魚類のエストロゲン受容体の環境化学物質に対する応答性とその多様性の解析

Analysis of mechanisms and diversity of fish estrogen receptors for endocrine disruptors

February 2016

Laboratory of Molecular Reproductive Biology
Graduate School of Integrated Pharmaceutical and Nutritional Sciences
University of Shizuoka

Saki Tohyama

Abbreviations

BPA	bisphenol A
DBD	DNA binding domain
E2	17β-estradiol
EDCs	endocrine disrupting chemicals
ERE	estrogen response elements
ESR	estrogen receptor
LBD	ligand binding domain
MOE	molecular operating environment
NP	4-nonylphenol
o,p'-DDT	*o,p'*-dichlorodiphenyltrichloroethane
VTG	vitellogenin
WGD	whole genome duplication

Contents

Summary

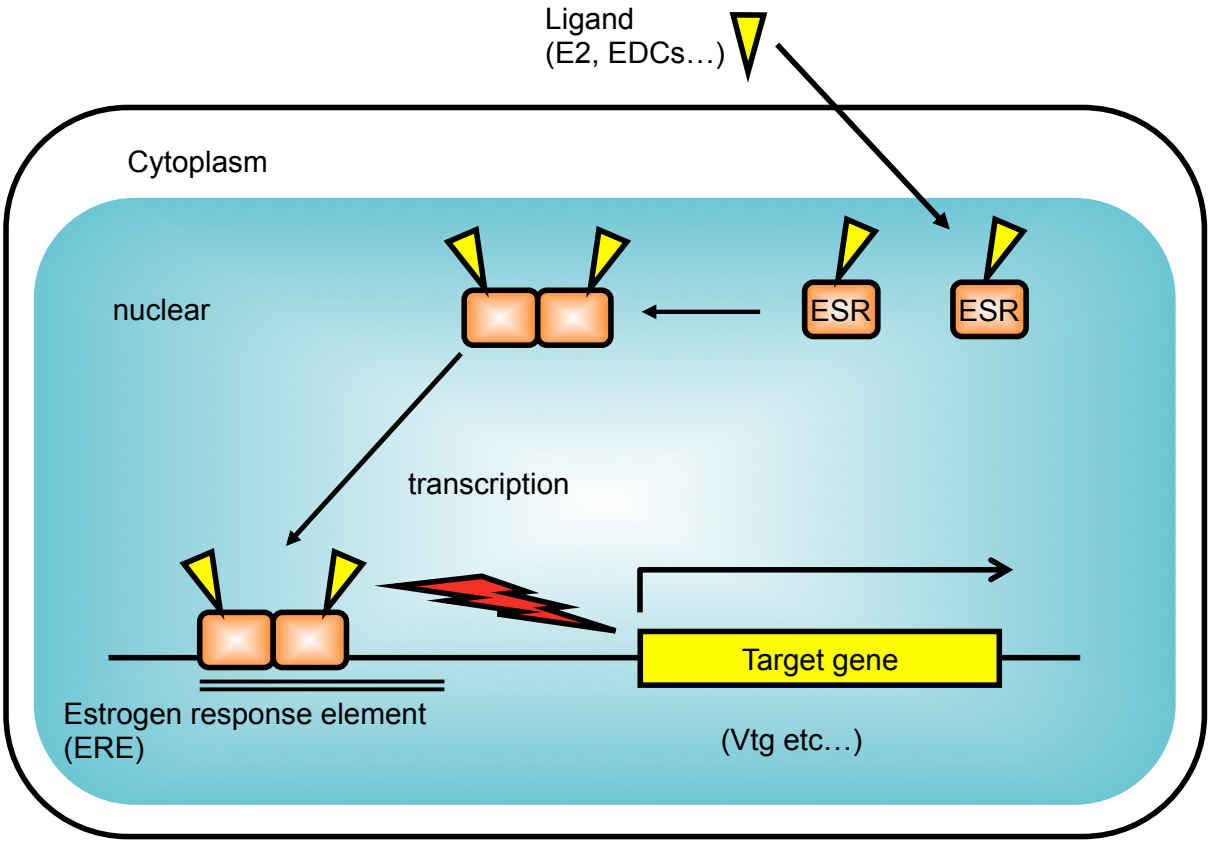
An integrated network of steroid hormones modulates the reproductive systems in most animals. Estrogens, one class of these steroid hormones, are responsible for the induction of behavioral and physiological processes involving many organ systems in vertebrates. Estrogens act via intracellular estrogen receptors (ESRs) that are members of the nuclear receptor superfamily of transcription factors. Upon ligand binding, ESR enhances the rate of transcriptional initiation by assembling and recruiting transcription regulatory complexes to the promoter regions of responsive genes (Summery Fig.1).

A wide range of environmental chemical pollutants are now known to mimic estrogenic activities by binding to ESRs and modulating endocrine systems in wild animals, especially aquatic species. Exposure to these chemicals, so called estrogenic endocrine disrupting chemicals (EDCs), cause disruptions in development, alter sexual differentiation and function, and impact adversely on reproduction in fish. For instance, testis-ova, the occurrence of oocytes in testis, have been used as an indicator of reproductive toxicity associating with exposure to estrogenic chemicals and are a well-documented phenomenon in a variety of wild fish species around the world. The formation of testis–ova is clearly accompanied with the disturbance of spermatogenesis, resulting in reduced fertility. To take effective measures to counter against threaten in wildlife by EDCs, mechanisms underlying EDCs-induced adverse effects in fish should be elucidated. Although, extensively studied in mammals, the evolution and molecular mechanisms associated with the ESRs in fish have been poorly understood. The receptor-dependent reporter gene assay using luciferase in mammalian cell lines are commonly used to characterize transactivation by steroid hormone receptors and allows for understanding on ESR-ligand interaction across different species.

Teleost species exhibit at least three subtypes of ESR, ESR1, ESR2a and ESR2b, thus estrogenic signaling pathways are complex. I applied *in vitro* reporter gene assays for ESRs in five fish species to investigate the ESR subtype-specificity for better understanding the signaling pathway of estrogenic EDCs. Responses to bisphenol A, 4-nonylphenol and *o,p'*-DDT varied among ESR subtypes, and the response pattern of ESRs was basically common among the different fish species; each receptor subtype responds similarly to natural estrogen 17β-estradiol (E2), but differentially to EDCs (e.g., ESR2a exhibits a weaker reporter activity compared with ESR2b). Using a computational *in silico* docking model, and through assays quantifying transactivation of the ligand-binding domain (LBD), I found that the LBD of the different ESR subtypes generally plays a key role in conferring responsiveness of the ESR subtypes to EDCs. These results also indicate that responses of ESR2s to EDCs cannot necessarily be predicted from the LBD sequence alone and an additional region is required for full transactivation of these receptors. My data thus provide advancing understanding on ESRs functioning for both basic and applied research (Chapter 1).

To investigate whether response patterns of ESR subtypes observed in Chapter 1 are common throughout ray-finned fish, I then investigated the functional diversity and molecular basis or ligand sensitivity of ESRs among ray-finned fish species (Actinopterygii), the most variable group within vertebrates. I cloned and characterized ESRs from several key species in the evolution of ray-finned fish including bichir (Polypteriformes, ESR1 and ESR2) at the basal lineage of ray-finned fish, and arowana (Osteoglossiformes, ESR1 and ESR2b) and eel (Anguilliformes, ESR1, ESR2a and ESR2b) both belonging to ancient early-branching lineages of teleosts, and suggest that ESR2a and ESR2b emerged through teleost-specific whole genome duplication, but an ESR1 paralogue has been lost in the early lineage of euteleost fish species. All cloned ESR isoforms showed similar responses to endogenous and synthetic steroidal estrogens, but they responded differently to non-steroidal estrogenic EDCs.

Thus, I show that variation in ligand sensitivity of ESRs can be attributed to phylogeny among species of different taxonomic groups in ray-finned fish. The molecular information provided contributes both to understanding of the comparative role of ESRs in the reproductive biology of fish and their comparative responses to EDCs (Chapter 2).



Summery Figure1. Schematic representation of the ligand-dependent ESR transactivation. Upon binding of a ligand to the ligand-binding domain (LBD) of ESR, The ligand-receptor complex binds as homo dimer to estrogen response elements (ERE) on the promoter regions of estrogen responsive target genes and stimulates their transcription.

Chapter 1

Understanding the molecular basis for differences in responses of fish estrogen receptor subtypes to environmental estrogens

Introduction

Steroid hormones play fundamental roles in regulating reproductive activities in vertebrates. Estrogens in particular regulate ovarian development, differentiation and maintenance, and oogenesis, as well as stimulating the hepatic synthesis of vitellogenin (VTG) and choriogenin, which are vital for oogenesis in fish [1, 2]. These effects are principally mediated through estrogen receptors (ESRs) which belong to the nuclear hormone receptor superfamily. After binding of a ligand to the ligand-binding domain (LBD) of ESR, this complex binds as homo dimer to estrogen response elements (ERE) in the promoter regions of estrogen responsive target genes, and regulates their transcription.

Two ESR subtypes (ESR1 and ESR2) have been cloned from amniotes. Despite similar *in vitro* ERE-binding capacities and comparable affinities for 17β-estradiol (E2) [3,4], ESR1 and ESR2 mediate distinct profiles of gene expression [5, 6]. Therefore, ESR1 and ESR2 have been considered to have specific roles and mediate responses to estrogens differently in mammals. In mice, ESR1 is essential for the development and function of reproductive organs, while ESR2 activity has more of a role associated with non-reproductive organs, although its presence (and activity) in the granulosa cells is required for fertility [7,8]. In most teleost species, three subtypes of ESR, namely ESR1 (formerly named ERα), ESR2a (formerly named ERβ2 except for medaka where it is known as ERβ1) and ESR2b (formerly named ERβ1, except for medaka where it is known as ERβ2) have been identified as being encoded by different genes, where ESR2a and ESR2b appear closely related, reflecting a gene duplication event [9-11]. In this thesis, I have adopted the ESR nomenclature for classifying different fish ESR subtypes to avoid confusion in comparisons across the study species (see Table 1 for more details on the nomenclature that has been adopted to describe ESRs in different fish species). The presence of three subtypes of ESR further complicates understanding of ESR subtype-specific roles. In medaka, the level of VTG induction by

several EDCs is positively correlated with expression of ESR2 subtypes[12]. Recent studies using gene knockdown in goldfish primary hepatocytes and zebrafish embryos have demonstrated that ESR2a and/or ESR2b are required for estrogen-mediated upregulation of ESR1 as well as VTG expression [13, 14]. These results suggest fundamental contributions of ESR2 subtypes in fish reproduction.

There is a global concern about the presence of EDCs in the environment and their health effects in both humans and wildlife. To date, particular emphasis has been on effects of estrogenic EDCs and more than 200 chemicals have been identified with estrogenic activity, including pharmaceuticals, industrial and agricultural compounds such as alkylphenols, pesticides, plasticizers and bisphenols. Most EDCs readily enter the aquatic and as a consequence, fish are especially at risk of exposure. Effects of some EDCs on fish are proven and they include delayed onset of sexual maturation, reduced gonadal growth, gonadal deformations, inhibition of spermatogenesis, reduced sperm counts, lowered egg production, skewed sex ratios and increased prevalence of intersex [15-19]. However, the involvement of different ESR subtypes in mediating the adverse effects of these estrogenic EDCs is so far poorly studied.

Risk of endocrine disruption on fish cannot necessarily be predicted for all species by simply examining receptor activation for a few model fish species [20-24] and comparative analyses are essential for understanding both mechanisms and differences in responsiveness to EDCs. *In vitro* reporter gene assays have been developed and applied successfully as screening methods to evaluate chemicals with estrogenic effects for a variety of model fish species [20, 21, 25]. These assays are now being applied to help inform on the similarities and differences between model and sentinel fish species in ESR activation for the different ESR subtypes [21, 25].

Here, I used custom developed *in vitro* ESR reporter gene assays for five fish species to analyze the ligand-, species-, and subtype-specificity for EDCs. The species adopted were medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) as laboratory 'model' species, three spined stickleback (*Gasterosteus aculeatus*), used for both laboratory and wild population studies, and carp (*Cyprinus carpio*) and roach (*Rutilus rutilus*) that have been used widely for environmental monitoring of EDCs impacts. The test compounds included bisphenol A (BPA), 4-nonylphenol (NP) and *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT), all widely recognized as estrogenic EDCs. I found that each ESR subtype showed distinct responses to EDCs, and these responses for the different ESR subtypes were comparable among five fish species. Given that the ligand sensitivity of ESR1 has been attributed previously to the LBD [20], I applied *in silico* docking model analysis and revealed a strong relationship between simulated ligand-LBD interaction potential and ESR activation in the transactivation assay. However, there were some exceptions to this, in particular for ESR2s, where I show no concurrence between simulated ligand-LBD interaction potential and ESR activation in the transactivation assay. Applying the use of GAL4-ESR-LBD fusion proteins and chimera proteins between ESR2a and ESR2b, I show that an additional region to the LBD was required for full transactivation of ESR2b by EDCs. My findings show that the activity of EDCs acting through ESR2s, cannot be necessarily predicted from the LBD sequence alone. The data presented further highlight the utility of transactivation assays for understanding ESR function and for informing on possible risk associated with EDCs acting via fish ESRs.

Materials and Methods

Source of fish

Zebrafish were kindly provided from Prof. Nagahama (Ehime University, Matsuyama, Japan). Three-spined stickleback were collected in Hokkaido, Japan and roach were raised at the University of Exeter (UK). Medaka were purchased from a local commercial supplier (Meito Suien, Nagoya, Japan).

Chemical reagents

E2 was purchased from Sigma-Aldrich (St. Louis, MO), and NP (purity >97.0%), BPA (purity >99.0%) and *o,p'*-DDT (purity >99.5%) were from Kanto-Kagaku (Tokyo, Japan). All compounds tested in the reporter gene assay were dissolved in dimethylsulfoxide (DMSO, Nacalai, Kyoto, Japan) and the concentration of DMSO in the culture medium did not exceed 0.1%.

Construction of ESRs

The expression plasmids for medaka ESR1, ESR2a; carp ESR1, ESR2a, ESR2b; zebrafish ESR1; stickleback ESR1, ESR2a; roach ESR1 and ESR2b have been described previously [20, 25-27]. The full-coding regions of medaka ESR2b (LC018711), zebrafish ESR2a (NM_180966) and zebrafish ESR2b (NM_174862) were amplified by PCR and subcloned into the mammalian expression vector pcDNA3.1 (Life Technologies, Carlsbad, CA). For the cloning of full length stickleback ESR2b (LC006094) and roach ESR2a (LC006093), RNA was isolated from liver tissues and reverse transcribed into cDNA which served as template for PCR using degenerate oligonucleotides as described previously [22]. The 5'- and 3'-ends of the ESRs were amplified by rapid amplification of the cDNA end (RACE) using the GeneRacer Kit (Life Technologies). A single full-length transcript of the

open reading frame was amplified using the primer set at the 5'-untranslated region and 3'-untranslated region (Fig. 1A).

Cell culture and transactivation assay

HEK293 cells (DS Pharma Biomedical, Osaka Japan) were seeded in 24-well plates (Thermo Scientific, Waltham, MA, USA) at 5 x 10^4 cells per 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, USA). After 24 h, the cells were transfected with 400 ng of pGL3-4xERE [20], 100 ng of pRL-TK (as an internal control containing the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter allowing normalization for variation in transfection efficiency) (Promega, Madison, WI, USA), and 200 ng of pcDNA3.1-ESR using Fugene HD transfection reagent (Promega) according to the manufacturer's instructions. After 4 h of incubation, E2 or EDCs were added to the medium at concentrations between 10^{-7} - 10^{-13} M or 10^{-5} - 10^{-10} M, respectively. After 44 h, 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 and results are presented as mean ± SEM from three separate experiments each consisting of three technical replicates per concentration tested. To average the background luciferase activity, data were normalized for responses of the different ESRs to the individual chemical (where zero and one hundred percent were defined as the lowest and the highest response against E2, respectively, for each data set) and dose-response data were analyzed by fitting three parametric nonlinear regression (slope=1) curves onto the normalized data and EC50

values were calculated from these curves using GraphPad Prism ver.5 (GraphPad Software, San Diego, CA, USA), The statistical analysis was performed with two-factor factorial ANOVA and significance was set at p=0.05.

Construction of GAL4-ESR-LBD and chimera ESR proteins

The hinge and LBD of each medaka (m)ESR subtype amplified by PCR were subcloned into a pBIND vector (Promega) containing the GAL4-DNA-binding domain. The hinge region was included as it is required for transactivation of GAL-LBD fusion proteins [28]. Medaka ESR2 chimeras in which the ESR2-LBDs were swapped were constructed using the In-Fusion cloning kit according to the manufacturer's protocols (Takara, Otsu, Japan) (Fig. 1B). The numbers of amino acid residues corresponding to each domain are shown in Figure 2.

The transactivation assays were performed as described above, with the exception that the GAL4-responsive pG5 vector (Promega) was used as a reporter. E2 was added to the medium at a concentration of 10^{-8} M which induces a maximal response, whereas EDCs were added at 10^{-5} M. This is the maximum concentration to avoid cytotoxicity as adopted in previous studies [20]. Results are presented as mean ± SEM from three separate experiments each consisting of three technical replicates per concentration tested. Data were normalized for responses of the different ESRs to the individual chemical, where zero and one hundred percent were defined as the vehicle control and the response against E2 (10^{-8}M), respectively, for each data set. The statistical analysis between the receptors was performed with two-way ANOVA with Bonferroni post test and significance was set at p=0.05.

Cellular Localization of mESR subtypes.

The full-coding regions of mESR1, mESR2a and mESR2b were amplified by PCR and subcloned into the pDsRed-Monomer-N1 vector (Takara; a mammalian expression vector

that encodes DsRed-Monomer (DsRed.M1), a monomeric mutant derived from the tetrameric *Discosoma sp*. Red fluorescent protein DsRed)(Fig. 1C). COS7 (DS Pharma Biomedical) cells were transiently transfected with 200 ng of DsRed-tagged ESR using Fugene HD transfection reagent. After 4 h of incubation, E2 was added to the medium at 10^{-8} M. After 20 h, cells were washed with PBS and fixed in 4% paraformaldehyde. Fluorescence images were taken using a Nikon Confocal Microscope A1Rsi (Nikon, Tokyo Japan).

Computational model and docking simulation for medaka ESR subtypes

The homology modeling of the ESR-LBD and the *in silico* analysis of the interaction potential between ligands and the ESR-LBD were performed using the programs of Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Canada). To build homology models of mESR-LBDs, the crystal structures of human (h)ESR1-LBD, hESR2 and the interaction between hESR1-LBD and BPA were obtained from the Protein Data Bank (PDB; entries 1A52, 2FSZ and 3UU7, respectively) [29-31]. The structures of the ESR-LBDs in the absence of the ligand were optimized by the AMBER12: EHT force field with an energy gradient of 0.056. The ESR-LBDs model was prepared with the Protonate 3D program by adjusting the protonation state of the running buffer to pH 7.0 in which the ligand-ESR interaction was monitored. These structures were then used to identify the ligand-binding sites using MOE Alpha Site Finder. The chemical structures of ligand were constructed, rendered, and minimized with the MMFF94x force field in MOE7. The systems were subjected to 20 ps heating and equilibration from 100 to 300 K, followed by 1 ns simulation at 300 K with 2 fs time steps. The molecular volumes of the resulting ligand structures were calculated with the AtomRegion program using a grid space of 0.1Å. Possible docking of ligands was searched with the ASEDock program. All ASEDock algorithms were coded using MOE Scientific Vector Language. Existing features implemented in MOE were

completely applied to realize the ASEDock functions. A total of 250 conformations were generated for each chemical by LowMode Molecular Dynamics8. The most stable ligand-binding modes with the ESR-LBD were determined based on the lowest U-total value (kcal/mol). Each docking simulation was evaluated with a U-dock score (kcal/mol) that was the sum of U_ele (electric energy), U_vdw (van der Waals energy), U_solv (solvation energy), and U_strain (strain energy). The most stable ligand-binding modes with the ESR-LBD were determined based on the lowest U-total value and each docking simulation was evaluated with a U-dock score (kcal/mol). The critical amino acids for the ligand interaction were determined using the ligand interaction module of MOE.

Results

Cloning and phylogeny of fish ESR subtypes

I collected information of all ESR subtypes from medaka, three-spined stickleback, zebrafish, common carp and roach from Genbank and Ensembl databases. Although most teleosts are predicted to have three ESRs (ESR1, ESR2a and ESR2b), information for only one ESR2 subtype was available in the database for both stickleback (ESR2a) and roach (ESR2b). To isolate stickleback ESR2b and roach ESR2a, partial DNA fragments were amplified from the liver of each fish species by RT-PCR using degenerate oligonucleotides [22]. DNA fragments similar to ESR were obtained from both stickleback and roach cDNAs and using the RACE technique, full length ESR2 cDNAs, including the ATG start site and TGA/TAA terminal signal were cloned. Based on the phylogenic analysis (see below), these isolated ESRs were identified as stickleback ESR2b and roach ESR2a. The stickleback ESR2b gene (LC006094) comprises 1956 nucleotides and encodes a protein of 651 amino acids, the roach ESR2a gene (LC006093) comprises 1653 nucleotides and encodes a protein of 550 amino acids.

Comparison of the amino acid sequences of the three ESR subtypes across the five fish species showed that all sequences could be subdivided into 5 domains as defined by Krust *et al* [32] (Fig. 2). The putative DNA-binding domain (DBD; C domain) and LBD (E domain) showed high similarities across the species analyzed (ESR1: DBD, 96-97%; LBD, 80-90%; ESR2a: DBD, 97%, LBD, 87-90% and ESR2b: DBD, 97-100%, LBD, 78-97%, all compared to the respective medaka sequence). The A/B-, D- and F-domains had lower homologies (ESR1: A-domain, 32-58%; D-domain, 32-49%; F-domain, 7-24%; ESR2a: A-domain, 43-62%; D-domain, 36-52%, F-domain, 16-32% and ESR2b: A-domain, 38-66%; D-domain, 40-62%, F-domain, 7-15%, all compared to the respective medaka sequence). On exclusion of the hypervariable A/B and F domains, the neighbor-joining phylogenetic tree for

ESRs resulted in the predicted two separate clades ESR1 and ESR2 (Fig. 3). In addition, the teleost ESR2 clade subdivides as a result of duplication of the ESR2 gene.

Estradiol-induced transcriptional activity of fish ESR subtypes

Differences in translational activities of ESR1s mediated by the natural estrogen, E2, were small among species with only a 3.2-fold difference in ESR1 sensitivity between the most (medaka, EC50=$1.31x10^{-10}$M) and the least (carp, EC50=$4.18x10^{-10}$M) sensitive species (Fig. 3A and Table 2). Similarly, the differences in ESR2a in response to E2 were also small with a 3.0-fold difference between the most (zebrafish, EC_{50}=$2.80x10^{-11}$M) and the least (stickleback, EC50=$8.44x10^{-11}$M) sensitive species (Fig. 4B and Table 2). In all species, ESR2a was more sensitive to E2 compared with ESR1. The EC50 of ESR2b showed an 88-fold difference in sensitivity between the most (zebrafish, EC50=$2.64x10^{-12}$M) and the least (roach, EC50=$2.33x10^{-10}$M) sensitive species (Fig. 4C and Table 2). Overall, with the exception of zebrafish, the EC50 values for ESR2b for E2 were between the EC50s for ESR1 and ESR2a. These results indicate that ESR subtype specificity and species differences are minimum in terms of the responses to E2.

Transactivation of fish ESRs exposed to EDCs

The dose-response curves and calculated EC50 values indicated species- and ESR subtype-differences in the sensitivity of ESRs to BPA, NP and *o,p'*-DDT (Fig. 5A-C and Table 2). Compared with E2, they all exhibited relatively weak estrogenic activities in inducing transactivation of all ESR subtypes examined.

BPA, NP and *o,p'*-DDT transactivated mESR1 and reached the maximum level of E2-induced transactivation at 10^{-5}M (Fig. 5A). However, they were found to be very weak estrogens for mESR2a; even at 10^{-5}M, BPA induced half of the maximum level of E2-induced

transactivation (Fig. 5B). In contrast, these chemicals activated mESR2b, and the order of responsiveness to the different chemicals (at 10^{-6} M) was NP< *o,p'*-DDT<BPA (Fig. 5C).

Although the pattern of transactivation of ESR1 in other fish species varied widely with the different chemicals, for all five species higher maximum activities EC50 values were generally observed for ESR1 compared with ESR2a (Fig. 5D-O, Table 2). When comparing maximum responses of ESR2a and ESR2b, the EDCs tested were more effective in activating ESR2b compared with ESR2a (stickleback; Fig. 5E, F, zebrafish; Fig. 5H, I and carp; Fig. 5K, L). Both forms of the roach ESR2 exhibited lower transactivation abilities in response to EDCs (Fig. 5N, O). Taken together, these results showed that each ESR subtype responded differently to EDCs, but that the comparative responses for any one ESR subtype seems to be conserved among the fish species.

Cellular localization of medaka ESR subtypes

I next investigated the possible reasons for the differential sensitivities among ESR subtypes using medaka as a model. Ligand-dependent nuclear translocation has been suggested to be a possible explanation for variable differences in transactivity between two medaka androgen receptor subtypes [33]. In contrast, experiments on cellular localization of mESRs showed a persistent nuclear localization of all three subtypes independent of the presence or absence of E2 under the culture conditions I adopted in COS7 cells (Fig. 6), suggesting that the nuclear localization step was not the cause of the differential sensitivities among subtypes.

In silico analysis of ligand-binding to mESRs

Our group have previously reported that ligand sensitivities of ESR1 can be attributed, at least in part, to the LBD [20]. The DBDs of all three mESR subtypes showed

high similarities (Fig. 7). These findings, together with the finding above of a persistent nuclear localization of all three subtypes independent of the presence or absence of E2, suggested that ligand-binding as the most likely step responsible for subtype selectivity rather than DNA-binding or nuclear translocation. To gain further insight into the ligand sensitivity among ESR subtypes relating to the LBD, we applied *in silico* analysis of all three mESR-LBD, which is based on a crystal structure of hESR-LBD as a template [29-31]. The homology models of mESR-LBD have close resemblance to hESRs-LBD, with low root-mean square deviation (RMSD) values (0.562Å for ESR1, 0.757Å for ESR2a and 0.820 for ESR2b).

Amino acid residues forming the ligand-binding pocket of the mESR1 for E2 were conserved with hESR1 [30, 34] (Fig. 8A). For example, the hydroxyl group at position C3 of E2 was hydrogen-bonded to the Leu390 of the mESR1, whereas the hydroxyl group at position C17 interacted with His527 (Fig. 8A). In addition, Glu356, which is known to form a hydrogen-bond with E2 in the hESR1 [30, 34] was located at a position close to C3, suggesting a possible electrostatic interaction between them. The interaction energy (U-dock) between mESR1 and E2 was -58.06 kcal/mol. Docking simulations between mESR1 and EDCs were performed, assuming that EDCs occupy the ligand-binding pocket with the lowest binding energy. The *in silico* analysis revealed that at least one side of residues (Glu356) interact with the hydrogen-bonded-, hydroxyl- or chloride-group between ESR1 and EDCs (Fig. 8B-D). In contrast, His527 in mESR1 did not interact with the hydrogen bonds with EDCs, suggesting a lower binding affinity. Given that both sides of the hydroxyl group participated in the hydrogen bond with Glu356 and Met424, the interaction energy between BPA and mESR1 was -48.03 kcal/mol, which was lower (suggesting a more stable binding) compared to NP (-39.58 kcal/mol) or *o,p'*-DDT (-33.17 kcal/mol) (Fig. 8C, D).

Amino acid residues of the ESR2s interacting with E2 were conserved between human and medaka [31]. In particular, Glu316 (mESR2a) and Glu355 (mESR2b) formed hydrogen bonds with the ligand (Fig. 8E, I), and His487 (mESR2a) and His526 (mESR2b) was located in close proximity to the hydroxyl group of C17 of E2. The interaction energy of mESR2a and mESR2b with E2 was estimated to be -53.87 and -55.95 kcal/mol, respectively, showing a similar binding potential compared to mESR1. For mESR2a, modeling suggests that BPA forms hydrogen bonds with Glu377 and Lys298 (Fig. 8F), both not participating in interactions with E2 (Fig. 8B). NP formed a hydrogen bond with Glu276, whereas *o,p'*-DDT did not form this bond (Fig. 8G, H). Accordingly, interaction energy of *o,p'*-DDT was the highest among the EDCs used in this experiment (-49.29, -47.37 and -36.88 kcal/mol for BPA, NP and *o,p'*-DDT, respectively). For mESR2b, a hydrogen bond was observed between Glu355 and the hydroxyl substitution of the BPA and NP (Fig. 8J, K), whereas no strong interaction was detected between *o,p'*-DDT and mESR2b (U-dock estimated as -30.85 kcal/mol; Fig. 8L).

Molecular mechanisms of differential response among ESR subtypes

The predicted interaction energies between ESRs and EDCs showed similar values among the subtypes, but this was not consistent with the transactivation assay, in which ESR2a showed lower transactivation abilities in response to EDCs than those of other ESRs. I then evaluated the above *in silico* simulation results through a series of empirical studies applying the GAL4 system. In this system, upon ligand-binding to the GAL4-ESR-LBD fusion protein, this complex interacts with the GAL4 binding site and activates the reporter transcription (Fig. 9A). The process is completely depending on the LBD and thus enables us to empirically evaluate the interaction between ESR-LBD and the ligands [35].

E2 similarly transactivated all GAL4-mESR-LBD fusion proteins. For ESR1, the EDCs tested induced transactivation of GAL4-mESR1-LBD with the order in responsiveness of BPA>NP>*o,p'*-DDT (Fig. 9A), consistent with the predicted interaction energy *in silico* (Figure 7B-D). On the other hand, NP activated GAL4-mESR2b-LBD weakly, but did not activate GAL4-mESR2a-LBD. BPA and *o,p'*-DDT failed to activate either of the GAL4-mESR2-LBD fusion proteins (Fig. 9A). This suggests that mESR2-LBD can interact with the EDCs but require additional domain for the EDC-driven transactivation in the GAL4 system. I therefore constructed chimera proteins in which LBDs were exchanged between mESR2a and mESR2b. All mESR variants, including the two chimera mESRs, showed ERE-driven reporter activity in response to E2. On the other hand, chimera mESR2s showed that the response to EDCs was much more comparable to the mESRs which the LBD originated from (Fig. 9B). These results indicated that: 1) differences in the transactivation among ESR subtypes largely depends on the LBD; 2) a region additional to the LBD is required for full transactivation of ESR2b by EDCs and; 3) such a region can be substituted by counterparts of mESR2 subtypes.

Discussion

Various EDCs have been reported to affect sex determining processes and alter reproductive output, particularly in fish and amphibian species. For fish, EDC exposures can be continuous and in some cases they occur at high levels. Thus, fish species have been used extensively as models for research into the effects of EDCs and as sentinel for endocrine disruption in wildlife populations. In assessments of the effects of chemicals, generally few studies have taken into consideration 'species diversity' and/or the 'genetic diversity'. A whole genome duplication event occurred coincident with the teleost radiation [9-11, 36] resulting in duplicated paralogous genes and subsequently induced neo-functionalization (i.e. acquiring a new function), sub-functionalization (the original gene function is split and distributed to different paralogues) or inactivation/gene loss [37]. As a consequence in teleosts, estrogen signaling is mediated through at least three ESR subtypes and each subtype will likely show differential responses to ligands. This complicates understanding of estrogen signaling pathways and the deleterious effects of the EDCs on those pathways to affect physiological function.

Species differences of ESR subtypes in response to EDCs

All ESR subtypes responded to the endogenous estrogen, E2, in a similar manner, except for zebrafish ESR2b that showed a high sensitivity compared with the other fish ESR2bs studied. This likely reflects functional conservation in the associated physiological processes during evolution. In contrast, there were clear differences in the responses to EDCs. ESR2a exhibited the weakest reporter activity based on the maximum response. ESR1 and ESR2b both responded to BPA, NP and *o,p'*-DDT, but the sensitivity (EC50) of ESR2b was generally lower compared with the responses for ESR1. My data thus show that these functional responses to estrogenic EDCs for each ESR have persisted during evolution in

different teleost species. I also found, however, that there are differences in the responsiveness of each ESR between fish species. For example, carp and roach ESRs (cyprinid fish) were relatively less sensitive to EDCs which might, at least in part, bear testimony to their ecological niche and tolerance to chemical contamination.

The gene network involved in ESR regulation and the interrelationships between the different ESRs has not been elucidated fully. However, a gene knockdown experiment in goldfish primary hepatocytes has revealed that ESR2a and/or ESR2b are required for estrogen-mediated induction of ESR1 [14, 38]. Furthermore, morpholino-oligonucleotide knockdown experiments in zebrafish embryos revealed interactive expression of ESRs[13]. Although morpholino knockdown experiments are now known to cause non-specific and/or off-target effects in some cases and this can potentially lead to misinterpretation of gene network analyses [39], it is not surprising that coordinated regulation processes of ESRs are required for the induction of estrogen target genes such as VTG. This illustrates that even though ESR1 may act a primary signaling pathway for responding to EDCs (and thus acts as a sensitive biomarker for exposure estrogenic EDCs), responses of the ESR2s to EDCs are also crucial for assessing the effects of these chemicals in exposed animals.

Identifying the source of variation in the responses of ESR to estrogenic EDCs

Our group previously reported that the species differences in activation of fish ESR1 by EDCs depends on the LBD [20]. The LBD confers the ligand-binding and here various cofactor interactions can affect the responses to estrogens [40]. The LBD, therefore, most likely contributes to subtype variation in response to EDCs.

To evaluate the mode of ligand-binding to ESRs, I applied a 3D structure-based computational method. The docking model analysis revealed that the overall architecture of the three mESR-LBDs is similar and amino acid residues interacting with E2 are highly

conserved. As a consequence, the interaction energies (U-dock) between E2 and the different mESR subtypes are comparable. This is consistent with the similar responses of all three mESRs to E2 in my transactivation assays. The hydroxyl group at position C3 of E2 forms a hydrogen-bond with Leu390 of mESR1, Glu316 of mESR2a and Glu355 of mESR2b, whereas the hydroxyl group at position C17 interacts with His527 of mESR1 and possibly with His487 of mESR2a and His526 of mESR2b. The electrostatic interactions between these amino acid residues and the hydroxyl groups at positions C3 and C17 are important for the maximum transactivation of mESRs. Because electrostatic interactions, such as hydrogen bonds, are much stronger than van der Waals forces, an electrostatic interaction network is likely to be the main element explaining the different preferences of EDCs to the ESR subtypes. In addition, lack of interactions between the histidine residues and the ligand induce a substantial reorientation of the imidazole ring of the histidine, possibly destabilizing the interaction between the ligand-binding pocket and the ligand [29, 41]. Nonetheless, two hydroxyl groups of BPA form a hydrogen bond with mESR1 whereas there was only one for NP, but no hydroxyl group in *o,p'*-DDT, resulting in the difference of an order of interaction energy between mESR1 and EDCs as follows: BPA << NP < *o,p'*-DDT. Importantly, this order is consistent with my empirical GAL4-mESR1-LBD assay, showing the robustness of my simulation.

Likewise, the two phenol groups of BPA possibly form hydrogen bonds with Glu377/Lys298 of mESR2a and Glu355/Gly523 of mESR2b. The interaction energy between mESR2s and BPA was estimated to be lower than that between mESR1 and BPA. ESR2a is generally predicted to interact stably with EDCs (lower interaction energy), although this is not consistent with a weak or even lack of mESR2a transactivation by EDCs.

To further investigate the contribution of LBD and to add empirical observation, I performed reporter gene assays using the GAL4 system, which can eliminate potential

dependence of the ESR on the N-terminus and DNA-binding domain of the protein. However, we did not observe any transactivation of GAL4-mESR2a-LBD or GAL4-mESR2b by the EDCs. This discrepancy between the GAL4-system and the intact receptor transactivation assay suggested that additional features are involved in EDC-induced activation of mESR2b. By constructing and applying chimera mESRs in which the LBD of mESR2a was replaced by the corresponding region of mESR2b and vice versa, I confirmed that such region can be replaced by counterparts of mESR2 subtypes. Taken together, my results suggest that although the LBD plays a major role for the differences seen in the transactivation among mESR subtypes, this does not account fully for the differences seen between the mESR2s. The N-terminus within AF-1 is indispensable for BPA-induced transactivation of hESRs [29] These results indicate that in addition to the LBD, highly structural modifications caused by ligand-binding and probably involving in AF-1 within the N-terminal domain, are necessary for transactivation responses, at least for the mESR2b. It remains to be established whether or not EDCs actually bind to ESR2a and, therefore, the mode of binding between them, if any, needs to be further addressed to establish this, which could be established though *in vitro* binding assays and/or crystal structural analysis.

The DBD is highly conserved among ESR subtypes and is thus likely to share largely overlapping properties of DNA-binding. However, ligand-receptor interactions may result in significant modulation within the C-terminal extension of the core DBD and this could affect DNA-binding of the ESRs. It has been also shown that ESR can activate signaling pathways and modulate gene expression independently of direct binding to DNA [43, 44]. It could be the case therefore that some EDCs may have potent effects by modulating ER via non-canonical pathways *in vivo*.

In this study, I show that the ESR reporter gene assay system can be usefully applied in the analysis of ligand-induced ESR transactivation across different fish species to

identify potentially sensitive species, to help understand the roles of different ESR subtypes in estrogen signaling, and to identify functionally important interactions that confer species and ESR subtype specificity for EDCs. These molecular analyses derived from the transactivation assays, together with the *in silco* analyses help in our understanding on the functional divergence of the ESR subtypes in fish and for informing on possible risk associated with exposure to EDCs in fish. Interactions of EDCs with the ESRs and the interactions between ESRs, however, may vary depending on stage of reproductive development, metabolism, season and abiotic factors, such as water temperatures and ultimately *in vivo* studies using life stages of interest are essential in the final risk analysis for estrogenic EDCs.

Table 1. Nomenclature of fish ESRs. It is important here to clarify the terms used for describing teleost ESRs, as the published nomenclature for classification has been confusing, particularly with regards to nomenclature for ESR2 (formerly ERβ) subtypes. To standardize the estrogen receptor subtype nomenclature across all species, here we have adopted the ESR. The medaka ERβ1 is orthologous to ERβ2 in other fish species, including carp and zebrafish, whereas medaka ERβ2 is orthologous to ERβ1 in zebrafish and carp. In human, the accepted nomenclature for ERs is ESR and this has subsequently also been adopted for zebrafish. With regards to the ESR2 orthologues in zebrafish the former ERβ1 subtype is now designated Esr2b, and the ERβ2 subtype is now designated Esr2a. Based on this and to standardize the ESR subtype nomenclature across all species, we have adopted the ESR nomenclature.

Species	ESR1		ESR2a		ESR2b	
	Previous nomenclature	Accession number	Previous nomenclature	Accession number	Previous nomenclature	Accession number
Medaka	ERα	P50241	ESR2	NM_001104702	ERβ2	NM_001128512
	ER	AB033491				
Zebrafish	ESR1	NM_152959	ESR2a	NM_180966	ESR2b	NM_174862
	ESR1	BC162466	ERβ2	AJ414567	ERβ1	AJ414566
	ER	AB037185	ERβa	AF516874	ERβb	AF349413
Carp	ERα	AB334722	ERβ2	AB334724	ERβ1	AB334723
			ERβ	AB083064		
Roach	ERα	AB190289	ESR2a*	LC006093	ERβ	AB190290
					ESR2a	GQ303561**
stickleback	ESR1	NM_001267672	ESR2a	ENSGACG00000007514	ESR2b*	LC006094

*cloned in this study

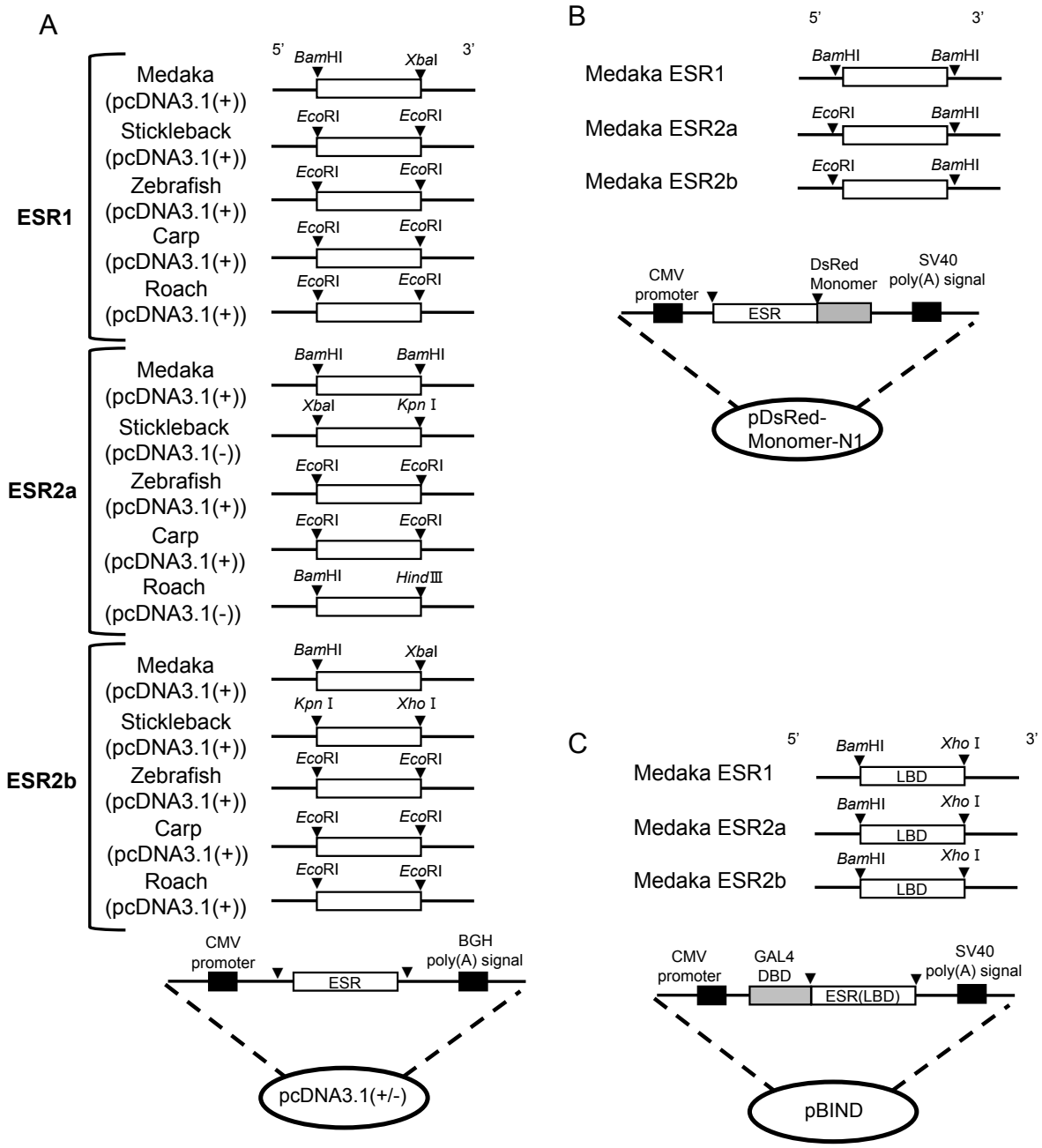


Figure 1. Schematic representation of the plasmid structures used in this study. (A) Each ESR cDNA was inserted into a conventional vector, pcDNA3.1, at the indicated restriction enzyme sites. (B) Medaka ESR cDNAs were inserted into the pDsRed-Monomer-N1 vector, which provide red rluorescent protein-fusion proteins. (C) Medaka ESR-LBD was inserted into the pBIND vector, which contains Gal4-DBD. ▼; restriction enzyme site.

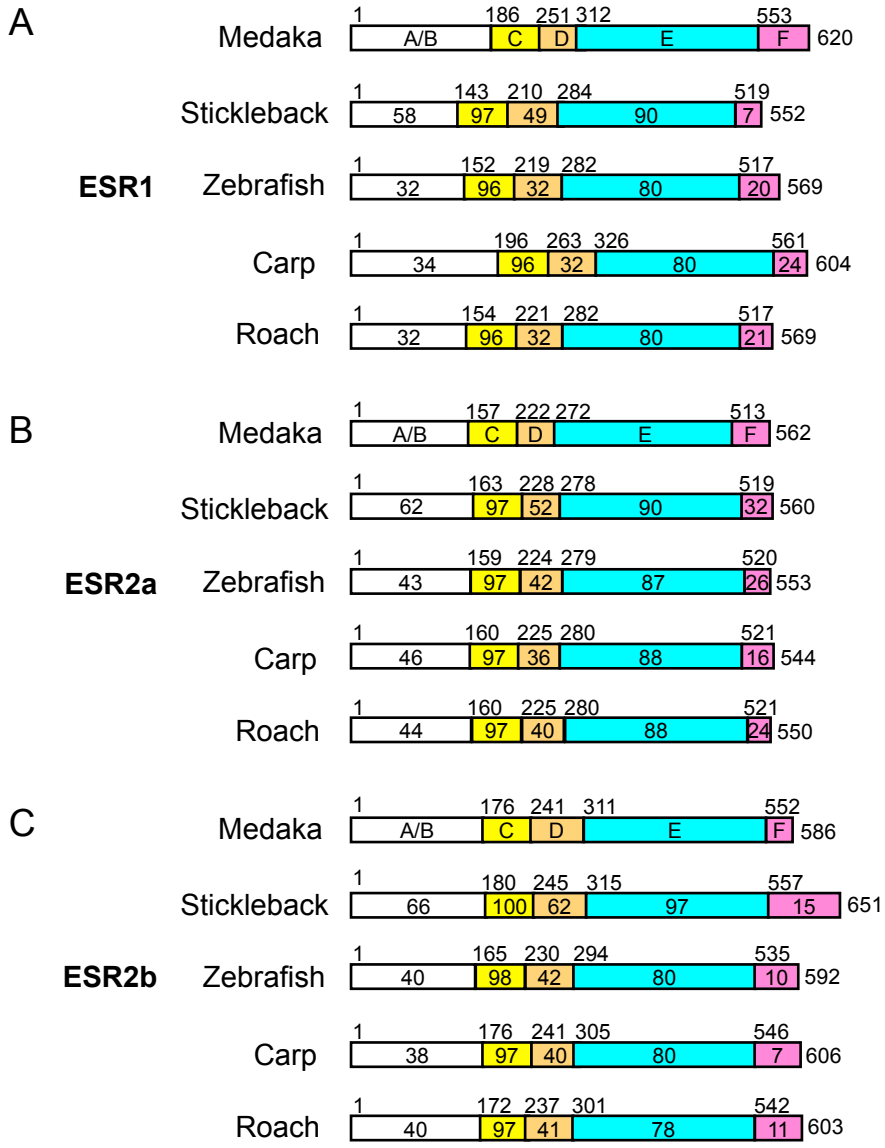


Figure 2. Domain structures of each ESR subtype in ESR1 (A), ESR2a (B), and ESR2b (C). Percent homology of each domain relative to the corresponding medaka ESR are depicted within the boxes, the numbers above each box refer to the position of amino acids in each domain.

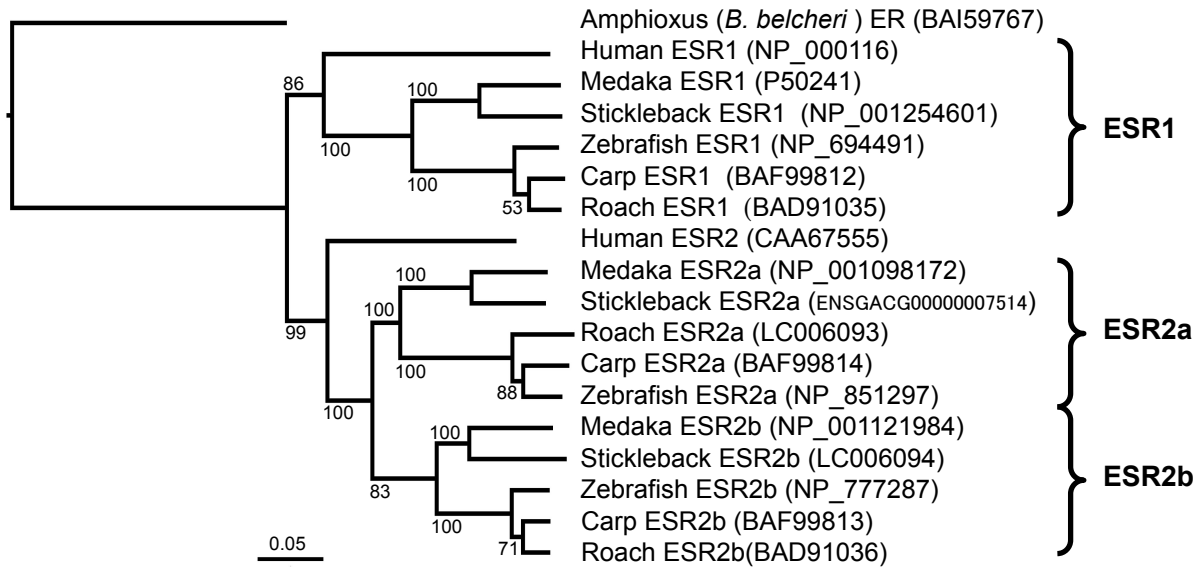


Figure 3. Evolutionary relationships among fish ESRs. The deduced amino acid sequences of the C-E domains of the ESRs were aligned using the Clustal X program. Alignments with questionable gaps were removed. A neighbor-joining tree was constructed from this alignment using a 1,000 replicate bootstrap analysis. FigTree version1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) was used to draw and view the rooted neighbor-joining tree. Amphioxus (*Branchiostoma belcheri*) ESR was used as outgroup of the phylogeny. Human (*Homo sapiens*) ESR1 and ESR2 were also included as the reference. The scale bar represents 0.05 substitutions per site.

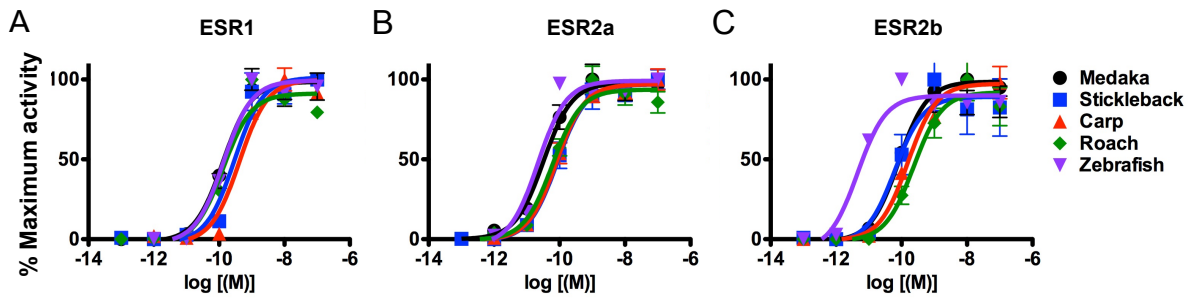


Figure 4. Concentration- response profiles of fish ESR1 (A), ESR2a (B) and ESR2b (C) activated by 17β-estradiol. Dose-response curve were fitted on data normalized between 0 and 100%, where zero and one hundred were defined as the smallest and the largest values in each data set, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

Table 2. Gene transcriptional activities for E2, BPA, NP and *o,p'*-DDT mediated by each ESR subtype from five fish species. For each species, potencies are presented as relative to the response to E2. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

		E2	BPA	NP	*o,p'*- DDT
Medaka ESR1	EC50 (M)	1.31×10^{-10}	7.88×10^{-7}	6.20×10^{-7}	9.63×10^{-7}
	95% CI (M)	$(0.92\text{-}1.9) \times 10^{-10}$	$(0.5\text{-}1.2) \times 10^{-6}$	$(0.37\text{-}1.0) \times 10^{-6}$	$(0.69\text{-}1.4) \times 10^{-6}$
	RP (%)	100	0.017	0.021	0.014
Medaka ESR2a	EC50 (M)	3.25×10^{-11}	6.11×10^{-7}		6.14×10^{-7}
	95% CI (M)	$(1.9\text{-}5.7) \times 10^{-11}$	$(4.8\text{-}7.7) \times 10^{-7}$		$(3.8\text{-}9.9) \times 10^{-7}$
	RP (%)	100	0.0053		0.0053
Medaka ESR2b	EC50 (M)	8.16×10^{-11}	1.84×10^{-5}	8.64×10^{-7}	2.11×10^{-6}
	95% CI (M)	$(0.63\text{-}1.1) \times 10^{-10}$	$(0.89\text{-}3.8) \times 10^{-5}$	$(0.63\text{-}1.2) \times 10^{-6}$	$(1.7\text{-}2.6) \times 10^{-6}$
	RP (%)	100	0.00044	0.0094	0.0039

		E2	BPA	NP	*o,p'*- DDT
Stickleback ESR1	EC50 (M)	2.94×10^{-10}	2.95×10^{-7}	9.63×10^{-8}	2.95×10^{-7}
	95% CI (M)	$(1.5\text{-}5.8) \times 10^{-10}$	$(0.06\text{-}1.5) \times 10^{-6}$	$(0.42\text{-}2.2) \times 10^{-7}$	$(0.08\text{-}1.1) \times 10^{-6}$
	RP (%)	100	0.10	0.31	0.10
Stickleback ESR2a	EC50 (M)	8.44×10^{-11}	1.74×10^{-6}	4.78×10^{-7}	5.33×10^{-7}
	95% CI (M)	$(0.51\text{-}1.4) \times 10^{-10}$	$(0.83\text{-}3.7) \times 10^{-6}$	$(0.16\text{-}1.4) \times 10^{-6}$	$(2.9\text{-}9.9) \times 10^{-7}$
	RP (%)	100	0.0049	0.018	0.016
Stickleback ESR2b	EC50 (M)	6.30×10^{-11}	7.30×10^{-6}	3.46×10^{-6}	4.65×10^{-6}
	95% CI (M)	$(0.18\text{-}2.3) \times 10^{-10}$	$(0.17\text{-}3.1) \times 10^{-5}$	$(1.5\text{-}7.9) \times 10^{-6}$	$(2.7\text{-}8.0) \times 10^{-6}$
	RP (%)	100	0.00086	0.0018	0.0014

		E2	BPA	NP	*o,p'*- DDT
Zebrafish ESR1	EC50 (M)	1.36×10^{-10}	7.88×10^{-7}	6.20×10^{-7}	8.73×10^{-6}
	95% CI (M)	$(0.79\text{-}2.3) \times 10^{-10}$	$(0.53\text{-}1.2) \times 10^{-6}$	$(0.37\text{-}1.0) \times 10^{-6}$	$(0.57\text{-}1.3) \times 10^{-5}$
	RP (%)	100	0.017	0.022	0.0015
Zebrafish ESR2a	EC50 (M)	2.80×10^{-11}	3.86×10^{-7}	-	1.73×10^{-7}
	95% CI (M)	$(1.7\text{-}4.6) \times 10^{-11}$	$(2.3\text{-}6.5) \times 10^{-7}$	-	$(0.62\text{-}4.8) \times 10^{-7}$
	RP (%)	100	0.0073	-	0.016
Zebrafish ESR2b	EC50 (M)	2.64×10^{-12}	1.82×10^{-6}	2.82×10^{-7}	1.00×10^{-6}
	95% CI (M)	$(1.1\text{-}6.5) \times 10^{-12}$	$(1.4\text{-}2.4) \times 10^{-6}$	$(0.92\text{-}8.6) \times 10^{-7}$	$(0.52\text{-}1.9) \times 10^{-6}$
	RP (%)	100	0.00016	0.00094	0.00027

		E2	BPA	NP	*o,p'*- DDT
Carp ESR1	EC50 (M)	4.18×10^{-10}	-	8.47×10^{-6}	-
	95% CI (M)	$(2.4\text{-}7.2) \times 10^{-10}$	-	$(0.60\text{-}1.2) \times 10^{-5}$	-
	RP (%)	100	-	0.0049	-
Carp ESR2a	EC50 (M)	8.01×10^{-11}	1.07×10^{-6}	8.96×10^{-7}	1.36×10^{-6}
	95% CI (M)	$(0.53\text{-}1.2) \times 10^{-10}$	$(0.62\text{-}1.8) \times 10^{-6}$	$(0.30\text{-}2.7) \times 10^{-6}$	$(0.47\text{-}3.9) \times 10^{-6}$
	RP (%)	100	0.0075	0.0089	0.0059
Carp ESR2b	EC50 (M)	1.57×10^{-10}	-	4.22×10^{-6}	9.20×10^{-6}
	95% CI (M)	$(0.90\text{-}2.7) \times 10^{-10}$	-	$(2.3\text{-}6.7) \times 10^{-6}$	$(0.49\text{-}1.7) \times 10^{-5}$
	RP (%)	100	-	0.0037	0.0017

		E2	BPA	NP	*o,p'*- DDT
Roach ESR1	EC50 (M)	1.32×10^{-10}	5.91×10^{-6}	4.24×10^{-7}	4.39×10^{-6}
	95% CI (M)	$(0.71\text{-}2.4) \times 10^{-10}$	$(3.7\text{-}9.5) \times 10^{-6}$	$(2.0\text{-}9.0) \times 10^{-7}$	$(0.07\text{-}2.9) \times 10^{-5}$
	RP (%)	100	0.0022	0.031	0.0030
Roach ESR2a	EC50 (M)	6.06×10^{-11}	2.94×10^{-6}	4.63×10^{-7}	9.36×10^{-7}
	95% CI (M)	$(0.33\text{-}1.1) \times 10^{-10}$	$(1.1\text{-}8.1) \times 10^{-6}$	$(0.02\text{-}8.8) \times 10^{-6}$	$(0.42\text{-}2.1) \times 10^{-6}$
	RP (%)	100	0.0021	0.013	0.0065
Roach ESR2b	EC50 (M)	2.33×10^{-10}	2.56×10^{-5}	-	-
	95% CI (M)	$(1.0\text{-}5.4) \times 10^{-10}$	$(0.01\text{-}4.9) \times 10^{-4}$	-	-
	RP (%)	100	0.00091	-	-

95% CI; 95% confidence intervals of EC50

RP, relative potency; (EC50 E2/EC50 chemical X) x 100

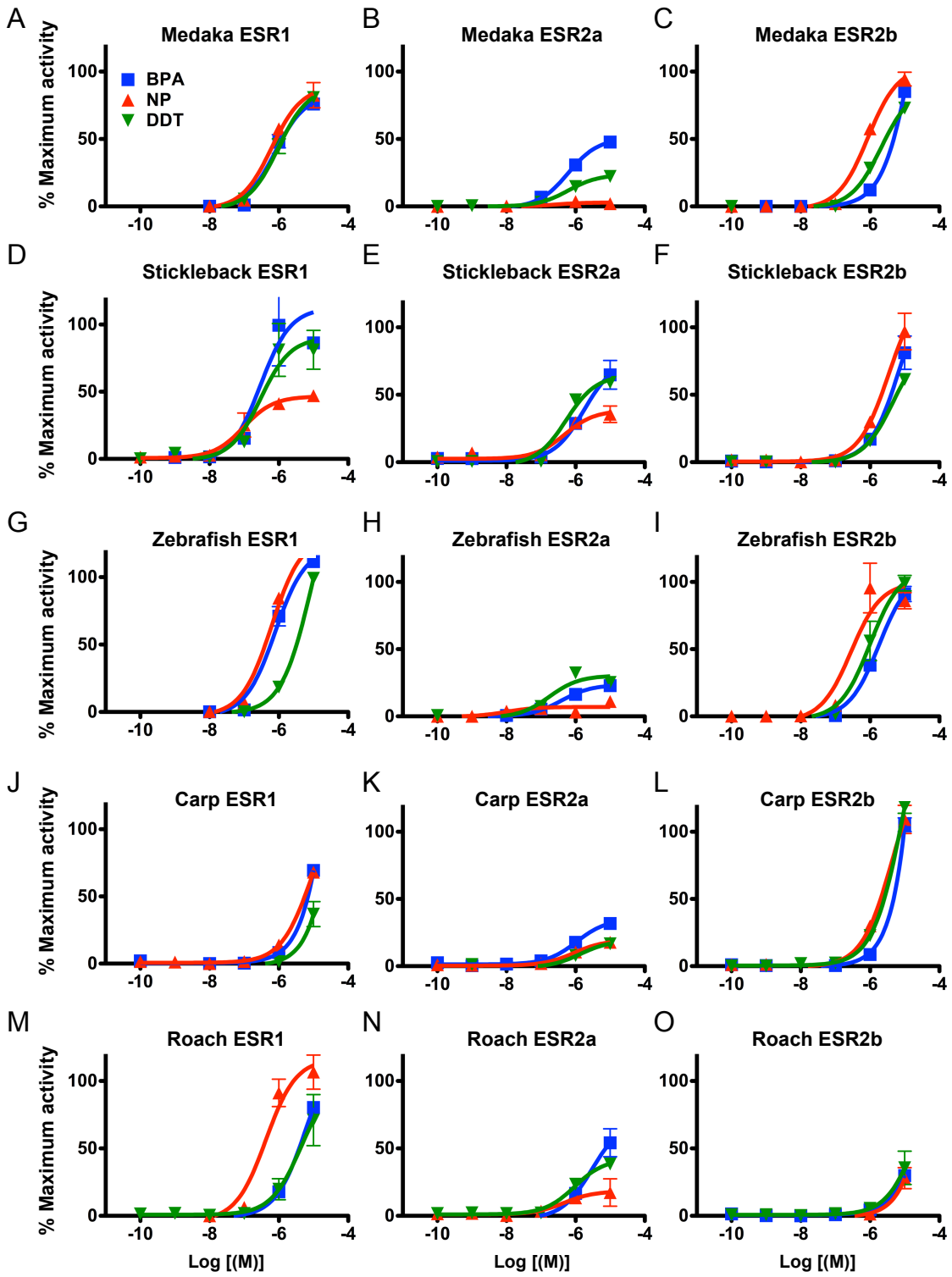


Figure 5. Concentration-response profiles of all ESR subtypes from five fish species activated by EDCs. Fish species investigated were medaka (A-C; the same figures as Figure 1A-C), stickleback (D-F), zebrafish (G-I), carp (J-L) and roach (M-O). Chemicals tested were BPA, NP, and *o,p'*- DDT. Dose-response curves fitted on data normalized between 0 and 100%, where zero and one hundred were defined as the smallest and the largest values of E2 response, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

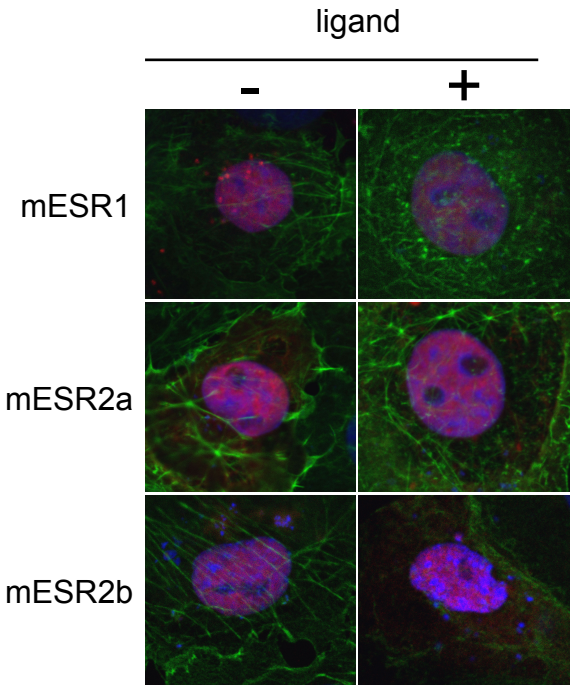


Figure 6. Cellular localization of mESR subtypes in COS7 cells. All mESR subtypes are localized in the nucleus in the presence or absence of E2, suggesting that nuclear localization step does not participate in the differential sensitivities among subtypes. The chromatin DNA and actin were stained with DAPI (blue) and Phalloidin (green), respectively.

Figure 7. Domain structures and sequence comparison of mESR subtypes. (A) Percent homology of each domain relative to mESR1 are depicted. (B) Sequence comparison for mESR subtypes. DBD and LBD are in red and blue, respectively. Note the highly conserved amino acid residues in the DBD (*).

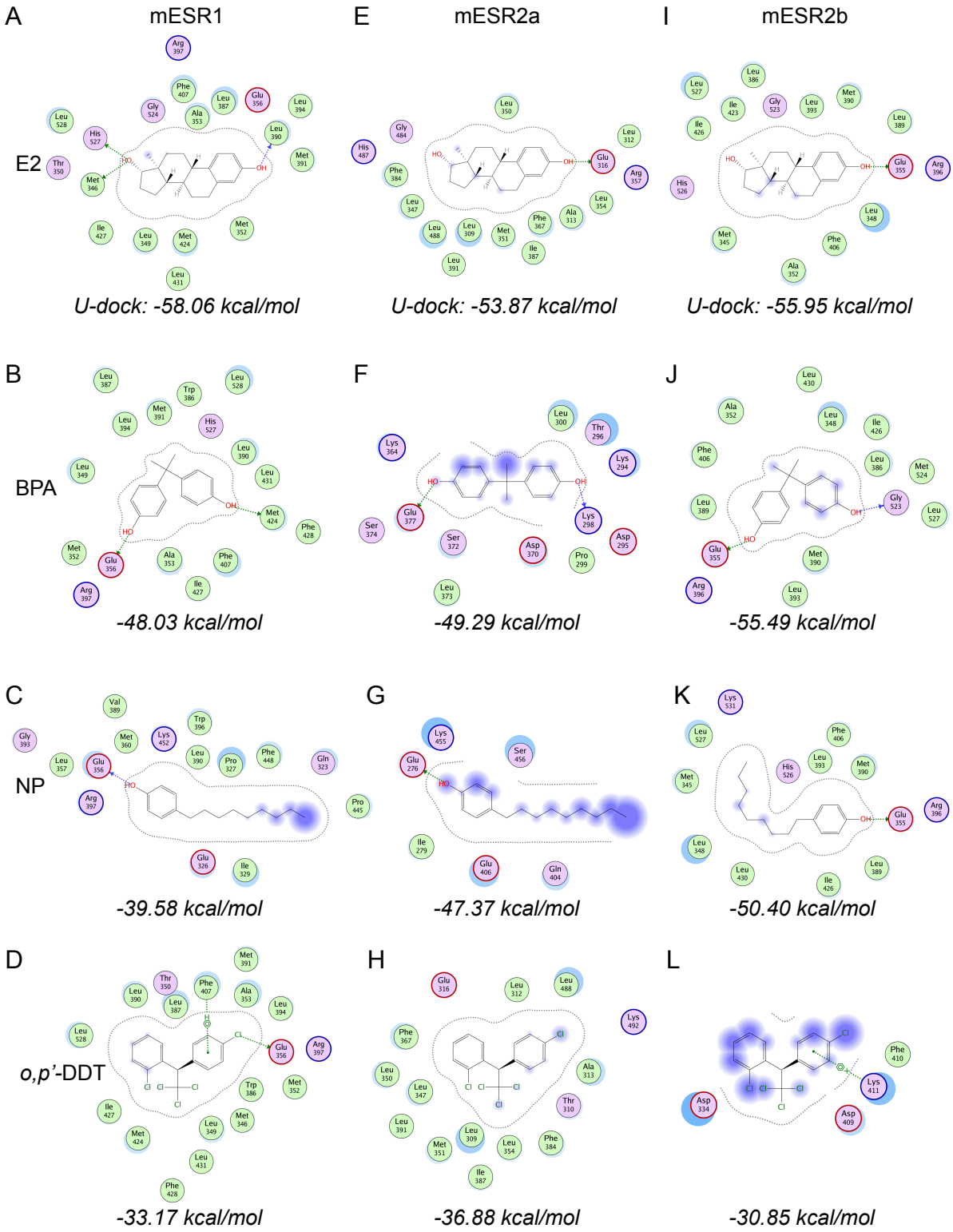


Figure 8. Predicted interaction of amino acid residues of mESRs and the ligands. Amino acid residues of mESR1 (A−D), mESR2a (E−H), and mESR2b (I−K) predicted to interact with E2 (A, E, I), BPA (B, F, and J), NP (C, G, and K), and *o,p'*-DDT (D, H, and L) are depicted. Polar (pink) and hydrophobic (green) amino acid residues interacting with ligands are indicated. Green dotted arrows indicate hydrogen bond. Purple circles indicate exposed region of the ligand.

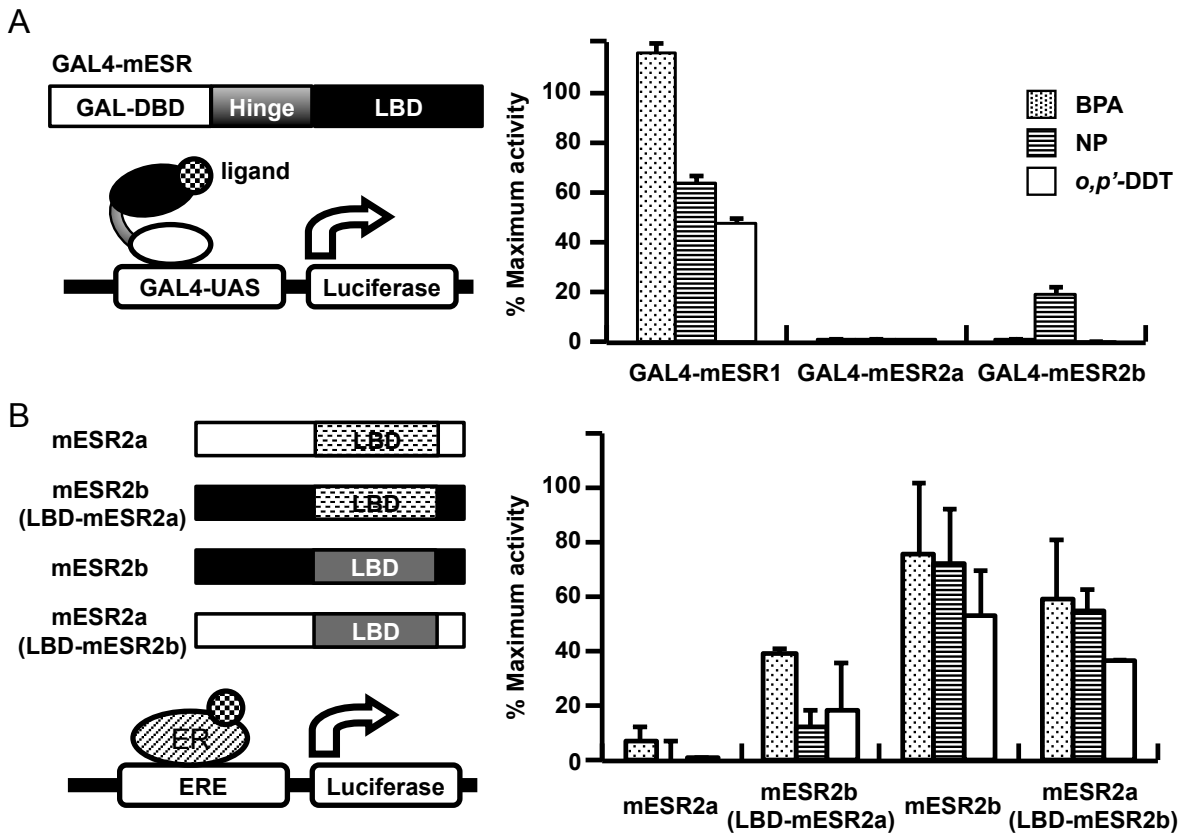


Figure 9. Ligand-binding domain (LBD)-dependent transactivation of mESR subtypes in response to EDCs. The contribution of LBDs of mESRs to differential responses to EDCs was investigated using the GAL4 system (A) and conventional assays with wild type and LBD-chimera mESRs (B). For ligands, 10^{-5} M of EDCs were added to the medium. Data normalized between 0 and 100%, where zero and one hundred were defined as the values for vehicle control and the E2 (10^{-8} M) in each data set, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

Chapter 2

Evolution of estrogen receptors in ray-finned fish and their comparative responses to estrogenic substances

Introduction

Estrogens play important roles in growth, development, reproduction and behavior in vertebrates and their effects are mediated principally through estrogen receptors (ESRs), members of the nuclear steroid hormone receptor superfamily which are activated by their cognate ligands [45, 46]. Upon ligand binding, the complex of receptor and ligand binds to a specific DNA sequence (estrogen response element; ERE), located in the regulatory regions of their target genes and subsequently activating gene transcription. Like other nuclear receptors, ESRs contain six distinct domains labeled from A to F, as defined previously [32]. The C-domain (DNA-binding domain; DBD) and E-domain (ligand-binding domain; LBD) are responsible for DNA-binding and ligand-binding, respectively, and are highly conserved among species as key functional domains [47-49].

Estrogen-responsive ESRs have been identified in all classes of vertebrates. To date, early-branching vertebrates including lamprey (Agnatha, a stem lineage of vertebrate), shark (Chondrichthyes, a sister group of Osteichthyes or bony fish) and lungfish (Diplocercidae, an early branching lineage of tetrapods) have been shown to have two distinct types of ESR, ESR1 and ESR2, probably originating from a duplication of the ESR gene early in the vertebrate lineage [48] (see also Fig. 16). However, in some lineages one isoform has been subsequently lost [50-52] (see also Discussion). Intriguingly, the largest extant group within the Actinopterygii (ray-finned fish), teleosts, have three forms of ESRs: ESR1, ESR2a and ESR2b [53], where ESR2a and ESR2b appear to be closely related to each other. Only one type of ESR2 has been reported previously in gar (Lepisosteiformes, a sister group of teleosts). These observations suggest that the duplication of ESR2 seen in teleosts reflects a teleost-specific whole genome duplication (WGD) event [55-58]. Detailed information relating to the characterization of ESRs in ray-finned fish however is still limited. Cladistia (e.g., bichir: Polypteriformes) are generally regarded as the most basal lineage of ray-finned

fish leading to the teleost lineage [59], whereas Osteoglossomorpha (e.g., arowana: Osteoglossiformes) and Elopomorpha (e.g., eel: Anguilliformes) belong to an ancient family and early-branching lineages of teleost. As such, these organisms are highly relevant fish species for investigating the evolution and functional divergence of the ESRs as they diverged at times prior to (bichir) and after (arowana, eel) the teleost-specific WGD. To date, however, ESRs in these species have not been characterized.

Disruption of the endocrine system is of concern globally both for humans and wildlife. Endocrine disrupting chemicals (EDCs) enter the aquatic environment where fish species are especially at risk of exposure from discharges emanating from wastewater treatment works and agricultural run-off. EDCs are extremely wide ranging in their derived sources and they include alkylphenols, pesticides, plasticizers, bisphenols [e.g., bisphenol A (BPA), 4-nonylphenol (NP)], *o,p'*-dichlorodiphenyltrichloroethane (DDT), natural and pharmaceutical estrogens [17β-estradiol (E2), estrone (E1), estriol (E3) and 17α-ethinylestradiol (EE2)]. Exposure to these EDCs, especially steroidal estrogens are known to induce a range of effects on fish including reproductive and developmental disorders such as skewed sex ratios and increased prevalence of intersex [16, 60-62]. Most of these investigations however are limited to a few model fish species. Teleosts show a lot of divergence within vertebrates and have experienced WGD. This has resulted in a high complexity of the genetic structure of ESRs and this needs due consideration in unraveling species responsiveness to environmental estrogens.

Our group have developed and applied *in vitro* reporter gene assays to evaluate comparative estrogenic potency of EDCs for a variety of fish species [20, 63]. Through these analyses, we clarified species-dependent transactivation of ESR1 where medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), bluegill (*Lepomis macrochirus*) and guppy (*Poecilia reticulata*) ESR1s show higher sensitivities to BPA, NP and *o,p'*-DDT compared

with those of cyprinids [carp (*Cyprinus carpio*), roach (*Rutilus rutilus*)] [20]. In addition, I have found subtype-specific responses of ESR2 to EDCs with weaker reporter activity of ESR2a compared with ESR2b and these responses were comparable among medaka, stickleback, carp, roach and zebrafish (*Danio rerio*) (Chapter 1 and [11]). In this chapter, to investigate whether such response patterns of ESR subtypes are common throughout ray-finned fish, and to understand the functional diversity of ESRs, I cloned ESRs from several key fish species of particular significance in terms of the evolution of ray-finned fish, including bichir, arowana and eel, and characterized the phylogenetic relationships and transactivities elicited by steroidal estrogens and estrogenic EDCs.

Materials and Methods

Chemical reagents

E1 (purity >99.0%), E2 (purity >98.0%), E3 (purity >99.0%) and EE2 (purity >98.0%) were purchased from Sigma-Aldrich, and 4-NP (purity >97.0%), BPA (purity >99.0%) and *o,p'*-DDT (purity >99.5%) were from Kanto-Kagaku. All compounds tested in the reporter gene assay were dissolved in dimethylsulfoxide (DMSO; Nacalai) and the concentration of DMSO in the culture medium did not exceed 0.1%.

Source of fish

Silver arowana (*Osteoglossum bicirrhosum*) and grey bichir (*Polypterus senegalus*) were purchased from a commercial supplier (Meito Suien). Japanese eel (*Anguilla japonica*) was kindly provided from Freshwater Resources Research Center, Aichi Fisheries Research Institute (Nishio, Japan). All animal handling procedures and protocols were approved by the institutional animal care and use committee at the National Institute for Basic Biology, Okazaki, Japan.

Cloning and construction of ESRs

For cloning bichir, arowana and eel ESRs, RNA was isolated from liver, brain and ovary, and reverse transcribed into cDNA which served as template for PCR using degenerate oligonucleotides designed at conserved amino acid region in the DBD and LBD [63]. The 5'- and 3'- ends of the ESRs were amplified by rapid amplification of the cDNA end (RACE) using the GeneRacer Kit (Life Technologies). A full-length transcript of the open reading frame was amplified using PrimeStar GXL polymerase (Takara). The PCR reaction was performed independently using the cDNA templates from liver, brain and gonads. The resulting amplification products were subcloned into pCR-Blunt II-TOPO vector (Life

Technologies) and at least 6 clones were sequenced for each PCR reaction. The PCR products were then subcloned into the pcDNA3.1 vector (Life Technologies). The full-coding region of eel ESR2b (AB003356) was amplified by a standard RT-PCR and subcloned into the pcDNA3.1 vector. The eel ESR2b cDNA isolated in the present study was identical to the previous sequence in the Genbank, with an amino acid substitution (glycine to serine at position 136)(Fig. 9).

Phylogenetic tree of ESRs

The deduced amino acid sequences of DBD and LBD, including hinge regions, were aligned using the Clustal X program. Alignments with questionable gaps were removed. A maximum likelihood tree based on the JTT-matrix-based model [64] was constructed from this alignment using a 1,000 replicate bootstrap analysis using MEGA6 software [65]. Amphioxus (*Branchiostoma belcheri*) ESR was used as an outgroup of the phylogeny. The accession numbers of the sequences used in the phylogenetic analyses are listed in Table 3.

Transactivation assay and data analysis

To examine the ligand-sensitivities with the ESRs, transactivation assays using pGL3-4xERE were performed as described in Chapter 1. All transfections were performed at least three times. Results are presented as mean ± SEM from three separate experiments each consisting of three technical replicates per concentration tested. Data were normalized for responses of the different ESRs to the individual chemical, where zero and one hundred percent were defined as the smallest and the largest response of the ESRs to E2, respectively, for each data set. EC50 values were calculated from these curves using GraphPad Prism version 5 (GraphPad Software).

Results

Cloning of bichir, arowana and eel ESRs

To isolate bichir ESRs, partial DNA fragments were amplified from bichir liver RNA by RT-PCR using degenerate oligonucleotides. Two DNA fragments similar to ESR were obtained, and the full length cDNAs of bichir ESR1 and ESR2 were successfully cloned by 5'- and 3'-RACE. Based on the phylogenetic analysis (see below), these isolated ESRs were identified as bichir ESR1 and ESR2 (Genbank accession numbers: bichir ESR1, LC057256; bichir ESR2, LC057257). The bichir ESR1 and ESR2 cDNAs are comprised of 1611 and 1758 nucleotides and encode proteins of 536 and 585 amino acids, respectively.

Likewise, two partial DNA fragments similar to ESR were amplified from arowana liver RNA. Although Osteoglossiformes are a representative of an early evolutionary lineage of teleosts, I was only able to isolate single genes of ESR1 and ESR2 in this study, even after using RNA from different tissues such as brain and gonad, and several different primer sets. Using 5'- and 3'-RACE, and subsequent phylogenetic analysis (see below), I identified these clones as arowana ESR1 and ESR2b, respectively (Genbank accession numbers: arowana ESR1, LC057258; arowana ESR2b, LC057259). The arowana ESR1 and ESR2b cDNAs are comprised of 1624 and 1680, nucleotides and encode proteins of 541 and 559 amino acids, respectively. During the cloning approach, I also found a splice variant of the ESR1 containing a 280 base pair insertion in the DBD, encoding for a truncated form of ESR1 (214 amino acids; data not shown). This truncation interrupts the DBD and as a consequence, this truncated form of ESR1 seemed to be non-functional.

For eel, the full length of ESR2b (AB003356), and a partial sequence of ESR1 (EU073125) had been registered previously. To isolate other types of ESRs, I performed RT-PCR and successfully isolated another type of ESR, and its sequence was similar to ESR2a. No ESR1-like sequence other than the registered one were isolated. Using 5'- and

3'-RACE, the full length cDNA of eel ESR1 and ESR2a were cloned (Genbank accession numbers: eel ESR1, LC057260; eel ESR2a, LC057261). The eel ESR1 and ESR2a cDNAs comprise of 1650 and 1866 nucleotides and encode proteins of 549 and 621 amino acids, respectively.

In several species, alternatively spliced ESR1 variants in the N-terminal have been identified, resulting in shorter and longer ESR1 variants truncated at the N-terminus [66, 67]. According to Genbank and Ensembl, several fish species have both longer and shorter variants, whereas the remaining species have one of either variants [66]. In all three fish species within the current study, we were only able to clone ESR1 forms that resembled the short ESR1 variant.

Comparison of the amino acid sequences of ESR subtypes across the three fish species showed that all sequences could be subdivided into A to F domains with a high degree of similarities in the putative DBD and LBD across the species analyzed (Fig. 10). Comparison of the predicted bichir ESRs with ESRs from arowana and eel, bichir ESRs revealed shared similarities of 94% (DBD) and 71-73% (LBD) for ESR1 and 94-98% (DBD) and 69-74% (LBD) similarities for ESR2 (Fig. 9). As expected, arowana and eel ESRs showed higher similarities (ESR1: DBD, 94%; LBD, 71-73%; ESR2: DBD, 94-98%; LBD, 69-74%; Fig. 9B). Taken together, the DBD consists of a highly conserved core with two asymmetric zinc fingers, including P-box and D-box [66, 67] (Fig. 11), suggesting conserved functional potency in terms of DNA-binding capacity [7]. The LBD, on the other hand, is less similar but most residues known to participate in E2 binding [68, 69] are well conserved (Fig. 12).

The organization of the phylogenetic tree is generally consistent with the previously described hypothesis of fish evolution [30] (Fig. 13, and Fig .14 for detail). Bichir ESR1 and ESR2 both appear to be ancestral forms of Actinopterygii ESR1 and ESR2, respectively.

However, bichir and sturgeon ESR2s form the same clade in the current phylogeny based on a highly similar sequence between them. The arowana and eel ESR1s were included in the cluster of teleost ESR1. Phylogenetic analysis showed that ESR2 could be divided into ESR2a and ESR2b groups after the divergence of the Actinopterygii lineage and two types of eel ESR2 were included separately in this group, respectively. The obtained arowana ESR2 was assigned to the ESR2b group, suggesting that ESR2a in this group might have been subsequently lost.

Transcriptional activation of ancient fish ER subtypes by natural and synthetic estrogens

Investigating transcriptional activation of the isolated ESRs by conventional transactivation assays using a synthetic ERE reporter, E2 was, as expected, the most potent estrogen among the natural estrogens examined and activated transcription in all ESR subtypes. The transcriptional activation of ESR1s mediated by E2 were similar between the species studied, with only a 2.2-fold difference in ESR1 sensitivity between the most (arowana, EC50 = 4.13×10^{-10} M) and the least (eel, EC50 = 7.64×10^{-10} M) sensitive species. E2 similarly activated ESR2s with EC50s similar to those of ESR1, however, in all species, ESR2s were more sensitive compared to the respective species ESR1s [bichir, EC50 = 1.12×10^{-10} M (ESR2) vs 3.88×10^{-10} M (ESR1), arowana, EC50 = 8.98×10^{-11} M (ESR2) vs 4.13×10^{-10} M (ESR1)] and particularly for both forms of eel ESR2s: The EC50s of eel ESR2a and ESR2b showed 6.26-fold (EC50 = 4.78×10^{-11} M) and 5.14-fold (EC50 = 3.93×10^{-11} M) differences compared with eel ESR1 (EC50 = 7.64×10^{-10} M) (Table 4 and Fig. 15).

The endogenous estrogens E1 and E3 were less efficient in activating all ESRs examined (Table 4 and Fig. 15). In particular, the maximum activity of eel ESR1 with E1 was limited (Fig. 15E). Efficiency of EE2-induced transactivation was comparable with E2, however, relative potencies of EE2 to E2 were higher in ESR1s compared to ESR2s in all

species.

Transcriptional activation of ancient fish ER subtypes by EDCs

The dose-response curves, obtained EC50 values, and subtype-dependent sensitivities of ESRs to BPA, NP and *o,p'*-DDT for the different fish species are shown in Table 4 and Fig. 15. Compared with endogenous estrogens, they all exhibited relatively weak estrogenic activities. BPA, NP and *o,p'*-DDT induced activation of ESR1, but even at exposure to the highest concentration (10^{-5} M), they did not reach the maximum level of E2-induced transactivation (Fig. 16). The pattern of transactivation of ESR2 varied widely. Compared with ESR1, bichir ESR2 was only minimally activated by EDCs (Fig.16). When comparing maximum responses of ESR2a and ESR2b, the EDCs tested were more effective in activating ESR2b (arowana, eel) compared with ESR2a (eel), consistent with previous observation seen in other teleost fish species [11].

The dose-response curves, calculated EC50 value, and subtype-dependent sensitivity of ESRs to BPA, NP and *o,p'*-DDT for the different fish species are shown in Table 1 and Fig. 4. Compared with endogenous estrogens, they all exhibited relatively weak estrogenic activities. BPA, NP and *o,p'*-DDT induced activation of ESR1, but even for exposure to the highest concentration (10^{-5}M), they did not reach the maximum level of E2-induced transactivation (Fig. 16). The pattern of transactivation of ESR2 varied widely. Compared with ESR1, bichir ESR2 was only minimally activated by EDCs (Fig. 16). When comparing maximum responses of ESR2a and ESR2b, the EDCs tested were more effective in activating ESR2b (arowana, eel) compared with ESR2a (eel), consistent with previous observation seen in other teleost fish species [16].

Discussion

As a primary mediator of estrogen signaling in vertebrates, ESR plays crucial roles in reproduction, development, and behavior. The ESR also mediates estrogenic effects of EDCs that mimic or block hormonal action. To determine the functional divergence and/or to identify any unique characteristic of ESRs in ray-finned fish, I isolated and characterized ESRs from bichir, arowana and eel.

Fish ESR evolution

Ancestral vertebrates diverged into two different lineages, Cyclostomata (jawless vertebrates; lamprey and hagfish) and Gnathostomata (jawed vertebrates) [70, 71] (Fig. 17). Subsequently, Gnathostomata were divided into Chondrichthyes (cartilaginous fishes) and Osteichthyes (bony fishes), then Osteichthyes further divided into Sarcopterygii (lobe-finned fishes and tetrapods) and Actinopterygii (ray-finned fish) in the early Devonian [70, 73]. The total number of Actinopterygii comprises of more than 30,000 species [74], the vast majority of which are teleosts. Several extant vertebrates species, located at the basal position of each lineage, are crucial for understanding the ancestral condition of diverse traits during the evolution. To date, in sea lamprey (*Petromyzon marinus*), one of the earliest-branching lineages in vertebrates, only one ESR gene has been identified [48]. By contrast, when I searched for ESR-like transcripts in the currently available database, I detected two ESR2-like partial sequences in the genome of sea lamprey (ENSPMAT00000006334 and ENSPMAT00000008391 in Ensemble). Two ESRs (ER1 and ER2) are also found in the Japanese lamprey (*Lethenteron japonicum*), and the BLAST search showed they are similar to ESR1 and ESR2, respectively. The current phylogenetic analysis excluded the Japanese lamprey ESR2-like protein (ER2) from the ESR2 clade, however, this is probably due to rapid substitution rates and is weakly supported in the phylogeny (Fig. 13). This is also a case for

hagfish (*Myxine glutinosa*) ESR, which is similar to ESR2 [75] and clustered with Japanese lamprey ESR2-like transcripts (Fig. 13). Catshark (*Scyliorhinus torazame*) and whale shark (*Rhincodon typus*) (Elasmobranchii, a subclass of Chondrichthyes) were identified to have only one ESR but they are categorized into ESR2 clade [51]. In addition, two ESR sequences similar to ESR1 (XM_007894405 and its splice variants) and ESR2 (XM_007910258) were found in elephant shark (*Callorhinchus milii*, Holocephali, a subclass of Chondrichthyes). Although it has yet to be determined if they are functional ESRs, these observations suggest that duplication of ESR into ESR1 and ESR2 occurred early in the vertebrate lineage [48]. However, additional ESR sequences from primitive fish species are required to draw definitive phylogenetic relationships.

The bichir (Polypteriforms), as the first diverging lineage within ray-finned fish, provides useful information for assessing the common ancestral state of vertebrates [56, 78, 79]. In this study, I cloned two types of ESRs, ESR1 and ESR2, from bichir which is the earliest Osteichthyes (bony fish) identified to have two sets of functional ESRs (ESR1 and ESR2). This composition persists in tropical gar (*Atractosteus tropicus*: Neopterygii, a sister group to teleosts containing gars and bowfin), that have single ESR1 and ESR2 subtypes [53]. Amur sturgeon (*Acipenser schrenckii*: Chondrostei) also has ESR1 and ESR2 with several isoforms, but the evolutionary relationships among the ESR genes in this group remains elusive due to complexity of polyploidy [53, 82].

In the teleost lineages examined so far, estrogen signaling is mediated through at least three ESR subtypes, namely ESR1, ESR2a and ESR2b. ESR2a and ESR2b appear to be closely related, reflecting a genome duplication event in the teleost ancestor (before the divergence of Osteoglossomorpha and Elopomorpha) [10]. Identification of the ESRs from the earliest diverging lineage among teleosts, Osteoglossomorpha (e.g., arowana Osteoglossiformes) and Elopomorpha (e.g., eel Anguilliformes) are of interest in terms of 1)

presence or absence of an ESR1 paralogue, i.e. a predicted second ESR1 gene which has never been found in teleosts, and 2) functional diversity between newly formed ESR2a and ESR2b. In the current study, I isolated only a single type of ESR1 and ESR2 DNA fragments from arowana; no ESR1 and ESR2 paralogues were amplified even after conducting PCRs under a wide range of conditions. In the eel, only one ESR1 subtype was obtained, whereas I obtained two types of eel ESR2, ESR2a and ESR2b. These results imply that a predicted ESR1 paralogue was lost rapidly at the basal lineage of teleosts. In all cases to date, only one ESR1 sequence has been identified in each tetrapod, except for rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), which have a second isoform of ESR1. This second isoform has been attributed to a WGD event that occurred in the salmonid ancestor [79-81]. By contrast, two types of ESR2 are persistent throughout the teleost lineage. Taken together, Elopomorpha (at least, eel Anguilliformes) is the earliest branched group exhibiting the genetic composition of ESRs (one ESR1 and two ESR2s) commonly seen in teleosts.

Teleosts show a considerable diversity, and the WGD event coincided with the teleost radiation, resulting in duplicated paralogous genes and subsequently induced neo-functionalization, sub-functionalization or inactivation/gene loss [44]. It has been inferred that for most pairs of homologous (duplicated) genes, gene loss (pseudogenization) of one gene copy occurs shortly after WGD [82]. In contrast, in a smaller number of cases, duplicated genes acquire a new function (neo-functionalization) and/or split the original gene function (sub-functionalization), resulting in both genes remaining as paralogues [37]. It has thus been proposed that the teleost-specific WGD enabled significant diversification of teleost lineage, and indeed teleosts occupy approximately half of all species of vertebrates, whereas less than 50 species are extant in pre-WGD lineages of Actinopterygii [35]. The functional diversity of ESR signaling likely contributes to the adaptive radiation of the teleost lineage, because estrogen signaling modulates the plasticity of sex and the variable mode of sex

determination. The highly conserved DBD and similar responses to E2 suggest that these ESRs may be undergoing neo- and/or sub-functionalization possibly by changing their expression pattern. To understand *in vivo* function of estrogen and ESR-mediating signaling, studies on the spatial and temporal expression pattern and gene regulation of each ESR subtypes are needed [25, 83, 84].

ESRs response to estrogens and EDCs

Amphioxus (Cephalochordates: invertebrate chordate groups and a sister subphylum of the vertebrates) ESR is the most ancestral type of ESR identified within the deuterostome lineage. This ESR seems to have secondly lost the capacity to bind E2 [9, 85, 86], whereas all fish ESRs from the descendant lineages, including lamprey, are capable of responding to E2 [9]. This likely reflects functional conservation in the associated physiological processes in vertebrates. Consistent with our previous data for other fish species, EC50 values among fish ESRs for E2 were in the low nanomolar range (<10 nM) [8, 10, 11, 21]. The natural endogenous estrogens, E1 and E3, were less potent in ESR transactivation. These tendencies are also consistent with findings reported previously for ESRs in other fish species [8, 10]. EE2, a synthetic estrogen and component of the contraceptive pill, showed similar efficiency with E2. EE2 is found in the surface water at 1-10 ng/L (e.g., downstream of wastewater treatment plants) [88, 89], and these concentrations are enough to induce feminized responses in some male fish species [22, 89].

A wide range of EDCs have been identified with concern that they may induce, or contribute to the induction of deleterious effects in wildlife [91]. Most EDCs readily enter the aquatic system, thus fish species are especially at risk of exposure. However, to date, few studies have examined the ESRs from early-branched teleosts. Here I provide clear evidence of differential responsiveness of different ESR subtypes to estrogenic EDCs in some of the

more ancient fish species. This is comparable to findings in more recently evolved fish where, for example Cypriniformes ESR1 is less sensitive to non-steroidal estrogenic EDCs than that of Neoteleostei ESR1 [21]. Bichir, arowana and eel ESR1s all showed relatively lower sensitivity to the estrogenic EDCs compared with that seen for Neoteleostei ESR1, suggesting that ESR1 became more sensitive to these EDCs during evolution to Neoteleostei. The findings from the transactivation assay further suggest that the efficacious responses of ESR2b to EDCs [11] have become more sensitized after the divergence from ancestral ESR2 into ESR2a and ESR2b. In general, ESR1 appears to be more responsive to EDCs than ESR2, and this does not only hold true for fish but also for human ESRs [91]. Thus, I show that the sensitivity among ESR subtypes is conserved between phylogenically different taxonomic groups.

In the current study, I show that all cloned ESRs are activated by estrogenic EDCs. However, the EC50s for these EDCs are high, and it should be addressed if this would be relevant to *in vivo* exposures. *In vivo* exposure scenarios are complicated by various factors such as duration of reproductive development, metabolism, seasonal effects and water temperatures. Furthermore, differences in experimental methodologies and procedures between laboratories complicate our understanding on the susceptibility of different fish species to EDCs effects, including sex reversal [92]. There are clearly possible differences between *in vivo* effects and *in vitro* reporter activity of ESRs, however our previously published data support a strong compatibility between the ability of specific EDCs to induce vitellogenin gene expression in male fish liver and responses induced in the *in vitro* reporter assay [22].

A major goal in studies on nuclear receptors is to identify the residues responsible for differences in transactivation between species. For example, the E2-independent, constitutive active ESR function in molluscs can be explained by two amino acid substitutions

[94]. Several studies have investigated the transactivation of ESRs for a range of EDCs in several fish species, however, no amino acid(s) that determine responses to chemicals have been identified by such a “horizontal” approach [94]. I thus suggest that the sensitivity to EDCs may not be determined by single amino acid mutation(s) but rather by the accumulation of permissive mutations, which had no apparent effect on the interaction between innate ligand (i.e., E2-binding and receptor function), but induce some change of the proteins and tolerate the large effect mutations that caused the shift in specificity for EDCs. The residues that are in direct contact to E2 should be highly conserved and such amino acid mutations must be restrictive, whereas permissive mutations accumulate and such subsequent allosteric changes of ligand pocket should be involved in chemical response. The current study shows that susceptibility to EDCs could be taxonomically constrained within ray-finned fish. My comparative analysis of ESRs sheds light on the functional diversity of fish endocrinology and is furthermore useful for supporting predictions for endocrine disruptive effects of EDCs in fish.

Table 3. Amino acid sequences and their accession numbers used in phylogenetic analysis.

Scientific Name	Common Name	Sequence Name	Genebank/Ensembl/Swiss-Prot accession number
Petromyzon marinus	Sea lamprey	Sea lamprey ESR	AAK20929
Protopterus dolloi	African lungfish	Lungfish ESR1	BAG82648
		Lungfish ESR2	BAG82649
Polypterus senegalus	Gray bichir	Bichir ESR1	LC057256
		Bichir ESR2	LC057257
Acipense schrenckii	Amur sturgeon	Sturgeon ESR1a	BAG82650
		Sturgeon ESR1b	BAG82651
		Sturgeon ESR2	BAG82652
Atractosteus tropicus	Tropical gar	Tropical gar ESR1	BAG82653
		Tropical gar ESR2	BAG82654
Lepisosteus oculatus	Spotted gar	Spotted gar ESR2	ENSLOCT00000012649
Anguilla japonica	Japanese eel	Eel ESR1	LC057260
		Eel ESR2a	AB003356
		Eel ESR2b	LC057261
Osteoglossum bicirrhosum	Silver arowana	Arowana ESR1	LC057258
		Arowana ESR2b	LC057259
Homo sapience	Human	Human ESR1	CAA27284
		Human ESR2	BO001428
Oryzias latipes	Medaka	Medaka ESR1	XP_004083548
		Medaka ESR2a	NP_001098172
		Medaka ESR2b	NP_001121984
Gasterosteus aculeatus	Stickleback	Stickleback ESR1	NP_001254601
		Stickleback ESR2a	ENSGACG00000007514
		Stickleback ESR2b	BAR64353
Danio rerio	Zebrafish	Zebrafish ESR1	ABS88330
		Zebrafish ESR2a	NP_851297
		Zebrafish ESR2b	NP_777287
Cyprinus carpio	Common carp	Carp ESR1	BAF99812
		Carp ESR2a	BAF99814
		Carp ESR2b	BFA99813
Rutilus rutilus	Roach	Roach ESR1	BAD91035
		Roach ESR2a	BAR64352
		Roach ESR2b	BAD91036
Rhincodon typus	Whale shark	Whale shark ESR2	BAJ15289
Scyliorhinus torazame	Cat shark	Cat shark ESR2	BAJ15288
Squalus acanthias	Spiny dogfish	Dogfish ESR2	AAK57823
Branchiostoma belcheri	Amphioxus (Belcher's lancelet)	Amphioxus (B.F.) ESR	AB510027
Branchiostoma floridae	Amphioxus (Florida lancelet)	Amphioxus (B.B.) ESR	ACF16007
Myxine glutinosa	Atlantic hagfish	Hagfish ESR	ACC85903
Gambusia affinis	Western mosquitofish	Mosquitofish ESR1	BAF76770
		Mosquitofish ESR2a	BAF76771
		Mosquitofish ESR2b	BAF76772
Ictalurus punctatus	Channel catfish	Channel catfish ESR1	NP_001187003
		Channel catfish ESR2	NP_001187012
Oncorhynchus mykiss	Rainbow trout	Rainbow trout ESR1a	NP_001117821
		Rainbow trout ESR1b	NP_001118030
		Rainbow trout ESR2a	NP_001118225
		Rainbow trout ESR2b	NP_001118042
Mugil cephalus	Flathead mullet	Mullet_ESR1	BAF43298
Odontesthes bonariensis	Pejerrey	Pejerrey ESR1	ABY19510
		Pejerrey ESR2a	ABY19511
		Pejerrey ESR2b	AHA46380
Conger myriaster	Whitespotted conger	Conger eel ESR2	BAD02929
Micropterus salmoides	Largemouth bass	Largemouth bass ESR1	AAG44622.2
		Largemouth bass ESR2a	AAO39211
		Largemouth bass ESR2b	AAO39210
Micropogonias undulatus	Atlantic croaker	Atlantic croaker ESR1	P57753
		Atlantic croaker ESR2a	P57783
		Atlantic croaker ESR2b	P57781
Haplochromis burtoni	Burton's mouthbrooder	Burtoni ESR1	NP_001273259
		Burtoni ESR2a	NP_001273264
		Burtoni ESR2b	XP_005948914
Pimephales promelas	Fathead minnow	Fathead minnow ESR1	AAU87498
		Fathead minnow ESR2	AAT45195
Kryptolebias marmoratus	Mangrove killifish	Mangrove killifish ESR1	ABC68615
		Mangrove killifish ESR2a	BAF03497
		Mangrove killifish ESR2b	ABC68616
Acanthogobius flavimanus	Yellowfin goby	Yellowfin goby ESR1	BAF46102
		Yellowfin goby ESR2	BAF46103
Alligator mississippiensis	American alligator	Alligator ESR1	NP_001274203
		Alligator ESR2	NP_001274193
Gallus gallus	Chicken	Chicken ESR1	ADQ38960
		Chicken ESR2	NP_990125
Elaphe quadrivirgata	Striped snake	Snake ESR1	BAJ15426
		Snake ESR2	BAJ15428
Rugosa rugosa	Wrinkled frog	Frog ESR1	BAJ04337
		Frog ESR2	ACZ51368
Hynobius tokyoensis	Tokyo salamander	Salamander ESR1	BAJ05027
		Salamander ESR2	BAJ05028
Lethenteron japonicum	Japanese lamprey	Japanese lamprey ER1	BAM48573
		Japanese lamprey ER2	BAM48574

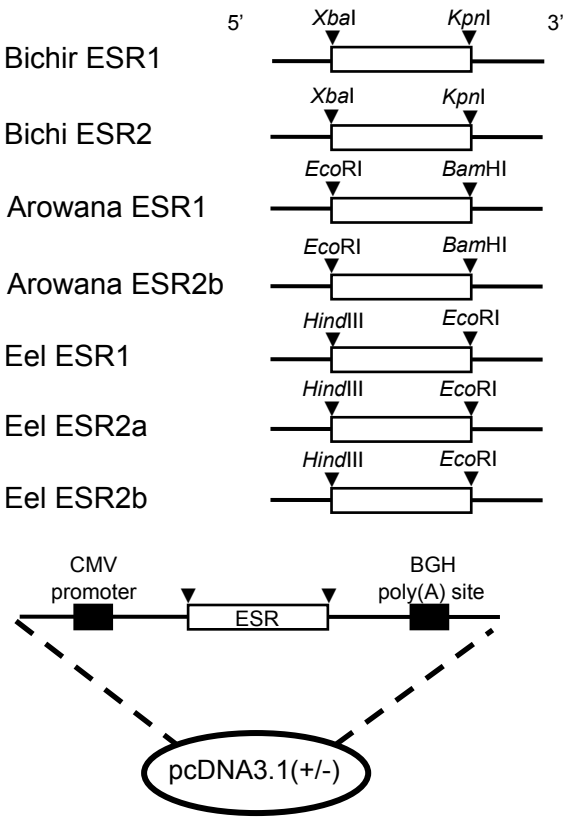


Figure 9. Schematic representation of the plasmid structures used in this study. Each ESR cDNA was inserted into a conventional vector, pcDNA3.1, at the indicated restriction enzyme site. ▼; restriction enzyme site.

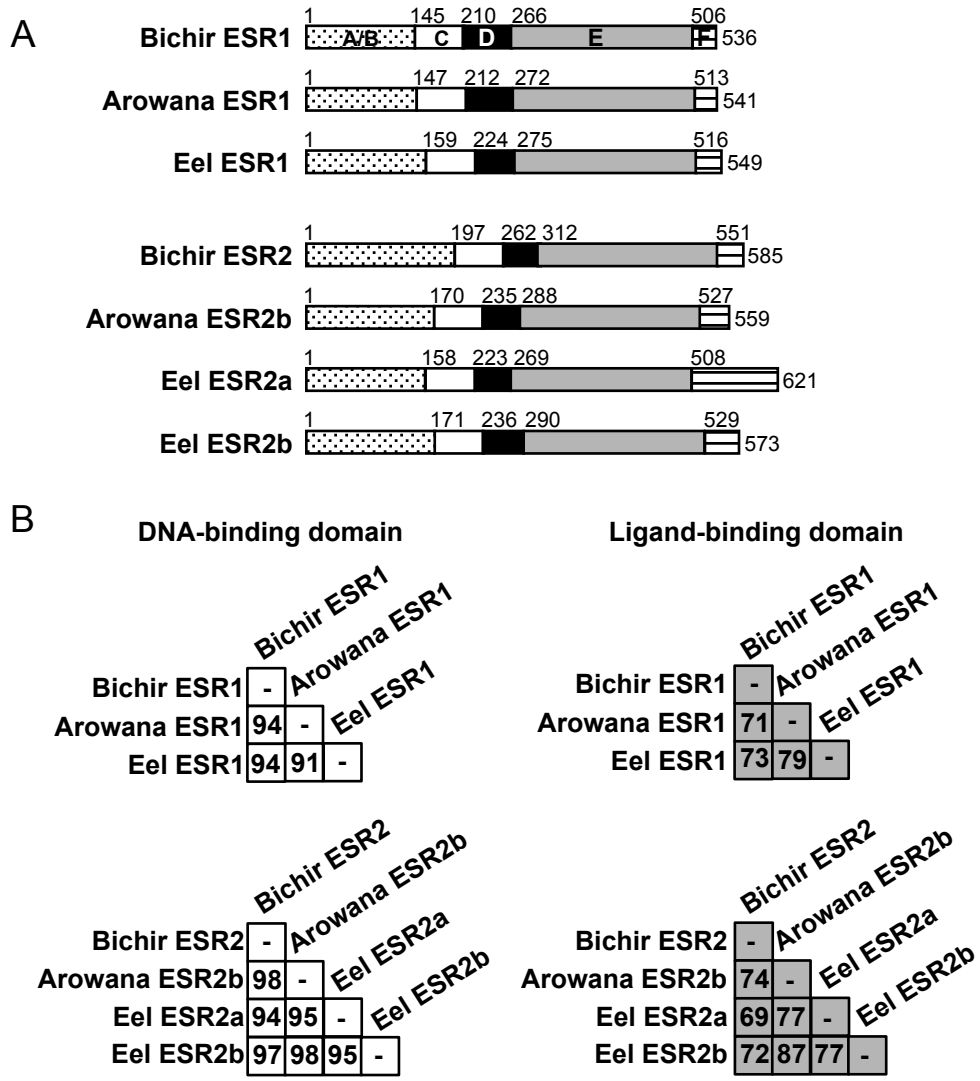


Figure 10. Domain structures and sequence comparison of fish ESRs. Domain structure of fish ESRs characterized in this study. The numbers above each box refer to the position of amino acids in each domain. (B) Percentage sequence identity of the individual domains between three fish ESRs. The numbers within each box indicate the percentage identity between species. The different colors represent the colors the domains are presented in (A).

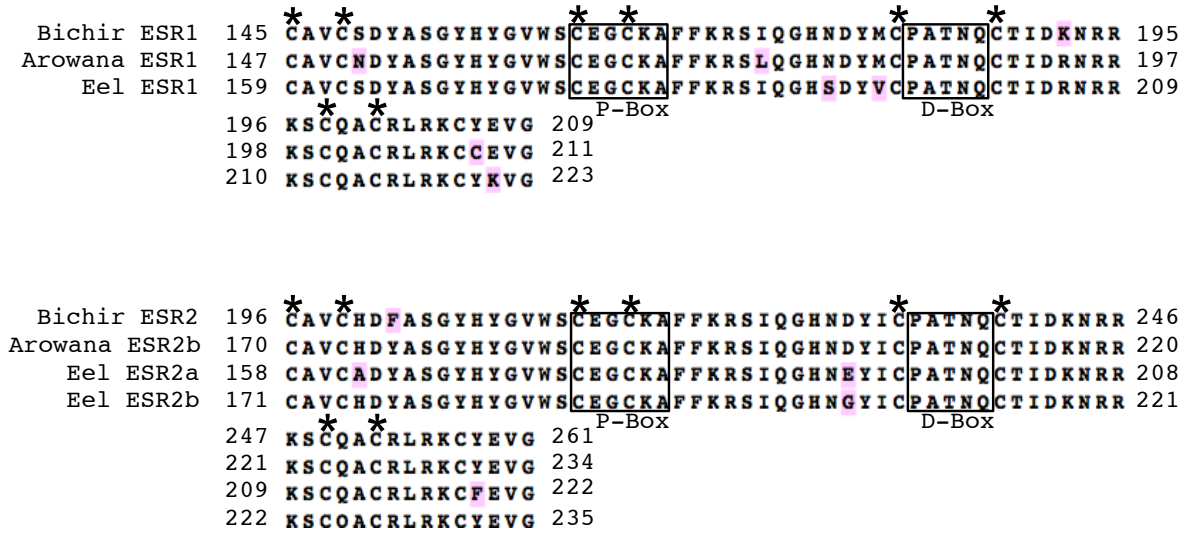


Figure 11. Multiple sequence alignments of the DBD of the ESRs among bichir, arowana and eel. Asterisks indicate the cysteines that constitute the tetrahedral coordination of two zinc ions. P-box (an alpha helix in the first zinc finger, which is responsible for high-affinity recognition of the ERE) and D-box (an alpha helix in the second zinc finger, and is a site that mediates receptor dimerization) are marked with boxes. Non-conserved amino acids residues are highlighted.

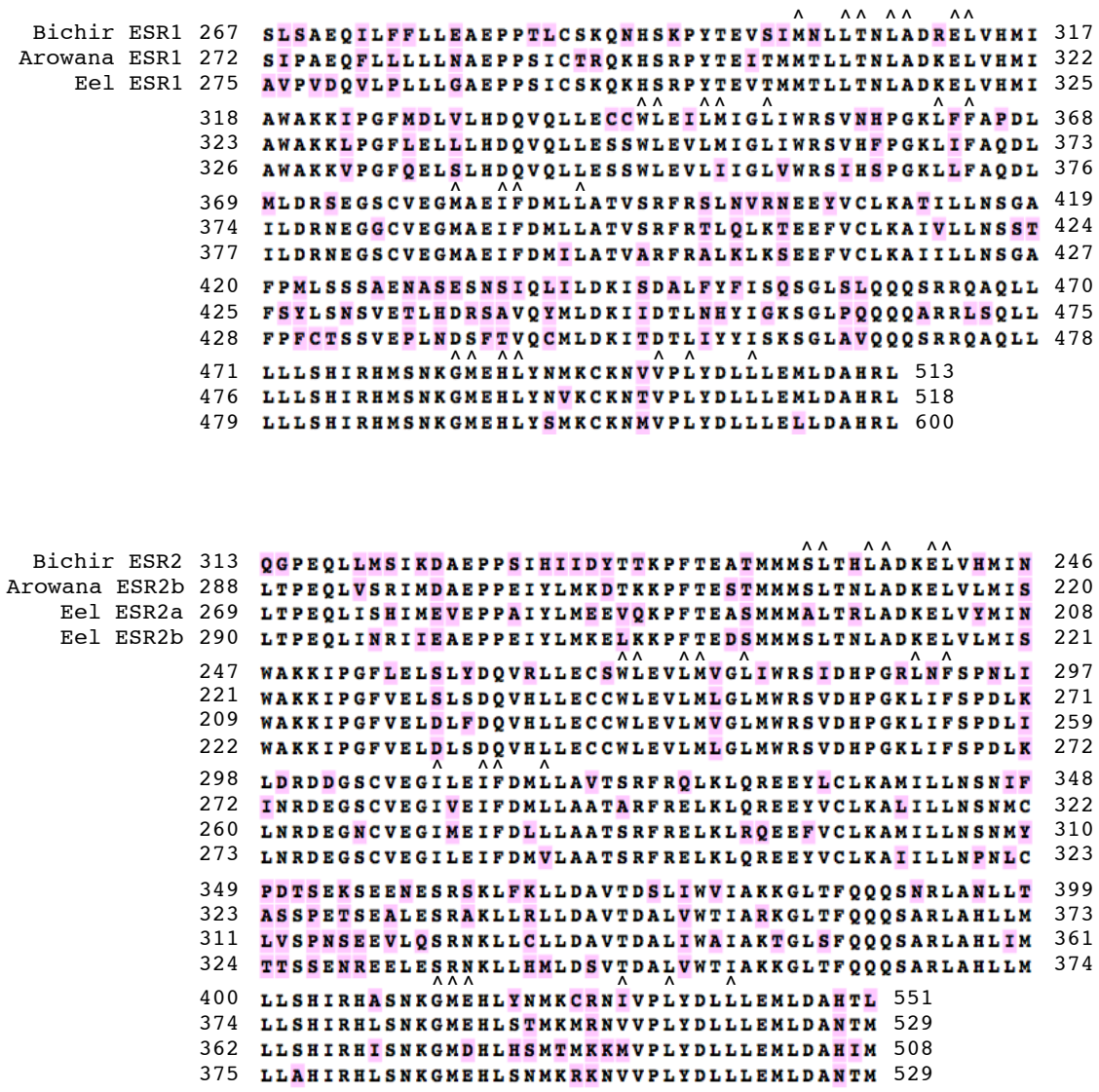


Figure 12. Multiple sequence alignments of the LBD of the ESRs among bichir, arowana and eel. The numbers correspond to the positions of amino acids. ^ indicates residues of the human ESR1 known to interact with E2. Non-conserved amino acids residues are highlighted.

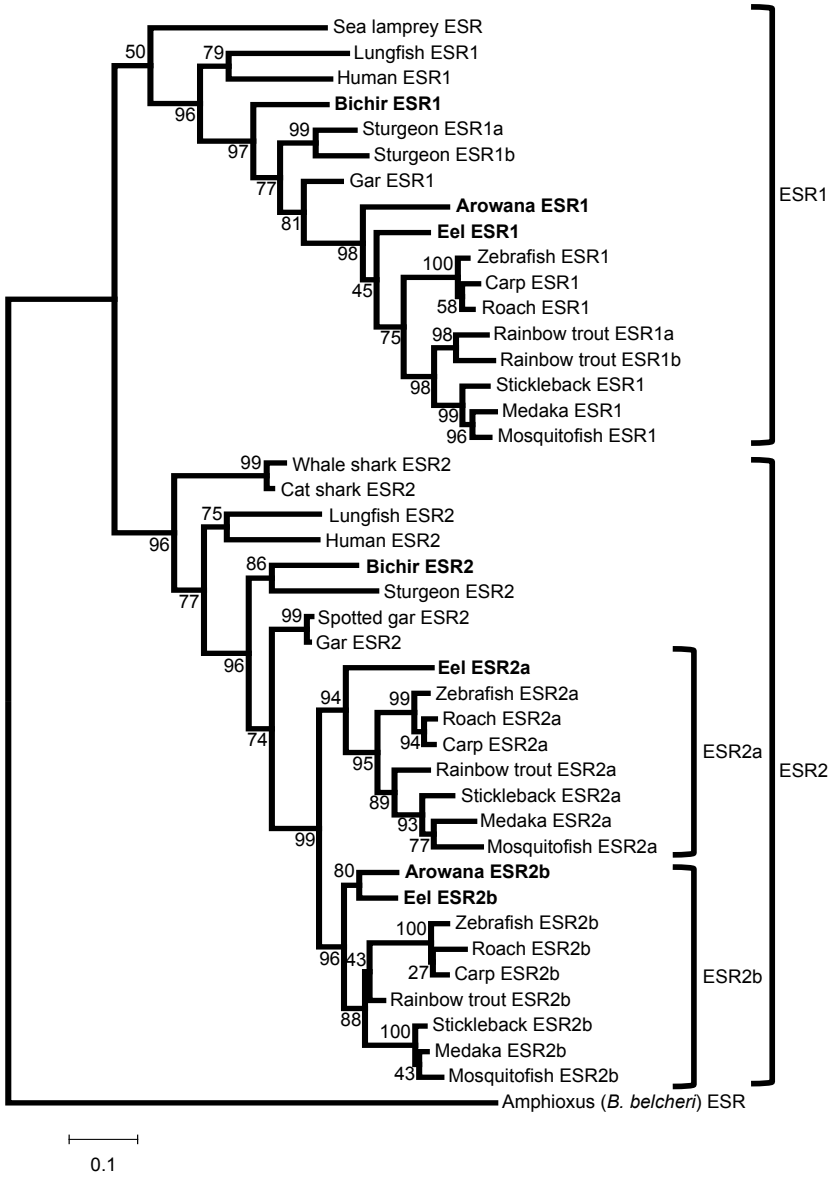


Figure 13. Evolutionary relationships between vertebrate ESRs. A maximum likehood tree was constructed using a 1,000 replicate bootstrap analysis and the tree with the highest (log) likehood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bichir, arowana and eel ESRs are highlighted in bold. The branch length reflects the estimated proportions of substitutions along each branch. The scale bar represents 0.1 substitutions per site. Amphioxus (*Branchiostoma belcheri*) ESR was used as outgroup of the phylogeny. Expanded phylogenies and a list of genes, species and accession number are in Fig. 13 and Table 3.

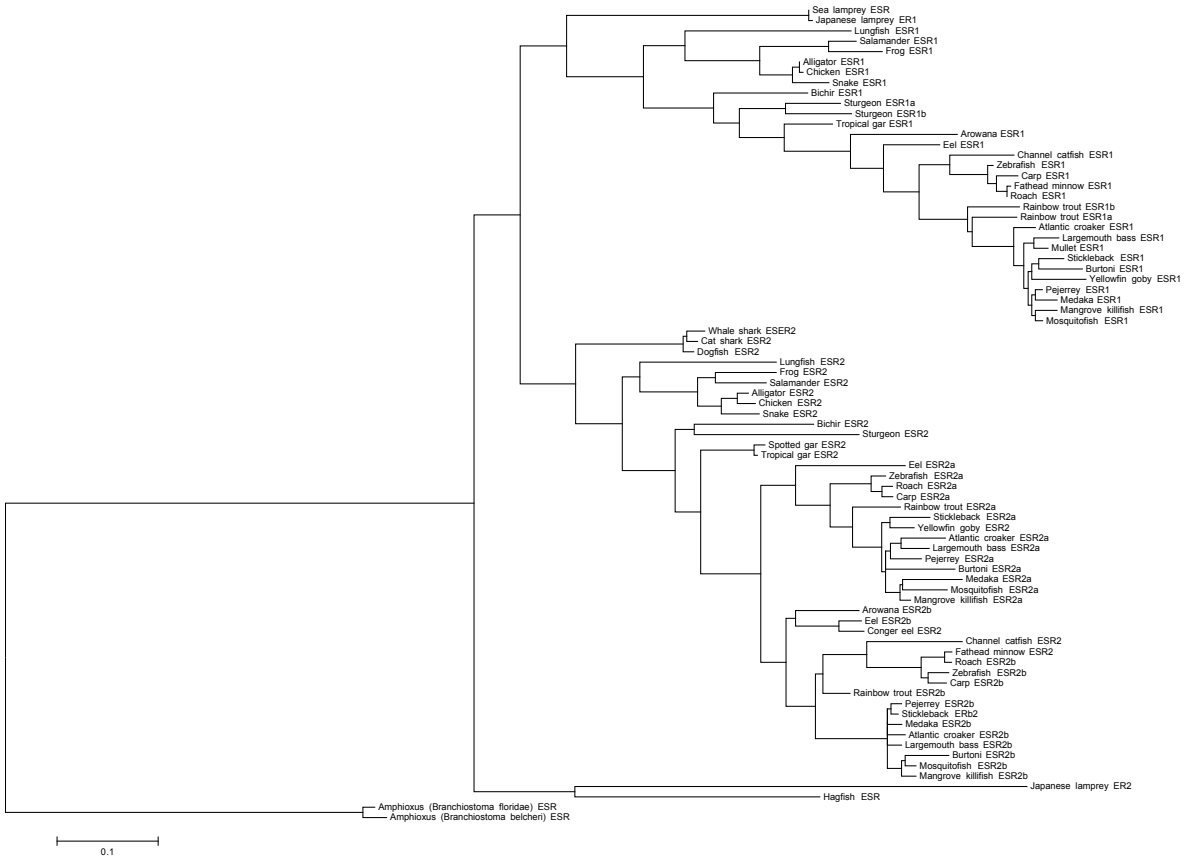


Figure 14. Maximum likehood ESR phylogeny in vertebrates. A maximum likehood tree was constructed using a 1,000 replicate bootstrap analysis and the trees with highest log likehood are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The branch length reflects the estimated proportion of substitutions along each branch. The scale bar represents 0.1 substitutions per site. Amphioxus (*Branchiostoma belcheri*) ESR was used as outgroup of the phylogeny. Note that phylogenetic relationship of ESRs in Agnatha (lamprey and hagfish) is less supported.

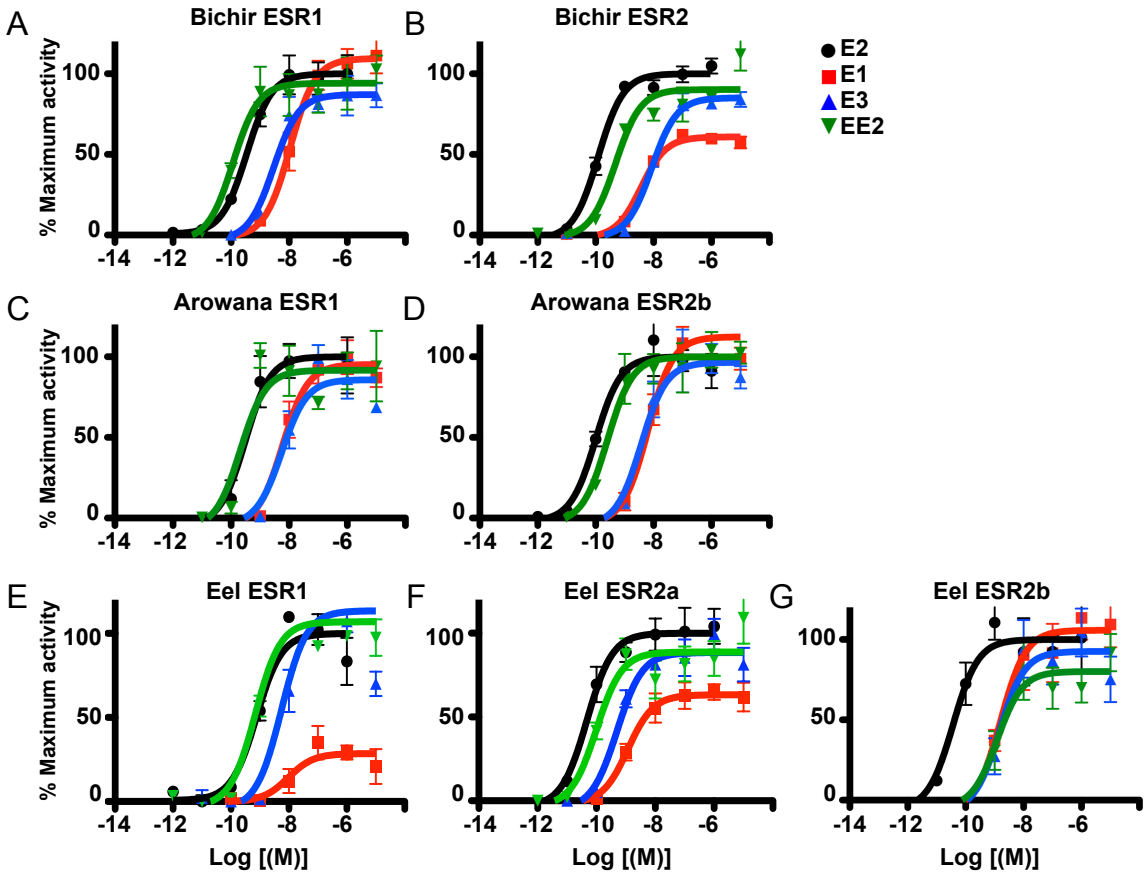


Figure 15. Concentration-response profiles of ESRs activated by natural and synthetic estrogens. Reporter activities of bichir ESR1 (A), bichir ESR2 (B), arowana ESR1 (C), arowana ESR2b (D), eel ESR1 (E), eel ESR2a (F) and eel ESR2b (G) by E1, E2, E3 and EE2 were examined. Dose-response curves fitted on data normalized between 0 and 100%, where zero and one hundred were defined as the smallest and the largest values of E2 response, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

Table 4. Gene transcriptional activities of estrogens and EDCs mediated by fish ESRs.

		E2	E1	E3	EE2	BPA	NP	*o,p'*- DDT
Bichir ESR1	EC50 (M)	$3.88x10^{-10}$	$9.75x10^{-9}$	$2.85x10^{-9}$	$1.19x10^{-10}$	$5.31x10^{-6}$	$9.60x10^{-7}$	$1.41x10^{-7}$
	95%CI (M)	$(1.84\text{-}8.15)x10^{-10}$	$(5.35\text{-}0.18)x10^{-9}$	$(1.28\text{-}6.34)x10^{-9}$	$(4.62\text{-}0.31)x10^{-10}$	$(2.18\text{-}0.13)x10^{-6}$	$(6.1\text{-}0.15)x10^{-7}$	$(9.08\text{-}0.22)x10^{-7}$
	RP (%)	100	3.98	13.6	325.1	0.0073	0.04	0.027
Bichir ESR2	EC50 (M)	$1.12x10^{-10}$	$3.78x10^{-9}$	$8.64x10^{-9}$	$4.83x10^{-10}$	-	-	-
	95%CI (M)	$(8.23\text{-}0.18)x10^{-11}$	$(2.49\text{-}5.74)x10^{-9}$	$(6.07\text{-}0.12)x10^{-9}$	$(2.17\text{-}0.11)x10^{-10}$	(Very wide)	(Very wide)	(Very wide)
	RP (%)	100	3.2	1.4	25.1	-	-	-
Arowana ESR1	EC50 (M)	$4.13x10^{-10}$	$5.97x10^{-9}$	$5.97x10^{-9}$	$2.45x10^{-10}$	-	$3.17x10^{-7}$	-
	95%CI (M)	$(1.42\text{-}0.12)x10^{-10}$	$(1.68\text{-}0.21)x10^{-9}$	$(2.12\text{-}0.23)x10^{-9}$	$(6.55\text{-}0.92)x10^{-10}$	(Very wide)	$(1.44\text{-}7.00)x10^{-6}$	(Very wide)
	RP (%)	100	6.9	5.9	186.1	-	0.013	-
Arowana ESR2b	EC50 (M)	$8.98x10^{-11}$	$6.83x10^{-9}$	$3.76x10^{-9}$	$2.46x10^{-10}$	$1.13x10^{-6}$	$7.44x10^{-7}$	$7.78x10^{-7}$
	95%CI (M)	$(4.84\text{-}0.17)x10^{-11}$	$(3.07\text{-}0.15)x10^{-9}$	$(1.50\text{-}9.43)x10^{-9}$	$(1.05\text{-}5.73)x10^{-10}$	$(2.12\text{-}0.61)x10^{-5}$	$(2.49\text{-}0.22)x10^{-7}$	$(8.15\text{-}0.074)x10^{-7}$
	RP (%)	100	1.3	2.4	36.6	$7.92x10^{-5}$	0.0012	$1.2x10^{-6}$
Eel ESR1	EC50 (M)	$7.64x10^{-10}$	$9.56x10^{-9}$	$5.52x10^{-9}$	$6.92x10^{-10}$	$2.84x10^{-7}$	$1.53x10^{-7}$	$1.62x10^{-7}$
	95%CI (M)	$(3.39\text{-}0.17)x10^{-10}$	$(1.14\text{-}0.80)x10^{-9}$	$(1.08\text{-}0.28)x10^{-9}$	$(2.68\text{-}0.18)x10^{-10}$	$(6.51\text{-}0.012)x10^{-8}$	$(6.05\text{-}0.39)x10^{-6}$	$(7.22\text{-}0.36)x10^{-7}$
	RP (%)	100	8	13.8	110.4	0.27	0.05	0.05
Eel ESR2a	EC50 (M)	$4.78x10^{-11}$	$1.17x10^{-9}$	$5.0x10^{-10}$	$1.0x10^{-10}$	$1.33x10^{-7}$	$8.14x10^{-7}$	$4.88x10^{-7}$
	95%CI (M)	$(2.07\text{-}0.11)x10^{-11}$	$(5.17\text{-}0.26)x10^{-10}$	$(2.22\text{-}0.11)x10^{-9}$	$(3.50\text{-}0.29)x10^{-10}$	$(3.53\text{-}0.50)x10^{-7}$	$(4.28\text{-}0.15)x10^{-7}$	(Very wide)
	RP (%)	100	4.1	9.6	47.5	$3.0x10^{-4}$	$5.9x10^{-4}$	$1.0x10^{-5}$
Eel ESR2b	EC50 (M)	$3.93x10^{-11}$	$1.51x10^{-9}$	$1.40x10^{-9}$	$1.29x10^{-10}$	$7.44x10^{-7}$	$8.14x10^{-7}$	$3.18x10^{-7}$
	95%CI (M)	$(1.14\text{-}0.14)x10^{-11}$	(5.30-4.32)x10-9	$(4.16\text{-}0.47)x10^{-10}$	$(3.11\text{-}0.54)x10^{-11}$	$(2.79\text{-}0.22)x10^{-7}$	$(4.28\text{-}0.15)x10^{-7}$	$(1.17\text{-}0.12)x10^{-7}$
	RP (%)	100	2.6	2.8	30.4	$5.3x10^{-4}$	$4.9x10^{-4}$	0.01

-, not determined because of very weak estrogenicity. 95% CI; 95% confidence intervals of EC50. RP, relative potency; (EC50 E2/EC50 chemical X) 100.

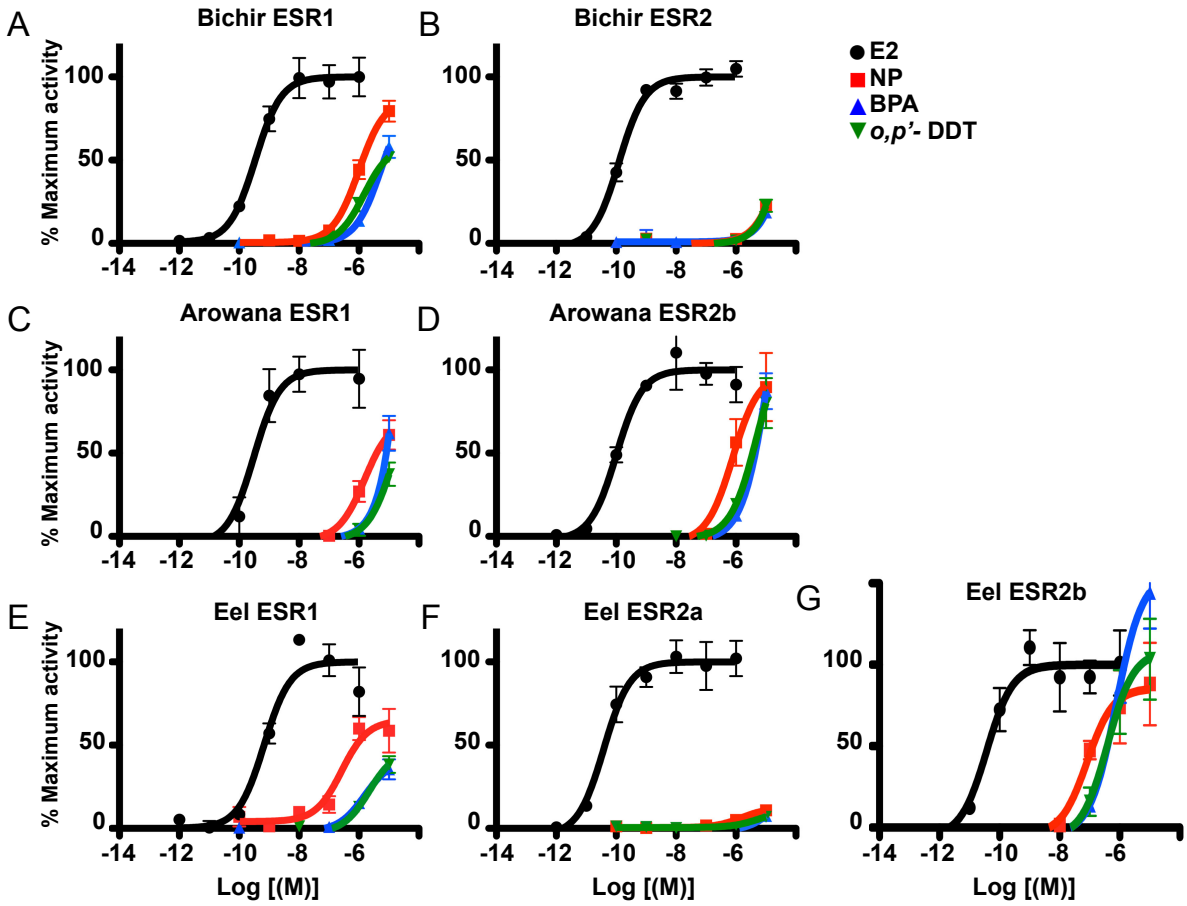


Figure 16. Concentration-response profiles of ESRs activated by estrogenic EDCs. Reporter activities of bichir ESR1 (A), bichir ESR2 (B), arowana ESR1 (C), arowana ESR2b (D), eel ESR1 (E), eel ESR2a (F) and eel ESR2b (G) by BPA, NP and *o,p'*-DDT were examined. Response to E2 is also represented as a reference. Dose-response curves were fitted on data normalized between 0 and 100%, where zero and one hundred were defined as the smallest and the largest values of E2 response, respectively. Data are presented as mean ± SEM from three independent assays each consisting of three technical replicates per concentration tested.

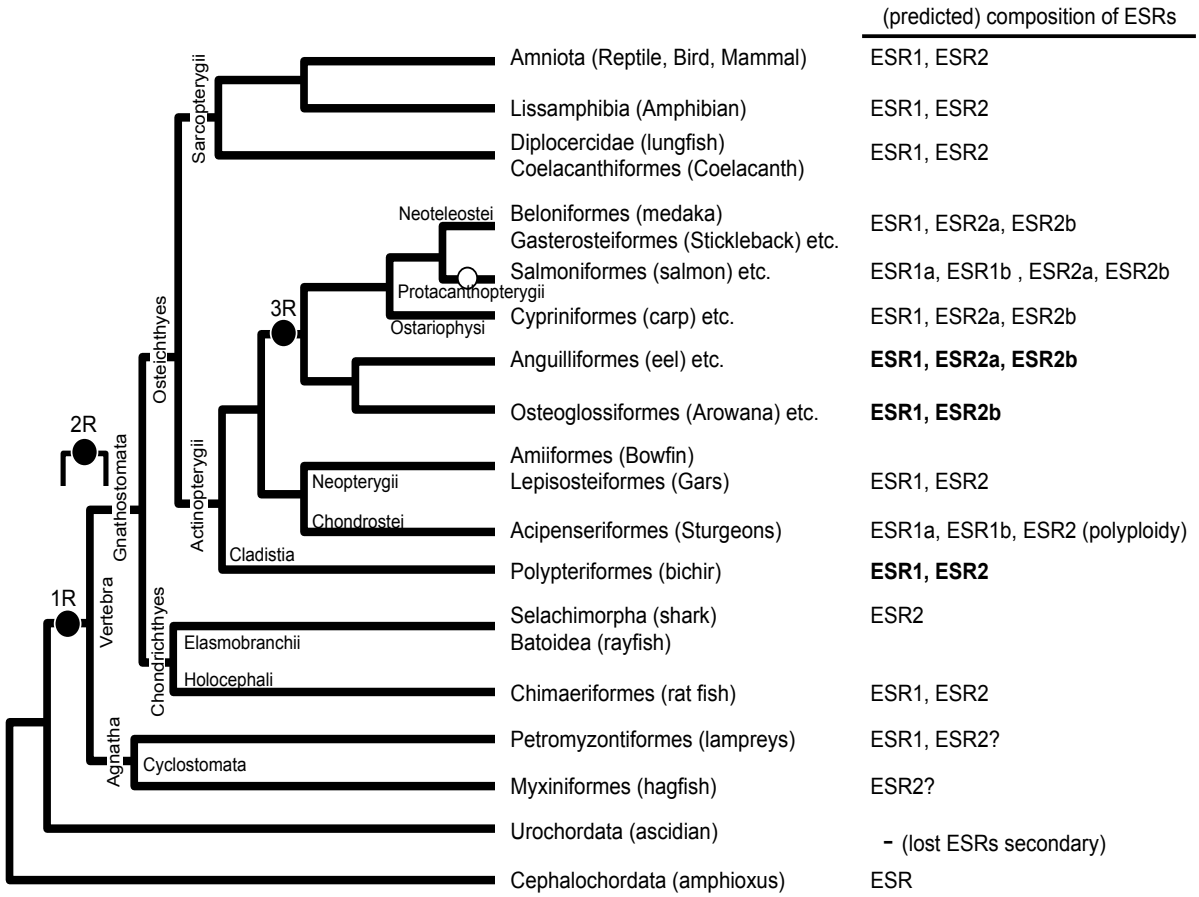


Figure 17. Schematics illustration of Predicted ESR evolution. A simple phylogeny is indicated according [54, 76], and this study. 1R, 2R and 3R indicate predicted timing for first, second and third (teleost-specific)-rounds of WGD, respectively. Salmonid ancestor is suggested to experience additional WGD (open circle). Bold indicate the ESRs cloned in this study. See details of predicted ESR composition in the text.

Conclusion

Steroid hormones such as estrogens and androgens play fundamental roles in regulating reproductive activities in vertebrates. Estrogens regulate ovarian development, differentiation and maintenance, and also stimulate the hepatic synthesis of vitellogenin and choriogenin in fish, which are all vital for reproduction. It has been reported that estrogenic environmental endocrine disrupting chemicals (EDCs) elicit disruptions in sexual development, alter sexual differentiation and function, and impact adversely on reproduction. These effects are principally mediated through estrogen receptors (ESRs).

Fish species have been widely used as experimental models and sentinels for evaluating the estrogenic effects of EDCs. For example, increasing concentration of VTG and formation of testis-ovo in male medaka have been utilized for screening for estrogenic effects of EDCs-induced effects. Apart from such apical phenotypes, however, basic biology (i.e., mechanisms of transcriptional regulation elicited by EDCs, and genetic and species diversity) of fish ESR subtypes, ESR1, ESR2a and ESR2b, remains elucidated. In my thesis, towards understanding the fish ESRs biology, I provide the basic mechanisms and differential responses of fish (medaka, stickleback, zebrafish, roach, carp) ESR subtypes to EDCs (Chapter 1) and molecular basis for evolution of ESRs in ray-finned fish (Chapter 2).

Each ESR subtype is differentially expressed *in vivo*, which depends on the timing of development, cell types and sexes. In addition, response to estrogens and EDCs *in vivo* is complicated by factors relating to incorporation and metabolism of the ligands. I am now establishing the loss-of-function mutant medaka for each ESR subtypes. The molecular information provided by such studies will help develop understanding on events at the molecular level that link with adverse outcome pathways (AOP) and the role of ESRs in reproductive biology in fish.

References

1. Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F.: Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *Gen. Comp. Endrocrinol*., **165**, 352-366 (2010).
2. Lubzens, E., Young, G., Bobe, J., Cerda, J.: Oogenesis in teleosts: how eggs are formed. *Gen. Comp. Endrocrinol*., **165**, 367-389 (2010).
3. Yi, P., Driscoll, M.D., Huang, J., Bhagat, S., Hilf, R., Bambara, R.A., Muyan, M.: The effects of estrogen-responsive element- and ligand-induced structural changes on the recruitment of cofactors and transcriptional responses by ER alpha and ER beta. *Mol. Endocrinol*., **16**, 674-693 (2002).
4. Bowers, J.L., Tyulmenkov, V.V., Jernigan, S.C., Klinge, C.M.: Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. *Endocrinology*, **141**, 3657-3667 (2000).
5. Chang, E.C., Charn, T.H., Park, S.H., Helferich, W.G., Komm, B., Katzenellenbogen, J.A., Katzenellenbogen, B.S.: Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding. *Mol. Endocrinol*., **22**, 1032-1043 (2008).
6. Williams, C., Edvardsson, K., Lewandowski, S.A., Strom, A. Gustafsson, J.A.: A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. *Oncogene*, **27**, 1019-1032 (2008).
7. Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., Mark, M.: Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development*, **127**, 4277-4291 (2000).
8. Couse, J.F., Korach, K.S.: Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr. Rev*., **20**, 358-417 (1999).

9. Vandepoele, K., De Vos, W., Taylor, J.S., Meyer, A., Van de Peer, Y.: Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. *Proc. Natl. Acad. Sci. U. S. A.*, **101**, 1638-1643 (2004).

10. Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P.: Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 10751-10756 (2000).

11. Meyer, A., Schartl, M.: Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr. Opin. Cell Biol.*, **11**, 699-704 (1999).

12. Yost, E.E., Lee Pow, C., Hawkins, M.B., Kullman, S.W.: Bridging the gap from screening assays to estrogenic effects in fish: potential roles of multiple estrogen receptor subtypes. *Environ. Sci. Technol.*, **48**, 5211-5219 (2014).

13. Griffin, L.B., January, K.E., Ho, K.W., Cotter, K.A., Callard, G.V.: Morpholino-mediated knockdown of ERalpha, ERbetaa, and ERbetab mRNAs in zebrafish (*Danio rerio*) embryos reveals differential regulation of estrogen-inducible genes. *Endocrinology*, **154**, 4158-4169 (2013).

14. Nelson, E.R., Habibi, H.R.: Functional significance of nuclear estrogen receptor subtypes in the liver of goldfish. *Endocrinology*, **151**, 1668-1676 (2010).

15. Scholz, S., Kluver, N.: Effects of endocrine disrupters on sexual, gonadal development in fish. *Sex. Dev.*, **3**, 136-151 (2009).

16. Mills, L.J., Chichester, C.: Review of evidence: are endocrine disrupting chemicals in the aquatic environment impacting fish populations? *Sci. Total Environ.*, **343**, 1-34 (2005).

17. Yokota, H., Seki, M., Maeda, M., Oshima, Y., Tadokoro, H., Honjo, T., Kobayashi, K.: Life-cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). *Environ. Toxicol. Chem*., **20**, 2552-2560 (2001).

18. Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., Potter, T.: Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem*., **20**, 297-308 (2001).

19. Andersen, H.R., Andersson, A.M., Arnold, S.F., Autrup, H., Barfoed, M., Beresford, N.A., Bjerregaard, P., Christiansen, L.B., Gissel, B., Hummel, R., Jørgensen, E.B., Korsgaard, B., Le, Guevel, R., Leffers, H., McLachlan, J., Nielsen, J. B., Olea, N., Oles-karasko, A., Pakdel, F., Pedersen, K.L., Perez, P., Skakkeboek, N.E., Sonnenschein, C., Soto, A.M., Sumpter, J.P., Thorpe, S.M., Grandjean, P.: Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environ. Health Perspect*., **107** (Suppl1), 89-108 (1999).

20. Miyagawa, S., Lange, A., Hirakawa, I.,Tohyama, S., Ogino, Y., Mizutani, T., Kagami, Y., Kusano, T., Ihara, M., Tanaka, H., Tatarazako, N., Ohta, Y., Katsu, Y., Tyler, C.R., Iguchi, T.: Differing species responsiveness of estrogenic contaminants in fish is conferred by the ligand binding domain of the estrogen receptor. *Environ. Sci. Technol*., **48**, 5254-5263 (2014).

21. Cosnefroy, A., Brion, F., Maillot-Marechal, E., Porcher, J. M., Pakdel, F., Balaguer, P., Ait-Aissa, S.: Selective activation of zebrafish estrogen receptor subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. *Toxicol. Sci*., **125**, 439-449 (2012).

22. Lange, A., Katsu, Y., Miyagawa, S., Ogino, Y., Urushitani, H., Kobayashi, T., Hirai, T., Shears, J. A., Nagae, M., Yamamoto, J., Ohnishi, Y., Oka, T., Tatarazako, N., Ohta, Y.,

Tyler, C.R., Iguchi, T.: Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. *Aquat. Toxicol*., **109**, 250-258 (2012).

23. Jobling, S., Casey, D.; Rogers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R.: Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquat. Toxicol*., **66**, 207-222 (2004).
24. Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F.: Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biol. Reprod*., **66** , 1881-1892 (2002).
25. Chakraborty, T., Katsu, Y., Zhou, L.Y., Miyagawa, S., Nagahama, Y., Iguchi, T.: Estrogen receptors in medaka (*Oryzias latipes*) and estrogenic environmental contaminants: an *in vitro-in vivo* correlation. *J. Steroid. Biochem. Mol. Biol*., **123**, 115−121 (2011).
26. Katasu, Y., Lange, A., Miyagawa, S., Urushitani, H., Tatarazako, N., Kawashima, Y., Tyler, C.R., Iguchi, T.: Cloning, expression and functional characterizeation of carp, *Cyprinus carpio*, estrogen receptors and their differential activations by estrogens. *J. Appl. Toxicol*., **33**, 41-49 (2013).
27. Katasu, Y., Lange, A., Urushitani, H., Tatarazako, H., Ichikawa, R., Paull, G.C., Cahill, L. L., Jobling, S., Tyler, C.R., Iguchi, T.: Functional associations between two estrogen receptors, environmental estrogens, and sexual disruption in the roach (*Rutilus rutilus*). *Environ. Sci. Technol*., **41**, 3368-3374 (2007).
28. Katsu, Y., Ichikawa, R., Ikeuchi, T., Kohno, S., Guillette, L.J.Jr., Iguchi, T.: Molecular cloning and characterization of estrogen, androgen, and progesterone nuclear receptors from a freshwater turtle (*Pseudemys nelsoni*). *Endocrinology*, **149**, 161-173 (2008).

29. Delfosse, V., Grimaldi, M., Pons, J.L., Boulahtouf, A., le Maire, A., Cavailles, V., Labesse, G., Bourguet, W., Balaguer, P.: Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. *Proc. Natl. Acad. Sci. U. S. A*., **109**, 14930-14935 (2012).

30. Tanenbaum, D.M., Wang, Y., Williams, S.P., Sigler, P.B.: Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl. Acad. Sci. U. S. A*., **95**, 5998-6003 (1998).

31. Wang, Y., Chirgadze, N.Y., Briggs, S.L., Khan, S., Jensen, E.V., Burris, T.P.: A second binding site for hydroxytamoxifen within the coactivator-binding groove of estrogen receptor beta. *Proc. Natl. Acad. Sci. U. S. A*., **103**, 9908-9911 (2006).

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

33. Ogino, Y., Katoh, H., Kuraku, S., Yamada, G.: Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. *Endocrinology*, **150**, 5415-5427 (2009).

34. Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., Carlquist, M.: Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, **389**, 753-758 (1997).

35. Kohno, S., Katsu, Y., Iguchi, T., Guillette, L.J.Jr.: Novel approaches for the study of vertebrate steroid hormone receptors. *Integr. Comp. Biol*., **48**, 527-534 (2008).

36. Nelson, J.S.: Fishes of the world, 3rd d., J. Wiley. New York (1994).

37. Ohno, S., Wolf, U., Atkin, N.B.: Evolution from fish to mammals by gene duplication. *Hereditas*, **59**, 169-187 (1968).

38. Nelson, E.R., Habibi, H.R.: Estrogen receptor function and regulation in fish and other vertebrates. *Gen. Comp. Endrocrinol*., **192**, 15-24 (2013).

39. Kok, F.O., Shin. M. Ni., C.W., Gupta, A., Grosse, A.S., van Impel, A., Kirchmaier, B.C., Peterson-Maduro, J., Kourkoulis, G., Male, I., DeSantis, D.F., Sheppard-Tindell, S., Ebarasi, L., Betsholtz, C., Schulte-Merker, S., Wolfe, S.A., Lawson, N.D.: Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev. Cell*, **32**, 97-108 (2015).

40. Danielian, P.S., White, R., Lees, J.A., Parker, M.G.: Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J*. **11**, 1025-1033 (1992).

41. Nettles, K.W., Bruning, J.B., Gil, G., Nowak, J., Sharma, S.K., Hahm, J.B., Kulp, K., Hochberg, R.B., Zhou, H., Katzenellenbogen, J.A., Katzenellenbogen, B.S., Kim, Y., Joachmiak, A., Greene, G.L.: NFkappaB selectivity of estrogen receptor ligands revealed by comparative crystallographic analyses. *Nat. Chem. Biol*., **4**, 241-247 (2008).

42. Khorasanizadeh, S., Rastinejad, F.: Nuclear-receptor interactions on DNA-response elements. *Trends Biochem. Sci*., **26**, 384-390 (2001).

43. Filardo, E. J., Quinn, J.A., Bland, K.I., Frackelton, A.R.Jr.: Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol. Endocrinol*., **14**, 1649-1660 (2000).

44. O'Lone, R., Frith, M.C., Karlsson, E.K., Hansen, U.: Genomic targets of nuclear estrogen receptors. *Mol. Endocrinol*., **18**, 1859-1875 (2004).

45. Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz. G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M.: The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835-839 (1995).

46. Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M, Strom, A. Treuter, E., Warner, M., Gustafsson, J.A.: Estrogen receptors: how do they signal and what are their targets. *Physiol. Rev*., **87**, 905-931 (2007).

47. Klinge, C.M.: Estrogen receptor interaction with estrogen response elements. *Nucleic. Acids Res*., **29**, 2905-2919 (2001).

48. Thornton, J.W.: Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proc. Natl. Acad. Sci. U. S. A*., **98**, 5671-5676 (2001).

49. Evans, R.M.: The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889-895 (1988).

50. Gray, K.A., Yates, B., Seal, R.L., Wright, M.W., Bruford, E.A.: Genenames.org: the HGNC resources in 2015. *Nucleic Acids Res*., **43**, D1079-1085 (2015).

51. Katsu, Y., Kohno, S., Narita, H., Urushitani, H., Yamane, K., Hara, A., Clauss, T.M., Walsh, M.T., Miyagawa, S., Guillette, L.J.Jr., Iguchi, T.: Cloning and functional characterization of Chondrichthyes, cloudy catshark, *Scyliorhinus torazame* and whale shark, *Rhincodon typus* estrogen receptors. *Gen. Comp. Endocrinol*., **168**, 496-504 (2010).

52. Paris, M., Pettersson, K., Schubert, M., Bertrand, S., Pongratz, I., Escriva, H., Laudet, V.: An amphioxus orthologue of the estrogen receptor that does not bind estradiol: insights into estrogen receptor evolution. *BMC Evol. Biol*., **8**, 219 (2008).

53. Katsu, Y., Kohno, S., Hyodo, S., Ijiri, S., Adachi, S., Hara, A., Guillette, L.J.Jr., Iguchi, T.: Molecular cloning, characterization, and evolutionary analysis of estrogen receptors from phylogenetically ancient fish. *Endocrinology*, **149**, 6300-6310 (2008).

54. Tohyama, S., Miyagawa, S., Lang, A., Ogino, Y., Mizutani, T., Tatarazako, N., Katsu, Y., Ihara, M., Tanaka, H., Ishibashi, H., Kobayashi, T., Tyler, C.R, Iguchi, T.: Understanding

the molecular basis for differences in responses of fish estrogen receptor subtypes to environmental estrogens. *Environ. Sci. Technol.*, **49**, 7439-7447 (2015).

55. Sato, Y., Nishida, M.: Post-duplication charge evolution of phosphoglucose isomerases in teleost fishes through weak selection on many amino acid sites. *BMC Evol. Biol.*, **7**, 204 (2007).

56. Hoegg, S., Brinkmann, H., Taylor, J.S., Meyer, A.: Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J. Mol. Evol.*, **59**, 190-203 (2004).

57. Chiu, C.H., Dewar, K., Wagner, G.P., Takahashi, K., Ruddle, F., Ledje, C., Bartsch, P., Scemama, J.L., Stellwag, E., Fried, C., Prohaska, S.J., Stadler, P.F., Amemiya, C.T.: Bichir HoxA cluster sequence reveals surprising trends in ray-finned fish genomic evolution. *Genome Res.*, **14**, 11-17 (2004).

58. Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker , M., Postlethwait, J.H.: Zebrafish hox clusters and vertebrate genome evolution. *Science*, **282**, 1711-1714 (1998).

59. Venkatesh, B., Erdmann, M. V., Brenner, S.: Molecular synapomorphies resolve evolutionary relationships of extant jawed vertebrates. *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 11382-11387 (2001).

60. Soffker, M., Tyler, C.R.: Endocrine disrupting chemicals and sexual behaviors in fish--a critical review on effects and possible consequences. *Crit. Rev. Toxicol.*, **42**, 653-668 (2012).

61. Corcoran, J., Winter, M.J., Tyler, C.R.: Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. *Crit. Rev. Toxicol.*, **40**, 287-304 (2010).

62. Scholz, S., Kluver, N.: Effects of endocrine disrupters on sexual, gonadal development in fish. *Sex. Dev*., **3**, 136-151 (2009).

63. Lange, A., Katsu, Y., Miyagawa, S., Ogino, Y., Urushitani, H., Kobayashi, T., Hirai, T., Shears, J.A., Nagae, M., Yamamoto, J., Ohnishi, Y., Oka, T., Tatarazako, N., Ohta, Y., Tyler, C.R., Iguchi, T.: Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. *Aquat. Toxicol*., **109**, 250-258 (2012).

64. Jones, D.T., Taylor, W.R., Thornton, J.M.: The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci*., **8**, 275-282 (1992).

65. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S.: MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*., **30**, 2725-2729 (2013).

66. Cotter, K.A., Yershov, A., Novillo, A., Callard, G.V.: Multiple structurally distinct ERalpha mRNA variants in zebrafish are differentially expressed by tissue type, stage of development and estrogen exposure. *Gen. Comp. Endocrinol*., **194**, 217-229 (2013).

67. Metivier, R., Petit, F. G., Valotaire, Y., Pakdel, F.: Function of N-terminal transactivation domain of the estrogen receptor requires a potential alpha-helical structure and is negatively regulated by the A domain. *Mol. Endocrinol*., **14**, 1849-1871 (2000).

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

69. Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., Sigler, P.B.: Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature*, **352**, 497-505 (1991).

70. Nelson, J.S.: Fishes of the world. 4th ed. J. Wiley, New York (2006).

71. Hedges, S.B.: Vertebrates, in: Hedges, S.B., Kumar, S. (Eds.) The Timetree of Life, Oxford university press, New York, pp. 309-314 (2009).

72. Kuraku, S., Kuratani, S.: Time scale for cyclostome evolution inferred with a phylogenetic diagnosis of hagfish and lamprey cDNA sequences. *Zoolog. Sci*., **23**, 1053-1064 (2006).

73. Basden, A.M., Young, M.I. Coates, M.I., Ritchie, A.: The most primitive osteichthyan braincase? *Nature*, **403**, 185-188 (2000).

74. Eschmeyer, W.N., Fricke, R.: Catalog of Fishes, California Academy of Sciences. San Francisco (2012).

75. Bridgham, J.T., Brown, J.E., Rodriguez-Mari, A., Catchen, J.M., Thornton, J.W.: Evolution of a new function by degenerative mutation in cephalochordate steroid receptors. *PLoS Genet*., **4**, e1000191 (2008).

76. Kikugawa, K., Katoh, K., Kuraku, S., Sakurai, H., Ishida, O., Iwabe, N., Miyata, T.: Basal jawed vertebrate phylogeny inferred from multiple nuclear DNA-coded genes. *BMC Biol*., **2**, 3 (2004).

77. Inoue, J.G., Miya, M., Tsukamoto, K., Nishida, M.: Basal actinopterygian relationships: a mitogenomic perspective on the phylogeny of the "ancient fish". *Mol. Phylogenet. Evol*., **26**, 110-120 (2003).

78. Fontana, F., Lanfredi, M., Congiu, L., Leis, M., Chicca, M., Rossi, R.: Chromosomal mapping of 18S-28S and 5S rRNA genes by two-colour fluorescent in situ hybridization in six sturgeon species. *Genome*, **46**, 473-477 (2003).

79. Allendorf, F.W., Thorgaard, G.H.: Tetraploidy and evolution of salmonid fishes, in: Turner, B.J. (Ed.), Evolutionary Genetics of Fish, Plenum Press, New York, pp. 1-53 (1984).

80. Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad, C.3rd.: The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERalpha2 and both ERbeta isoforms. *Gene*, **392**, 164-173 (2007).

81. Nikoleris, L., Hansson, M.C.: Unraveling the estrogen receptor (er) genes in Atlantic salmon (*Salmo salar*) reveals expression differences between the two adult life stages but little impact from polychlorinated biphenyl (PCB) load. *Mol. Cell. Endocrinol*., **400**, 10-20 (2015).

82. Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noel, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., Aury, J.M., Louis, A., Dehais, P., Bardou, P., Montfort, J., Klopp, C., Cabau, C., Gaspin, C., Thorgaard, G.H., Boussaha, M., Quillet, E., Guyomard, R., Galiana, D., Bobe, J., Volff, J.N., Genet, C., Wincker, P., Jaillon, O., Crollius, H.R, Guiguen, Y.: The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nat. Commun*., **5**, 3657 (2014).

83. Katsu, Y., Lange, A., Miyagawa, S., Urushitani, H., Tatarazako, N., Kawashima, Y., Tyler, C.R., Iguchi, T.: Cloning, expression and functional characterization of carp, *Cyprinus carpio*, estrogen receptors and their differential activations by estrogens. *J. Appl. Toxicol.*, **33**, 41-49 (2013).

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

85. Callard, G.V., Tarrant, A.M., Novillo, A., Yacci, P., Ciaccia, L., Vajda, S., Chuang, G.Y., Kozakov, D., Greytak, S.R., Sawyer, S., Hoover, C., Cotter, K.A.: Evolutionary origins of the estrogen signaling system: insights from amphioxus. *J. Steroid. Biochem. Mol. Biol*., **127**, 176-188 (2011).

86. Katsu, Y., Kubokawa, K., Urushitani, H., Iguchi, T.: Estrogen-dependent transactivation of amphioxus steroid hormone receptor via both estrogen and androgen response elements. *Endocrinology*, **151**, 639-648 (2010).

87. Vulliet, E., Cren-Olive, C.: Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. *Environ. Pollut*., **159**, 2929-2934 (2011).

88. Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M.: Behavior and occurrence of estrogens in municipal sewage treatment plants--I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ*., **225**, 81-90 (1999).

89. Rose, J., Holbech, H., Lindholst, C., Norum, U., Povlsen, A., Korsgaard, B., Bjerregaard, P.: Vitellogenin induction by 17beta-estradiol and 17alpha-ethinylestradiol in male zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. C Toxicol .Pharmacol*., **131**, 531-539 (2002).

90. Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins. G.S., Soto, A.M., Zoeller, R.T., Gore, A.C.: Endocrine-disrupting chemicals: an Endocrine statement. *Endocr. Rev*., **30**, 293-342 (2009).

91. Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A.: Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*, **139**, 4252-4263 (1998).

92. Senior, A.M., Nakagawa, S.: A comparative analysis of chemically induced sex reversal in teleosts: challenging conventional suppositions. *Fish Fisheries*, **14**, 60-76 (2013).

93. Bridgham, J.T., Keay, J., Ortlund, E.A., Thornton, J.W.: Vestigialization of an allosteric switch: genetic and structural mechanisms for the evolution of constitutive activity in a steroid hormone receptor. *PLoS Genet*., **10**, e1004058 (2014).

94. Harms, M.J., Thornton, J.W.: Analyzing protein structure and function using ancestral gene reconstruction. *Curr. Opin. Struct. Biol*., **20**, 360-366 (2010).

Acknowledgements

I am grateful to Professor Tohru Kobayashi (University of Shizuoka), for his encouragement and kind support during this study. I also would like to express my appreciation to Professors Atsukazu Kuwahara (University of Shizuoka), Kimiko Yamakawa-Kobayashi (University of Shizuoka) and Masakazu Makino (University of Shizuoka) for reviewing this thesis.

I am grateful to Professor Taisen Iguchi (National Institute for Basic Biology) for his supervising the entire study. I am deeply grateful to Assistant Professor Shinichi Miyagawa (National Institute for Basic Biology), for all his help providing me with the technical assistance and valuable advice, Assistant Professor Yukiko Ogino (National Institute for Basic Biology), for her excellent advice and warm encouragement, and Mr. Takeshi Mizutani (National Institute for Basic Biology) and all my laboratory members for their assistance and warm encouragement.

I wish to express my appreciation to Dr. Anke Lange (University of Exeter, United Kingdom) and Professor Charles R. Tyler (University of Exeter), for their critical readings of the manuscript.

I wish to thank Drs. Norihisa Tatarazako (National Institute for Environmental Studies), Masaru Ihara (Kyoto University), Professors Hiroaki Tanaka (Kyoto University), Yoshinao Katsu (Hokkaido University), and Hiroshi Ishibashi (Ehime University), for their kind cooperation throughout the collaborative research.

Lastly, I wish to express my sincere thanks to my parents supporting my student life.