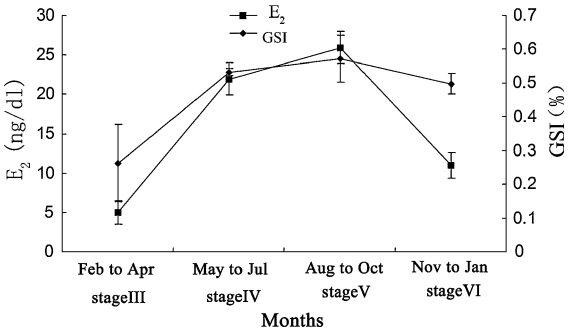

Fig. 6 Serum 17b-Estradiol (E_2) and Gonadosomatic (GSI) levels in male half-smooth tongue sole. Values are expressed as mean ± SE of mean. *Different letters* indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test)

hstsERβ were poorly conserved in aa sequence compared with other ERβ 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 ERβ which was conserved in all the ERs has been shown to form the dimer interface in both ERα and ERβ homodimers (Pike et al. 1999).

Tissue distribution of hstsERβ 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 ERβ 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 ERβ1 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 ERβ 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 hstsERβ expression levels varied with reproductive stages in the testis, brain and liver of males.

The expression of hstsERβ 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 ERβ may had an important function during gonadal development and/or maturation (Halma et al. 2004), and studies by Rodríguez et al. (2001) illustrated that ERβ have initiated spermatogenesis and even spermiate. Variation of ERβ 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 ERβ decreased, simultaneously the serum of E_2 appeared to increase. However, during the stage VI, the level of ERβ 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 ERβ might not the predominant receptor for estrogen binding during the spermatogenesis in male.

By RT-PCR, the expression files of hstsERβ in brain were detected, and result showed that the ERβ was expressed in all stages during the gonadal development in half-smooth tongue sole. Some studies illustrated that the expression of ERβ-mRNA was found in many regions of brain, suggesting the important role of ERβ 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 ERβ in brain was founded in the stage IV, and the serum level of E_2 was increased at that stage, then the expression level of ERβ tended to decrease, but the serum level of E_2 still increased until highest. It was seemed that the ERβ 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 ERβ 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 ERβ expression in liver was not significantly affected by E_2 of fathead minnow, but the ERα was (Filby and Tyler 2005), the observation may suggest the ERβ and ERα 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 ERβ were cloned and characterized in this study, and it was classified phylogenetically. The expression of hstsERβ 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 ERβ and expression profiles are a prerequisite for investigating the regulation of estrogen action of half-smooth tongue in more details.

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References

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

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BlAST and PSI–Blast: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bairoch A, Bucher P, Hofmann K (1997) The PROSITE database its status in 1997. Nucleic Acids Res 25:217–221

Bernard DJ, Bentley GE, Balthzart J, Turek FW, Ball G (2001) Soy isoflavone supplements antagonize reproductive behavior and estrogen receptor α and β dependent gene expression in the brain. Biochimie 142:2946–2952

Cavaco JEB, Vilrokx C, Trudeau VL, Schulz RW, Goos HJT (1998) Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). Am J Physiol 44:1793–1802

Chang X, Kobayashi T, Todo T (1999) Molecular cloning of estrogen receptors d and 8 in the ovary of a teleost fish, the tilapia (*Oreochromis niloticus*). Zool Sci 16:653–658

Chen CF, Wen HS (2012) The characteristic analysis of P450c17 mRNA expression during the reproductive cycle in male *Cynoglossus semilaevis Günther*. J Fish china 36:1019–1025 (Chinese with English abstract)

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)

Choi CY, Habibi HR (2003) Molecular cloning of estrogen receptor beta and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol Cell Endocrinol 204:169–177

Danielian PS, White R, Lees JA, Parker MG (1992) Identification of a conserved region required for hormone dependent transcriptional activation by steroid-hormone receptors. EMBO J 11:1025–1033

Filby AL, Tyler CR (2005) Molecular characterization of estrogen receptors 1,2a and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). Biol Reprod 73:648–662

Gomez JM, Weil C, Ollitrault M, LeBail PY, Breton B, LeGac F (1999) Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 113:413–428

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J (1986) Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150–1154

Guiguen Y, Baroiller JF, Ricordel MJ, Iseki K, McMeel OM, Martin SAM, Fostier A (1999) Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a Tilapia (*Oreochromis niloticus*). Mol Reprod Dev 54:154–162

Halm S, Martınez-Rodrıguez G, Rodrıguez L, Prat F, Mylonas CC, Carrillo M, Zanuy S (2004) Cloning, characterisation, and expression of three oestrogen receptors (ERα, ERβ1 and ERβ2) in the European sea bass, *Dicentrarchus labrax*. Mol Cell Endocrinol 223:63–75

Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P (2000) Identification of a third estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci USA 97:10751–10756

Koike S, Sasaki M, Muramatsu M (1987) Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res 15:2499–2513

Krust A, Green S, Argos P, Kumar V, Walter P, Bornert J, Chambon P (1986) The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J 5:891–897

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930

Kumar V, Green S, Stack G, Berry M, Jin J, Chambon P (1987) Functional domains of the human estrogen receptor. Cell 51:941–951

Lannigan DA (2003) Estrogen receptor phosphorylation. Steroids 68:1–9

Lassiter CS, Kelley B, Linney E (2002) Genomic structure and embryonic expression of estrogen receptor beta a (ER beta a) in zebrafish (*Danio rerio*). Gene 299:141–151

Levin ER (2005) Integration of the extranuclear and nuclear actions of estrogen. 2005. Mol. Endocrinal 19:1951–1959

Liu XZ, Zhuang ZM, Ma AJ, Chen SQ, Sun ZZ, Liang Y, Xu YJ (2005) Reproductive biology and breeding technology of *Cynoglossus semilaevis* Günther. Mar Fish Res 26:7–14 (Chinese with English abstract)

Ma CH, Dong KW, Yu KL (2000) cDNA cloning and expression of a novel estrogen receptor β-subtype in goldfish (*Carassius auratus*). Biochim Biophys Acta 1490:145–152

Maitre JL, Mercier L, Dolo L, Valotaire Y (1985) Characterization of specific receptors for estradiol, induction of vitellogenin and its mRNA in the liver of rainbow trout. Biochimie 67:215–225

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the second decade. Cell 83:835–839

Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, Pakdel F (2002) Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol Reprod 66:1881–1892

Miura C, Miura T, Kudo N, Yamashita M, Yamauchi K (1999) cDNA cloning of a stage-specific gene expressed during HCG-induced spermatogenesis in the Japanese eel. Develop Growth Differ 41:463–471

Montano MM, Muller V, Trobaugh A, Katzenellenbogen BS (1995) The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. Mol Endocrinol 9:814–825

Mosselman S, Polman J, Dijkema R (1996) ERb: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53

Nagler JJ, Cavileer T, Sullivan J, Cyr DG, Rexroad C (2007) The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERa2 and both ERβ isoforms. Gene 392:164–173

Nakamura M, Kobayashi T, Chang XT, Nagahama Y (1998) Gonadal sex differentiation in teleost *fish*. J Exp Zool 281:362–372

O'donnell L, Robertson KM, Jones ME, Simpson ER (2001) Estrogen and spermatogenesis. Endocr Rev 22:289–318

Pakdel F, Guellec Le, Vaillant C, Le Roux MG, Valotaire Y (1989) Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. Mol Endocrinol 3:44–51

Patino R, Xia Z, Gale WL, Wu C, Maule AG, Chang X (2000) Novel transcripts of the estrogen receptor alpha gene in channel catfish. Gen Comp Endocrinol 120:314–325

Patisaul HB, Dindo M, Whitien PL, Young LJ (1985) Characterization of specific receptors for estradiol, induction of vitellogenin and its mRNA in liver of rainbow trout (*Salmo gairdnerii*). Biochimie 67:215–225

Pennie WD, Aldridge TC, Brooks AN (1998) Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. J Endocrinol 158:11–14

Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M (1999) Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618

Price RH, Butler CA, Webb P, Uht R, Kushner P, Handa RJ (2001) A splice variant of estrogen receptor beta missing exon 3 displays altered subnuclear localization and capacity for transcriptional activation. Endocrinology 142:2039–2049

Rodríguez L, Zanuy S, Carrillo M (2001) Influence of daylength on the age at first maturity and somatic growth in male sea bass (*Dicentrarchus labrax*, L.). Aquaculture 196:159–175

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

Saunders PT, Maguire SM, Gaughan J, Millar MR (1997) Expression of oestrogen receptor β (ERβ) in multiple rat tissues visualized by immunohistochemistry. J Endocrinol 154:13–16

Schwabe JW, Chapman L, Finch JT, Rhodes D (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75:567–578

Socorro S, Power DM, Olson PE, Canario AV (2000) Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution. J Endocrinol 166:293–306

Sumpter JP, Jobling S (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103:173–178

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

Tan NS, Lam TJ, Ding JL (1996) The first contiguous estrogen receptor gene from a fish, *Oreochromis aureus*: evidence for multiple transcripts. Mol Cell Endocrinol 120:177–192

Tchoudakova A, Pathak S, Callard GV (1999) Molecular cloning of an estrogen receptor b subtype from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 113:388–400

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

Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P (1989) The human estrogen receptor has two independent non-acidic transcriptional activation functions. Cell 59:477–487

Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP (1994) Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8:21–30

Weinberger C, Hollenberg SM, Rosenfeld MG, Evans RM (1985) Domain structure of human glucocorticoid receptor and its relationship to the v-erbA oncogene product. Nature 318:670–672

White R, Lees JA, Needham M, Ham J, Parker M (1987) Structural organization and expression of the mouse estrogen receptor. Mol Endocrinol 1:735–744

Wu C, Patino R, Davis KB, Chang X (2001) Localization of estrogen receptor α and β RNA in germinal and nongerminal epithelia of the channel catfish testis. Gen Comp Endocrinol 124:12–20

Xia Z, Patino R, Gale WL, Maule AG, Densmore LD (1999) Cloning, in vitro expression and novel phylogenetic classification of a channel catfish estrogen receptor. Gen Comp Endocrinol 113:360–368