

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

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Objectives: In order to identify the possibility of striped 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 striped 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 striped 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 ER α of other fish, and was more closely related to zebrafish ER α (88% identity) than to the ER α 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 striped bitterling could provide basic information in toxicological studies of EEDCs in the striped bitterling.

Key words: Estrogen receptor, Estrogenic endocrine disrupting effects, Gene cloning, Striped 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 striped 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 striped 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, ER α and ER β , have been reported in humans and mice [6-11], while three functional ER forms, ER α , ER β 1

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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 ER β 2, 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 striped bitterling. We therefore cloned and sequenced the ER gene from striped bitterlings to identify characteristics of this gene that could play important roles in the toxicity of EEDCs.

MATERIALS AND METHODS

1. Fish and RNA Isolation

Striped 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 °C. 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 striped 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 striped 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 μ L, which contained 4 μ L of 5×RT Buffer, 9 U AMV reverse transcriptase, 20 U of an RNase inhibitor, 1 /L of 10 mM dNTPs and 0.5 /g/mL of random hexamers. The following RT conditions were used: room temperature for 10 minutes, 42 °C for 50 minutes, and 94 °C for 5 minutes. The PCR was conducted with 2 μ L of the first-strand cDNA in a final volume of 25 μ L, which also contained 2.5 μ L of 10×Ex-Taq Buffer, 1 μ L of 2.5 mM dNTPs, 2.5 units of Ex-Taq polymerase, and the primers (NF/NR). The following PCR conditions were used: 94 °C for 2 minutes followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 minutes, and a final extension step of 72 °C 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°C for 1 minutes followed by 30 cycles of 94°C for 30 seconds, and 68°C 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 striped 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 striped 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

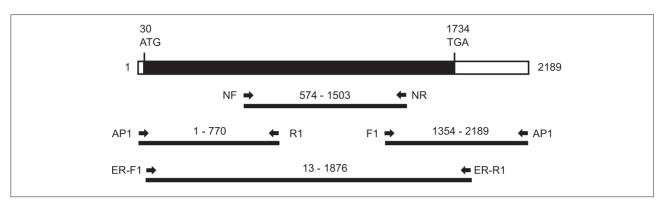


Figure 1. Cloning strategy and schematic view of SB ER cDNAs. The open reading frame is represented by a thick black line. The positions of the start codon (ATG) and the stop codon (TGA) are indicated. The binding sites of two degenerated primers (NF and NR) and four gene-specific primers (R1, F1, ER-F1, 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: striped 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°C 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 μ L 2×SYBR[®] Premix Ex-TaqTM (Takara Bio Inc., Japan), and 1 μ L 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 °C, 10 seconds at 51 °C (β -actin, 200 bp) or 42 $^{\circ}$ C (ER, 258 bp), and 15 seconds at 72 $^{\circ}$ C 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

1. Identification of ERs from the Striped Bitterling

Liver poly (A)+from the striped 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 ER α 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 striped 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.

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nt		
-29	LAB domain	
1	ATGTACCCCAAGGAGGAGGAGCACAGCGCAGCGGACATCAGCTCCTCTGTCAATTACCTTGATGGAGCCTATGAGTACCCAGAACCCACACAG	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	ACCTACGCCACCTCATCACCCGCAGAGCCTGCCTCTGTCGGATACTACCCCGGCTCCCATGGACCCCCCGCAGCCCCCAGTGAAGAACAT	
	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	
074	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	CACCACTCGACCCACCAGGTGTCCTACTACCTGGACACCTCTTCTAGCACAGGTGTACAGGTCCAGTGTGCTGTCCTCTCAGCAGGCAG	100
	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
361		domain
301	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	GAGACGCGCCTCTGTGCGGTGTGCAGCGACTACGCCTCTGGCCTATCATTATGGAGTCTGGTCCTGTGAGGGATGCAAAGCTTTCTTCAAG	100
	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	AGAAGCATTCAAGGTCACAATGACTATGTTTGTCCAGCAACCAAC	
	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	AGACTACGCAAGTGTTATGAAGTAGGCATGATGAAAGGAGGATATTCGTAAGGACCGCGGTGGCCGCACTATCAGGCGTGAGAGAGGAGG	
	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 R	240
704	E doma	in
721	AGTGGTAATGAGGATCGTGACAAGAGCTACAGTGAGCAGTCGAGCCGTACTGGACTGAGGACAGCTCAGAACAAGAGGAAGÃAGAGCAGC	070
811	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 Agcggggtggttagcgcttatgccatgccacctgaccaggtgctggtgttgcttctggggtgcagagcctccggctgttcatgtcacgtcag	270
011	S G V V S A L C M P P D Q V L V L L L G A E P P A V C S R Q	300
901	AAGCACAGCCGCCCGTACACTGAGATCACCCTGATGATGTCCCTACTCACAAACATGGCTGACAAGGAACTCGTCCACATGATTGCCTGGGCT	300
001	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	ATATGGAGGTCCATTCACTCACCTGGAAAACTCATCTTTGCTCAGGATCTCATCCTTGATAGGAGGCGAAGGAGAATGTGTCGAGGGGGATG	
	IWRSIHSPGKLIFAQDLILDRSEGECVEGM	390
1171	GCTGAGATTTTCGACATGCTTTTGGCGACTGTGGCTCGATTTCGCAACCTTAAACTCAAGCTGGAGGAATTTGTATGCCTTAAAGCCATC	
1001	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	ATACTTCTCAATTCTGGTGCATTTTCATTCTGCTCCAGTCCAGTCGAGCCCCCTGATGGGCAGCTTCATGGTGCAGTGTATGCTGGACAAC	450
1351	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 Atcactgatgccctcatttactgcatcagtacatcaggtgcctcactgcagtccgcagtcccgccgtcaggcgcagctcctgctgctgctc	450
1301	ITDALIYCISTSGASSQATAATAATAATAATAATAATAATAATAATAATAATAATA	480
1441	TCCCACATCAGACACATGAGCAACAAAGGAATGGAGCACTTATACCATATGAAAATGTAAGAATCGAGTCCCGCTGTACGATCTTTTGCTG	400
1111	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	GAGATGCTGGATGCCCAGCGATTCCAACCCCCAGGAACGGTGCAGCGACTGTGGGTACAGAGTGACAAAGACGGCCCTTCTACACCCCACA	
	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 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	TTTTGAAGGTGGAATTGAATTTGAGTCTCTCCGTGTGCCTTATCAGAGTCCACTTTAGATTTCCGTCCAGAAATGAACACACATCTGAAAAT	
1891	GAACCTATTTGTAAAACACACAGGTTAGGTTTTCAAATAGTAACAAGGCTCCCTAAAACTATACATCTATGGTCACTTGATTCATGAGTT	
1981	GTTGTCAAATCTTATTACAGGGATTCAAAGGACATTTTCTCGTAGATTTATGAAAAAGAGCTCTTTTTGGAGAGAGA	
2071	GCTGCTAGTTCTTATTTTCTTTTAACATCTATTGAGCGGCGGTTTTAACGTCACAAATAAAAAAAA	

Figure 2. Nucleotide and deduced amino acid sequences of the striped 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 ER α and the other branch clusters with the ER β . The SB ER clustered with zebrafish ER α , goldfish ER α , and channel catfish ER α .

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 ER α of goldfish (*Carassius auratus*) and the closest amino acid identity (88.0%) with the ER α of zebrafish (*Danio rerio*) (Table 2). The amino acid sequences derived from the SB ER consisted of the nuclear receptorcharacteristic 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 striped 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 striped 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

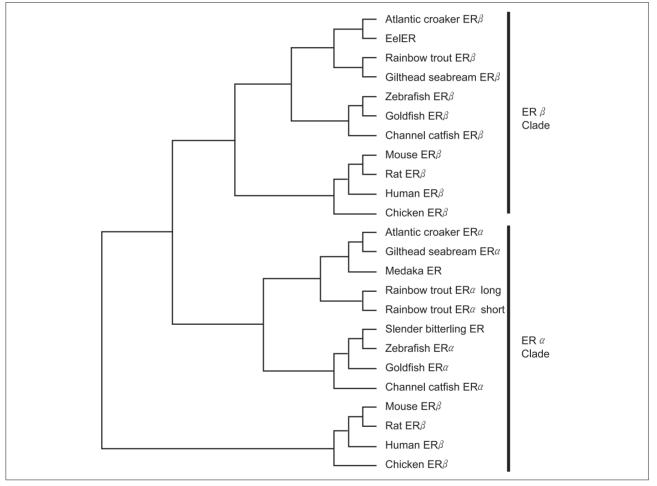


Figure 3. Phylogenetic analysis showing the relationships of the SB ER with other estrogen receptors from vertebrates. The phylogenetic analysis was carried out using Clustral W. Vertebrate ER sequences were obtained from the Genebank database. Genebank accession numbers of the sequences are: goldfish ER β , AF177465; zebrafish ER β , AJ275911; channel catfish ER β , AF185568; Atlantic croaker ER β , AF298181; eel ER, AB003356; rainbow trout ER β , D0177439; gilthead seabream ER β , AF136980; mouse ER β , NM207707; rat ER β , NM012754; human ER β , AF051427.1; chicken ER β , NM204 794; gilthead seabream ER α , AF136979; medaka ER, AB033491; Atlantic croaker ER α , AF298183; rainbow trout ER α long, AJ242740; rainbow trout ER α short, AJ242741; zebrafish ER α , AF349412; goldfish ER α , AY344444; channel catfish ER α , NM001200074; rat ER α , NM012689; mouse ER α , NM007956; human ER α , NM00125; chicken ER α , NM205183; striped bitterling ER, JF275853.

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

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

Primer	Striped bitterling ER		
length (bp)	Nucleotide (%)	Deduced amino acid (%)	
Goldfish ER α	87.5	85.0	
Goldfish ER β	43.8	41.0	
Zebrafish ER α	86.7	88.0	
Zebrafish ER β	42.4	43.0	
Human ER α	45.0	47.0	
Human $\text{ER}\beta$	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 ER α (11.4%), but the SB ER shared a higher degree of identity with the zebrafish ER α (83.6%) and the goldfish ER α (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 ER α is required for potential mitogen-activated protein kinase (MAPK) activity and a potential MAPK phosphorylation site (PX1 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

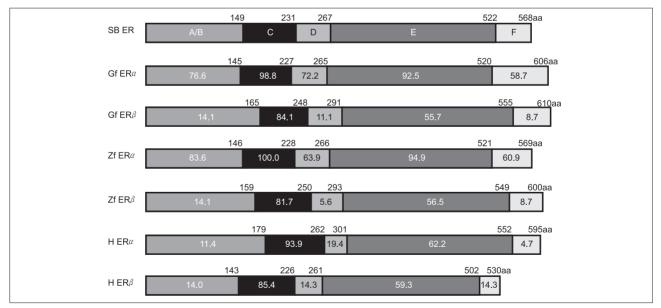


Figure 4. Schematic alignment of the striped bitterling ER and the ERs of other species. The domains of A/B, C, D, E, and F are indicated according to Krust et al. [7] and the numbers above the sequences indicate the positions of the amino acid residues for each region. The percentage identity of each domain relative to the SB ER is indicated within the box representing the corresponding domain. Data for comparisons are: goldfish ER α (AY344444), goldfish ER β (AF177465), zebrafish ER α (AF349412), zebrafish ER β (AJ275911), human ER α (NM_000125), human ER β (AF051427.1), striped bitterling ER (JF275853).

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

	↓ A/B domain
SB ER Gf ER α Gf ER β Zf ER α H ER α H ER β	- M Y P K E E H S A A D I S S S V N - - - - - - - - - - 17 - M Y P K E E H S V G G I S S S V N - - - - - - 17 - M S S T G P A P A S A S P V Q A N R G N S P N I L P L L Y T S - - - 32 - M Y P K E E H S A G G I S S S V N - - - - 32 - M Y P K E E H S A G G I S S S V N - - - - - 32 - M Y P K E E H S A G G I S S S V N - - - - - 32 - M S S S P G P A P V L D S S K A D R G A S P A L L P R L Y A S - - - 31 M T M T L H T K A S G M A L L H Q I Q G N E L E P L N R P Q L K I P L E R P L G 40 - M D I K N S P S S L N S P - - S S Y N C S Q S I L P - - - 24
SB ER Gf ER α Zf ER β Zf ER α H ER α H ER β	Y L D G A Y E Y P E - P T Q T Y A T S S P A E P A S V G Y Y P A P M 50 Y L D G S Y E F P N - P T Q T Y G T S S P A E P A S V G Y Y P A P P 50 Q L G M D S Q T I C I P S P Y V E A C Q D Y S P P H G G E I S H G A L T L 69 Y L D G A Y E Y P N - P T Q T F G T S S P A E P A S V G Y Y P A P P 50 P L G M D N Q T V C I P S P Y V E A C Q D Y S P P H G G E F N H G A L T L 68 E V Y L D S S K P A V Y N Y P E G A A Y E F N A A A A A N A Q V Y G Q T G L P Y 80 L E H G S I Y P S S Y V D S H H E Y - P - A M T F Y S P A V M N 55 MAPK
SB ER Gf ER α Zf ER β Zf ER α H ER α H ER β	D L HA A P S E E H L Q T I D A G S S S P I M F A P A S P Q L S P Y 84 D P H V P P V E E H L Q S L G S P R I F A P S S P Q L S P Y 80 Y S P V S S P V L G Y T H P P V S E S L V P L N S A I F W P P H P T H S T P 107 D P H E E H L Q T L G G G S S S P L M F A P S S P Q L S P Y 80 Y S P V S S A V L G F H R P P V S E S L V P L N S A I F W P P H P T H S T P 107 G P G S E A A A F G S N G L G G F P P L N S V S P S P L M L L H P P P Q L S P F 120 Y S - I P S N V T N L E G G P G R Q T T S P N V L W P T P G H L S P L 89
SB ER Gf ER α Zf ER β Zf ER α H ER α H ER α H ER β	PSHHGGHHST-HQVSYYLDTSSSTVYRSSVLSSQQAGVGL 123 LSHPGGHPST-HQASYYLDTPSSSLYRSSVVSSQQAGGGL 119 SLHCPSPLAYRETHAHTTWEDAKTH-INQSSSV 139 LSHHGGHHTTPHQVSYYLDSSSSTVYRSSVVSSQQAAVGL 120 -LHCP
SB ER Gf ER α	CEVLCSATNRQELYAGSRAAGGFDSEKETRFCAVCSDYAS 163 CEELCSASDRQEMYSGSRGTGGFDSEKETRFCAVCSDYAS 159
Gf ER β Zf ER α Zf ER β H ER α H ER β	L T H A K L L G Q Q L D G D D G L N P S P G I L G K G D T H F C A V C H D Y A S 179 C E E L C S A T D R Q E L Y T G S R A A G G F D S G K E T R F C A V C S D Y A S 160 L S H T K L L G Q Q L E G D N G L N P S A S I V G K G D T H F C A V C H D Y A S 173 S D N R R Q G G R E R L A S T N D K G S M A M E S A K E T R Y C A V C N D Y A S 193 P V N R E T L K R K V S G N R C A S P V T G P G S K R D A H F C A V C S D Y A S 157
Gf ER β Zf ER α Zf ER β H ER α	L T H A K L L G Q Q L D G D D G L N P S P G I L G K G D T H F C A V C H D Y A S 179 C E E L C S A T D R Q E L Y T G S R A A G G F D S G K E T R F C A V C S D Y A S 160 L S H T K L L G Q Q L E G D N G L N P S A S I V G K G D T H F C A V C H D Y A S 173 S D N R R Q G G R E R L A S T N D K G S M A M E S A K E T R Y C A V C N D Y A S 193 P V N R E T L K R K V S G N R C A S P V T G P G S K R D A H F C A V C S D Y A S 193 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 199 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 199 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 203 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 204 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 205 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 205 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 205 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K S R 219 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K S R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y U C P A T N Q C T I D K S R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y U C P A T N Q C T I D K N R 203 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y U C P A T N Q C T I D K N R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R 233 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R 233 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R 233 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R 233 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R 233
Gf ER β Zf ER α H ER α H ER β SB ER Gf ER α Gf ER β Zf ER β Zf ER β H ER α	L T H A K L L G Q Q L D G D D G L N P S P G I L G K G D T H F C A V C H D Y A S 179 C E E L C S A T D R Q E L Y T G S R A A G G F D S G K E T R F C A V C S D Y A S 160 L S H T K L L G Q Q L E G D N G L N P S A S I V G K G D T H F C A V C H D Y A S 173 S D N R R Q G G R E R L A S T N D K G S M A M E S A K E T R Y C A V C N D Y A S 193 P V N R E T L K R K V S G N R C A S P V T G P G S K R D A H F C A V C S D Y A S 193 F V N R E T L K R K V S G N R C A S P V T G P G S K R D A H F C A V C S D Y A S 193 C Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 199 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D R N R 199 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 199 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 219 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 219 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 219 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 219 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 210 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y V C P A T N Q C T I D K R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y U C P A T N Q C T I D K R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y U C P A T N Q C T I D K R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y U C P A T N Q C T I D K R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y W C P A T N Q C T I D K R 213 G Y H Y G V W S C E G C K A F F K R S I Q G H N D Y W C P A T N Q C T I D K R 233

Figure 5. Amino acid alignment of SB ER (JF275853), Gf ER α (AY344444), Gf ER β (AF177465), Zf ER α (AF349412), Zf ER β (AJ275911), H ER α (NM_000125), and H ER β (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: striped bitterling estrogen receptor, Gf: goldfish, Zf: zebrafish, H: human, AP-1: activating protein-1.

	SB ER Gf ER $\boldsymbol{\alpha}$ Gf ER $\boldsymbol{\beta}$ Zf ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\beta}$	E domain K K S S 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 K H S R P Y K K S S S G G V S A L C L P P D Q V L L L L E A E P P A V C S R Q K H S R P F G D R A E G - C G Q N L S P E Q L V N C I L E A E P P Q I Y L R E P I K K P Y K K R S G G V V S T L C M S P D Q V L L L L G A E P P A V C S R Q K H S R P Y G G R A E G - R A L N Y S P E Q L V S C I L E A E P P Q I Y L R E P V K K P Y S K K N S L A L S L T A D Q M V S A L L D A E P P I L Y S E Y D P T R P F T A L S P E Q L V L T L L E A E P P H V L I S R P S - A P F	306 304 328 305 330 337 289
	SB ER Gf ER α Gf ER β Zf ER α Zf ER β H ER α H ER β	T E I T M M S L L T N M A D K E L V H M I A W A K K V P G F Q D L S L H D Q V Q T E I T M M S L L T N M A D K E L V H M I A W A K K V P G F Q D L S L H D Q V Q T E A S M M M S L T N L A D K E L V L M I S W A K K I P G F V E L T L S D Q V H T E I T M M S L L T N M A D K E L V L M I S W A K K V P G F Q D L S L H D Q V Q T E A S M M M S L T S L A D K E L V L M I S W A K K I P G F V E L T L S D Q V H S E A S M M M S L T N L A D R E L V H M I N W A K R V P G F V E L T L S D Q V H T E A S M M M S L T N L A D K E L V H M I S W A K K I P G F V E L T L S D Q V H S E A S M M M S L T K L A D K E L V H M I S W A K K I P G F V E L S L F D Q V R	346 344 368 345 370 377 329
	SB ER Gf ER α Gf ER β Zf ER α Zf ER β H ER α H ER β	L L E S S W L E V L M I G L 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 L L E S S W L E V L M I G L 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 L L E C C W L D I L M L G L M W R S V D H P G K L I F S P D L K L N R D E G T C L L E S S W L E V L M I G L 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 L L E C C W L D I L M L G L M W R S V D H P G K L I F A Q D L I L D R S E G E C L L E C C W L D I L M L G L M W R S V D H P G K L I F T P D L K L N R E E G N C L L E C C W L D I L M L G L M W R S V D H P G K L I F T P D L K L N R E E G N C L L E C A W L E I L M I G L V W R S M E H P G K L L F A P N L L L D R N Q G K C L L E S C W M E V L M M G L M W R S I D H P G K L I F A P D L V L D R D E G K C	386 384 408 385 410 417 369
	SB ER Gf ER α Gf ER β Zf ER α H ER α H ER β	V E G M 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 I L L N S G V E G MT E I F D M L L A T V A R F R S L K L K L E E F V C L K A I I L L N S G V E G I M E I F D M L L A T T S R F R E L K L Q R E E Y V C L K A MI L L N S S V E G M A E I F D M L L A T V A R F R S L K L K L E E F V C L K A I I L I N S G V E G I M E I F D M L L A T T S R F R E L K L Q R E E Y V C L K A MI L L N S G V E G I M E I F D M L L A T T S R F R E L K L Q R E E Y V C L K A MI L L N S N V E G M Y E I F D M L L A T T S R F R E L K L Q R E E Y V C L K A MI L L N S G V E G M V E I F D M L L A T T S R F R E L K L Q H K E Y L C V K A MI L L N S G	426 424 448 425 450 457 409
	SB ER Gf ER α Gf ER β Zf ER α Zf ER β H ER α H ER β	A F S F C S S P V E P L M G S F M V Q C M L D N I T D A L I Y C I S T S G A S L A F S F C S S S V E P L M D S F M V Q C M L D N I T D A L I Y C I S K S G A T P N C S R L P Q T P E D V E S R G K V L R L L D S V T D A L V W T I S R T G L S S A F S F C S S P V E P L M D N F M V Q C M L D N I T D A L I Y C I S K S G A S L N C S S L P Q T P E D V E S R G K V L N L L D S V T D A L V W I I S R T G L S S V Y T F L S S T L K S L E E K D H I H R V L D K L T D T L I H L M A K A G L T L M Y P - L V T A T Q D A D S S R K L A H L L N A V T D A L V W V I A K S G I S S	466 464 488 465 490 497 448
	SB ER Gf ER $\boldsymbol{\alpha}$ Gf ER $\boldsymbol{\beta}$ Zf ER $\boldsymbol{\alpha}$ Zf ER $\boldsymbol{\beta}$ H ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\beta}$	Q L Q S R R Q A Q L L L L L 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 Q L Q S R R Q A Q L L L L L S H I R H M S N K G M E H L Y R M K C K N R V P L Y H Q Q S I R L A H L L M L L S H I R H L S N K G I E H L S T M K R K N V V L L Y Q L Q S R R Q A Q L L L L L S H I R H M S N K G M E H L Y R M K C K N R V P L Y Q Q Q S I R L A H L L M L L S H I R H M S N K G M E H L Y R M K C K N V V L L Y Q Q Q S I R L A H L L M L L S H I R H M S N K G M E H L Y S M K C K N V V L L Y Q Q Q S M R L A Q L L L I L S H I R H M S N K G M E H L Y S M K C K N V V P L Y Q Q Q S M R L A N L L M L L S H V R H A S N K G M E H L L N M K C K N V V P V Y	506 504 528 505 530 537 488
	SB ER Gf ER α Gf ER β Zf ER α Zf ER β H ER α H ER β	DLLLEMLDAQRFQPPG	535 533 568 534 561 565 510
	SB ER Gf ER $\boldsymbol{\alpha}$ Gf ER $\boldsymbol{\beta}$ Zf ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\alpha}$	P S T P T 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 W S T P T A G S S S P S R G P R A M Q P N - T A R V Q T P D L C P V Q Q K Q Q P A L K E S N Q D T R H S P Q A E G T V D K T L H R V D K T L H R V D P S T P T T S S S S N N S P R G G A A A I Q S N G A C H S H S P D P R P Q P D L K D S D Q E T P H S P R A E E M V N K T L H S S L L R E D S H L A T A G S T S S H S L Q K Y Y I T G E A E G F P A T V E C S P A E D S K S K E G S Q N P Q S Q	568 568 606 569 596 595 530
	SB ER Gf ER $\boldsymbol{\alpha}$ Gf ER $\boldsymbol{\beta}$ Zf ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\alpha}$ H ER $\boldsymbol{\beta}$	L K N V K T V N Q S V L A N R F E G G T E V S L C A L S E N T L D S R R P I V D T D M D T N	568 606 610 569 600 595 530
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Figure 5. Continued.

(192 - 196aa) (Figure 5), the last two being crucial for DNAbinding [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 - 512and amino acid sequence (DY) positions 160 - 161 (Figure 2,5). The DBD of the ER is required for activation at the AP-

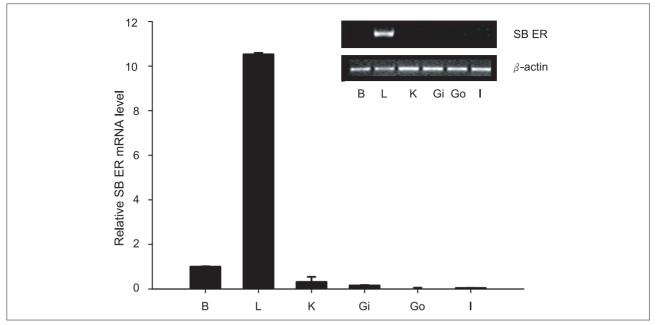


Figure 6. Tissue-specific expression of in ER mRNA in the striped bittering. Gene-specific primers were used to quantify mRNAs using real-time PCR. The results for the ER were normalized to β -actin and are shown as the average of two striped bitterlings. The relative mRNA expression for the ER was calculated as the fold change compared with brain ER levels. The results are expressed as mean \pm 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 $ER\alpha$, zebrafish ER α and the human ER α , respectively. The LBD of the SB ER had a low percentage identity (< 60%) with the $ER\beta$ 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. hER α 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 ER α involved in receptor dimerization and estrogendependent 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 $ER\alpha$ [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 ER α of all vertebrates [16]; in particular, the tyrosine at position 506 is conserved in the ER α and ER β of all vertebrates (Figure 5).

The striped 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 striped bitterlings showed a significant increase upon exposure to ethinyl estradiol. These results indicate that the striped 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 striped 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 ER α of other fish, and they showed an 88% identity with zebrafish ER α . 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 striped bitterling

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CONFLICT OF INTEREST

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

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