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ORIGINAL PAPER

Molecular cloning and tissue distribution of three estrogen receptors from the cyprinid fish *Varicorhinus barbatulus*

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Abstract We present molecular cloning and tissue expression analysis of three estrogen receptor (ER) subtypes, *vbERα*, *vbERβ*1 and *vbERβ*2, from liver of the cyprinid fish *Varicorhinus barbatulus* through reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The sequence alignment and phylogenetic analysis reconfirmed the evolutionary relationship of *V. barbatulus* within the family Cypriniformes. Directional constraints for subtype-specific substitution of critical amino acids were observed in the E2 binding region. For amino acid substitution, *vbERβ* exhibited a M517L change in the ligand-dependent transactivation region. The tissue distributions were investigated using RT-PCR with subtype-distinguishable primers. Both *vbERα* and *vbERβ*1 were most highly expressed in liver, while *vbERβ*2 was higher in intestine. Here we demonstrate that the identification and cloning of *ER* subtypes using PCR is feasible in wildlife in that the temporal and spatial observations are consistent with those from phylogeny analysis and crystal structural investigation by others.

Keywords Estrogen receptor subtype cloning · Phylogenetic analysis · Tissue distribution · RT-PCR · *Varicorhinus barbatulus*

Abbreviations

AF	Activation function
CK-II	Casein-kinase II
DBD	DNA binding-domain
ER	Estrogen receptor
ERR	Estrogen-related receptor
LBD	Ligand binding-domain
MAPK	Mitogen-activated protein kinase
NCBI	National Center for Biotechnology Information
PKC	Protein kinase C
RACE	Rapid amplification of cDNA end
RT-PCR	Reverse transcription-polymerase chain reaction
vbER	Estrogen receptor of *Varicorhinus barbatulus*

Introduction

Estrogen receptors (ERs) are members of the steroid hormone receptor family that are capable of binding ligands as receptors and transactivating genes as transcription factors involved in growth, development and differentiation of many reproductive and non-reproductive tissues. In response to endocrine signals such as 17*β*-estradiol, *ER*s can act through transcription activation (genomic) and/or cytosolic (non-genomic) signaling pathways (Nilsson et al. 2001). Stereotypic structures of *ER*s are recognizable by their A to F domains from N- to C-termini (Krust et al. 1986). The A/B domain is the first region to exhibit cell

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type- and promoter-specific transactivation functions (AF-1). The C domain (also called the DNA binding-domain; DBD) is the most conserved region, and contains two zinc-finger motifs responsible for the recognition and binding of ERE regulatory elements in the target promoters. The D domain, which is poorly conserved, is a hinge linking C and E domains. The E domain (also called ligand binding-domain; LBD) is rich in hydrophobic residues and is important for specific binding of steroid hormones or xenoestrogens. The E/F domain exhibits the second transactivation function (AF-2) for target genes.

In vertebrate *ER*s, α, *β* and *γ* (or *β*2) subtypes are found in fish while only α and *β* subtypes are found in mammals. Subtypes of *ER* in mammals are encoded by different genes that differ in their affinity for estrogenic or anti-estrogenic ligands (Barkhem et al. 1998), the transcription mechanisms for their target genes (Tremblay et al. 1997), spatial/temporal expression patterns (Kuiper et al. 1997), and even ontogenic development (Enmark and Gustafsson 1999). It would be interesting to know how functional differences in *ER*s in reproduction, differentiation, development, metabolism, metamorphosis and homeostasis are related to *ER* paralogs and how the additional *γ* (or *β*2) subtype in fish contributes to the *ER* functional picture.

Estrogen receptors have attracted increasing attention in relation to the monitoring of environmental pollutants (Garcia-Reyero et al. 2001). Xenoestrogens are known to adversely affect normal endocrine physiology of humans and wildlife by binding with the *ER*, resulting in interference with normal estrogen responsive genes (Sonnenschein and Soto 1998). Exposure to xenoestrogens results in poor growth and reproductive performance in both male and female trout (Ashfield et al. 1998; Bjerselius et al. 2001). However, differential expression patterns have been observed in goldfish (Ma et al. 2000) more than in most other bony fishes (Hawkins et al. 2000; Menuet et al. 2002; Filby and Tyler 2005), suggesting that species from different niches (or taxa) may employ different estrogenic mechanisms. The higher concentrations of xenoestrogens detected in aquatic environments in Taiwan than in other countries (Yuan et al. 2002) has led to the search for a sentinel species able to faithfully reflect these estrogenic impacts. The indigenous fish *Varicorhinus barbatulus* was selected for our study because it is a ubiquitous resident often found in the upper and mid reaches of Taiwan rivers and is sensitive to most contaminants. Here we report on the cloning and sequence analysis of three subtypes of *ER* genes from the liver of *V. barbatulus*. The tissue distribution of *vbER* subtypes was investigated in both males and females. The results provide additional understanding of *ER* functional mechanisms and phylogeny, and may lay the ground for better assessment of potential estrogenic risks to wildlife of different ecosystems.

Materials and methods

Sample collection and RNA extraction

Taiwanese cyprinid fishes (*V. barbatulus*) ranging in length from 18 to 20 cm were reared and maintained in a semi-recirculating tank at room temperature. After treating with 40 μg/l 17*β*-estradiol for 2 weeks to over-express the *ER* transcripts, the fish were anesthetized and killed by decapitation, and their livers were removed and frozen immediately in liquid nitrogen. Total RNA was extracted using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) following the manufacturer's instructions. The RNA concentration was determined by absorbance at 260 nm, and its quality was monitored both by its integrity on agarose gel and by A_{260nm}/A_{280nm} ratios >1.8.

Cloning of V. barbatulus *ERα*, *ERβ*1, and *ERβ*2 cDNA

Two consecutive steps were used to clone the subtype genes: reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) from both 5′ and 3′ ends (Fig. 1). In the first step, *ER* core regions conserved in all three subtypes were obtained by RT-PCR. For original core region amplification, two degenerate primers (primer 1 and 2; see Fig. 1) were designed based on the overlapping DNA and hormone binding domains of *ER* from six teleost fish species including *ERβ* of goldfish (GenBank accession no. AF061269), *ERα* of channel catfish (AF061275), *ERα* of gilthead seabream (AJ006039), *ERα* of medaka (D28954), *ERβ* of Atlantic croaker (AF298181), and *ERα* of rainbow trout (AJ242740). Total RNA from fish liver was reverse-transcribed using the Superscript II (Invitrogen) one-step reverse transcriptase PCR kit and random primers. Polymerase chain reaction (PCR) amplification of the core region with two degenerate primers (primers 1 and 2; see Fig. 1) was carried out as follows: initial denaturation at 94°C for 3 min, then 30 cycles of denaturation for 30 s at 94°C, annealing at 55°C for 30 s, and extension for 1 min at 72°C. A 0.9-kb fragment of the core region was amplified and cloned into the pGEM-T Easy vector (Promega) for sequencing. The sequence was confirmed using BLAST on NCBI (National Center for Biotechnology Information, National Institutes of Health). These new *ER* genes from *V. barbatulus* were given the nomenclature *vbER*s.

In the second step, the conserved *ER* core regions were extended toward both 5′ and 3′ ends using 5′ RACE and 3′ RACE techniques. The 5′ end of *vbERα* or *vbERβ*1 was then amplified using subtype specific primers (primers 3, 4 for *vbERα* and primers 3, 8 for *vbERβ*1, see Fig. 1) for 5′-RACE with the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). A 3′-RACE procedure

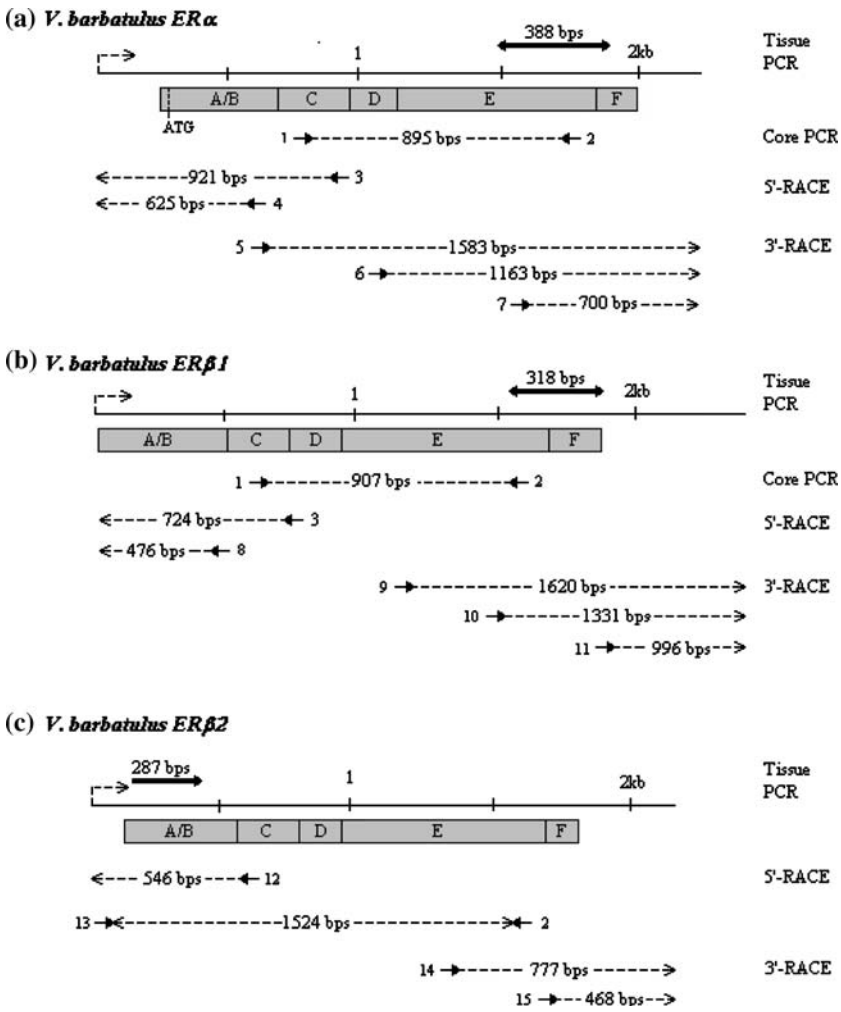

Fig. 1 The cloning strategy for three *ER* cDNAs, including *vbERα*, *vbERβ*1 and *vbERβ*2, in the cyprinid fish (*V. barbatulus*) using RT-PCR and 5′/3′ RACE. The positions of the structural domains relative to the putative open reading frames are marked as *grey bars*. The lengths of corresponding fragments obtained are shown in base pairs (bps). *Arrows* indicate the location and direction of primers for cloning procedures

using subtype specific primers (primers 5, 6, 7 for *vbERα*; primers 9, 10, 11 for *vbERβ*1, see Fig. 1) was also performed to obtain the full 3′ extensions of both genes. According to the goldfish *ERβ*1 sequence (GenBank accession no. AF061269), a subtype specific primer 12 was designed and amplified to the 5′ end of *vbERβ*2 using a 5′-RACE procedure. The full 3′ extension of *vbERβ*2 was identified using designated primers (primers 13, 14, 15) for 3′-RACE procedure. The full-length cDNAs of three *ER* subtypes were assembled by overlapping these sequences. A list of the sequences of primers used is provided in Table 1 and the primer sets with the position of these primers according to gene maps are shown in Fig. 1.

Sequence analysis and phylogenetic classification

Sequence results of vbERs were compared with the Genbank/EMBL database by basic BLAST similarity search. The sequence identity for total cDNA nucleotides and the inferred amino acid sequences of six domains derived from Krust et al. (1986) were analyzed using the package of DNAMAN software. The nucleotide sequences were translated to protein sequences using Translate Tool (http://www.expasy.org/tools/dna.html) and aligned using the Clustal W program (http://www.ebi.ac.uk/clustalw). The phylogenetic tree was also produced by the same software in which the distance matrix was calculated using the Neighbor-Joining algorithm with the PAM matrix model.

Tissue specimens and RT-PCR

Total RNA was extracted using the Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) from brain, eye, heart, liver, intestine, ovary, and testes of six male and six female fish. Purified total RNA was reverse transcribed with an oligo(dT) primer and M-MLV reverse transcriptase (Toyobo). The expression levels of different subtypes were PCR amplified using gene-specific primer sets (see Fig. 1) which span mainly the E domain for *vbERα*, E/F domain junction *vbERβ*1, and A/B domain for *vbERβ*2. Expression of *β*-actin was used as the endogenous control to confirm that target sequence amplified at the same efficiency, and the *β*-actin primers used are 5′-GACATCAAGGAGA AGCTGTGC-3′ and 5′-TCCAGACGGGGTATTTACG

Table 1 The sequences and the relative positions of oligonucleotide primers used for the cloning the full-length cDNAs of *vbERα*, *vbERβ*1 and *vbERβ*2

Primer	Target gene	Cloning step	Primer sequence (5′→3′)	Location
1	*vbERα* *vbERβ*1 *vbERβ*2	Conserved core region	CA(G/A)GG(T/A)CACAATGA(T/C)TA(C/T)AT	830–849(F) 679–698(F) 633–652(F)
2	*vbERα* *vbERβ*1 *vbERβ*2	Conserved core region	TG(C/G)TCCATGCCTTTGTT(A/G)CT	1,745–1,764(R) 1,579–1,598(R) 1,560–1,579(R)
3	*vbERα* *vbERβ*2	5′ RACE	CATCATGCCCACTTCATAGCAC	922–943(R) 725–746(R)
4	*vbERα*	5′ RACE	CCAACACCTGCCTGCTGAGA	626–645(R)
5	*vbERα*	3′ RACE	TGTACTCTGGATCAAGAGCCG	687–707(F)
6	*vbERα*	3′ RACE	GTCAGTGCTTTATGTATGCCTC	1,106–1,127(F)
7	*vbERα*	3′ RACE	TCATTCTGCTCCAGTCCAGT	1,571–1,590(F)
8	*vbERβ*1	5′ RACE	ACCATCACCATCCAGTTGCTG	477–497(R)
9	*vbERβ*1	3′ RACE	GAAACTCATGTTCTCACCTGACC	1,208–1,230(F)
10	*vbERβ*1	3′ RACE	CAGCAACAGTCCATCCGGCT	1,500–1,519(F)
11	*vbERβ*1	3′ RACE	CATCGAGTGGACATGGACACAG	1,833–1,854(F)
12	*vbERβ*2	5′ RACE	CAAAGAGGTAGAAGTCTCCTCT	546–567(R)
13	*vbERβ*2	3′ RACE	CTCTCTCGCAAGTACAGTCT	35–54(F)
14	*vbERβ*2	3′ RACE	CGTCGGAGGGAGGAGAGGAGC	1,412–1,432(F)
15	*vbERβ*2	3′ RACE	CCCAAAGAGAGCAAAGCTGTC	1,720–1,741(F)
16	*vbERα*	Tissue PCR	GGCTCGCTTCCGTAGTCTCA	1,489–1,508(F)
17	*vbERα*	Tissue PCR	TGCTGCTGGTTGTGGGTGTA	1,897–1,916(R)
18	*vbERβ*1	Tissue PCR	GTCAACCATGAAGAGAAAAAAC	1,583–1,604(F)
19	*vbERβ*1	Tissue PCR	CCCTACATTGGAAAAGCGGCA	1,923–1,943(R)
20	*vbERβ*2	Tissue PCR	TCTCGCAAGTACAGTCTACGAA	38–59(F)
21	*vbERβ*2	Tissue PCR	CTGCCGCACTGATGGGCAGT	347–366(R)

C-3′. The PCR condition was set to run 35 cycles at 94°C for 30 s for denaturing, 58°C for 30 s for annealing, and 72°C for 40 s for extension. The RT-PCR for β-actin was used to normalize the viability produced by enzyme efficiency in all experiments, which were performed in triplicate. A number of controls were performed to ensure proper PCR amplification. Negative controls consisting of no template and PCR performed on samples not subjected to reverse transcription were run in every test. The PCR amplicons were electrophoresed on a 1.5% agarose gel and the bands were visualized by ethidium bromide staining. The staining images were obtained by high-resolution camera and the band densities were calculated by QuantityOne software (Bio-Rad).

Statistical analysis

Statistical analyses were performed using SigmaStat 2.0 software (Jandell ScientiWc). Differences in mRNA expression for the three *ER* subtypes among different tissues were analyzed by ANOVA with all pairwise comparisons performed by the Tukey Test.

Usage of common names

Common names were used for convenience and referred to their official taxonomy as the following: sea bream, *Sparus auratus*; largemouth bass, *Micropterus salmoides*; sea bass, *Dicentrarchus labrax*; eelpout, *Zoarces viviparus*; killifish, *Fundulus heteroclitus*; rainbow trout, *Oncorhynchus mykiss*; channel catfish, *Ictalurus punctatus*; goldfish, *Carassius auratus*; zebrafish, *Danio rerio*; fathead minnow, *Pimephales promelas*; Atlantic croaker, *Micropogonias undulatus*; medaka, *Oryzias javanicus*; carp, *Cyprinus carpio*; Japanese eel, *Anguilla japonica*.

Results

Sequence analysis

We cloned the full-length cDNAs for *ERα*, *ERβ*1 and *ERβ*2 from livers of *V. barbatulus* using consecutive techniques of PCR amplification by degenerate primers designed to match the conserved core regions and 5′-RACE/3′-RACE

techniques for the terminal extension (Fig. 1). Following the definition of official nomenclature of nuclear receptors (Nuclear Receptors Nomenclature Committee 1999) based on results of sequence alignments, the deduced amino acid sequences were 612 residues for *vbERα*; 612 residues for *vbERβ*1, and 558 residues for *ERβ*2. The sequences were deposited in GenBank with the accession numbers AJ547632 (*vbERα*), AJ314603 (*vbERβ*1) and AJ547633 (*vbERβ*2). Interestingly, the *vbERα* possessed a putative internal ATG (at position 132 bp downstream of the first ATG) co-aligning with the translation starts of *ERα* from goldfish and zebrafish, whereas the most 5′ ATG producing a longer form aligned with the translation starts of *ERα* from rainbow trout, sea bass, and channel catfish (Fig. 2).

The highest identities of the three subtypes showed that *vbERα* is 93% similar with minnow *ERα*, *vbERβ*1 is 92% similar with goldfish *ERβ*2, and *vbERβ*2 is 95% similar with carp *ERβ*; overall of 88–90% with zebrafish. For inter-subtype sequence comparison within *V. barbatulus*, there was 42% identity between *vbERα* and *vbERβ*1, 41% between *vbERα* and *vbERβ*2, and 50% between *vbERβ*1 and *vbERβ*2. The shorter sequence of *vbERα* than *vbERβ*1 and *vbERβ*2 was also observed in other teleosts (Filby and Tyler 2005; Pakdel et al. 1990). The *vbER*s contained the *ER* signature domains that separated them from other classes of nuclear receptors. As expected, there was 80–91 and 57–69% identity, respectively, in the conserved domains C and E/F between *vbER*s and human *ER*s.

We then examined the conservation of functional residues (Fig. 3). Four residues, L349, M421, Y526, and C530, important for E2 ligand binding (Hawkins and Thomas 2004; Filby and Tyler 2005), showed subtype-specific sub-

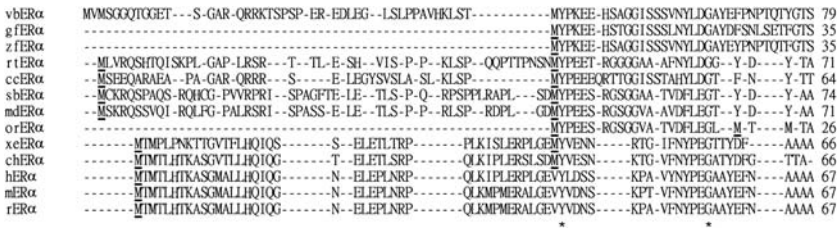

Fig. 2 The optimal alignment of 5′-terminal amino acids for different *ERα* subtypes between the cyprinid fish (*V. barbatulus*) in this study and other organisms. The sequences used are from goldfish (gfERα; AY055725), zebrafish (zfERα; BAB16893); rainbow trout (rtERα; AJ242740), channel catfish (cc*ERα*; AF253505), sea bass (sb*ERα*; AJ505009), medaka (mdERα; D28954), *Oreochromis aureus* (orERα; CAA63774); *Xenopus laevis* (xeERα; P81559), chicken (chERα; X03805), human (hERα; P03372), mouse (mERα; NM_007956), and rat (rERα; NM_012689). *Asterisks* indicate the conserved residues shared in these species. The possible initiator methionines (M) are *underlined*

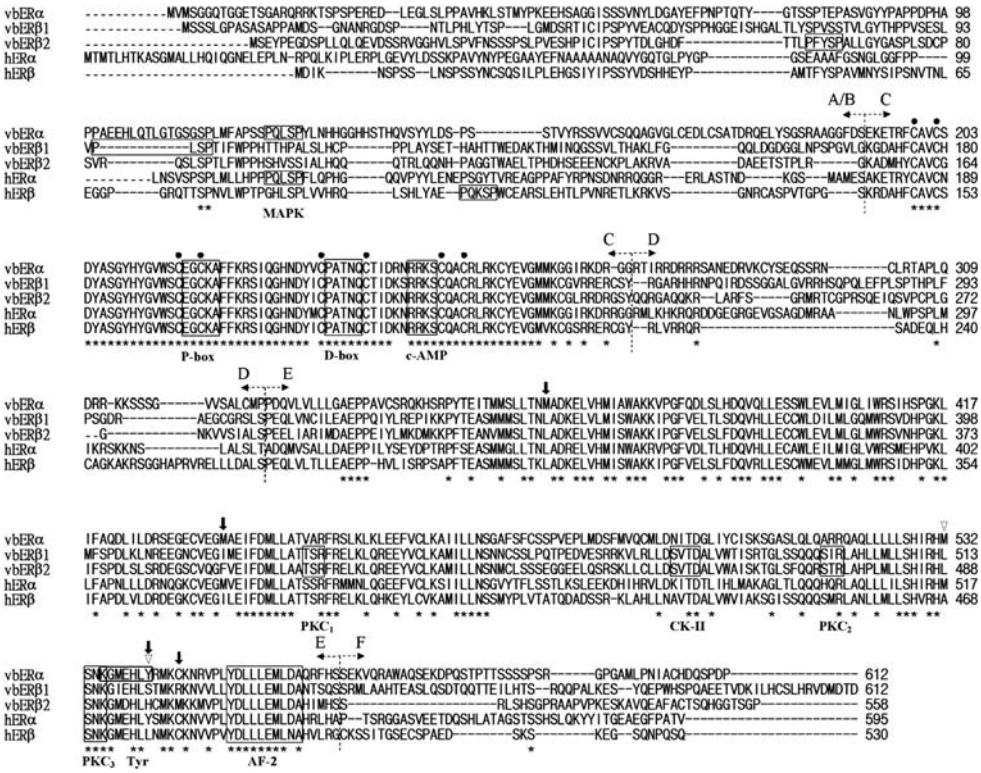

Fig. 3 Alignment of amino acid sequences of *vbERα*, *vbERβ*1, *vbERβ*2 with human *ER* subtypes (hERα and hER*β*). The consensus residues are marked by *asterisks* at the bottom. The functional domains from A to F are annotated with *broken arrows*. The eight-cysteine residues consisting of two zinc-finger motifs are designated by *filled circles*. The P- and D-box, the AF-2, the potential CK-II, and PKC phsophorylation sites are *boxed*. The tyrosine kinase phosphorylation site is marked with Tyr. The functional residues presented in three *vbER* subtypes are also identified in term of E2 dependent activation (*open triangle*) and the E2 ligand binding (*arrows pointing downward*) as proposed by human *ER*s

stitution such that *ERα*, *ERβ*1 and *ERβ*2 display particular residues in the aligned positions. The two residues, M517, and Y526, important for ligand-dependent transactivation (Ekena et al 1996) appeared to show species-specific substitution such that *ERβ* exhibits a M517L change in *V. barbatulus*, while there is a M517V change in Perciformes.

Phylogenetic analysis

To elucidate how these *vbER*s compare with their vertebrate orthologs, 42 related *ER*s (in amino acid sequences) of species including fish and mammals were collected to construct a phylogenetic tree (Fig. 4). Human estrogen-related receptor (ERR) was used as an outgroup to root the tree since ERR is an orphan nuclear receptor deviating from *ER*s for their constitutive activities without a known ligand (Gaillard et al. 2007). Most nodes of this phylogenetic tree were well supported and showed the evolutionary relationship in the generally accepted taxonomic groupings. Common trends were found such that two main clades of *ERα* and *ERβ* subtypes constituted the first separation and each subtype clade was further divided into sister clades including teleost *ER*s and mammal *ER*s. The teleost *ERβ* clade showed a further duplication in *ERβ* to produce *ERβ*1 and *ERβ*2 (or *ERγ*), as was observed by others (Robinson-Rechavi et al. 2001). The *vbERα*, *vbERβ*1 and *vbERβ*2 pos-

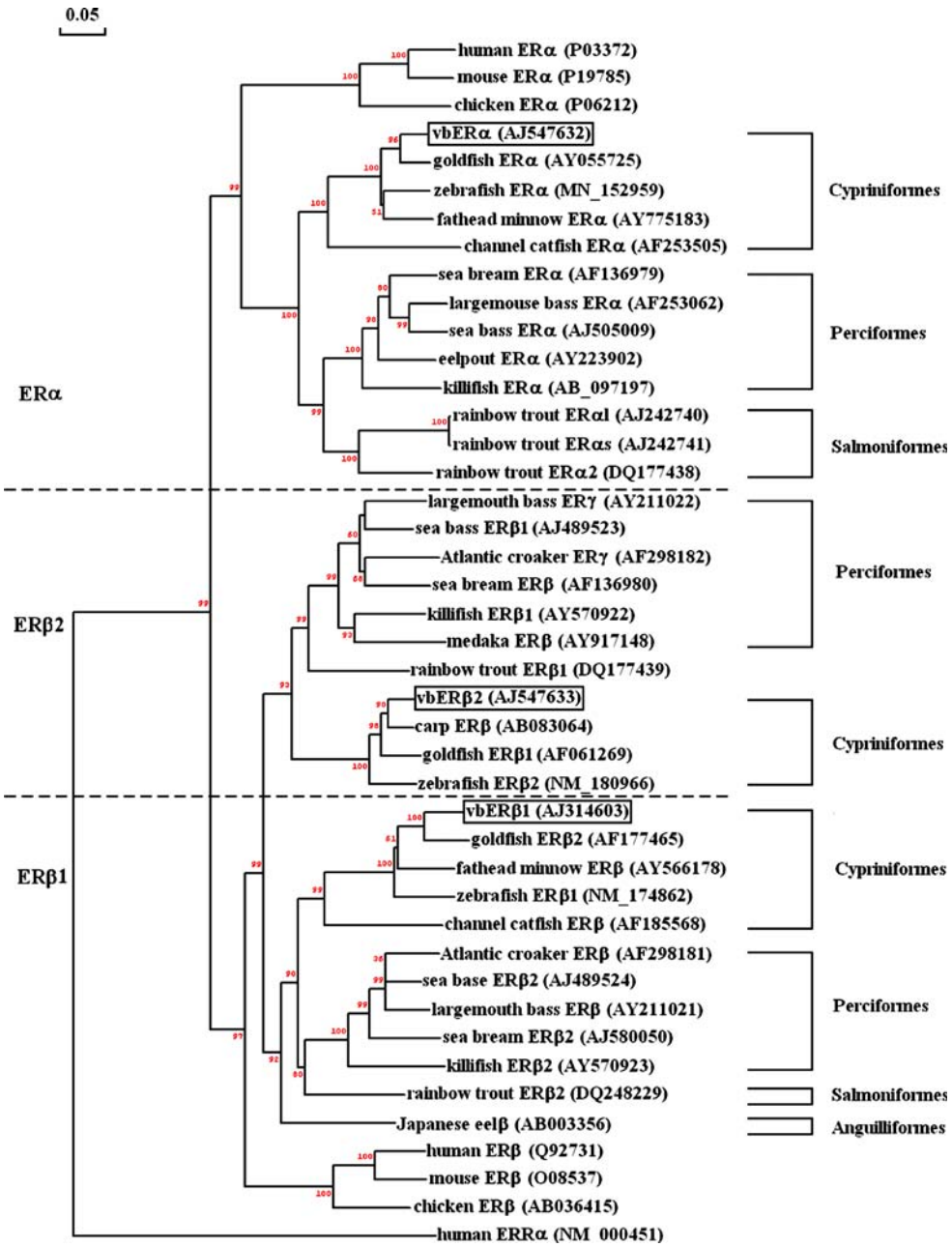

Fig. 4 Molecular phylogeny of *vbERα*, *vbERβ*1 and *vbERβ*2 based on protein sequences. The deduced amino acid sequence of *vbER* subtypes were aligned with ER proteins from teleost fish and representative mammals with sequences available. Human ERR is used as an outgroup to root the tree. NCBI database accession numbers of the sequences are indicated in brackets. Numbers at nodes are support in percentage of 1,000 bootstrap replicates

sessed lineages within the Euteleostei, were grouped together with their Cypriniformes relatives including goldfish, carp and zebrafish, and branched out from Perciformes including bass, croaker, seabream, killifish and medaka. The results of phylogenetic analysis were consistent with the results of sequence identity analysis from multiple sequence alignment, indicating that target species exhibiting close resemblance in sequence of *vbER* paralogs supported the relationship of *V. barbatulus* in Euteleostei phylogeny.

Tissue distributions of *vbERα*, *vbERβ*1 and *vbERβ*2

The tissue distribution of the three *ER* subtypes in male and female fish was investigated by semi-quantitative RT-PCR technique (Fig. 5). The expression levels of *ER* mRNA were normalized against that of *β*-actin to eliminate the sporadic variation caused by differences in enzyme efficiency. Expression of the *vbER*s was not confined to reproductive tissues. Moderate (0.2–0.7-fold of *β*-actin) expression was observed in brain, eye, heart, liver, intestine and gonads for all *vbER*s. The highest transcription level of *vbERα* was found in liver among six tissues in male and female fish. The *vbERβ*1 was detected most strongly in liver, followed by intestine, then gonad and brain, with the lowest expression in heart and eye, in both males and females. The *vbERβ*2 expressed most highly in liver, intestine and gonad. In general, the highest expression levels of *vbERα* and *vbERβ*1 were found in liver, and of *vbERβ*2 in intestine. The relative expression of each *vbER* subtype in male tissues mirrored expression levels in females.

Discussion

This study presents the cloning of members of the estrogen receptor family and the expression of their mRNA in adult tissues of the cyprinid fish *V. barbatulus*. The fact that the highest identities were found in *ER*s of goldfish and zebrafish matches well with the close taxonomic grouping of goldfish and zebrafish with *V. barbatulus* in the Cypriniformes. This also confirmed that the design of teleost degenerate primers is effective. For inter-subtype sequence comparison, there was 42–50% identity between *vbERα* and *vbERβ*, indicating that these *ER*s are from different loci of the genome, making *vbERβ*1 and *vbERβ*2 unlikely to be alleles on the same locus, or products of alternative splicing. These *vbER* genes are functional as shown in our recent study (Fu et al. 2007) in which we explored the transcriptional abilities of *vbER*s transformed into yeast expression vector to detect the potential estrogenic effects of xenoestrogens.

Putative protein functional sites for *vbER*s showed consistency with all *ER*s. Regions that are conserved in all *vbER*s are cysteine residues for two zinc fingers, P-box, D-box, and a cAMP site in the DBD domain, an AF-2 site, and a PKC phosphorylation site in the LBD domain (Fig. 3). The P- and D-boxes were shown to be crucial for

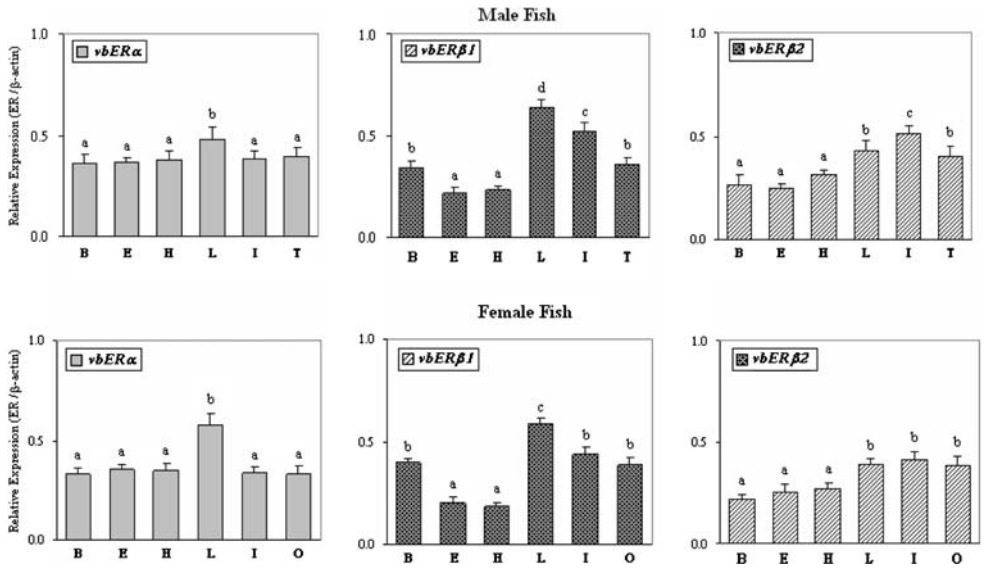

Fig. 5 The tissue distributions of *vbERα*, *vbERβ*1, and *vbERβ*2 using semi-quantitative RT-PCR. Gene-specific primers for each *ER* subtype were used to investigate the relative expression levels of *vbER* paralogs in males (the *upper panel*) and females (the *lower panel*) with *β-actin* as an internal control. The tissues were collected from brain (*B*), eye (*E*), heart (*H*), liver (*L*), intestine (*I*), testis (*T*), and ovary (*O*). Different *letters* above the bars indicate significant differences in levels of *ER* transcriptions. Data are shown as mean ± standard deviation ($n = 6$) from six males and six females fish after normalization to *β-actin* activity

DNA-binding by Härd and Gustafsson (1993). Conservation in aspects of DNA binding specificity, ligand-dependent transcriptional activation, and kinase regulation for the three *vbER*s is hence inferred. The functional significance of PKC sites in all *ER*s was shown by Cho and Katzenellenbogen (1993) in that activation of PKC markedly enhances *ER*-mediated transcriptional activation in a ligand-dependent manner. As for subtype-specific conservation, the putative MAPK phosphorylation site in the A/B domain were conserved only in the *ERα* subtype both in sequence and position, as observed by others (Kato et al. 1995; Socorro et al. 2000). The MAPK pathway was reported capable of influencing ligand-independent transcriptional activity of *ER* in both mammalian *ERα* and *ERβ* (Lannigan 2003). The potential tyrosine (Tyr) kinase phosphorylation sites (KGMEHLY) were found uniquely in *vbERα* and casein-kinase II phosphorylation site (CK-II) only in *vbERβ* subtypes, consistent with observations of others (Le Goff et al. 1994). These phosphorylation sites suggest that *vbER* subtypes might adopt different functions coupled with distinctive regulation mechanisms.

In mammals, transcription variants have arisen for *ERα* from alternative usage of exons (reviewed by Herynk and Fuqua 2004). In human, usage of internal initiator methionine (ATG) generates a shorter form of hERα without exon 1 with defect function in domain A/B. The short form *ERα* was reported to exhibit dominant negative effects by forming a heterodimer with the longer (wild-type) *ERα* that interfered with the AF-1 transcription activity of the *ER* dimer in osteoblasts (Sanyal et al. 2005). It was suggested that the short form in trout *ERα* exhibits 15–25% of the total receptor activity in a cell-, promoter-specific and hormone-independent manner (Nagler et al. 2000). Transcriptional variants from each *vbERβ* subtype of this study were not detectable.

The conservation of putative functional residues in the core region of the E domain was examined, and showed subtype- and even species-specific substitution. First, residues M421, Y526, and C530, (Hawkins and Thomas 2004; Filby and Tyler 2005) are important for ligand binding. We found that the three residues substituted as a set in a subtype-specific manner such that the (M, Y, C) residue set is substituted to be (I, S, R) in *ERβ*1; while (F, H, M) is found in *ERβ*2. Crystal structural evidence showed that interchanges of hydrophobic M, F, and I residues in hERα M421 could mimic other subtypes, resulting in changes of binding affinities toward diethylstilbestrol and tamoxifen (Hawkins and Thomas 2004). Secondly, residue M517, which is important for ligand-dependent transactivation (Ekena et al. 1996) showed that *ERβ* exhibited a M517L change in *V. barbatulus*, and a M517V change in Percomorpha.

While the sequence of *vbER*s shared greater similarity with data from minnow *ERα*, goldfish *ERβ*2 and carp *ERβ*, the expression profile of *vbER*s resembled more closely results from fathead minnow in the family Cyprinidae. The highest expression in liver for *vbERα* and *vbERβ*1 and in intestine for *vbERβ*2 matched subtype expression patterns in fathead minnow (Filby and Tyler 2005). Although goldfish *ERβ*1, zebrafish *ERβ*1 and *ERβ*2, and gilthead seabream *ERα* were mainly expressed in gonads, the general expression in heart, liver, intestine and kidney showed largely overlapping expression patterns with our cyprinid fish *V. barbatu*lus (Socorro et al. 2000). The overlapping but not identical expression patterns of the *vbER*s might suggest that subtype-specialization has occurred. For example, paralogs of nuclear receptors were shown to adopt separate expression patterns, which might lead to new functions. *PPARα* (peroxisome proliferator-activated receptor alpha) was expressed in a large spectrum of but not all tissues, *PPARβ* was expressed ubiquitously, and *PPARγ* was expressed only in fat tissues (Michalik et al. 2006). We also noticed that moderate (0.2–0.7-fold of *β-actin*) expression was observed in all tissues inspected for all *vbERs*. Likewise, expression of *ERβ*1 and *ERβ*2 in sea bass was widespread, with similar levels among tissues (Halm et al. 2004). The similar levels of expression are probably because the technique prevents sharp distinction of fold change under the nearly saturated amplification of PCR. The other possibility was that when non-genomic mechanisms were examined, activity enhancement instead of changes in amounts served as the critical index for *ER* function.

When the phylogenetic tree of *vbER*s was analyzed, there was consistency with other trees in euteleostei fish *ER* evolution in that no *vbER* subtype was found to redirect into clades of different families. Each *vbER* was placed in the same clade with, and sister to its goldfish ortholog. Generally, the *vbERα*, *vbERβ*1 and *vbERβ*2 that had lineages within the Euteleostei were grouped together with their Cypriniformes relatives and branched out from the Perciformes. The position of *V. barbatulus* in Euteleostei phylogeny supports the resultant target species by sequence alignment. The presence of three duplicated *vbER*s was consistent with the hypothesis that a single primitive invertebrate ERR underwent gene duplication events, one after divergence between vertebrates and Amphioxus, and one before the divergence between lamprey, hagfish and gnathostomes (Ohno 1999).

In conclusion, we present molecular cloning and tissue expression analysis of three estrogen receptor subtypes, *vbERα*, *vbERβ*1 and *vbERβ*2, from the cyprinid fish *V. barbatulus*. The sequence alignment and phylogenetic analysis reconfirmed the evolutionary relationship of *V. barbatulus* within the Cypriniformes. The overlapping but not identical expression patterns of *vbER*s suggest that subtype-specialization may have occurred. Here, we demonstrate that the cloning of *ER* subtypes from wildlife using PCR is feasible

in that temporal and spatial observations are consistent with those from phylogeny analysis and crystal structural investigation by others. Furthermore, we were able to show that estrogenic chemicals affect a sentinel species at the molecular level.

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