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Molecular Cloning and Characterization of the Estrogen Receptor from the Slender Bitterling (*Acheilognathus yamatsutae*)

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Objectives: In order to identify the possibility of slender bitterling (SB) (*Acheilognathus yamatsutae*) being used as a test species for estrogenic endocrine disrupting chemicals (EEDCs), we carried out the cloning and sequence characterization of the estrogen receptor (ER).
Methods: The ER from a slender bitterling was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR), 5'- and 3'-rapid amplification of cDNA ends (5'-RACE and 3'-RACE) and T-vector cloning. The expression of ER mRNA was also analyzed in six tissues (brain, liver, kidney, gill, gonad, and intestines) by real-time PCR.
Results: We obtained an ER from the slender bitterling. The SB ER cDNA was 2189 base pairs (bp) in length and contained a 1707 bp open reading frame that encoded 568 amino acid residues. The SB ER amino acid sequence clustered in a monophyletic group with the ERα of other fish, and was more closely related to zebrafish ERα (88% identity) than to the ERα of other fish. The SB ER cDNA was divided into A/B, C, D, E and F domains. The SB ER has conserved important sequences for ER functions, such as the DNA binding domain (D domain), which are consistent with those of other teleosts.
Conclusions: The ER of the slender bitterling could provide basic information in toxicological studies of EEDCs in the slender bitterling.

Key words: Estrogen receptor, Estrogenic endocrine disrupting effects, Gene cloning, Slender bitterling

INTRODUCTION

Since the aquatic environment serves as the ultimate sink for many environmental pollutants such as endocrine disrupting chemicals (EDCs) [1], particular attention has been given to evaluate the ecological effects of EDCs (a wide variety of developmental, morphological and reproductive abnormalities: feminization or intersex) on indigenous aquatic populations such as fish [2,3]. Recently, the dominant species distributed over a wide range of habitats have been used extensively in aquatic toxicity research. In this study, we used slender bitterling (*Acheilognathus yamatsutae*), which is an indigenous species widely distributed throughout both polluted and unpolluted areas of Korea. It has a suitable individual size (about 8 cm) and sex ratio (female : male = about 1:1) for laboratory scale studies. The slender bitterling is a particularly good species for studying endocrine disrupting effects of xenoestrogens because the male has a nuptial coloration and the ovipositor of the female appears during the spawning season. Estrogenic EDCs (EEDCs) can mimic the physiological action of estrogens at estrogen receptors

(ERs), causing reproductive impairments in animals and humans [4,5]. In the classic estrogen-mediated pathway, estrogen binds to the ligand-binding domain of the ER followed by the formation of a liganded receptor homodimeric complex. The complex then binds to the distal estrogen response elements (ERE) in the 5'-promoter regions of the E2-responsive genes, and further interacts with the components of the general transcription factor complex and coactivators or adaptors, and finally induces transcription. Therefore, knowledge of the interactions of EEDCs with estrogen receptors is important for understanding their mechanisms of action and for developing effective risk management strategies. Generally, two ERs, ERα and ERβ, have been reported in humans and mice [6-11], while three functional ER forms, ERα, ERβ1

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Table 1. Nucleotide sequences and positions of primers used in the polymerase chain reactions

Primer	Length (bp)	Position*	Sequence (5'-3')
NF	20	574 - 593	GCATHCARGGTCACAATGAC
NR	20	1484 - 1503	CCATKCCTTTGTTGCTCATG
R1	25	746 - 770	GTCACGATCCTCATTACCACTCCTC
F1	26	1354 - 1379	TCATGGTGCAGTGTATGCTGGACAAC
ER-F1	20	13 - 32	CCCACAAACTCTCACCTATG
ER-R1	20	1857 - 1876	TCTGATAAGGCACACGGAGA

*Positions reflect nucleic acid locations in Figure 1.

and ERβ2, have been identified in fish species such as the zebrafish and goldfish, for example. [12-14]. However, there is little information on the ER-mediated toxic effects stimulated by EEDCs in the slender bitterling. We therefore cloned and sequenced the ER gene from slender bitterlings to identify characteristics of this gene that could play important roles in the toxicity of EEDCs.

MATERIALS AND METHODS

I. Fish and RNA Isolation

Slender bitterlings (*Acheilognathus yamatsutae*) ranging from 7.0 to 9.5 cm in length were obtained from the Inland Fisheries Ecological Research Institute (Chungpyoung, Korea) and kept in a semi-recirculating tank at 20-22℃. For ER gene cloning, liver samples were removed and immediately stored in liquid nitrogen until needed. Six organs (brain, liver, kidney, gill, gonad and intestines) from the slender bitterlings were used to determine the tissue distribution of ERs. Total RNA was isolated from 100 mg of samples using Trizol reagent (Invitrogen, CA, USA).

II. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

The slender bitterling ER (SB ER) was cloned using an RT-PCR approach with degenerated primers (NF and NR, Figure 1) that were designed based on known teleost ER sequences, including red seabream (*Pagrus major*) ER (accession number AB007453) [15], gilthead seabream (*Sparus aurata*) ER (accession number AF136979) [16], zebrafish (*Danio rerio*) ER (accession number AF349412), and killifish (*Oryzias sp.*) ER (accession number D28954). These sequences were retrieved from GenBank. The primer sequences used were synthesized from Bioneer (Daejeon, Korea) and are shown in Table 1. The first-strand cDNA was generated by reverse transcribing 1 μg of mRNA in a final volume of 20 μL, which contained 4 μL of 5×RT Buffer, 9 U AMV reverse transcriptase, 20 U of an RNase inhibitor, 1 μL of 10 mM dNTPs and 0.5 μg/mL of random hexamers. The following RT conditions were used: room temperature for 10 minutes, 42℃ for 50 minutes, and 94℃ for 5 minutes. The PCR was conducted with 2 μL of the first-strand cDNA in a final volume of 25 μL, which also contained 2.5 μL of 10×Ex-Taq Buffer, 1 μL of 2.5 mM dNTPs, 2.5 units of Ex-Taq polymerase, and the primers (NF/NR). The following PCR conditions were used: 94℃ for 2 minutes followed by 30 cycles of 94℃ for 30 seconds, 50 ℃ for 30 seconds, 72℃ for 1 minutes, and a final extension step of 72℃ for 10 minutes. The PCR-amplified products were electrophoretically analyzed on 1.2% agarose gel.

### III. Rapid Amplification of cDNA Ends (RACE)

The unknown sequences at the 5'- and 3'-ends of SB ER cDNA were determined by performing 5'- and 3'-RACE using a Marathon cDNA Amplification kit (Clontech Lab, CA, USA). For 5'-RACE and 3'-RACE, the adaptor-ligated, oligo (dT)-primed and double-stranded liver cDNA was synthesized, and the gene-specific primers (GSPs) of SB ER (R1, F1, ER-F1 and ER-R1) were coupled with the adaptor primers (AP1) in the PCR reactions. The GSPs of SB ER were designed according to the partial sequence defined by RT-PCR and are shown in Table 1. The following PCR conditions were used: 94℃ for 1 minutes followed by 30 cycles of 94℃ for 30 seconds, and 68℃ for 3 minutes. All primers were synthesized from Bioneer (Daejeon, Korea). The RACE PCR products were analyzed by electrophoresis on 1.2% agarose gel.

IV. cDNA Cloning and Sequencing

The PCR-amplified products were excised from 1.2% agarose gel using the SV Gel and PCR Clean Up System (Promega, WI, USA), and then cloned into the pGEM T-Easy vector (Promega, WI, USA). Recombinant clones were selected by blue-white screening. The white colonies were cultured, and the plasmids were purified using a plasmid purification kit (Promega, WI, USA). For determination of the SB ER sequence, three clones from each of the three slender bitterling samples were sequenced completely (forward and reverse) using an ABI 3730XL (Applied Biosystems Inc., CA, USA) and analyzed using Data collection 3.0 software (Applied Biosystems Inc., CA, USA).

V. Phylogenetic Analysis

The ER amino acid and nucleotide sequences of the slender bitterling were aligned with those of other teleosts using ClustalW Multiple alignment version 1.7 [17]. The sequence alignment, percentage identity analysis and phylogenetic trees of the SB ER were performed with the software MegAlign from DNASTAR® (Lasergene v8.1, Madson, WI, USA). The aligned sequences were used to construct phylogenetic trees using a distance method within
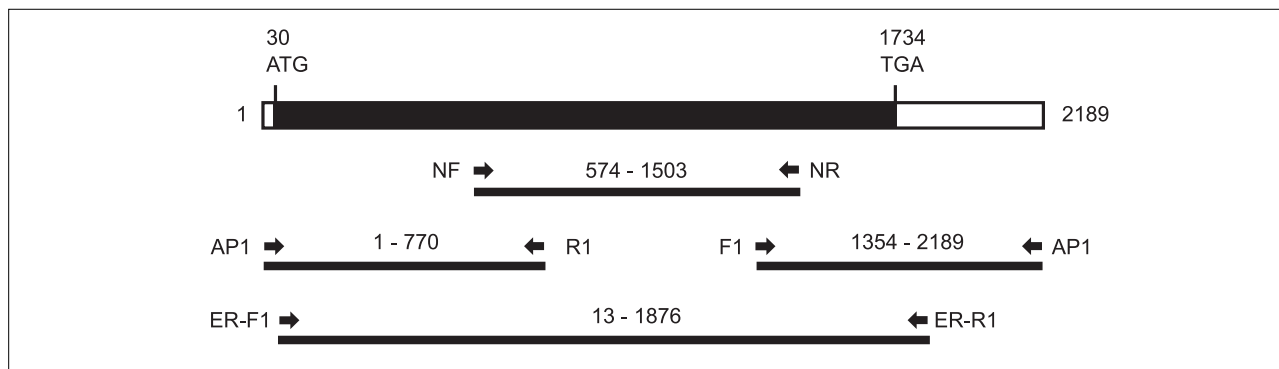
Figure 1. Cloning strategy and schematic view of SB ER cDNAs. The open reading frame is represented by a thick black line. The positions of the start codon (ATG) and the stop codon (TGA) are indicated. The binding sites of two degenerated primers (NF and NR) and four gene-specific primers (R1, F1, ER-F1, and ER-R1) are shown. The AP1 primer was provided in the RACE kit (Clontech Lab, CA, USA). The complete cDNA sequence was determined by overlapping the PCR products using the Chromas 3.2 analysis program.

SB ER: slender bitterling estrogen receptor, AP1 primer: adaptor primer, RACE kit: Marathon cDNA Amplification kit.

phylogenetic analysis under parsimony (PAUP). Gaps and missing data were completely excluded from the analysis and bootstrap analysis was performed using MegAlign, Lasergene v8.1 (DNASTAR®, Madson, WI, USA).
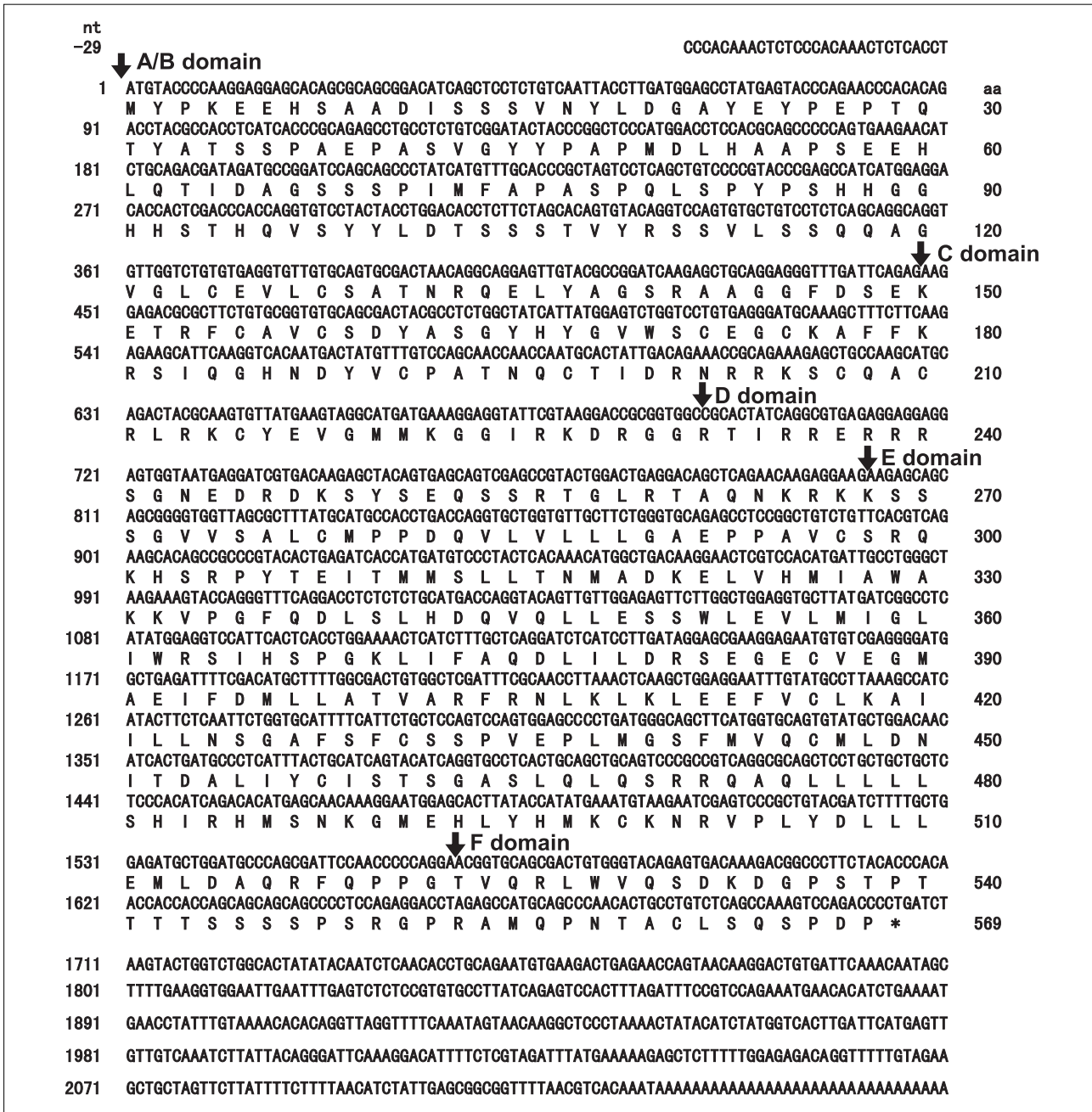
VI. Quantitative Real-Time PCR for ER mRNA Expression

Total RNA was purified using Trizol reagent (GIBCO, BR) according to the manufacturer's protocol and stored at -80℃ until needed. The primers of the ER were designed according to the SB ER using SES (Scientific & Educational Software, version 3.0). β-actin, designed according to carp actin [18], was used to normalize the mRNA levels. The following primers were used: ER REAL-F (Forward) 5'-GCC TCT GGC TAT CAT TAT GGA GTC T-3'; ER REAL-R (Reverse), 5'-GTC ACG ATC CTC ATT ACC ACT CCT C-3'; β-actin-F (Forward), 5'-CAC TGT GCC CAT CTA CGA G-3'; β-actin-R (Reverse), 5'-CCA TCT CCT GCT CGA AGT C-3'. The primers were synthesized from Bioneer (Daejeon, Korea). The RT was performed as described above. Real-time PCR analysis was performed at least three times. The real-time fluorescence signal generated by the nonspecific double-stranded DNA binding dye, SYBR Green I, was analyzed using Smart Cycler software. The PCR was performed using 0.4 μM of the sense and antisense oligonucleotide primers, 12.5 μL 2×SYBR® Premix Ex-Taq™ (Takara Bio Inc., Japan), and 1 μL template DNA in a final volume of 25 μL. The PCR amplification profile for the assays consisted of 10 seconds at 95℃ followed by 45 cycles (single assays) of three steps consisting of 5 seconds at 95℃, 10 seconds at 51℃ (β-actin, 200 bp) or 42℃ (ER, 258 bp), and 15 seconds at 72℃ using a Smart Cycler (Cepheid, Sunnyvale, CA, USA). The fluorescence signals were measured at the end of each extension step. The threshold cycle (Ct) was determined for

each sample using the exponential growth phase and the baseline signal from the fluorescence versus the cycle number plots. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

RESULTS

I. Identification of ERs from the Slender Bitterling

Liver poly (A)+from the slender bitterling was used to clone and sequence the ER cDNA. One sense (NF) and antisense primers (NR) for the PCR reactions were derived from two consensus regions identified by an alignment of nucleotide sequences from known teleost ERs. The primer pair, NF/NR, amplified a 930 bp fragment of ER, which was similar to goldfish ERα sequences. The sequence information was used to design specific primers for 5'- and 3'-RACE, which were used to obtain the complete ER cDNA sequence of the slender bitterling (Figure 1). The 5'- and 3'-RACE PCR reactions were performed with AP1/R1 and AP1/F1 to obtain a 770 bp fragment and an 836 bp fragment of SB ER, respectively (Figure 1). The sequences derived from the 5'- and 3'-RACE PCR products were aligned with the cloned fragments to construct the full-length cDNA sequence. As shown in Figure 2, the full-length cDNA of the SB ER was 2189 bp. The SB ER (Genebank Accession No. JF275853) of the 2189 bp total sequence contained a 29 bp 5'-untranslated region (UTR), a 1707 bp open reading frame encoding a 568 amino acid protein, a stop codon, and a 453 bp 3'-UTR. The SB ER gene has a theoretical molecular weight (MW) of 63.0 kDa.

```
 nt
−29                                                          CCCACAAACTCTCCCACAAACTCTCACCT
   ↓ A/B domain
 1 ATGTACCCCAAGGAGGAGCACAGCGCAGCGGACATCAGCTCCTCTGTCAATTACCTTGATGGAGCCTATGAGTACCCAGAACCCACACAG   aa
   M Y P K E E H S A A D I S S S V N Y L D G A Y E Y P E P T Q   30
 91 ACCTACGCCACCTCATCACCCGCAGAGCCTGCCTCTGTCGGATACTACCCGGCTCCCATGGACCTCCACGCAGCCCCCAGTGAAGAACAT
   T Y A T S S P A E P A S V G Y Y P A P M D L H A A P S E E H   60
181 CTGCAGACGATAGATGCCGGATCCAGCAGCCCTATCATGTTTGCACCCGCTAGTCCTCAGCTGTCCCCGTACCCGAGCCATCATGGAGGA
   L Q T I D A G S S S P I M F A P A S P Q L S P Y P S H H G G   90
271 CACCACTCGACCCACCAGGTGTCCTACTACCTGGACACCTCTTCTAGCACAGTGTACAGGTCCAGTGTGCTGTCCTCTCAGCAGGCAGGT
   H H S T H Q V S Y Y L D T S S S T V Y R S S V L S S Q Q A G   120
                                                              ↓ C domain
361 GTTGGTCTGTGTGAGGTGTTGTGCAGTGCGACTAACAGGCAGGAGTTGTACGCCGGATCAAGAGCTGCAGGAGGGTTTGATTCAGAGAAG
   V G L C E V L C S A T N R Q E L Y A G S R A A G G F D S E K   150
451 GAGACGCGCTTCTGTGCGGTGTGCAGCGACTACGCCTCTGGCTATCATTATGGAGTCTGGTCCTGTGAGGGATGCAAAGCTTTCTTCAAG
   E T R F C A V C S D Y A S G Y H Y G V W S C E G C K A F F K   180
541 AGAAGCATTCAAGGTCACAATGACTATGTTTGTCCAGCAACCAACCAATGCACTATTGACAGAAACCGCAGAAAGAGCTGCCAAGCATGC
   R S I Q G H N D Y V C P A T N Q C T I D R N R R K S C Q A C   210
                                                    ↓ D domain
631 AGACTACGCAAGTGTTATGAAGTAGGCATGATGAAAGGAGGTATTCGTAAGGACCGCGGTGGCCGCACTATCAGGCGTGAGAGGAGGAGG
   R L R K C Y E V G M M K G G I R K D R G G R T I R R E R R   240
                                                        ↓ E domain
721 AGTGGTAATGAGGATCGTGACAAGAGCTACAGTGAGCAGTCGAGCCGTACTGGACTGAGGACAGCTCAGAACAAGAGGAAGAAGAGCAGC
   S G N E D R D K S Y S E Q S S R T G L R T A Q N K R K K S S   270
811 AGCGGGGTGGTTAGCGCTTTATGCATGCCACCTGACCAGGTGCTGGTTCTTCTGGGTGCAGAGCCTCCGGCTGTCTGTTCACGTCAG
   S G V V S A L C M P P D Q V L V L L G A E P P A V C S R Q   300
901 AAGCACAGCCGCCCGTACACTGAGATCACCATGATGTCCCTACTCACAAACATGGCTGACAAGGAACTCGTCCACATGATTGCCTGGGCT
   K H S R P Y T E I T M M S L L T N M A D K E L V H M I A W A   330
991 AAGAAAGTACCAGGGTTTCAGGACCTCTCTCTGCATGACCAGGTACAGTTGTTGGAGAGTTCTTGGCTGGAGGTGCTTATGATCGGCCTC
   K K V P G F Q D L S L H D Q V Q L L E S S W L E V L M I G L   360
1081 ATATGGAGGTCCATTCACTCACCTGGAAAACTCATCTTTGCTCAGGATCTCATCCTTGATAGGAGCGAAGGAGAATGTGTCGAGGGGATG
   I W R S I H S P G K L I F A Q D L I L D R S E G E C V E G M   390
1171 GCTGAGATTTTCGACATGCTTTTGGCGACTGTGGCTCGATTTCGCAACCTTAAACTCAAGCTGGAGGAATTTGTATGCCTTAAAGCCATC
   A E I F D M L L A T V A R F R N L K L K L E E F V C L K A I   420
1261 ATACTTCTCAATTCTGGTGCATTTTCATTCTGCTCCAGTCCAGTGGAGCCCCTGATGGGCAGCTTCATGGTGCAGTGTATGCTGGACAAC
   I L L N S G A F S F C S S P V E P L M G S F M V Q C M L D N   450
1351 ATCACTGATGCCCTCATTTACTGCATCAGTACATCAGGTGCCTCACTGCAGCTGCAGTCCCGCCGTCAGGCGGCAGCTCCTGCTGCTGCTC
   I T D A L I Y C I S T S G A S L Q L Q S R R Q A Q L L L L L   480
1441 TCCCACATCAGACACATGAGCAACAAAGGAATGGAGCACTTATACCATATGAAATGTAAGAATCGAGTCCCGCTGTACGATCTTTTGCTG
   S H I R H M S N K G M E H L Y H M K C K N R V P L Y D L L L   510
                   ↓ F domain
1531 GAGATGCTGGATGCCCAGCGATTCCAACCCCCAGGAACGGTGCAGCGACTGTGGGTACAGAGTGACAAAGACGGGCCCTTCTACACCCACA
   E M L D A Q R F Q P P G T V Q R L W V Q S D K D G P S T P T   540
1621 ACCACCACCAGCAGCAGCAGCCCCTCCAGAGGACCTAGAGCCATGCAGCCCAACACTGCCTGTCTCAGCCAAAGTCCAGACCCCTGATCT
   T T T S S S S P S R G P R A M Q P N T A C L S Q S P D P *   569
1711 AAGTACTGGTCTGGCACTATATACAATCTCAACACCTGCAGAATGTGAAGACTGAGAACCAGTAACAAGGACTGTGATTCAAACAATAGC
1801 TTTTGAAGGTGGAATTGAATTTGAGTCTCTCCGTGTGCCTTATCAGAGTCCACTTTAGATTTCCGTCCAGAAATGAACACATCTGAAAAT
1891 GAACCTATTTGTAAAACACACAGGTTAGGTTTTCAAATAGTAACAAGGCTCCCTAAAACTATACATCTATGGTCACTTGATTCATGAGTT
1981 GTTGTCAAATCTTATTACAGGGATTCAAAGGACATTTTCTCGTAGATTTATGAAAAAGAGCTCTTTTTGGAGAGACAGGTTTTTGTAGAA
2071 GCTGCTAGTTCTTATTTTCTTTTAACATCTATTGAGCGGCGGTTTTAACGTCACAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 2. Nucleotide and deduced amino acid sequences of the slender bitterling ER. The nucleotides and amino acids are numbered on the left-hand and right-hand sides of each line, respectively. The boundaries of the five distinct domains, A/B, C, D, E, and F are indicated by arrows according to Krust et al. [7]. The stop codon, TGA, is marked with an asterisk. The GenBank accession number is JF275853.

ER: estrogen receptor.

II. Sequence Analysis

In order to determine the relationship between the various ERs, phylogeny analysis was carried out using the deduced whole receptor protein sequences. Analysis based on the SB ER amino acid sequences was subsequently performed to determine the evolutionary relationships between the various ERs. The consensus trees showed two main clades (Figure 3); one of them clusters with the ERα and the other branch clusters with the ERβ. The SB ER clustered with zebrafish ERα, goldfish ERα, and channel catfish ERα.

When the nucleotides and deduced amino acid sequences of SB ER were aligned to those from the other known species, the SB ER shared the closest nucleotide similarity (87.3%) with the ERα of goldfish (*Carassius auratus*) and the closest amino acid identity (88.0%) with the ERα of

zebrafish (*Danio rerio*) (Table 2). The amino acid sequences derived from the SB ER consisted of the nuclear receptor-characteristic domains A/B, C, D, E, and F (Figure 4). The DNA-binding domain (DBD) C shared the highest amino acid identity (81.7 - 100.0%) with the other ERs, whereas the ligand-binding domain (LBD) E and the other domains (A/B, D and F) were poorly conserved (55.7 - 94.9%) (Figure 4,5).

III. Tissue–Specific Expression of SB ER

We compared the expression pattern of SB ER in a variety of tissues such as brain (B), liver (L), Gill (Gi), gonads (Go; ovary or testis) and intestines (I) of the slender bitterling by real-time PCR. The SB ER mRNA was detected in the brain, liver, kidney, gill, gonads, and intestines (Figure 6). In particular, the liver showed the highest transcription level of ER (Figure 6).

DISCUSSION

Within the Organization for Economic Co-operation and Development (OECD) and the US Environmental Protection Agency (US EPA), zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*), for example, have been used as test species for the development of test guidelines for EDCs [19]. In this study, in order to identify the possibility of the slender bitterling as test species for EEDCs, in particular, we carried out a cloning and sequence characterization of the ER. Estrogens are important hormonal regulators of reproductive
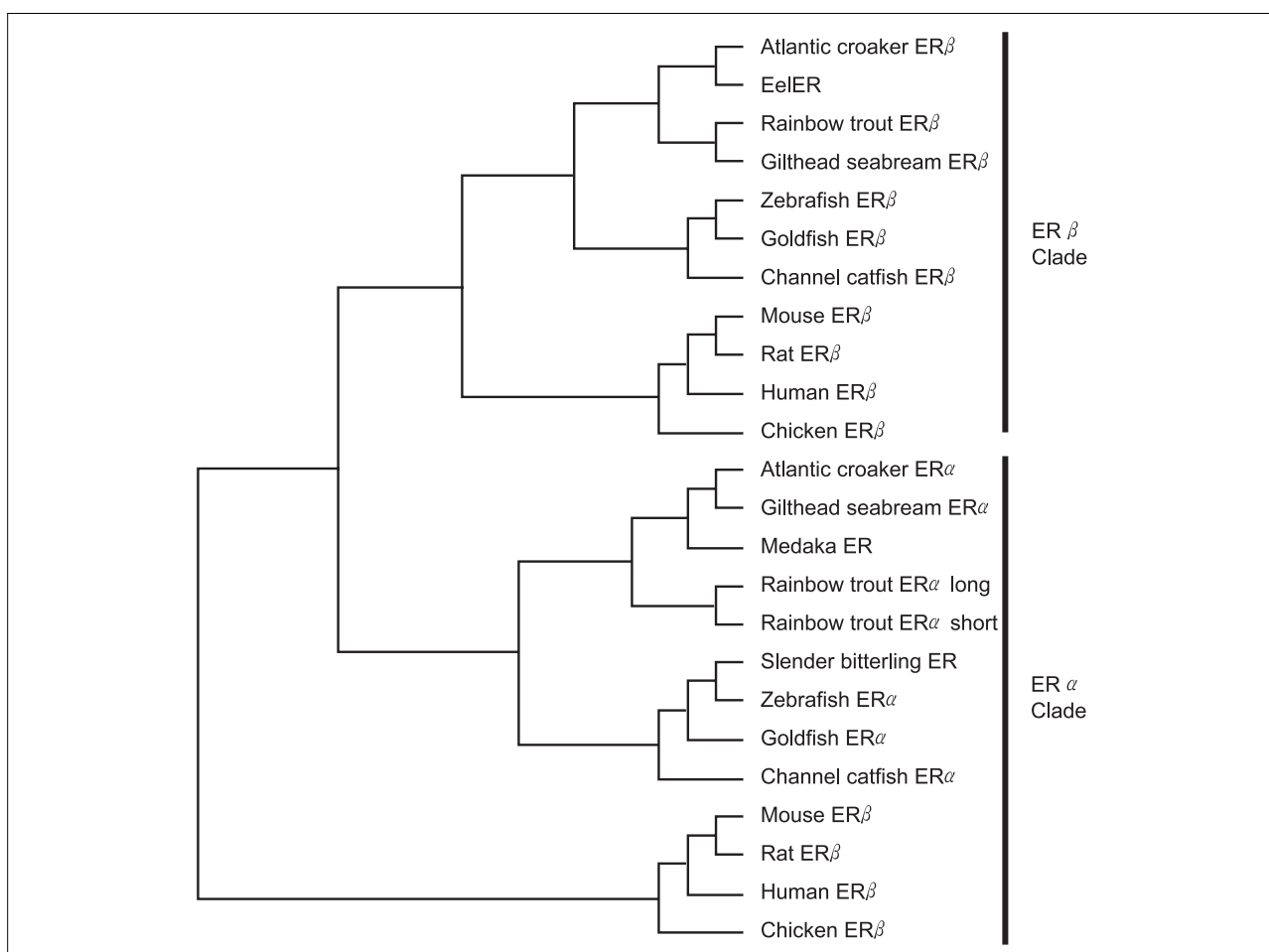
Figure 3. Phylogenetic analysis showing the relationships of the SB ER with other estrogen receptors from vertebrates. The phylogenetic analysis was carried out using Clustral W. Vertebrate ER sequences were obtained from the Genebank database. Genebank accession numbers of the sequences are: goldfish ERβ, AF177465; zebrafish ERβ, AJ275911; channel catfish ERβ, AF185568; Atlantic croaker ERβ, AF298181; eel ER, AB003356; rainbow trout ERβ, DQ177439; gilthead seabream ERβ, AF136980; mouse ERβ, NM207707; rat ERβ, NM012754; human ERβ, AF051427.1; chicken ERβ, NM204 794; gilthead seabream ERα, AF136979; medaka ER, AB033491; Atlantic croaker ERα, AF298183; rainbow trout ERα long, AJ242740; rainbow trout ERα short, AJ242741; zebrafish ERα, AF349412; goldfish ERα, AY344444; channel catfish ERα, NM001200074; rat ERα, NM012689; mouse ER α, NM007956; human ERα, NM_000125; chicken ERα, NM205183; slender bitterling ER, JF275853.

SB ER: slender bitterling estrogen receptor, ER: estrogen receptor.

Table 2. Nucleotide (open reading frame) and deduced amino acid identities (%) of the selected ER gene

Primer length (bp)	Slender bitterling ER	
	Nucleotide (%)	Deduced amino acid (%)
Goldfish ERα	87.5	85.0
Goldfish ERβ	43.8	41.0
Zebrafish ERα	86.7	88.0
Zebrafish ERβ	42.4	43.0
Human ERα	45.0	47.0
Human ERβ	44.9	45.3

ER: estrogen receptor.

processes in teleost fishes, including gonadal differentiation and development [20], and they mediate these activities through binding to specific intranuclear receptor proteins, the ERs [21].

In this study, we obtained a SB ER of 2189 bp in length, which contained a 1707 bp open reading frame that encoded 568 amino acid residues (Figure 2,5). The SB ERs are classified into six distinct domains depending on their function; A/B, C, D, E, and F domains [7,22]. The SB ER cDNA has also the ER-characteristic domains A/B, C, D, E, and F (Figure 2,4).

The N-terminal domain (A/B region) of nuclear receptors encodes a ligand-independent activation function (AF1), a region of the receptor involved in protein-protein interactions and transcriptional activation of target-gene expression [23]. The amino-terminal modulating A/B region (residues 1 - 149) of SB ER was found to have a low percentage of identity with human ERα (11.4%), but the SB

ER shared a higher degree of identity with the zebrafish ERα (83.6%) and the goldfish ERα (76.6%) (Figure 4). The A/B domain in the SB ER was also found to be rich in S and P amino acid residues, which was reported for other ER subtypes [24]. Phosphorylation of the S residue contained in the ERα is required for potential mitogen-activated protein kinase (MAPK) activity and a potential MAPK phosphorylation site ($PX_{1 \text{ or } 2} SP$) conserved in the A/B domain [24,25]. The SB ER A/B domain conserved a potential MAPK phosphorylation site (79-PQLSP-83), indicating the involvement of the MAPK pathway in the SB ER-induced response (Figure 5).

The C-terminal, E/F, of the LBD is ligand-dependent activation function (AF2), which mediates ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene expression. The C or DBD located in the middle of the ER is the most conserved region and contains two zinc-binding motifs that interact with specific DNA sequences known as ERE in the regulatory region of target genes [23].

The DBD, the C domain, is the most conserved region in ERs. The DBD of SB ER (residues 150 - 231) showed the most conserved region (81.7 - 100% identity) when compared to the corresponding regions of goldfish, zebrafish, and humans (Figure 4). The DBD contained the conserved features of this region, i.e. the eight cysteine residues (155aa, 158aa, 172aa, 175aa, 191aa, 197aa, 207aa, and 210aa; indicated by asterisks in Figure 5) of the two zinc-finger motifs and the P box (173 - 179aa) and D box
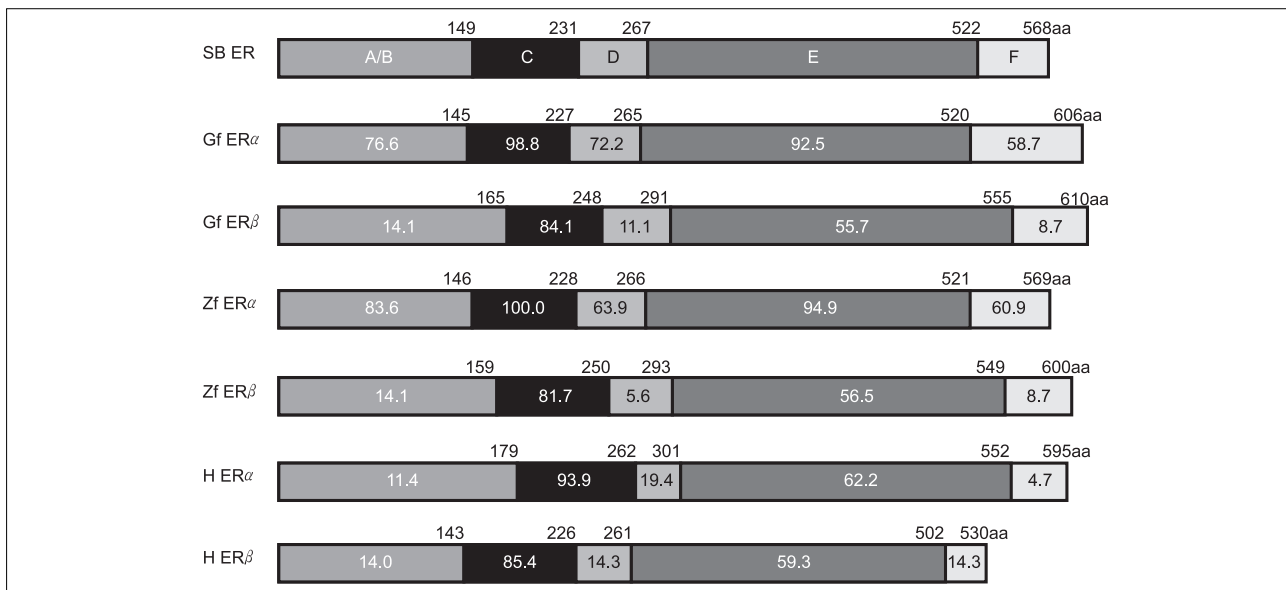
Figure 4. Schematic alignment of the slender bitterling ER and the ERs of other species. The domains of A/B, C, D, E, and F are indicated according to Krust et al. [7] and the numbers above the sequences indicate the positions of the amino acid residues for each region. The percentage identity of each domain relative to the SB ER is indicated within the box representing the corresponding domain. Data for comparisons are: goldfish ERα (AY344444), goldfish ERβ (AF177465), zebrafish ERα (AF349412), zebrafish ERβ (AJ275911), human ERα (NM_000125), human ERβ (AF051427.1), slender bitterling ER (JF275853).

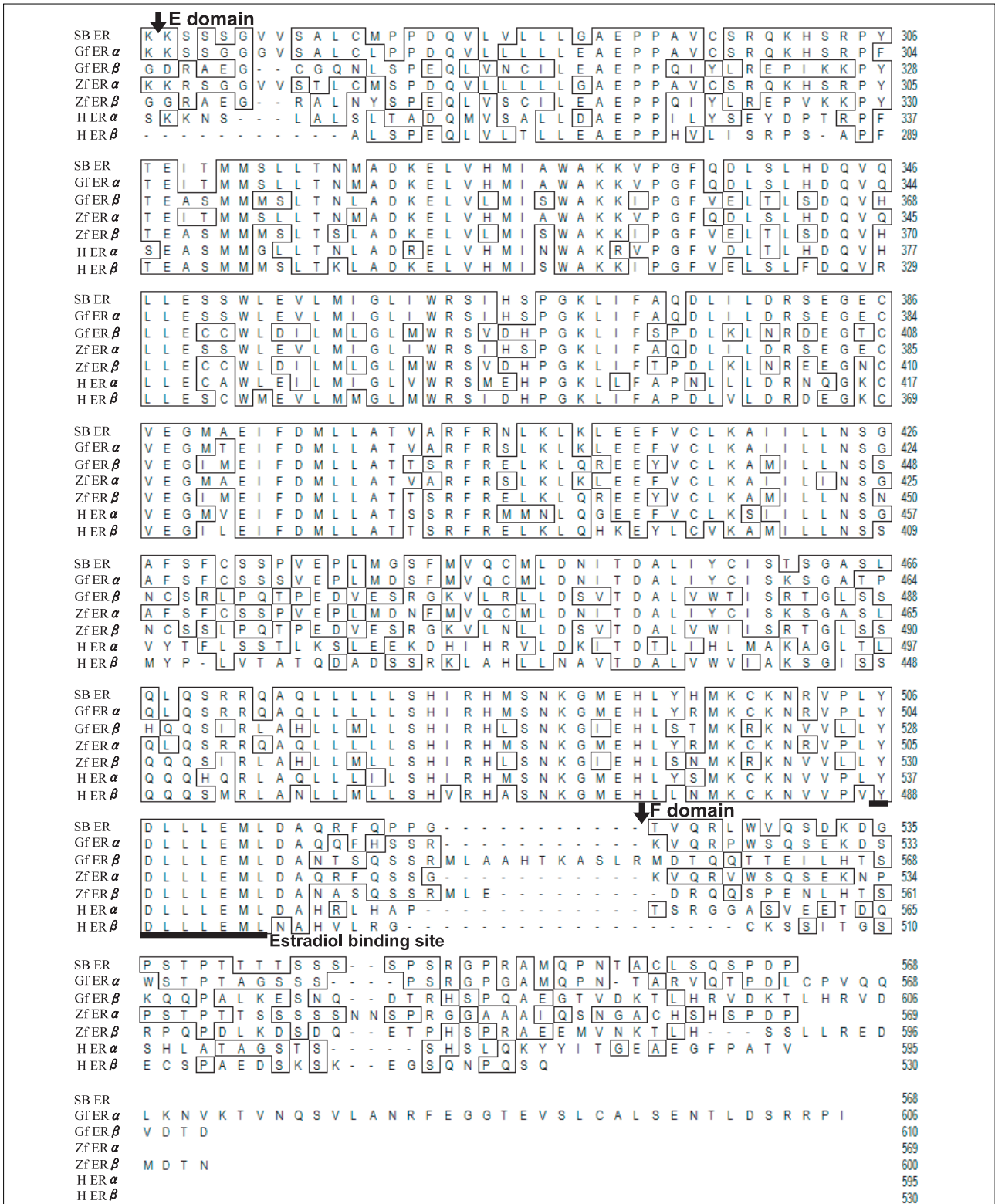ER: estrogen receptor, ERs: estrogen receptors, SB ER: slender bitterling estrogen receptor.

Figure 5. Amino acid alignment of SB ER (JF275853), Gf ERα (AY344444), Gf ERβ (AF177465), Zf ERα (AF349412), Zf ERβ (AJ275911), H ERα (NM_000125), and H ERβ (AF051427.1). The boundaries of the five distinct domains, A/B, C, D, E, and F are indicated by arrows according to Krust et al. [7]. In the A/B domains, the potential MAPK phosporylation (mitogen-activated protein kinase) site is upperlined. The potential AP-1 binding site is boxed in by a dashed line. The zinc-binding motifs containing eight cysteine residues (asterisks) are underlined (C domains). The P- and D-boxes are also indicated in the DNA-binding C domain. In the E/F binding domain, the estradiol binding site (YDLLLEML) is underlined.

SB ER: slender bitterling estrogen receptor, Gf: goldfish, Zf: zebrafish, H: human, AP-1: activating protein-1.

↓ E domain

↓ F domain

Estradiol binding site

Figure 5. Continued.

(192 - 196aa) (Figure 5), the last two being crucial for DNA-binding [26]. The SB ER also conserved a potential activating protein (AP)-1 binding site (CGACTAC instead of the TGA(GC)T(C/A)A) located at nucleotides 506 - 512 and amino acid sequence (DY) positions 160 - 161 (Figure 2,5). The DBD of the ER is required for activation at the AP-
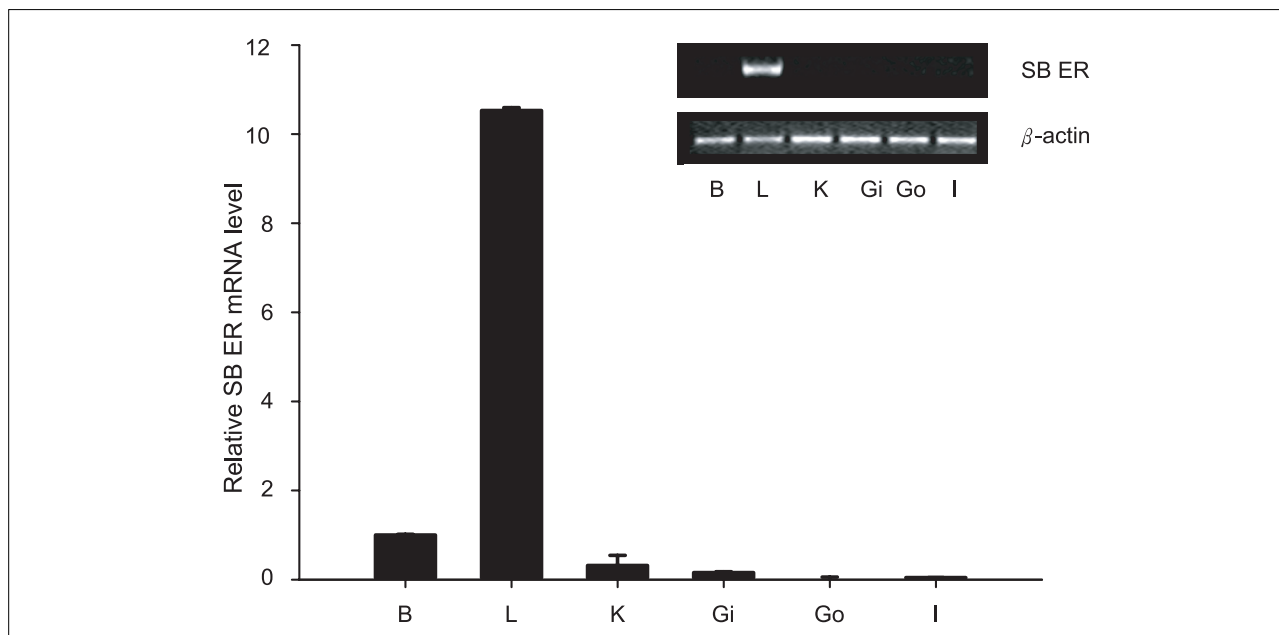
Figure 6. Tissue-specific expression of in ER mRNA in the slender bittering. Gene-specific primers were used to quantify mRNAs using real-time PCR. The results for the ER were normalized to β-actin and are shown as the average of two slender bitterlings. The relative mRNA expression for the ER was calculated as the fold change compared with brain ER levels. The results are expressed as mean ± standard deviation of three separate experiments for each data point. Brain (B), liver (L), kidney (K), gill (Gi), gonad (Go) and intestines (I).

ER: estrogen receptor, PCR: polymerase chain reaction.

1 site [27]. It is possible that the ER mediates synthesis of the AP-1 protein via protein-protein interactions and ER activation at the AP-1 site [27,28].

The LBD of the SB ER between 268aa and 522aa had 92.5%, 94.9% and 62.2% identity with the goldfish ERα, zebrafish ERα and the human ERα, respectively. The LBD of the SB ER had a low percentage identity (< 60%) with the ERβ of other species. In the LBD, the estrogen-dependent activation domain AF2 was present in the SB ER. The AF2 domain sequence of the SB ER, YDLLLEML, was located in the LBD at amino acid residues 506 - 513 (Figure 2,5), indicating that transcriptional activation is dependent on ligand binding as reported previously [22]. In addition, amino acids that have been shown to be crucial for estrogen binding in human ERs, i.e. hERα G521, H524, L525, and M528 [29,30], were all conserved in the SB ER (G490, H493, L494, and M497). Furthermore, amino acids of the human ERα involved in receptor dimerization and estrogen-dependent activation (i.e. H513 and Y537) [31,32] were observed in the SB ER (H482 and Y506, Figure 5). Migliaccio et al. [33] reported that the phosphorylation of tyrosine residues in the LBD may be coupled to ligand binding. Similar to mammalian ERα [16], which contains five tyrosine residues in goldfish (Accession number, AY055725) [34], the SB ER also contains five tyrosine residues (250aa, 307aa, 457aa, 495aa and 506aa) in the LBD (Figure 5). These tyrosine residues are conserved in similar positions among teleosts, and the tyrosine at position 495 is

conserved in the ERα of all vertebrates [16]; in particular, the tyrosine at position 506 is conserved in the ERα and ERβ of all vertebrates (Figure 5).

The slender bitterling showed the highest ER transcription level in the liver (Figure 6). The ER expression showed a similar pattern to that of goldfish [34]. The liver ER may play an important role in estrogenic reproductive activity via the ER-ERE pathway, such as vitellogenin synthesis. Kim et al. [35] reported that the vitellogenin mRNA level in the liver of slender bitterlings showed a significant increase upon exposure to ethinyl estradiol. These results indicate that the slender bitterling might be a suitable candidate species for toxicological research of EEDCs, although further studies are needed.

CONCLUSIONS

We obtained a full-length estrogen receptor (SB ER) cDNA sequence from the slender bitterling. The SB ER has an open reading frame of 1707 bp that encodes a 568 amino acid protein and has a theoretical molecular weight of 63.0 kDa. The SB ER amino acid sequences were found to cluster in a monophyletic group with the ERα of other fish, and they showed an 88% identity with zebrafish ERα. The SB ER cDNA was divided into A/B, C, D, E and F domains. The SB ER has conserved important sequences for ER functions such as the DNA binding domain (D domain),

which are consistent with those of other teleosts. The SB ERs identified in this study could provide basic information on toxicological studies of EEDCs in the slender bitterling

CONFLICT OF INTEREST

The authors have no conflict of interest to declare on this study.

REFERENCES

1. Matthiessen P, Sumpter JP. Effects of estrogenic substances in the aquatic environment. In: Braunbeck T, Hinton DE, Streit B, editors. Fish ecotoxicology. Basel: Birkhäuser; 1998. p. 319-335.
2. Sumpter JP, Johnson AC. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ Sci Technol 2005; 39(12): 4321-4332.
3. de Vlaming V, Biales A, Riordan D, Markiewicz D, Holmes R, Otis P, et al. Screening California surface waters for estrogenic endocrine disrupting chemicals (EEDC) with a juvenile rainbow trout liver vitellogenin mRNA procedure. Sci Total Environ 2007; 385(1-3): 66-79.
4. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 1995; 103(Suppl 7): 113-122.
5. Fu KY, Chen CY, Chang WM. Application of a yeast estrogen screen in non-biomarker species Varicorhinus barbatulus fish with two estrogen receptor sutypes to assess xenoestrogens. Toxicol In Vitro 2007; 21(4): 604-612.
6. Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, et al. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 1986; 320(6058): 134-139.
7. Krust A, Green S, Arogos P, Kumar V, Walter P, Bornert JM, et al. The chicken oestrogen receptor sequence: homology with v-er bA and the human oestrogen and glucocorticoid receptors. EMBO J 1986; 5(5): 891-897.
8. Koike S, Sakai M, Muramatsu M. Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res 1987; 15(6): 2499-2513.
9. Pakdel F, Féon S, Le Gac F, Le Menn F, Valotaire Y. In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol Cell Endocrinol 1991; 75(3): 205-212.
10. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996; 93(12): 5925-5930.
11. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 1996; 392(1): 49-53.
12. Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci USA 2000; 97(20): 10751-10756.
13. Ma CH, Dong KW, Yu KL. cDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (Carassius auratus). Biochem Biophys Acta 2000; 1490(1-2): 145-152.
14. Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, et al. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol Reprod 2002; 66(6): 1881-1892.
15. Touhata K, Kinoshita M, Toyohara H, Sakaguchi M. Sequence and expression of a cDNA encoding the red seabream estrogen receptor. Fish Sci 1998; 64(1): 131-135.
16. Muñoz-Cueto JA, Burzawa-Gérard E, Kah O, Valotaire Y, Pakdel F. Cloning and sequencing of the gilthead seabream estrogen receptor cDNA. DNA Seq 1999; 10(2): 75-84.
17. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22(22): 4673-4680.
18. Zhang YB, Hu CY, Zhang J, Huang GP, Wei LH, Zhang QY, et al. Molecular cloning and characterization of crucian carp (Carassius auratus L.) interferon regulatory factor 7. Fish Shellfish Immunol 2003; 15(5): 453-466.
19. Organization for Economic Cooperation and Development (OECD). Fish early life-stage toxicity test, test guideline 2010. OECD guidelines for the testing of chemicals. Paris: OECD; 1992.
20. Strüssmann CA, Nakamura M. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol Biochem 2002; 26(1): 13-29.
21. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 1999; 20(3): 358-417.
22. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 1987; 51(6): 941-951.
23. Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. Physiol Rev 2001; 81(4): 1535-1565.
24. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, et al. Activation of the estrogen receptor through phosphorylation by mitogen activated protein kinase. Science 1995; 270(5241): 1491-1494.
25. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, et al. Cloning, chromosomal localization, and functional analysis of the murine estrogen recetor beta. Mol Endocrinol 1997; 11(3): 353-365.
26. Härd T, Gustafsson JA. Structure and function of the DNA-binding domain of the glucocorticoid receptor and other

members of the nuclear receptor supergene family. Acc Chem Res 1993; 26(12): 644-650.

27. Webb P, Lopez GN, Uht RM, Kushner PJ. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 1995; 9(4): 443-456.

28. Philips A, Chalbos D, Rochefort H. Estradiol increases and anti-estrogens antagonize the growth factor-induced activator protein-1 activity in MCF7 breast cancer cells without affecting c-fos and c-jun synthesis. J Biol Chem 1993; 268(19): 14103-14108.

29. Ekena K, Weis KE, Katzenellenbogen JA, Katzenellenbogen BS. Identification of amino acids in the hormone binding domain of the human estrogen receptor important in estrogen binding. J Biol Chem 1996; 271(33): 20053-20059.

30. Halm S, Martinez-Rodriguez G, Rodriguez L, Prat F, Mylonas CC, Carrillo M, et al. Cloning, characterization, and expression of three oestrogen receptors (ERalpha, ERbeta1 and ERbeta2) in the European sea bass, Dicentrarchus labrax. Mol Cell Endocrinol 2004; 223(1-2): 63-75.

31. Fawell SE, Lees JA, White R, Parker MG. Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell 1990; 60(6): 953-962.

32. Arnold SF, Melamed M, Vorojeikina DP, Notides AC, Sasson S. Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. Mol Endocrinol 1997; 11(1): 48-53.

33. Migliaccio A, Di Momenico M, Green S, de Falco A, Kajtaniak EL, Blasi F, et al. Phosphorylation on tyrosine of in vitro synthesized human estrogen receptor activates its hormone binding. Mol Endocrinol 1989; 3(7): 1061-1069.

34. Choi CY, Habibi HR. Molecular cloning of estrogen receptor alpha and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol Cell Endocrinol 2003; 204(1-2): 169-177.

35. Kim HR, Park YJ, Kim JG, Chung KH, Oh SM. Molecular cloning of vitellogenin gene and mRNA expression by 17alpha-ethinylestradiol from slender bitterling. Gen Comp Endocrinol 2010; 168(3): 484-495.