Cloning and functional characterization of Chondrichthyes, cloudy catshark, *Scyliorhinus torazame* and whale shark, *Rhincodon typus* estrogen receptors

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Short title: Molecular cloning and characterization of shark estrogen receptor

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Abstract

Sex-steroid hormones are essential for normal reproductive activity in both sexes in all vertebrates. Estrogens are required for ovarian differentiation during a critical developmental stage and promote the growth and differentiation of the female reproductive system following puberty. Recent studies have shown that environmental estrogens influence the developing reproductive system as well as gametogenesis, especially in males. To understand the molecular mechanisms of estrogen actions and to evaluate estrogen receptor ligand interactions in Elasmobranchii, we cloned a single estrogen receptor (ESR) from two shark species, the cloudy catshark (*Scyliorhinus torazame*) and whale shark (*Rhincodon typus*) and used an ERE-luciferase reporter assay system to characterize the interaction of these receptors with steroidal and other environmental estrogens. In the transient transfection ERE-luciferase reporter assay system, both shark ESR proteins displayed estrogen-dependent activation of transcription, and shark ESRs were more sensitive to 17β-estradiol compared with other natural and synthetic estrogens. Further, the environmental chemicals, bisphenol A, nonylphenol, octylphenol and DDT could activate both shark ESRs. The assay system provides a tool for future studies examining the receptor-ligand interactions and estrogen disrupting mechanisms in Elasmobranchii.

Keywords: shark; estrogen receptor; transactivation; evolution; environmental chemicals

1. Introduction

Estrogens play important roles in the reproductive biology of all vertebrates studied to date, including fish. Currently, the majority of the known actions of estrogens are mediated by specific receptors that are localized in the nucleus of target cells. These nuclear estrogen receptors (ESRs) belong to a superfamily of nuclear transcription factors that include all other steroid hormone receptors including those for progestogens, androgens, glucocorticoids, mineralocorticoids, the vitamin D receptor, and the retinoic acid receptor (Blumberg and Evans, 1998). To date, in most vertebrates, two distinct forms of ESR, ESR1 (ERα) and ESR2 (ERβ), have been isolated whereas in teleost fish, one ESR1 and two ESR2 (ESR2a and ESR2b) have been identified. The ESR2b-form appears to be closely related to ESR2a, suggesting a

gene duplication event that has occurred within the teleosts (Katsu et al., 2008). Thus, the ancestral condition for the jawed vertebrates (Gnathostomata) is considered to have had two forms of ESR, corresponding to ESR1 and ESR2 (Hawkins et al., 2000). Chondrichthyes are jawed fish with skeletons consisting of cartilage rather than bone. They are divided into two subclasses; Elasmobranchii including sharks, rays and skates, and Holocephali (chimaera). Early study on steroids in Elasmobranchii ovaries identified 17β-estradiol, estrone and estriol (Wotiz et al., 1960; Chieffi and Lupo di Prisco, 1963; Simpson et al., 1964). Further, Resse and Callard (1991) reported the presence of estradiol-binding protein and characterized its ligand-specificity. However, we were able to identify only one full length of ESR sequence of Chondrichthyes, *Squalus acanthias*, in GenBank.

In vertebrates, 17β-estradiol is the principle estrogen in circulation and appears essential for normal ovarian development and function (Wallace, 1985). Embryonic exposure to inhibitors of aromatase, the enzyme responsible for the conversion of testosterone to 17β-estradiol, causes genetic females to become phenotypic males in chicken and at least one species of turtle (Elbrecht and Smith, 1992; Dorizzi et al., 1994). Likewise, embryonic exposure of various fishes, amphibians or reptiles to 17β-estradiol or estrogenic chemicals, pharmaceutical agents or environmental contaminants can induce skewed sex ratios toward females (for reviews, see Crews, 1996; Guillette et al., 1996; Tyler et al., 1998; Iguchi et al., 2001). A number of studies strongly suggest that endogenous 17β-estradiol acts as a natural inducer of ovarian differentiation in non-mammalian vertebrates, including Elasmobranchii (Devlin and Nagahama, 2002; Sinclair et al., 2002; Koob and Callard, 1999). However, the mechanisms of estrogen action on ovarian differentiation have not been determined.

Disruption of the endocrine system has been shown to occur in wild fish populations across the globe (Vos et al., 2000). Although the evidence for endocrine disruption in wild fish is extensive, there are few verifications of a causal relation between the presence of specific endocrine disrupting chemicals (ECDs) and a functional effect (disruption). Several studies have now shown that wild freshwater fish living in rivers heavily contaminated with treated wastewater treatment works effluent have altered reproductive development and function (Hecker et al., 2002; Jobling et al., 2002), and other investigations have shown that estrogens and estrogenic

chemicals are present in those effluents (Desbrow et al., 1998; Snyder et al., 2001) and likely involved in these disruptions (Jobling et al., 2006; Katsu et al., 2007b). In marine ecosystem, there are some reports of endocrine disruption in fish (Scott et al., 2006, 2007), and the accumulations of various environmental chemicals with suspected endocrine activity have been reported in sharks (Gelsleichter et al., 2005, 2006; Storelli et al., 2006; Silva et al., 2007; Haraguchi et al., 2009). The mechanisms by which estrogenic chemicals have an effect on sexual development and function in Elasmobranchii, such as shark, however, are still to be determined.

In this study, we isolated cDNA clones encoding shark orthologs of ESR. The transactivation function of shark ESR was subsequently determined by expressing these cDNAs in transiently transfected HEK293 cells which were then used to determine ligand-specificity of shark ESR with natural, synthetic and environmental estrogens. The extensive global distribution of sharks in tropical to polar aquatic ecosystems and their capability to tolerate highly contaminated environments make these animals an interesting biological model for assessing endocrine disruptors in a wide range of aquatic environments.

2. Methods

2.1. Animals

Cloudy catshark, *Scyliorhinus torazame*, were purchased from a local supplier. Whale shark (*Rhincodon typus*) tissues were obtained for research purposes from the Georgia Aquarium's Correll Center for Aquatic Animal Health. All experiments in this study involving sharks were carried out under the guidelines specified by the Institutional Animal Care and Use Committee at the Gergia Aquarium's Correll Center and Hokkaido University.

2.2. Chemical reagents

17β-Estradiol (E2), estrone (E1), estriol (E3), ethinylestradiol (EE2) and diethylstilbestrol (DES) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Bisphenol A (BPA) was purchased from Nacalai Tesque (Kyoto, Japan). The ERα □ selective agonist, propyl pyrazole triol (PPT) and ERβ-selective agonist, diarylpropionitrile (DPN) were obtained from Tocris Bioscience (Ellisville, MO). 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) were purchased from Tokyo Kasei

(Tokyo, Japan). Purity of BPA, NP and OP were over 99%. DDT and its metabolites were purchased from Chem Service (West Chester, PA). Purity of *p,p'*-DDD, *p,p'*-DDE, *o,p'*-DDE and *p,p'*-DDT were 99%, and *o,p'*-DDD and *o,p'*-DDT were 99.5%. All chemicals were dissolved in dimethylsulfoxide (DMSO). The concentration of DMSO in the culture medium did not exceed 0.1%.

2.3. Molecular cloning of estrogen receptors

Two conserved amino acid regions in the DNA binding domain (GYHYGVW) and the ligand binding domain (NKGM/IEH) of vertebrate ESRs were selected for the design of degenerate oligonucleotides. The second PCR using the first PCR amplicon, and nested primers that were selected in the DNA binding domain (CEGCKAF) and the ligand binding domain (NKGM/IEH). As a template for PCR, the first-strand cDNA was synthesized using total RNA isolated from liver. The amplified DNA fragments were subcloned with TA-cloning plasmids pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The 5' and 3' ends of ER cDNAs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech., Palo Alto, CA). Sequencing was performed using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and analyzed on the ABI PRISM 377 automatic sequencer (Applied Biosystems).

2.4. Database and sequence analysis

All sequences generated were searched for similarity using Blastn and Blastp at web servers of the National Center of Biotechnology Information (NCBI). Phylogenetic analysis was demonstrated with the amino acid sequences for estrogen receptor (ESR) from selected vertebrates and invertebrates as shown in Table S1. The regions from immediately outside of the DNA binding domain through the ligand binding domain of the sequences were trimmed according to the Conserved Domains Database in NCBI. The trimmed sequences were aligned using the PRANK program, a probabilistic multiple alignment program, which is good at insertions and deletions (Löytynoja and Goldman, 2008). The aligned sequences were used for the estimation of phylogenetic trees by the maximum likelihood analysis with the PhyML 3.0 program with JTT substitution model, NNI (Nearest Neighbor Interchange) and SH-like aLRT (approximately Likelihood-Ratio Test) branch supports (Guindon and Gascuel, 2003).

The estimated tree was edited on MEGA4 (Tamura et al., 2007). The aligned sequences were also used for the rate shift analysis (Knudsen et al., 2003).

2.5. Construction of plasmid vectors

The full-coding region of shark ESR was amplified by PCR with KOD DNA polymerase (TOYOBO Biochemicals, Osaka, Japan). The PCR product was gel-purified and ligated into the pcDNA3.1 vector (Invitrogen). An estrogen-regulated reporter vector containing four estrogen-responsive elements (4xERE), named pGL3-4xEREtkLuc was constructed as described previously (Katsu et al., 2006).

2.6. Transactivation assays

To examine ligand (e.g., environmental estrogen) interactions with the cloned estrogen receptors, HEK293 cells were seeded in 24-well plates at $5x10^4$ cells/well in phenol-red free Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone, South Logan, UT). After 24 h, the cells were transfected with 400 ng of pGL3-4xEREtkLuc, 100 ng of pRL-TK (as an internal control to normalize for variation in transfection efficiency; contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter; Promega, Madison, WI), and 200 ng of pcDNA3.1-ESR using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. After 4 h of incubation, various steroid hormones were applied to the medium. After 44 h, the cells were collected, and the luciferase activity of the cells was measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*)-luciferase activity / sea pansy (*Renilla reniformis*)-luciferase activity. All transfections were performed at least three times, employing triplicate sample points in each experiment. The values shown are mean ± SEM from three separate experiments, and dose-response data and EC50 were analyzed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA).

3. Results

3.1. Cloning of shark ER cDNAs

Using standard PCR techniques described above, partial DNA fragments were amplified from catshark and whale shark liver RNA. For each species, only one type of DNA fragment was obtained, and sequence analysis showed that these fragments had similarity to spiny dogfish ESR2. Using the RACE technique, we were able to clone full-length catshark and whale shark ESR cDNAs (GenBank Accession numbers, catshark ESR: AB551715, and whale shark ESR: AB551716). The cDNA for catshark ESR (*Scyliorhinus torazame* ESR, StESR) predicted 536 amino acids protein with a calculated molecular mass of 60.5 kDa, whereas the whale shark ESR (*Rhincodon typus* ESR, RtESR) is also predicted to be 536 amino acids but with a calculated molecular mass of 60.8 kDa (Fig. 1). Using the nomenclature of Krust et al. (1986), the shark ESR sequence can be divided into five domains based on its sequence identity to other vertebrate ESRs (Fig. 2). When our catshark sequence (RtESR) is compared with ESR1 from five other species (human, chicken, *Xenopus*, zebrafish, lungfish), catshark ESR1 shared 29-28, 95-92, 33-27, 65-63, 19-13% identities to the A/B, C, D, E, and F domains, respectively (Fig. 2A). In contrast, when RtESR is compared with the ESR2 of seven other vertebrate species, RtESR shared 45-32, 95-93, 38-29, 74-67, 28-16% identities to the A/B, C, D, E, and F domains, respectively (Fig. 2B). Sequence homology indicates that shark ESRs are more similar to the vertebrate ESR2 form than the vertebrate ESR1 form. Using phylogenetic analysis, based on the protein sequences of the C-, D- and E-domain, we were able to determine the relationship of shark ESRs relative to other vertebrates (Fig. 3; Fig. S1). All three shark ESRs were in the vertebrate ESR2 clade, and formed a group separate from bony vertebrates. No ESR1 type receptor has been identified in cartilaginous fishes in the NCBI database nor could we isolate one during this study. Lancelet ESR appears to be an ancient ESR and ancestral to both forms (ESR1 and ESR2) of vertebrate ESRs (Fig. 3; Fig. S1). Multiple sequence alignment and rate shift analysis revealed that most of the functionally important sites were identical among the vertebrate ESRs except some of the sites for the dimer interface (Fig. 4). Three of five non-conserved sites at 388, 416 and 442 for the dimer interface among vertebrates were positive at the rate shift analysis, which indicates the potential sites to make the functional characteristics of each subfamily, ESR1 and ESR2. Interestingly, sharks and hagfish (*Myxine*) ESR2 have the tetrapod type residue (S) at 388 instead of the bony fish type (N) (Fig. 4).

3.2. Transcriptional activities of shark estrogen receptors by estrogens

A transactivation assay was used to determine whether shark ESRs display estrogen-dependent transcriptional activity. ESRs activate transcription in an estrogen-dependent manner through a variety of response sites, the most typical of which is composed of two head-to-head AGGTCA half sites separated by three nucleotides, an estrogen-response element, ERE (Zilliacus et al., 1995; Gruber et al., 2004). To analyze the transcriptional activities of shark ESRs, we co-transfected a reporter plasmid containing four EREs in front of a minimal tk promoter driving the luciferase reporter gene (pGL3-4xEREtkLuc), together with a shark ESR encoding expression plasmid and pRL-TK control plasmid (Kohno et al., 2008). We examined the abilities of natural and synthetic estrogens to induce ESR-dependent transcriptional activity. Both shark ESRs activated the expression of the luciferase reporter gene in a dose-dependent manner for all estrogens examined (Fig. 5A and 5B). As shown in Figure 5A and 5B, both shark ESRs exhibited significant transcriptional activity at a concentration of 10^{-11} M E2. Concentration-response relationships were examined for two other natural estrogens, E1 and E3 using the same assay system (Fig. 5A and 5B). Both E1 and E3 activated shark ESR transcription, but were less effective (less potent) compared with E2. Both synthetic estrogens, EE2 and DES, also stimulated luciferase activity through the whale shark and catshark ESRs in a concentration-dependent manner (Fig. 5). Based on EC50 values, both shark ESRs were more sensitive to E2 than to E1, E3, EE2 or DES (Table 1). Propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) are ESR1- and ESR2-selective agonists in mammals, respectively (Stauffer et al., 2000; Meyers et al., 2001). These subtype selective ESR agonists were used to examine and characterize shark ESRs. We found that both shark ESRs are activated by DPN, the ESR2-agonist, but not PPT, the ESR1-agonist (Fig. 5C and 5D). These results suggest the ligand-sensitivity of the shark ESRs isolated in this study is similar to mammalian ESR2, but not ESR1.

3.3. Transcriptional activities of shark estrogen receptors by estrogenic chemicals

Considering the global contamination of aquatic ecosystems with various persistent chemicals, such as organochlorines (e.g. DDT and its metabolites such as DDE) and phenolic compounds (4-nonylphenol, 4-tert-octylphenol, and bisphenol A), and the fact that a number of these chemicals have been shown to bind to the ESRs of

various vertebrates, we tested whether a similar phenomenon could be documented for the shark ESRs identified here. First, we compared the ability of bisphenol A (BPA), 4-nonylphenol (NP), and 4-tert-octylphenol (OP) to induce shark ESR-dependent reporter gene expression. The ESRs from both shark species activated the transcription of the reporter gene when exposed to these three estrogenic chemicals; shark ESRs were slightly more sensitive to NP than BPA and OP (Fig. 6). Next, we examined whether the highly persistent and bioaccumulated/biomagnified environmental contaminant DDT and its metabolites (*p,p'*-DDD, *o,p'*-DDD, *p,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, and *p,p'*-DDT) could induce shark ESR-dependent reporter gene expression. Both shark ESRs were more sensitive to *o,p'*-forms than *p,p'*-forms (Figs. 7). Furthermore, *o,p'*-DDT was a more potent activator of shark ESRs than *o,p'*-DDD and *o,p'*-DDE. These results are consistent with other vertebrate where ESR-induced transcription by DDT and its metabolites has been reported. In order to truly understand the implications of these assay results, the effects of estrogenic environmental chemicals need to be studies using *in vivo* exposures on a model shark species so that in vivo sensitivity can be fully assessed.

4. Discussion

Estrogens are implicated in a wide array of reproductive activities in vertebrates, including gonadal differentiation, maturation of female reproductive tracts, and reproductive behaviors (Iguchi et al., 2001; McLachlan, 2001; Moore et al., 2005). In vertebrates, estrogens appear to induce both genomic actions through the nuclear ERs and non-genomic cellular actions via ERs localized at the cell membrane that activate G-protein coupled (Bjornstrom and Sjoberg, 2005). Reese and Callard (1991) reported specific estrogen-interacted protein, perhaps estrogen receptor, from the little skate, *Raja erinacea*. In 1997, a partial sequence for the nuclear estrogen receptor was reported from Chondrichthyes, the smaller spotted catshark, *Scyliorhinus canicula* (Escriva et al., 1997). Since then, only one additional sequence has been registered for the spiny dogfish (GenBank accession No. AF147746). We report here the sequences of two beta type ERs (ESR2) from the Elasmobranchii, one from the cloudy catshark and the other from the whale shark. Although a growing literature exists on sequence and evolutionary phylogeny for various vertebrate estrogen receptors (Bury and Strum, 2007; Howarth et al., 2008; Hu and Funder, 2006), few studies have examined the

estrogen receptor from Chondrichthyes, Elasmobranchii and Holocephali.

Shark ESRs isolated in this study belong to the vertebrate ESR2 family based on sequence similarity, phylogenetic analysis, and characterization using specific agonists. To date, there are no reports of an alpha-type ER (ESR1) nucleic and amino acid sequences from Chondrichthyes. Do Chondrichthyes have alpha-type ER? Currently, we have no answer of this question. Recently the elephant shark (*Callorhinchus milii*) genome sequence project was initiated (Venkatesh et al., 2006; Venkatesh et al., 2007). The elephant shark, also known as the elephant fish or ghost shark, is of the Holocephali belonging to the Order Chimaeriformes and Family Callorhynchidae. When we searched for ESR-like sequences in this database, we detected two amino acid sequences, CAVCNDFASGY and CAVCSDYASGY corresponding to the DNA-binding domain of the two vertebrate ESRs. The ESRs from three species of shark (spiny dogfish, catshark, and whale shark) have the CAVC**N**D**F**ASGY sequence. The CAVC**S**D**Y**ASGY sequence, belonging to the ESR1 form, has two different amino acid residues, S instead of N, and Y instead of F, that the elephant shark may express both forms of ESR. Therefore, analysis of the Holocephali ESR will be useful for further understanding estrogen receptor evolution in the Chondrichthyes. Moreover, two types of ESRs in vertebrate were appeared by the gene duplication after the blanching of lancelet ESR in our study, but it isn't seen in Chondrichthyes. Since lamprey and hagfish are very primitive vertebrates, we expected they could reveal how two types of ESRs were appeared. However, lamprey and hagfish ESR belonged to ESR1 and ESR2, respectively, although the support value was not high. To search both type of ESR in these species or primitive vertebrates might be the key species to understand the occurrences of ESR1 and ESR2 in vertebrates.

We examined the ability of newly identified shark ERs to transactivate and ERE-dependent reporter gene. Both shark ESRs responded similarly to E2. Relatively high concentrations of E1 and E3 were necessary for transactivation of the shark ESRs compared to the induction by E2. This is similar to what we found other vertebrates (Katsu et al., 2007a; 2007b; Naidoo et al., 2008; Katsu et al., 2010). We found that E2 is the most potent transcriptional activator of shark ESR compared with other natural and synthetic estrogens (Fig. 4A and 4B, Table 1). We found that DPN, an ESR2-agonist, but not an ESR1-agonist, activated transcription of both shark ESRs, suggesting that the shark ESRs are similar to mammalian ESR2. The receptor

dependent reporter gene assay system that we have used is suitable for the analysis of ligand-induced ESR transactivation from a wide array of species. Additional studies that focus on ontogenic and sexually dimorphic responses in several Elasmobranchii species could provide insight into the function of these steroids during the life history of Elasmobranchii species (Koob and Callard, 1999; Hamlett and Koob, 1999).

A significant number of contaminants have been shown to interact with the ESRs of mammals and other vertebrates (Guillette et al., 2006). Furthermore, many of these contaminants are persistent globally and potentially affect the developmental and reproductive biology of various animal species from numerous ecosystems. Concentrations of DDT and its metabolites were readily detectable in shark species (Storelli et al., 2005, 2006; Silva et al., 2007). We examined possible interactions between this pesticide and its metabolites (*p,p'*-DDD, *o,p'*-DDD, *p,p'*-DDE, *o,p'*-DDE, *p,p'*-DDT, *o,p'*-DDT) and the ESRs from shark using our transactivation system. We observed that *o,p'*-DDT could activate ESRs from both species of shark, consistent with our findings with ESRs from other vertebrate species (Katsu et al., 2008). Further studies should examine the molecular interactions between a species' steroid receptors and endogenous ligands as well as common contaminants in that species' environment. Our studies provide important tools with which to study the activation of ESRs and the potential effects of environmental chemicals. For example, estrogens are not only important for adult reproductive function but also for sex determination in many species, thus a better understanding of the potential role of various contaminants as environmental estrogens is critical for the ecotoxicology and conservation biology of wildlife species.

In summary, we cloned and sequenced an ESR from two Elasmobranchii species and then examined their ability to be activated by endogenous and xenobiotic estrogens. This is the first report to characterize the ligand-dependent activation of cloned ESR from Elasmobranchii. The Reproductive biology and endocrinology of Elasmobranchii were reviewed in order to derive a working hypothesis that explaines the complex nature of endocrine patterns observed in species utilizing disparate reproductive modes (Koob and Callard, 1999, Pierantoni et al., 2002). Our data provide useful tools for future studies examining the basic endocrinology of Elasmobtranchii steroid hormone receptors. We have demonstrated that transactivation assays, using ESRs from non-traditional model species, such as these

Elasmobranchii, provide important initial insights into potential risks from environmental xenoestrogens and confirm that such an approach could provide important data to aid in species conservation and ecological risk assessments. Our study has provided basic molecular data useful in examining the role of ESRs in future studies, such as those examining gonadal development, reproductive biology and evolutionary endocrinology.

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Figure Legends

Figure 1. Amino acid sequence comparison of ESR proteins from shark. Full amino acid sequences of spiny dogfish (*Squalus acanthias*, SaESR; Genbank accession No. AF147746), catshark (*Scyliorhinus torazame*, StESR; Genbank accession No. AB551715), and whale shark (*Rhincodon typus*, RtESR; Genbank accession No. AB551716) were aligned with Clustal W software. DNA-binding domain, DBD and ligand-binding domain, LBD are enclosed within a box. BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used to make this figure.

Figure 2. Domain structure of whale shark ESR (RtESR), and homology with ESR from other species. (A) Domain structure of ESR in whale shark (RtESR), and identity with catshark ESR (StESR), human ESR1 (HsESR1), chicken ESR1 (GgESR), western clawed frog ESR1 (XlESR1), zebrafish ESR1 (DrESR1), and lungfish ESR1 (PdESR1). (B) Domain structure of ESR in whale shark (RtESR), and identity with human ESR2 (HsESR2), chicken ESR2 (Gg ESR2), western clawed frog ESR2 (Xl ESR2), zebrafish ESR2a (DrESR2a), zebrafish ESR2b (DrESR2b), and lungfish ESR2 (Pd ESR2). The functional A/B to F domains are schematically represented with the numbers of amino acid residues indicated. The numbers within each box indicated the percent identity of the domain compared to whale shark ESR.

Figure 3. Phylogenetic tree of vertebrate and invertebrate ESRs using predicted amino acid sequences. Two of our new ESRs belonged to ESR2 group. The phylogenetic tree was constructed by using the maximum likelihood analysis on PhyML 3.0 with JTT substitution model, NNI (Nearest Neighbor Interchange) and SH-like aLRT (approximate Likelihood-Ratio Test) for branches blanch supports indicating but the numbers on the blanches. Two of our new ESRs were highlighted. The scale bar indicates 0.2 expected amino acid substitutions per site. For complete phylogeny and accessions, see supplemental Fig. S1 and supplemental Table S1.

Figure 4. Multiple sequence alignments of amino acid for two types of ESRs among vertebrates. The results suggest the sites of dimer interface at 388, 416 and 442 in this alignment would be important to become the different type of ESR functionally. Shark ESR2 has tetrapod type of amino acid at 338 in this alignment. The sequences were

aligned by PRANK program, a probabilistic multiple alignment program. Highlighted sites are the possible critical residues for functional similarities and difference estimated by rate shift analysis: Magenta, type-I faster; blue, type-I slower; red, type-I & II faster; cyan, type-I & II slower; green, type-II. The functionally important sites from NCBI Conserved Domains Database were indicated above the alignment with inversed letters and bold letters in alignment: Z, zinc binding site; D, DNA binding site; 2, dimer interface; L, ligand binding site; C, coactivator recognition site. Symbols under the alignment indicate the degree of conservation at each column: *, identical; :, conserved substitutions ; ., semi-conserved substitutions.

Figure 5. Transcriptional activities of shark ESR exposed to estrogens. Concentration-response profile for whale shark ESR (A and C), and catshark ESR (B and D) for natural and synthetic estrogens (A and B), and ER agonists (C and D). Natural estrogens, E1 (estrone), E2 (17β–estradiol), E3 (estriol), synthetic estrogens, EE2 (ethinylestradiol), DES (diethylstilbestrol), and ER agonist, PPT (propyl pyrazole triol, ESR1-selective agonist), and DPN (diarylpropionitrile, ESR2-selective agonist) are examined. Each point represents the mean of triplicate determinations, and vertical bars represent the mean±SEM.

Figure 6. Transcriptional activities of shark ESR exposed to bisphenol A (BPA), nonylphenol (NP), and 4-*tert*-octylphenol (OP). Concentration-response profile for whale shark ESR (A) and catshark ESR (B) for BPA, NO, and OP. Each point represents the mean of triplicate determinations, and vertical bars represent the mean± SEM.

Figure 7. Transcriptional activities of shark ESR exposed to DDT and its metabolites. Concentration-response profile for whale shark ESR (A and C) and catshark ESR (B and D) for *p,p'*-DDD, *p,p'*-DDE, or *p,p'*-DDT (A and B), and *o,p'*-DDD, *o,p'*-DDE, or *o,p'*-DDT (C and D). Each point represents the mean of triplicate determinations, and vertical bars represent the mean±SEM.

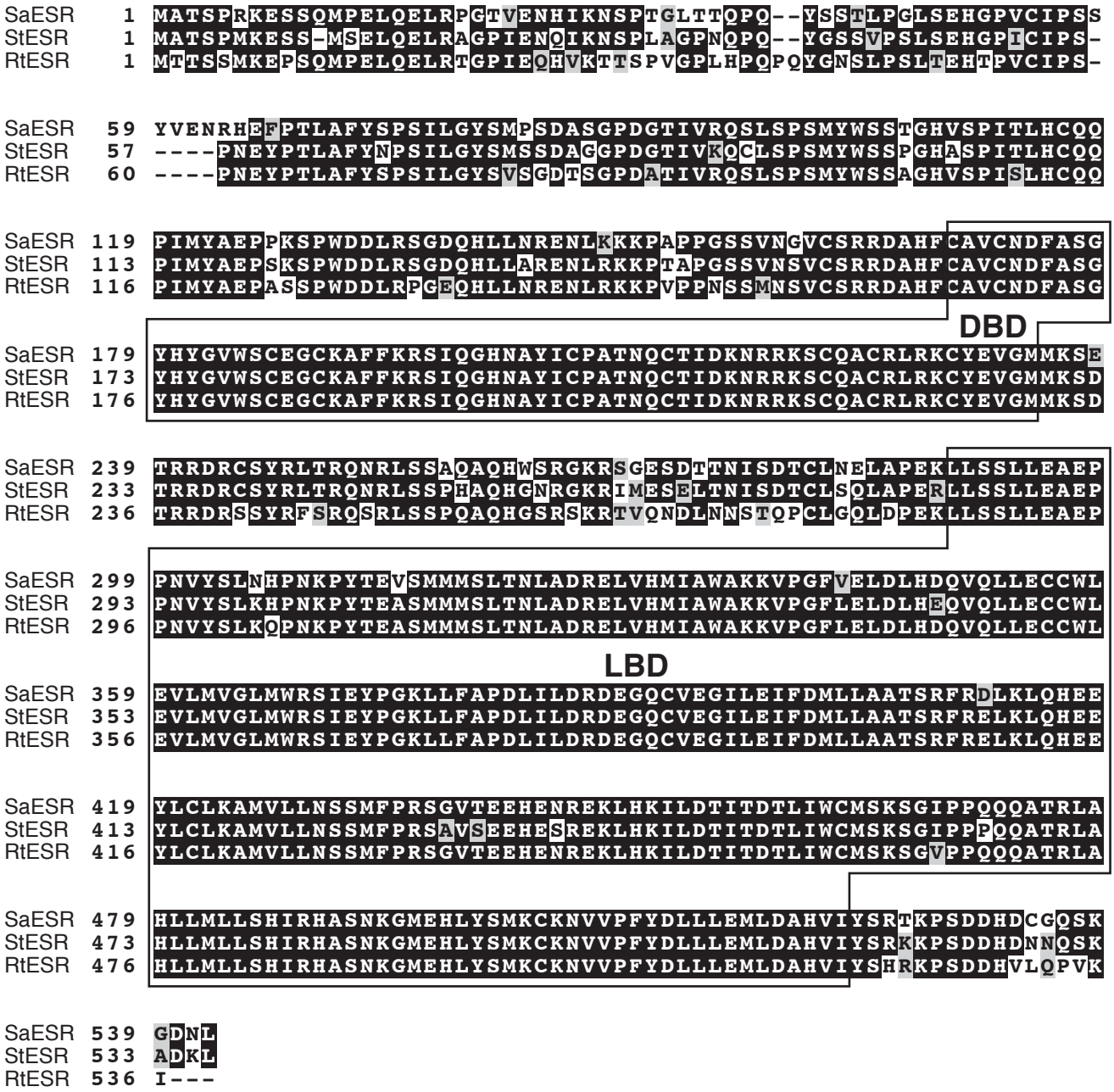
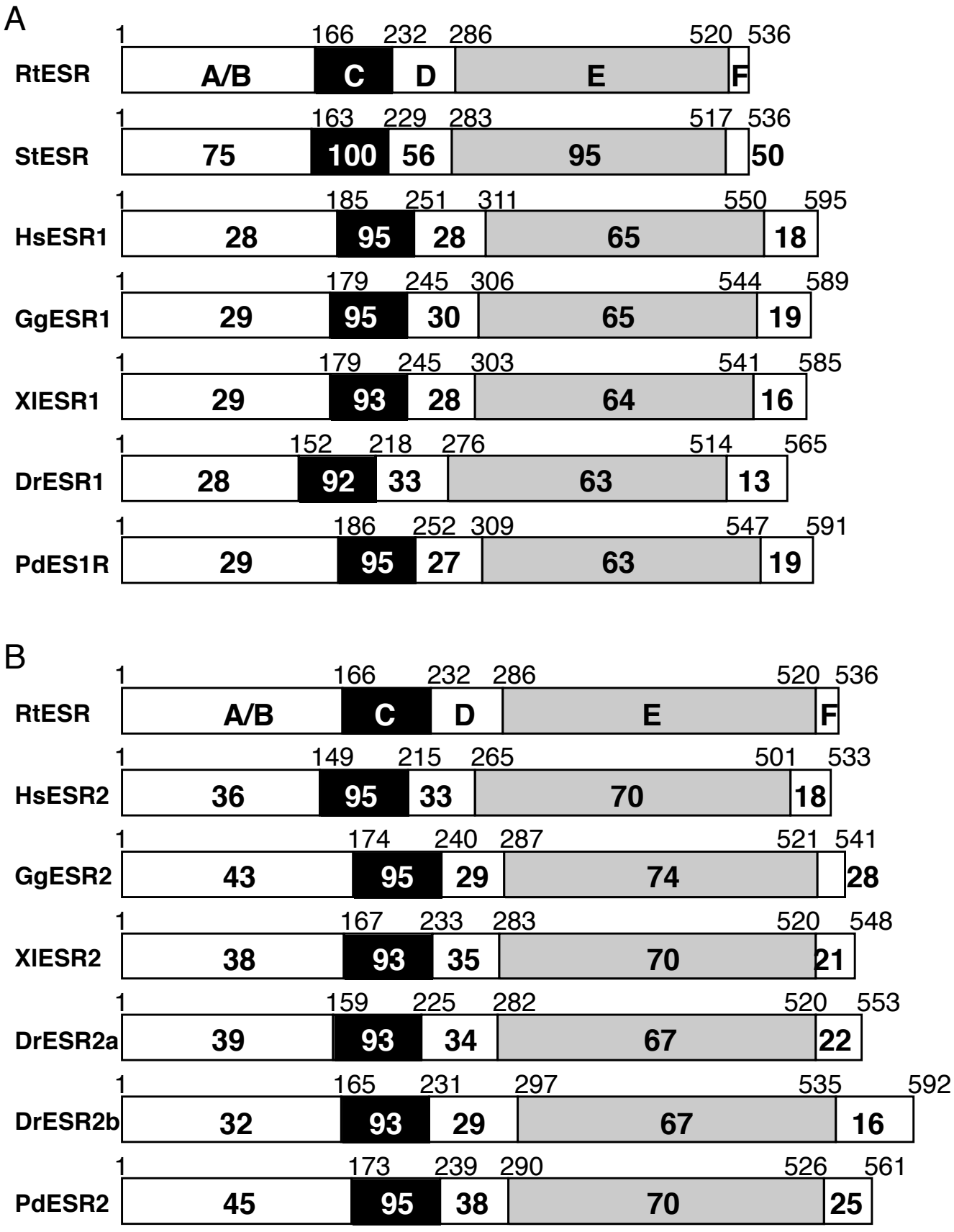


Figure 1; Katsu et al.



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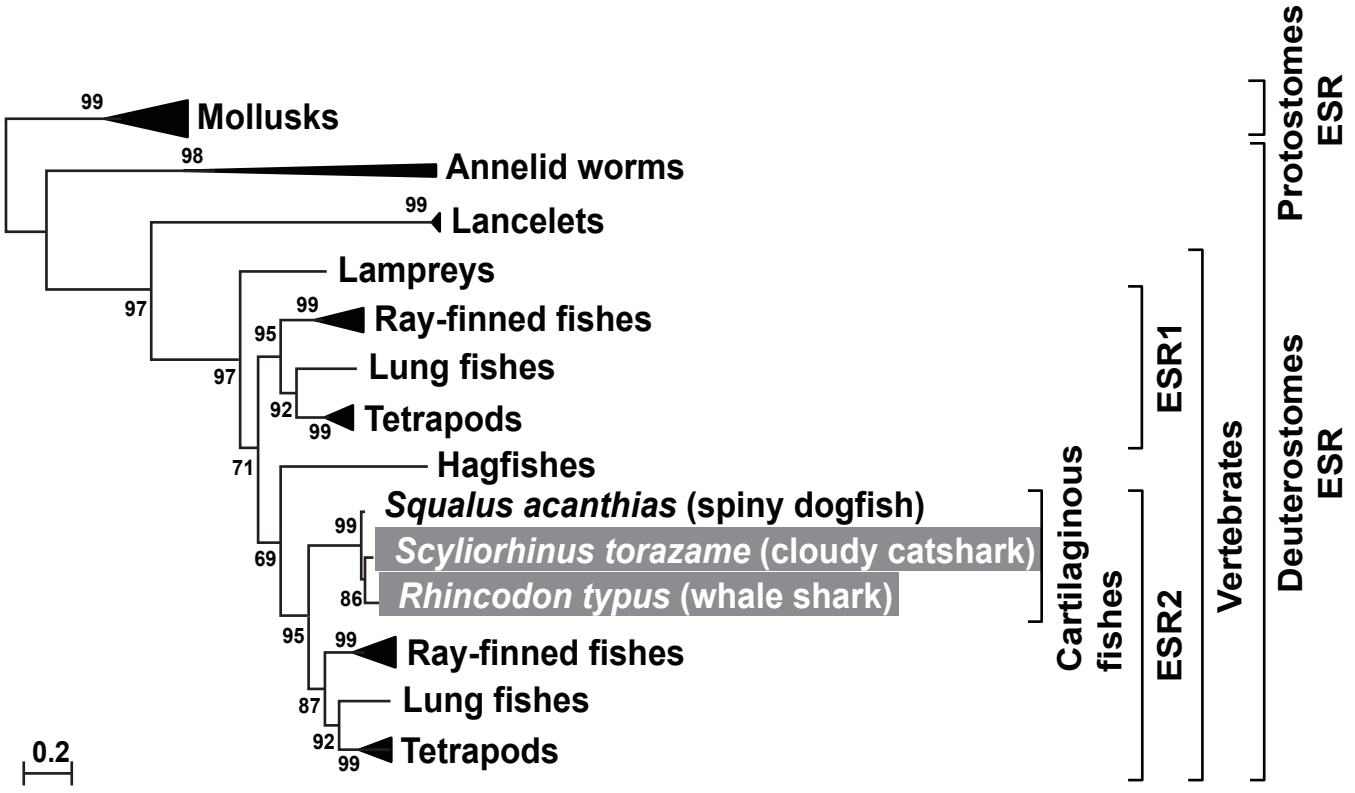

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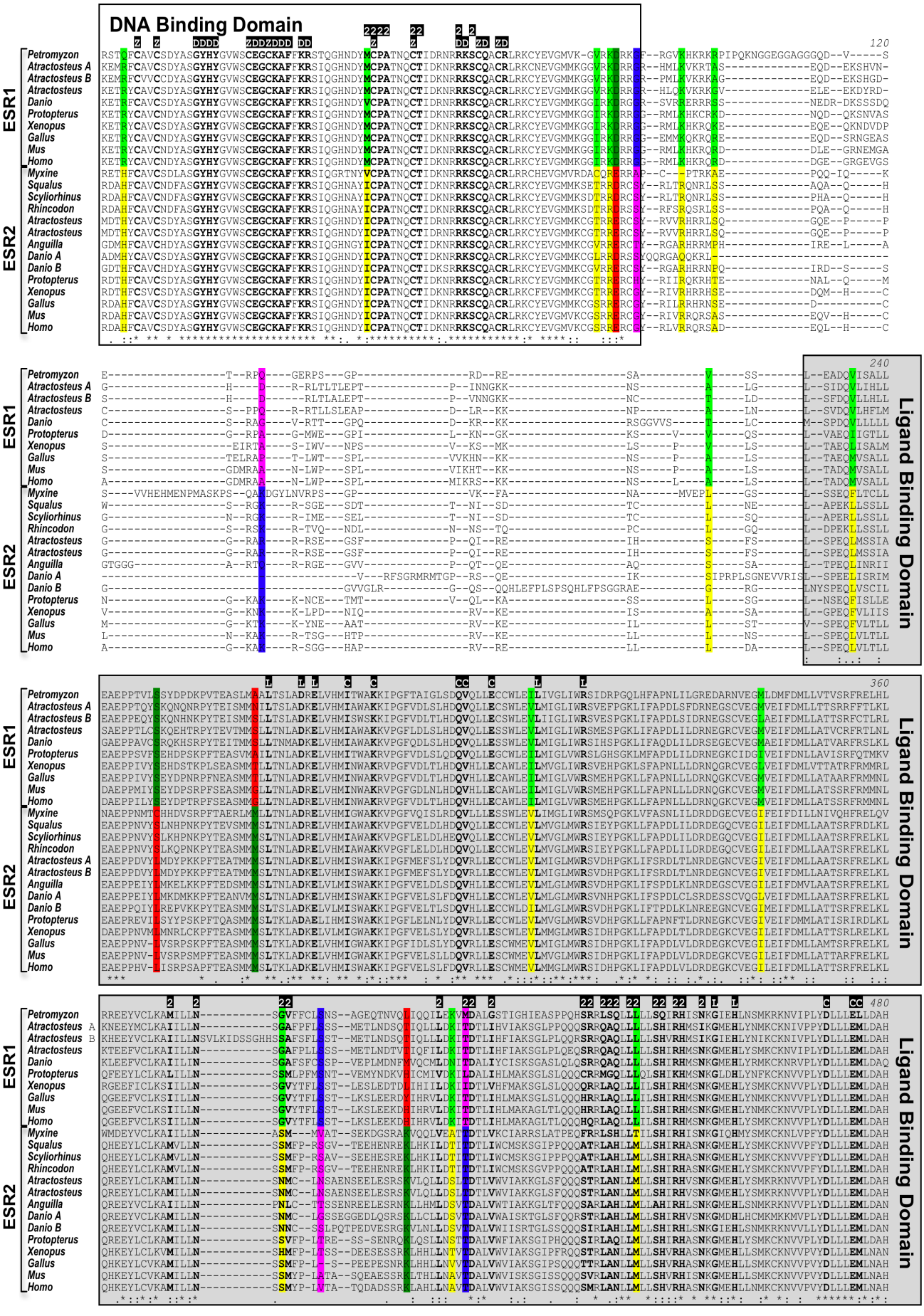

Figure 4; Katsu et al.

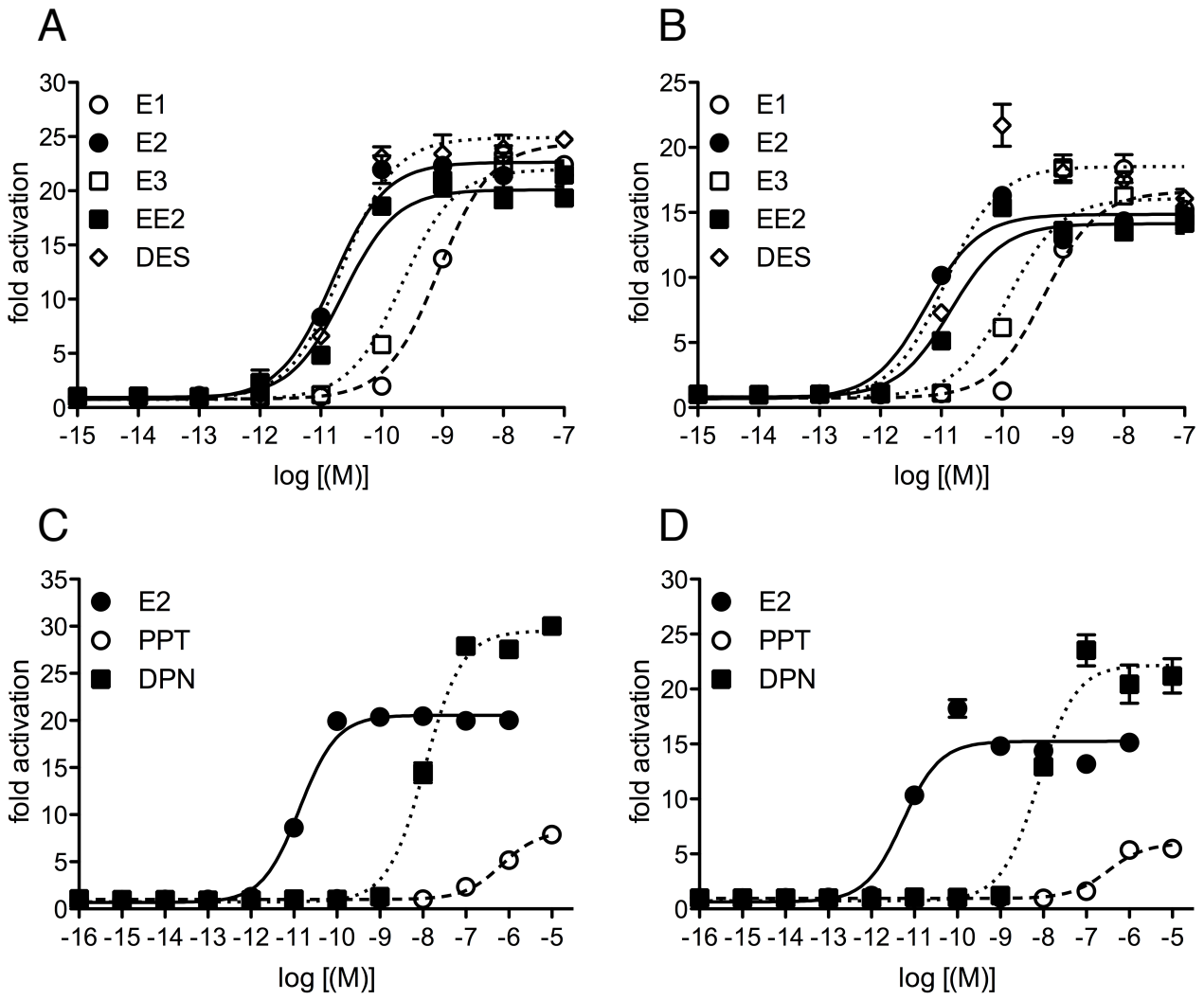

Figure 5; Katsu et al.

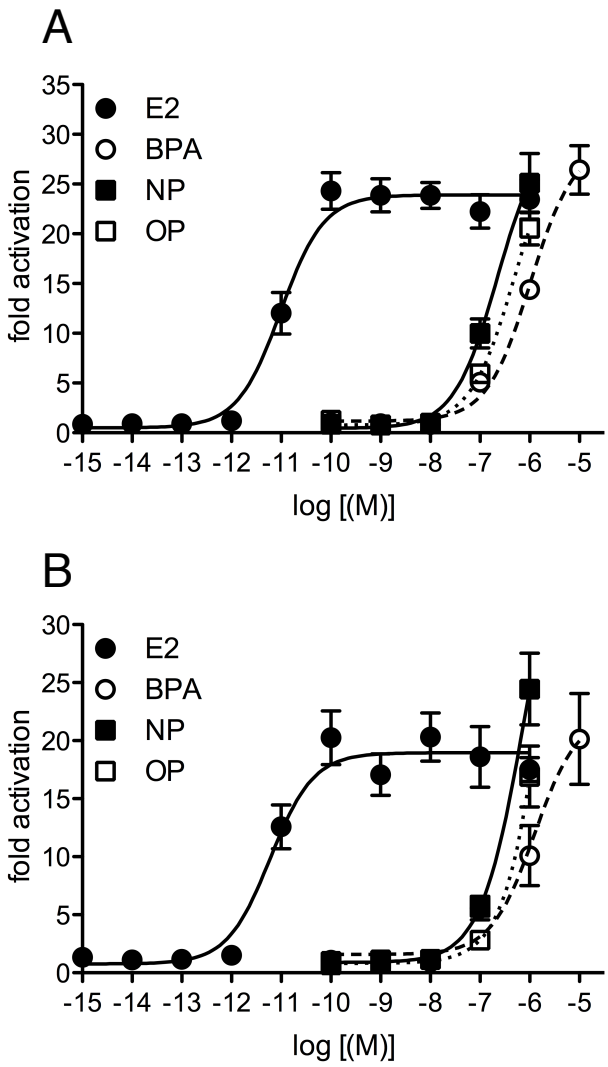

Figure 6; Katsu et al.

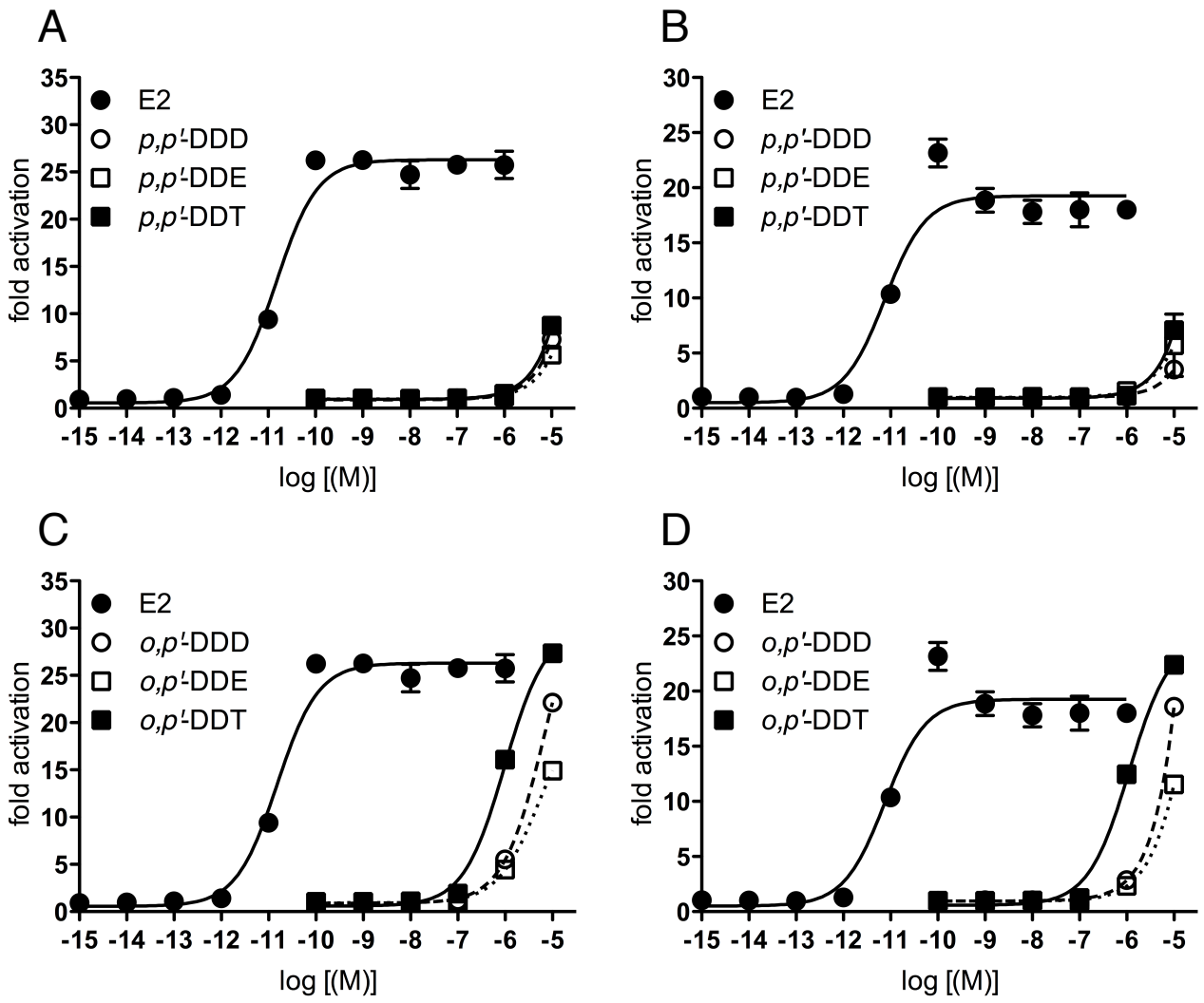

Figure 7; Katsu et al.

Table 1. Gene Transcriptional Activities of Estrogens Mediated by shark ESR

RtESR	EC50 (M)	95% CI[a](M)	RP[b] (%)
E1	8.636×10^{-10}	6.851×10^{-10} to 1.089×10^{-9}	1.1
E2	1.561×10^{-11}	1.114×10^{-11} to 2.186×10^{-11}	100
E3	2.151×10^{-10}	1.539×10^{-10} to 3.006×10^{-10}	4.5
EE2	2.364×10^{-11}	1.674×10^{-11} to 3.338×10^{-11}	39.6
DES	2.239×10^{-11}	1.623×10^{-11} to 3.087×10^{-11}	48.2

StESR	EC50 (M)	95% CI (M)	RP (%)
E1	4.976×10^{-10}	3.135×10^{-10} to 7.898×10^{-10}	1.8
E2	5.659×10^{-12}	3.418×10^{-12} to 9.374×10^{-12}	100
E3	1.254×10^{-10}	7.429×10^{-11} to 2.118×10^{-10}	7.3
EE2	1.430×10^{-11}	9.175×10^{-12} to 2.228×10^{-11}	66.0
DES	1.173×10^{-11}	6.777×10^{-12} to 2.031×10^{-11}	69.7

[a]95% CI: 95% confidence intervals of EC50.

[b]RP: relative potency = (EC50 E2/ EC50 chemical X) x 100.

RtESR: whale shark ESR, StESR: catshark ESR

Table S1. Amino acid sequences and their accession numbers used in phylogenetic analysis.

Sequence Name	Scientific Name	Common Name	Genbank or Swissprot accession numbers		
			ESR	ESR1	ESR2
*Octopus*_ESR	*Octopus vulgaris*	common octopus	Q19AB0		
*Marisa*_ESR	*Marisa cornuarietis*	Snail	ABI97119		
*Nucella*_ESR	*Nucella lapillus*	Snail	ABQ96884		
Thais ESR	*Thais clavigera*	Snail	BAC66480		
*Aplysia*_ESR	*Aplysia californica*	California sea hare	Q6VU64		
*Crassostrea*_ESR	*Crassostrea gigas*	Pacific oyster	BAF45381		
*Platynereis*_ESR	*Platynereis dumerilii*	Dumeril's clam worm	EU482033		
*Capitella*_ESR	*Capitella capitata*	polychaete worm	EU497681		
Branchiostoma_f ESR_A, B	*Branchiostoma floridae*	Florida lancelet	ACF16007 EEN69229		
Branchiostoma_b ESR_A	*Branchiostoma belcheri*	Japanese lancelet	BAI59767		
*Myxine*_ESR	*Myxine glutinosa*	Atlantic hagfish	ACC85903		
*Petromyzon*_ESR	*Petromyzon marinus*	Sea lamprey	AAK20929		
*Squalus*_ESR2	*Squalus acanthias*	spiny dogfish			AAK57823
*Rhincodon*_ESR2	*Rhincodon typus*	whale shark			AB551716
Scyliorhinus ESR2	*Scyliorhinus torazame*	cloudy catshark			AB551715
*Acipenser*_ESR1A, 1B, 2	*Acipenser schrenckii*	Amur sturgeon		BAG82650 BAG82651	BAG82654
*Atractosteus*_ESR1, 2	*Atractosteus tropicus*	tropical gar		BAG82653	BAG82654
*Anguilla*_ESR2	*Anguilla japonica*	Japanese eel			O13012
*Danio*_ESR1, 2A, 2B	*Danio rerio*	zebrafish		NP_694491	NP_851297 NP_777287
*Protopterus*_ESR1, 2	*Protopterus dolloi*	slender lungfish		BAG82648	BAG82649
*Xenopus*_ESR1, 2	*Xenopus laevis*	African clawed frog		NP_001083086	NP_001124426
*Gallus*_ESR1, 2	*Gallus gallus*	chicken		NP_990514	NP_990125
*Mus*_ESR1, 2	*Mus musculus*	house mouse		NP_031982	NP_034287
*Homo*_ESR1, 2	*Homo sapiens*	human		NP_000116	NP_001428