Characterization of evolutionary trend in squamate estrogen receptor sensitivity

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Highlights

- Estrogen receptors from three squamates were functionally characterized.
- Comparisons of ligand sensitivities suggest an evolutionary trend in vertebrate ERα.
- Squamates have high ligand sensitivity, while urodeles have low sensitivity.
- Homology models also suggest similar trend in molecular ligand binding affinity.

Abstract

Steroid hormones are a key regulator of reproductive biology in vertebrates, and are largely regulated via nuclear receptor families. Estrogen signaling is regulated by two estrogen receptor (ER) subtypes alpha and beta in the nucleus. In order to understand the role of estrogen in vertebrates, these ER from various species have been isolated and were functionally analyzed using luciferase reporter gene assays. Interestingly, species difference in estrogen sensitivity has been noted in the past, and it was reported that snake ER displayed highest estrogen sensitivity. Here, we isolated additional ER from three lizards: chameleon (*Bradypodion pumilum*), skink (*Plestiodon finitimus*), and gecko (*Gekko japonicus*). We have performed functional characterization of these ERs using reporter gene assay system, and found high estrogen sensitivity in all three species. Furthermore, comparison with results

from other tetrapod ER revealed a seemingly uniform gradual pattern of ligand sensitivity evolution. *In silico* 3D homology modeling of the ligand-binding domain revealed structural variation at three sites, helix 2, and juncture between helices 8 and 9, and caudal region of helix 10/11. Docking simulations indicated that predicted ligand-receptor interaction also correlated with the reporter assay results, and overall squamates displayed highest stabilized interactions. The assay system and homology modeling system provides tool for in-depth comparative analysis of estrogen function, and provides insight toward the evolution of ER among vertebrates.

Keywords: Squamates; Estrogen receptor; Estrogen sensitivity; Evolution of estrogen receptor

1. Introduction

Steroid hormones operate as powerful endogenous regulators in many of the vertebrate biological processes. Sex steroid hormones, such as progestagens, androgens, and estrogens, play prominent roles in reproductive biology, including development of sexual organs, behavior and reproductive cycles. Estrogens, in particular, are associated with development of female-characteristics in vertebrates, such as ovarian development, oogenesis, and secondary sex characteristics, in addition to metabolic and adipose regulation (Heldring et al., 2007, Hess, 2003 and Nilsson et al., 2001). Taken together, study of estrogen action remains one of most crucial component in understanding vertebrate reproductive biology. Currently, much of the insight on vertebrate molecular estrogen mechanism derives from mammalian and aquatic species, such as teleosts (Nelson and Habibi, 2013 and Tohyama et al., 2015). However, researches in other vertebrates, namely reptiles, are comparatively lacking.

Endogenous estrogens include 17β-estradiol (E2), estrone (E1), and estriol (E3), and are biosynthesized from androgens via aromatase enzyme in the gonad and the brain. Of these, E2 is widely regarded as the dominant estrogen among vertebrates. In addition to endogenous estrogens, several synthetic compounds with estrogenic activity are also reported; these include pharmaceutical drugs such as diethylstilbestrol (DES) and 17α-ethinylestradiol (EE2). Estrogen signalings are rapidly dispersed internally through circulatory systems, inducing gene expression changes in target cells. They are relayed in target cells via canonical nuclear receptors, estrogen receptors (ERs), for the most part and also via membrane signaling to some extent (Bjornstrom and Sjoberg, 2005). Two main ER subtypes are present: ER alpha (ERα) and ER beta. (ERβ). Similar to other nuclear steroid receptors, these two receptors are structurally composed of six domains, including the DNA-binding domain (DBD), that allows ER binding to DNA at estrogen response element (ERE), and ligand-binding domain (LBD), which forms a ligand-binding pocket for reception of estrogenic and various other compounds (Klinge, 2001). ERα is primarily considered to have higher significance in biological roles than ERβ, and have been associated with multitudes of physiological functions (Couse and Korach, 1999).

Functionality of nuclear receptors, such as ER, can be comprehensively assessed by various methods, such as *in silico* molecular simulations, and more commonly, with *in vitro* luciferase reporter gene assay system (Katsu et al., 2004, Kohno et al., 2008 and Tohyama et al., 2015). ER sensitivity to various administrations of estrogenic compounds has been previously investigated, and quantified in the form of effective concentration, EC_{50} values. In

current nuclear receptor studies, EC_{50}s are instrumental in evaluating the functionality, chemical sensitivity, and evolution of ERs in many organisms. Cross-species comparative analyses from wide spectrum of vertebrates, including reptiles, revealed species difference in ERα sensitivity in response to various ligands. Snakes displayed the lowest recorded EC_{50} values in all studied vertebrates, followed by other reptile and avian species (Katsu et al., 2008a and Katsu et al., 2010a; Naidoo et al., 2008). The biological implication of such species difference is yet to be elucidated, and lowered EC_{50} values may indicate a reptile-specific evolutionary trend.

Here, we report additional detail on for three more reptiles, Cape dwarf chameleon (*Bradypodion pumilum*), Japanese skink (*Plestiodon finitimus*), and Japanese gecko (*Gekko japonicus*), and provide more comprehensive description of ERα in reptiles, focusing particularly on squamates. In order to fully characterize the evolutionary trend in reptile ER sensitivity, we have isolated and performed *in vitro* luciferase reporter assay on these lizard ERs. Interestingly, a potential evolutionary trend was observed as ERα from squamates were found to be highly sensitive to estrogen compared to other vertebrate clades. Furthermore, *in silico* modeling of tetrapod ERαs was utilized to analyze the structural changes that may have lead to species differences in ER sensitivity.

2. Materials and methods

2.1. Animals and chemical reagents

The Japanese skink (*Plestiodon finitimus*) and the Japanese gecko (*Gekko japonicus*) were collected in the field in Okazaki, Aichi, Japan. Cape dwarf chameleon (*Bradypodion pumilum*) tissues were obtained from South Africa. All estrogenic compounds used in the study (E2, E1, E3, EE2, and DES) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The chemicals were dissolved in dimethylsulfoxide (DMSO) for use in reporter assay, and did not exceed 0.1% concentration in the culture medium.

2.2. Molecular cloning of estrogen receptors

Total RNA was extracted from ovary, and liver tissues, using RNeasy kit (Qiagen, Valencia, CA, USA) for chameleon and skink, and from tail tissue using ISOGEN reagent (Nippon Gene, Toyama, Japan) for gecko. Full coding region of the estrogen receptor from chameleon and skink was determined by standard procedure using SmartRACE kit (Takara, Ohtsu, Japan), and GeneRacer kit (Life Technologies, Carlsbad, CA, USA) for gecko. Full-length ERs of each species were cloned with KOD+ polymerase (Toyobo, Osaka, Japan). Primer information is reported in Supplementary Table S1. The amplified full-length ER cDNA products were then subcloned into pcDNA3.1 vector (Life Technologies). Estrogen regulated reporter vector with four estrogen-responsive elements (pGL3-4xERE) was also constructed as described previously (Katsu et al., 2004 and Katsu et al., 2006).

2.3. Sequence analysis

Multiple sequence alignment and amino acid similarity was calculated for various vertebrate ER homologues using CLUSTAL OEMGA (Goujon et al., 2010 and Sievers et al., 2011). Phylogenetic relationships of ER were then examined using predicted ER amino acid sequences from current study and previously reported sequences from GenBank database, summarized in Supplementary Table S2. Phylogenetic tree was constructed based on ER

conserved sites, which include the DNA binding domain (DBD), hinge region, and the ligand binding domain (LBD), with all the alignment gap sites eliminated using the maximum-likelihood methods with Jones-Taylor-Thornton model using MEGA 6 software (Tamura et al., 2013). The statistical confidence was then computed by bootstrap method with 1000 replications.

2.4. Transactivation assays

HEK293 cells (DS Pharma Biomedical, Osaka, Japan) were transfected with reporter vectors and pcDNA3.1-ER constructs, and estrogen-induced transcriptional activity was recorded using reporter gene assay in three technical replicates per each dosage concentration as previously described (Katsu et al., 2008a, Katsu et al., 2008b and Katsu et al., 2010b). Luciferase activity was recorded with Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA) under manufacturer's protocol, using GLOMAX 20/20 Luminometer (Promega).

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (Version 5.0b; GraphPad Software, Inc., San Diego, CA, USA). Dosage-response curve was constructed from normalized transactivation assay results, using three parametric non-linear regression fit, and EC_{50} values were subsequently calculated. For correlation analysis, average EC_{50} values were used for species with multiple previous data. Linear regression analysis was performed and graphed with 95% confidence interval of the best-fit line.

2.6. *In silico* modeling

Computational 3D homology modeling of ERα-LBD, and subsequent analyses were performed using Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Quebec, Canada), using protocol previously described (Tohyama et al., 2015). The homology models were constructed using the crystal structure of human ERα-LBD with E2 (1A52 in Protein Data Bank), and optimized using AMBER12:EHT force field (Labute, 2008 and Tanenbaum et al., 1998). Protonate three-dimensional program was used to prepare the model, and the protonation state of running buffer was adjusted to pH 7.0, same as the luciferase transactivation assays environment. Structural homologies between the constructed models were analyzed using Protein Consensus program. Ligand binding pockets were identified using MOE Alpha Site Finder. Most stable interaction potential between E2 and LBD models were predicted based on lowest calculated U-total value (kcal/mol), using ASEDock program with 250 conformations generated by low-mode molecular dynamics (Labute, 2010). Each docking simulations were evaluated with energy sum of electric, van der Waals, solvation and strain energy (U-dock score).

3. Results

3.1. Cloning of squamate ERs

Partial DNA fragments of ERs from the three lizard species, chameleon (*B. pumilum*), skink (*P. finitimus*), and gecko (*G. japonicus*) were amplified from liver, ovary and tail tissues. These lizard species were selected to represent the squamates broadly. Using the RACE technique, 5′ and 3′ region were amplified with the exception of gecko ERβ, which we were

not able to successfully amplify. Finally, full-length cDNAs of the ERs (three ERαs and two ERβs) were cloned and amino acid sequences were predicted. ERαs were predicted with 621, 612 and 588 aa, respectively. Difference in sequence length was due to notably extended 5′ sequence in chameleon ERα, and extended 3′ sequences in skink. ERβs amplified from chameleon and skinks were predicted with 549 and 553 aa, respectively. Similarity of the predicted amino acid sequences of both subtypes were fairly conserved among the lizard species, and overall, chameleon ERα shared 82%, and 79% with skink and gecko ERαs, while chameleon ERβ shared 84% with skink ERβ. Comparison with other vertebrate ER orthologs indicated high similarity as well, and was especially conserved in DNA- and ligand-binding domains (Fig. 1). Overall, chameleon ERα shared 80% (chicken), 73% (human), 72% (Western clawed frog), and 50% with medaka ERα. As for chameleon ERβ, 82% (chicken) 72% (human), 69% (Western clawed frog) and 52, 56% (medaka ERβ1 and ERβ2, respectively) amino acid similarity was observed. Phylogenetic analysis showed both of the newly predicted lizard ERα and ERβ to be more closely related to snakes than other reptiles such as turtles and alligators, consistent with the evolutionary divide between lepidosaurias (lizards, snakes) and archelosauria (birds, crocodilians, turtles) (Supplementary Fig. S1A, B). In contrast, while archelosauria ERαs displayed strong sequence conservation, lizard ERαs tended to display higher genetic distance, especially in chameleon ERα, indicating evolutionary divergence among lepidosaurias.

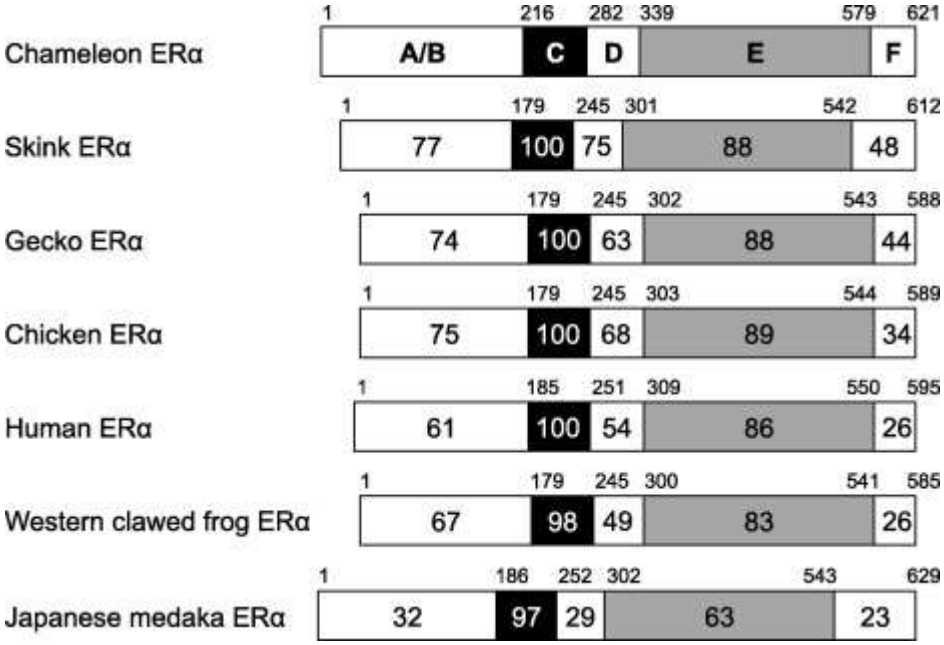


Fig. 1. Comparison of ERα similarity. Schematic of the ERα domain structure of chameleon, skink, gecko, chicken, human (*Homo sapiens*), African clawed frog (*Xenopus laevis*), and Japanese medaka (*Oryzias latipes*). Each box represents functional domain A/B to F. Percent amino acid identity in comparison to chameleon ER*α* is presented within each domain per species. The numbers above each domain boundary indicates amino acid residue position.

3.2. Transcriptional activity of lizard ERs

Next, functional analysis was performed on the isolated lizard ERs using *in vitro* luciferase reporter assay. HEK293 cells were co-transfected with an ER isolate and reporter vector with ERE, and transcriptional activity of ERs were assayed. Administration of each natural and synthetic estrogen induced luciferase expression in the ER-transfected cells, and clear dose-dependent curves were elicited (Fig. 2; Supplementary Fig. S2). Overall, the maximum fold

activation levels were relatively higher in chameleon ERs, while gecko and skink ERs resulted in similar fold activation levels. Concentration-response relationships were analyzed, and the calculated EC_{50}s of each natural estrogens were found to be similar among the lizards for both subtypes (Table 1; Supplementary Table S3). In contrast, differential transcriptional activity in response to synthetic estrogen administration was present between the three species. Chameleon ERα and skink ERα both displayed higher sensitivity toward EE2 than DES. EE2 was more potent than DES by 2.9-fold in chameleon ERα, and by 154.7-fold in skink ERα. In gecko ERα, however, DES was more potent than EE2 by approximately 2.5-fold. Furthermore, EE2 presence was highly potent to skink ERα, and had the lowest EC_{50} of 2.5×10^{-13} M with relative potency to E2 at 711.8%.

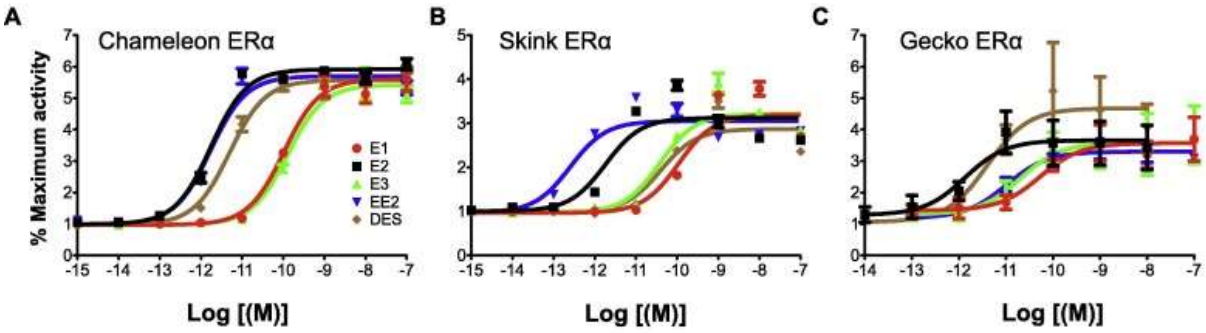


Fig. 2. Functional characterization of lizard ERα. Transcriptional activities of lizard ERα exposed to estrogens. Dose response curves for (A) chameleon, (B) skink, and (C) gecko are shown. Each point indicated is an average of triplicate determination; ±SE. Three natural estrogens, E1 (indicated in red), E2 (black), E3 (green), and two synthetic estrogens, EE2 (blue), and DES (brown) were examined.

Table 1. Gene transcriptional activities of estrogens mediated by lizard ERα.

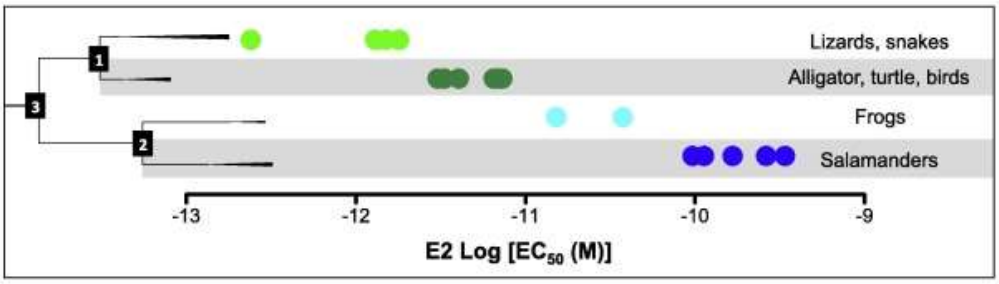
Chameleon ERα	**EC_{50} (M)**	**95% CI (M)**	**RP (%)**
E1	1.0×10^{-10}	7.4×10^{-11}–1.4×10^{-10}	1.7
E2	1.8×10^{-12}	1.3×10^{-12}–2.3×10^{-12}	100
E3	1.3×10^{-10}	8.5×10^{-11}–1.9×10^{-10}	1.4
EE2	1.7×10^{-12}	1.2×10^{-12}–2.4×10^{-12}	101.6
DES	5.0×10^{-12}	3.7×10^{-12}–6.7×10^{-12}	35.0
Skink ERα	**EC_{50} (M)**	**95% CI (M)**	**RP (%)**
E1	1.1×10^{-10}	4.8×10^{-11}–2.4×10^{-10}	1.7
E2	1.8×10^{-12}	7.2×10^{-13}–4.5×10^{-12}	100
E3	3.8×10^{-11}	1.8×10^{-11}–8.0×10^{-11}	4.8
EE2	2.5×10^{-13}	1.3×10^{-13}–5.0×10^{-13}	711.8
DES	3.9×10^{-11}	1.8×10^{-11}–8.8×10^{-11}	4.6
Gecko ERα	**EC_{50} (M)**	**95% CI (M)**	**RP (%)**
E1	5.3×10^{-11}	9.1×10^{-12}–3.0×10^{-10}	2.6
E2	1.3×10^{-12}	1.5×10^{-13}–1.2×10^{-11}	100
E3	2.0×10^{-11}	1.8×10^{-12}–2.3×10^{-10}	6.7
EE2	1.0×10^{-11}	3.1×10^{-12}–3.4×10^{-11}	13.1
DES	4.0×10^{-12}	5.2×10^{-13}–3.1×10^{-11}	33.3

Gene transcriptional activities of estrogens mediated by chameleon, skink, and gecko ERα. 95% CI: 95% Confidence intervals of EC_{50}. RP: Relative potency = (EC_{50} E2/EC_{50} chemical X) × 100.

3.3. Comparison within tetrapod ERα-LBDs

EC_{50} values from E2 action on lizard ERαs from current study, and on other vertebrate ERαs from previous experiments by our research group using same methodologies, were summarized for a potential species-wide comparison (Fig. 3A; Table 2) (Katsu et al., 2008a, Katsu et al., 2008b, Katsu et al., 2010a and Katsu et al., 2010b; Miyagawa et al., 2014 and Naidoo et al., 2008). Reported EC_{50} values indicated an extensive species variation in estrogen responsiveness among tetrapods. EC_{50} from fish species, in contrast, exhibited very little variance, and the further analyses focused solely upon tetrapods. Five squamates (lizards and snakes), four archelosauria (birds, alligator, and turtle), two anurans (frogs), and four urodeles (salamander) species were used for comparative study, and no mammalian data were available for this analysis. Consistent with the previous experiments, we show that lizards analyzed in current study have low EC_{50} values, similar to those of snakes. Additionally, species-specific EC_{50} pattern was also observed, with salamanders and squamates as the two ends of the seemingly EC_{50} spectrum. In fact, ER responsiveness varies by around 1000 fold between the highest recorded EC_{50} (*Cynops pyrrhogaster*) and the lowest recorded EC_{50} (*Protobothrops flavoviridis*). Same patterns of EC_{50} values for other endogenous estrogenic compounds were also seemingly present, though not enough data were available to make an assertion. The pattern was not observed from EC_{50}s derived from ERβ experiments (data not shown), although only a few studies are available at this stage. Amino acid sequences of ERαs were next investigated to analyze the structural change that allowed these EC_{50} differences. Ligand sensitivities (EC_{50}), in most part, can be attributed to the LBD, as previously documented (Miyagawa et al., 2014). Hence, multiple sequence alignment of the LBD was conducted with the investigated tetrapod ERα orthologs, with inclusion of human ERα-LBD as well (Fig. 3B). Several sites of squamate-, reptile- and anuran-specific mutations were observed.

A



B

Homo	SLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILM	388
Protobothrops	..SV.....E.....V.....N...N.....T.....	379
Elaphe	..S.....E.....V.....N.S.N.....T.....	381
Plestiodon	....E.....VV.....G...N...V.T.....T.....A.....	380
Bradypodion	...EE.....E.....V.....AG...N.....S.....	418
Gekko	.....VV.....G...N.....S.....A.....	381
Gallus	....E.....E.....V.....N...N.....T.....	382
Gyps	....E.....E.....V.....N...N.....T.....	380
Alligator	....E.....E.....V.....N...N.....T.....	382
Pseudemys	....E.....E.....V.....N.....T.....	381
Amietophrynus	....E.LI...ME.....V...H.S.K.....T.....K.....	377
Xenopus	....E.LI...ME.....V...H.S.K.L.....T.....K.....	379
Cynops	AM..E.LL...IE.....V...H..GKLL.....T.....K.....	378
Hynobius	.M..E.LL...IE.....V...H..SKLL.....T.....K.....S.....	378
Ambystoma	AM..E.LLT..IE.....V...H..NKQL.....T.....K.....	380
Andrias	.M.VE.LL...IE.....V...H...K.L.....T.....K.....	378
Homo	IGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKS	468
Protobothrops	.....V.....V.....I.....	459
Elaphe	.....V.....L.....S.....V.....I.....	461
Plestiodon	V..I.....M....F.....I.....I.....R.	460
Bradypodion	...I...VD.....G...R....LG.....T..T...V..VR.....I.....-.F..	497
Gekko	...C.....A.....AT.....I.....S.R.	461
Gallus	.....AA.....	462
Gyps	.....AA.....	460
Alligator	.....V.....AA.....	462
Pseudemys	.....AA...V.....	461
Amietophrynus	V..I...V.....S.....R....L.....VT.AT.....R.R....I.....E.	457
Xenopus	V..I...V.....S.....R....L.....VT.AT.....R.R....I.....E.	459
Cynops	V.....V.....S.....M.....F.....VT.AA...A.K.E....M.....E.	458
Hynobius	V.....V.....S.....R....F.....VT.AA...V.K.E....M.....E.	458
Ambystoma	V.....V.....S.....M.....R....F.....V..AA.....K.E....M.....E.	460
Andrias	V.....V.....S.....M.....R....F.....VT.A....V.K.E....M.....I.....N.VE.	458
Homo	LEEKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRL	549
Protobothrops	....E.....M.....S..SQ....R.....N.....	540
Elaphe	....E.T.....M.....S..SQ....R.....N.....H.	542
Plestiodon	....E.....T.....S..S.....R....F..M.....N.....	541
Bradypodion	VD...Y.....I...M.....S..SQ....R.....M.....N.....	578
Gekko	.....T.....S..S.....R.....F.....N.....	542
Gallus	...R.Y.....S..S.....R.....N.....	543
Gyps	...R.Y.....S..S.....R.....N.....	541
Alligator	.....Y.....S..S.....R.....N.....	543
Pseudemys	....E.....S..SPD...R.....N.....	542
Amietophrynus	..DT.Q..LI....I...V.F.G.T..S....QR.....QM	538
Xenopus	..DT.L..II....I...V.F...S..S....QR.....M	540
Cynops	P..TEQ..LI....I...V.....S..S.....R.....I	539
Hynobius	P..TEQ..LI....I...V.....S..S....QR.....I	539
Ambystoma	P..TEQ..LI....I...V.....S.HS....QR.....I	541
Andrias	P..TEQ.RLI.....V.....HS.....R.....N.....I	539

Fig. 3. Comparison of ligand sensitivity among tetrapod ERαs. (A) Species comparison of EC_{50} (M) in response to E2 exposure. Fifteen species consisting of five squamates (*Protobothrops flavoviridis*, *Elaphe quadrivirgata*, *Plestiodon finitimus*, *Bradypodion pumilum*, *Gekko japonicus*), four archelosauria (*Gallus gallus*, *Gyps africanus*, *Alligator mississippiensis*, *Pseudemys nelsoni*), two anurans (*Amietophrynus rangeri*, *Xenopus tropicalis*), and four urodeles (*Cynops pyrrhogaster*, *Hynobius tokyoensis*, *Ambystoma mexicanum*, *Andrias japonicus*) are compared, and arranged phylogenic manner. Figures in phylogeny indicates the evolutionary divergence that corresponds to split in E2 sensitivity in ERα (1. Squamate-archelosauria, 2. anuran-urodeles, and 3. amphibian-reptile divergence). (B) Multiple sequence alignment for tetrapod ERα-LBD.

Table 2. Species comparison of EC_{50} (M) in response to E2 exposure.

Common Name	Scientific name	EC_{50} (M)	Reference
Okinawa habu	*Protobothrops flavoviridis*	2.4×10^{-13}	Katsu et al. (2010a)
Japanese striped snake	*Elaphe quadrivirgata*	1.5×10^{-12}	Katsu et al. (2010a)
Japanese five-lined skink	*Plestiodon finitimus*	1.8×10^{-12}	This study
Cape dwarf chameleon	*Bradypodion pumilum*	1.8×10^{-12}	This study
Japanese gecko	*Gekko japonicus*	1.3×10^{-12}	This study
Chicken	*Gallus gallus*	4.1×10^{-12}	Naidoo et al. (2008)
White-backed vulture	*Gyps africanus*	3.0×10^{-12}	Naidoo et al. (2008)
		3.3×10^{-12}	Naidoo et al. (2008)
American alligator	*Alligator mississippiensis*	6.5×10^{-12}	Katsu et al. (2010a)
Red-belly turtle	*Pseudemys nelsoni*	7.1×10^{-12}	Katsu et al. (2008a)
		6.8×10^{-12}	Naidoo et al. (2008)
		7.3×10^{-12}	Katsu et al. (2010a)
African raucous toad	*Amietophrynus rangeri*	1.5×10^{-11}	Katsu et al. (2010b)
Western clawed frog	*Xenopus tropicalis*	3.8×10^{-11}	Katsu et al. (2010b)
Japanese firebelly newt	*Cynops pyrrhogaster*	3.4×10^{-10}	Katsu et al. (2010b)
Tokyo salamander	*Hynobius tokyoensis*	9.7×10^{-11}	Katsu et al. (2010b)
Axolotl	*Ambystoma mexicanum*	2.6×10^{-10}	Katsu et al. (2010b)
Japanese giant salamander	*Andrias japonicus*	1.1×10^{-10}	Katsu et al. (2008b)
		1.7×10^{-10}	Naidoo et al. (2008)
African lungfish*	*Protopterus dolloi*	2.1×10^{-10}	Katsu et al. (2008b)
Goldfish*	*Carassius auratus*	2.8×10^{-10}	Miyagawa et al. (2014)
Bluegill*	*Lepomis macrochirus*	2.5×10^{-10}	Miyagawa et al. (2014)
Guppy*	*Poecilia reticulata*	1.5×10^{-10}	Miyagawa et al. (2014)
Roach*	*Rutilus rutilus*	1.7×10^{-10}	Miyagawa et al. (2014)
Carp*	*Cyprinus carpio*	3.6×10^{-10}	Miyagawa et al. (2014)
Stickleback*	*Gasterosteus aculeatus*	3.7×10^{-10}	Miyagawa et al. (2014)
Zebrafish*	*Danio rerio*	1.2×10^{-10}	Miyagawa et al. (2014)
Japanese medaka*	*Oryzias latipes*	1.7×10^{-10}	Miyagawa et al. (2014)
Fathead minnow*	*Pimephales promelas*	2.3×10^{-10}	Miyagawa et al. (2014)

EC_{50} values of vertebrate ERα in response to E2 exposure. Species indicated in * are not included in the final graph (Fig. 3A).

3.4. *In silico* analysis of tetrapod ERα-LBDs

In silico modeling of ERα-LBD was performed to assess the structural basis for differential E2 sensitivity in tetrapods. Using the crystal structure of human ERα-LBD with E2 as a template, 3D models of the ERα-LBD for each species were constructed with MOE homology-modeling program (Tanenbaum et al., 1998). Protein consensus analysis was performed comprehensive aligning of generated 3D models (Fig. 4A). Overall, model was highly conserved, as predicted from amino acid sequence similarity (Fig. 1). Cross species-comparison of structural resemblance was assessed using root-mean square deviation (RMSD) values. RMSD values were fairly low, indicating high resemblance, and the values

ranged from 0.27 to 1.09, with *Xenopus tropicalis* having the least similar structure compared to all other receptor models. The main structural variation was observed at three points, at helix 2, junction between helices 8 and 9, and caudal region of helix 10/11.

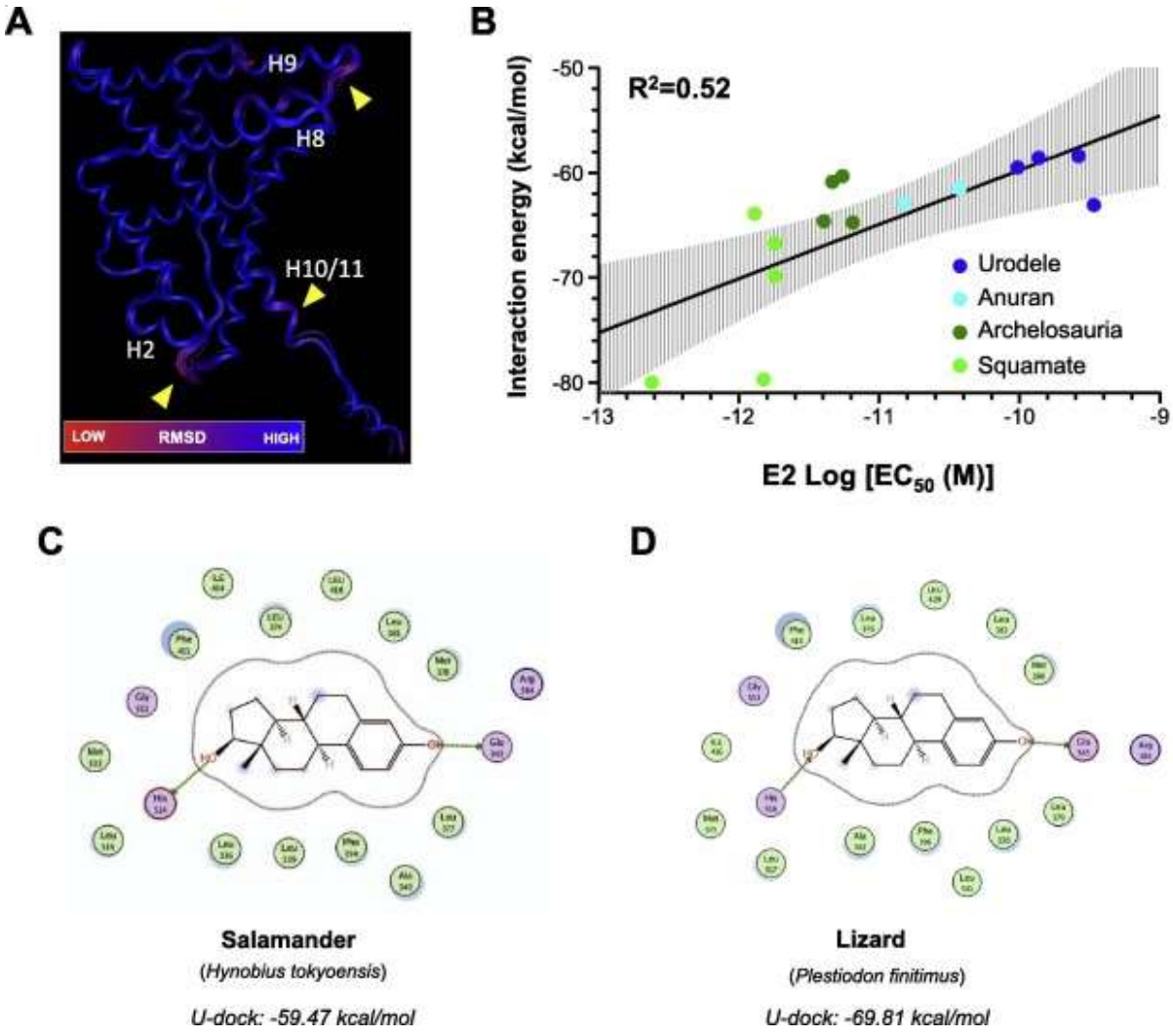


Fig. 4. Computer modeling of tetrapod ERαs. (A) Protein consensus among the constructed 3D homology models of ERα-LBD. Red-Blue scale indicates root-mean square deviation (RMSD) of the structure, with indicating low or high conservation in protein structure, respectively. (B) The relationship between the EC_{50} (M) in response to E2 exposure and interaction energy (kcal/mol) between ERα-LBD and E2 based on 15 tetrapod species. Regression line and 95% confidence interval is displayed. R^2 coefficient is indicated in the figure. Predicted ligand-receptor interactions in (C) salamander (*Hynobius tokyoensis*), and (D) skink (*Plestiodon finitimus*) ERα is presented, with U-dock score (kcal/mol). Amino acid residues are labeled in the ligand-binding pocket is presented (polar and hydrophobic amino acids are indicated in pink or green, respectively). Exposed ligand region is indicated in purple haze. Green arrow indicates hydrogen bond.

Docking simulations with E2 and the homology models were performed, and the predicted interaction energy was calculated. In all studied models, the general orientation of the ligand, and amino acid residues forming the binding pocket was consistent with previous documentation. Functionally important polar amino acids were in close proximity to the ligand, and hydrogen-bond or other interaction between hydroxyl group in C3 of E2 and glutamic acid and arginine (corresponding to human Glu353, Arg394) and C17-histidine (corresponding to human His524) was predicted in all species (Tanenbaum et al., 1998). In agreement with the EC_{50} data, increasingly stable binding of E2 to ERα was predicted along

the tetrapod phylogeny, with squamates having lowest interaction energy (U-dock) at −79.99 kcal/mol. Squamates were also predicted to have larger predicted ligand-binding pocket. Linear regression line analysis between EC_{50} and the interaction potential revealed a positive correlation between the two ($R^2 = 0.52$).

4. Discussions and conclusions

The profound role of estrogen and other steroid hormones is a universal element in the vertebrate endocrinology. As one of the main regulators of estrogen signaling, ERs pose great importance in understanding the molecular basis of the developmental, reproductive, and behavioral patterns of an organism. Here, we reveal a potential evidence for estrogen sensitivity as an evolutionary trait among tetrapod ERαs, by utilizing two independent methods. Both functional comparative analysis using traditional *in vitro* reporter gene assay system and *in silico* molecular docking simulations suggested the differential EC_{50} among tetrapods, with urodeles having least E2 sensitivity and squamates to have some of the highest. Both results from the two employed methods ultimately relied upon human data (use of HEK293 cell cultures, and use of template human ERα model). However, past studies also validated these methods as useful tools in understanding steroid hormone receptor functionality (Miyagawa et al., 2015 and Tohyama et al., 2015). Differing species responsiveness to estrogenic compounds has been previously described among teleost using same reporter gene assay system, although the differential responsiveness to E2 exposure was not found among them (Miyagawa et al., 2014), and the species differences were not as pronounced trend as the one seen in tetrapods. Indeed, EC_{50} by E2 exposure in the teleosts and lungfish ERs were generally similar to that of salamanders, and suggest conservation in E2 sensitivity within ancestral vertebrates. Three major gaps in E2 sensitivities were observed in the tetrapods, at the split between amphibians and reptiles, and secondary split between anuran and urodeles and between archelosauria and squamate. The phylogeny analysis implies that the anurans and squamates have independently acquired heightened E2 sensitivity, although, only two species have been investigated in anurans. Inclusion of mammalian data in future studies would shed further light on the overall evolution.

The exact details on the molecular mechanisms that permit such dramatic shift in EC_{50} are to be determined. ERs are widely receptive to multitudes of exogenous compounds directly or indirectly, in part due to the large ligand-binding pocket, and the increasing affinity in the hydrophobic ligand-binding pocket, as predicted by the docking modeling, certainly provides the explanation to a certain degree (Diamanti-Kandarakis et al., 2009). The actual amino acid residues corresponding to the binding pocket were conserved, and structural consensus analysis of the receptor models indicated most of the species variation to be focused around the protein bend between the major helices. Although they do not directly interact with ligand, they may influence the bending of the helices during the protein conformational change after the ligand binding, and affect the overall transcription efficiency (Paige et al., 1999).

The biological implication of such species differences in estrogen sensitivity is yet to be determined. Many benefits can be predicted, such as faster response to estrogen signaling and lower demand for hormone amounts. Major gap in EC_{50} occurred between reptiles-amphibians split, and the evolution of amniotic eggs, and the more efficient ER may have facilitated spread of habitat range from mainly aquatic to terrestrial environment. Furthermore, the apparent change may be to further differentiate ERα and ERβ functions, considering that this particular trend was not observed in ERβ. Significant distinctions in ERα

function and ERβ have been previously reported in mammals, including ligand selectivity and unique physiological roles (Heldring et al., 2007 and Kuiper et al., 1997).

Evolution of the steroid hormone receptors is a much-discussed theme in the field of comparative endocrinology (Harms et al., 2013, Thornton, 2001 and Thornton et al., 2003). Divergence of various contemporary steroid hormone receptors, including ERs, from the ancestral steroid hormone receptors was in theory initiated by discrete evolutionary shift in the receptor's ligand specificity through few amino acid mutations in the ligand-binding pocket region (Harms et al., 2013). Here, we present evidence for a potential evolution of ER in nature. However, our results also seem to suggest that the change in EC_{50} is not due to direct amino acid mutations in the pocket, but due to slight conformational change in the LBD structure as a whole. Emergence of tetrapod ER only represents a small portion of the ER evolution. In contrast to the discrete punctuated evolutionary shift in ligand sensitivity in a large timescale, however, ERα appears to undergo a uniform gradual evolution in this relatively short timespan. ERs are associated with regulation of reproductive actions in vertebrates, and most likely are subject to pressure from directional selection (Kubokawa et al., 2010). ERα, in particular, have been demonstrated to be integral for sex determination in the American alligator (Kohno et al., 2015).

Reptiles represent pivotal group in higher vertebrate evolution, and provide insights toward ancestral origin and evolution of many of the physiological mechanisms in higher vertebrates. Their wide range of global habitat and species diversity lead to large variation in endocrine control of development, physiology, and behavior. These unique qualities make reptiles an ideal subjects for study. If the predicted ERα evolutionary trend were of any indication, it would then imply that reptiles may be more vulnerable to estrogen presence than previously thought, and warrants further understanding in this relatively unexplored clade. Importance of its role in the reptile ecology has already been extensively documented in the past, including reproductive cycle, courtship behavior, and sex determination (Crews, 1979, Crews and Fitzgerald, 1980 and Ramsey and Crews, 2009). Furthermore, endocrine disruptions by xenoestrogen presence have also been extensively reported in reptiles; most famously, American alligator population that experienced an exposure to estrogenic pesticide displayed various lowered reproductive capability, including reduced fertility, hormonal imbalance, and abnormal development of accessory organs (Guillette et al., 1994 and Guillette et al., 1996). Endocrine disrupting chemical action has been documented in several other reptile groups as well (Bergeron et al., 1994 and Parsley et al., 2015).

In conclusion, here we have isolated three lizard ERs and assayed their transcriptional activities using *in vitro* luciferase reporter assay system. Using *in silico* modeling, we were able to model a potential evolutionary trend among tetrapod ERs and suggest the structural changes that allowed change in transcriptional capabilities. This work is extension of previous comparative ER function studies, and provides novel insight on the vertebrate ER mechanism and its evolution. The comparative analyses were made possible by the steady efforts to characterize various vertebrate ERs that are seldom studied. The apparent evolutionary pattern would have gone unnoticed without these studies, and hence there is a need for more comprehensive research on vertebrate ERs from variety of species for further understanding.

5. Additional information

Chameleon, skink, and gecko cDNA sequence data have been deposited in DNA Data Bank of Japan and National Center for Biotechnology Information. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S.M. (miyagawa@nibb.ac.jp).

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References

Bergeron, J.M., Crews, D., Mclachlan, J.A., 1994. PCBs as environmental estrogens – turtle sex determination as a biomarker of environmental contamination. Environ. Health Perspect. 102, 780–781.

Bjornstrom, L., Sjoberg, M., 2005. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol. Endocrinol. 19, 833–842.

Couse, J.F., Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. 20, 358–417.

Crews, D., 1979. The hormonal control of behavior in a lizard. Sci. Am. 241, 180–187.

Crews, D., Fitzgerald, K.T., 1980. ‘‘Sexual” behavior in parthenogenetic lizards (Cnemidophorus). Proc. Natl. Acad. Sci. U.S.A. 77, 499–502.

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

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., Lopez, R., 2010. A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res. 38, W695–W699.

Guillette Jr., L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodward, A.R., 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102, 680–688.

Guillette Jr., L.J., Pickford, D.B., Crain, D.A., Rooney, A.A., Percival, H.F., 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen. Comp. Endocrinol. 101, 32–42.

Harms, M.J., Eick, G.N., Goswami, D., Colucci, J.K., Griffin, P.R., Ortlund, E.A., Thornton, J.W., 2013. Biophysical mechanisms for large-effect mutations in the evolution of steroid hormone receptors. Proc. Natl. Acad. Sci. U.S.A. 110, 11475– 11480.

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

Hess, R.A., 2003. Estrogen in the adult male reproductive tract: a review. Reprod. Biol. Endocrinol. 1, 52.

Katsu, Y., Bermudez, D.S., Braun, E.L., Helbing, C., Miyagawa, S., Gunderson, M.P., Kohno, S., Bryan, T.A., Guillette Jr., L.J., Iguchi, T., 2004. Molecular cloning of the estrogen and progesterone receptors of the American alligator. Gen. Comp. Endocrinol. 136, 122–133.

Katsu, Y., Kohno, S., Oka, T., Mitsui, N., Tooi, O., Santo, N., Urushitani, H., Fukumoto, Y., Kuwabara, K., Ashikaga, K., Minami, S., Kato, S., Ohta, Y., Guillette Jr., L.J., Iguchi, T., 2006. Molecular cloning of estrogen receptor alpha (ERa; ESR1) of the Japanese giant salamander, Andrias japonicus. Mol. Cell. Endocrinol. 257–258, 84–94.

Katsu, Y., Ichikawa, R., Ikeuchi, T., Kohno, S., Guillette Jr., L.J., Iguchi, T., 2008a. Molecular cloning and characterization of estrogen, androgen, and progesterone nuclear receptors from a freshwater turtle (Pseudemys nelsoni). Endocrinology 149, 161–173.

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

Katsu, Y., Matsubara, K., Kohno, S., Matsuda, Y., Toriba, M., Oka, K., Guillette, L.J., Ohta, Y., Iguchi, T., 2010a. Molecular cloning, characterization, and chromosome mapping of reptilian estrogen receptors. Endocrinology 151, 5710–5720.

Katsu, Y., Taniguchi, E., Urushitani, H., Miyagawa, S., Takase, M., Kubokawa, K., Tooi, O., Oka, T., Santo, N., Myburgh, J., Matsuno, A., Iguchi, T., 2010b. Molecular cloning and characterization of ligand- and species-specificity of amphibian estrogen receptors. Gen. Comp. Endocrinol. 168, 220–230.

Klinge, C.M., 2001. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 29, 2905–2919.

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

Kohno, S., Bernhard, M.C., Katsu, Y., Zhu, J., Bryan, T.A., Doheny, B.M., Iguchi, T., Guillette Jr., L.J., 2015. Estrogen receptor 1 (ESR1; ERa), not ESR2 (ERb), modulates estrogen-induced sex reversal in the American alligator, a species with temperature-dependent sex determination. Endocrinology 156, 1887–1899.

Kubokawa, K., Tando, Y., Roy, S., 2010. Evolution of the reproductive endocrine system in chordates. Integr. Comp. Biol. 50, 53–62.

Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J.Å., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138, 863–870.

Labute, P., 2008. The generalized Born/volume integral implicit solvent model: estimation of the free energy of hydration using London dispersion instead of atomic surface area. J. Comput. Chem. 29, 1693–1698.

Labute, P., 2010. LowModeMD-implicit low-mode velocity filtering applied to conformational search of macrocycles and protein loops. J. Chem. Info. Model. 50, 792–800.

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., 2014. 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.

Miyagawa, S., Yatsu, R., Kohno, S., Doheny, B.M., Ogino, Y., Ishibashi, H., Katsu, Y., Ohta, Y., Guillette Jr., L.J., Iguchi, T., 2015. Identification and characterization of the androgen receptor from the American alligator, Alligator mississippiensis. Endocrinology 156, 2795–2806.

Naidoo, V., Katsu, Y., Iguchi, T., 2008. The influence of non-toxic concentrations of DDT and DDE on the old world vulture estrogen receptor alpha. Gen. Comp. Endocrinol. 159, 188–195.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24.

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., Gustafsson, J.Å., 2001. Mechanisms of estrogen action. Physiol. Rev. 81, 1535–1565.

Paige, L.A., Christensen, D.J., Gron, H., Norris, J.D., Gottlin, E.B., Padilla, K.M., Chang, C.Y., Ballas, L.M., Hamilton, P.T., McDonnell, D.P., Fowlkes, D.M., 1999. Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. Proc. Natl. Acad. Sci. U.S.A. 96, 3999–4004.

Parsley, L.M., Wapstra, E., Jones, S.M., 2015. Atrazine disrupts gonadal development in a live-bearing lizard. Endocr. Disrupt. 3, e1006071.

Ramsey, M., Crews, D., 2009. Steroid signaling and temperature-dependent sex determination-reviewing the evidence for early action of estrogen during ovarian determination in turtles. Semin. Cell Dev. Biol. 20, 283–292.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

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

Thornton, J.W., 2001. 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.

Thornton, J.W., Need, E., Crews, D., 2003. Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. Science 301, 1714–1717.

Tohyama, S., Miyagawa, S., Lange, A., Ogino, Y., Mizutani, T., Tatarazako, N., Katsu, Y., Ihara, M., Tanaka, H., Ishibashi, H., Kobayashi, T., Tyler, C.R., Iguchi, T., 2015. Understanding the molecular basis for differences in responses of fish estrogen receptor subtypes to environmental estrogens. Environ. Sci. Technol. 49, 7439–7447.

Appendix A. Supplementary data

Supplementary Figure S1.

Phylogenetic relationship among (A) ERα and (B) ERβ amino acid sequences for selected higher vertebrate species. Phylogenetic tree was constructed based on conservative domains including DNA binding domain and ligand binding domain, using maximum-likelihood methods. Bootstrap value above 70 is indicated beside the respective branch. Database accession numbers of genes used is tabulated in Supplementary Table S2.

Supplementary Figure S2.

Transcriptional activities of lizard ERβ exposed to estrogens. Dose response curves for (A) chameleon, and (B) skink are shown. Each point indicated is an average of triplicate determination; ± SE. Three natural estrogens, E1 (indicated in red), E2 (black), E3 (green), and two synthetic estrogens, EE2 (blue), and DES (brown) were examined.

Supplementary Table S1.

Primer information

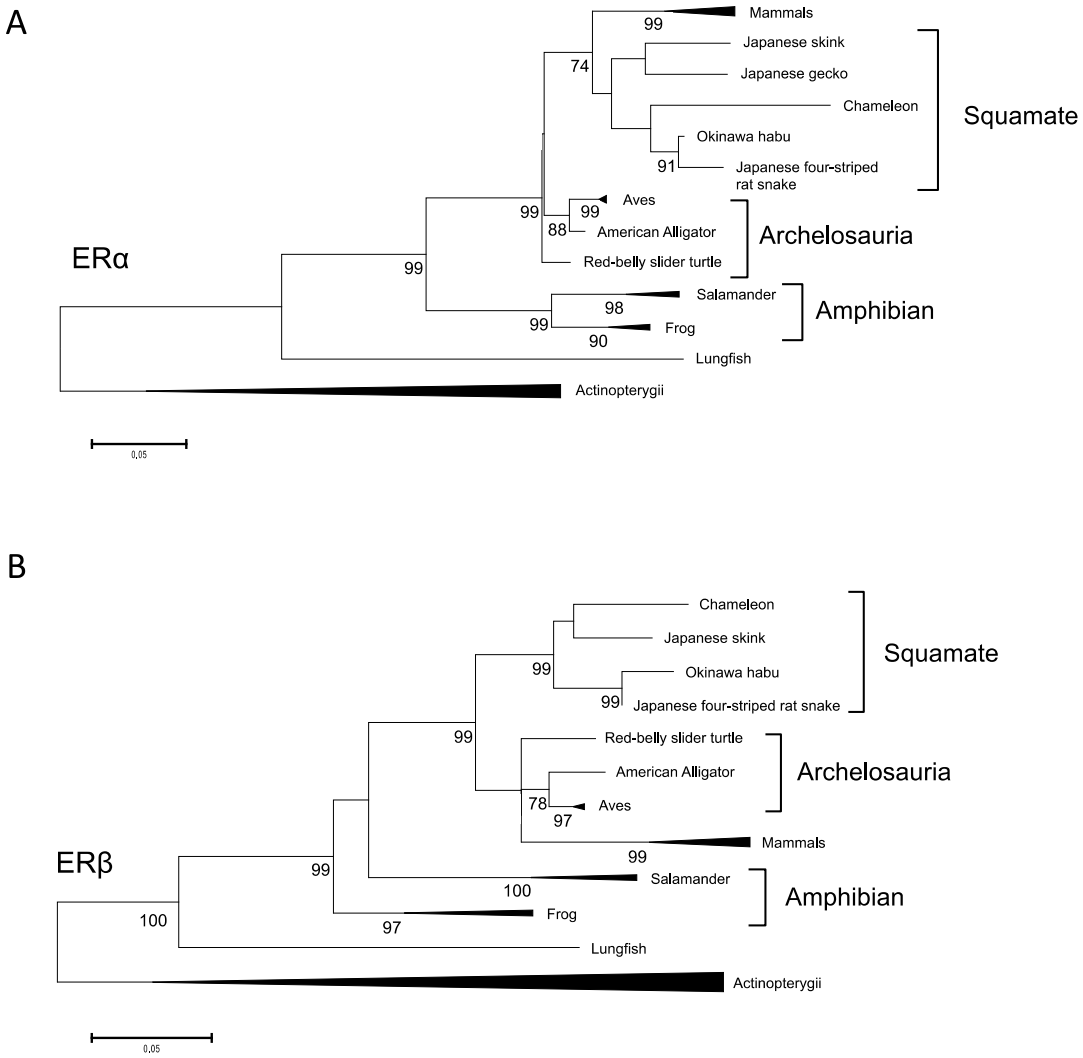
Supplementary Table S2.

Species name and GenBank accession number for the estrogen receptor sequence used for construction of the phylogeny tree.

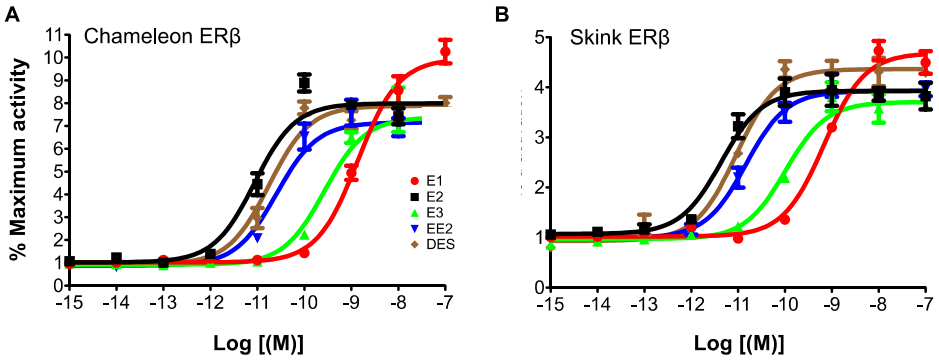
Supplementary Table S3.

Gene transcriptional activities of estrogens mediated by chameleon and skink ERβ. 95% CI: 95% Confidence intervals of EC_{50}. RP: Relative potency = (EC_{50} E2/ EC_{50} chemical X) x 100.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Table S1. Primers for RT-PCR of lizard ERs.

Chameleon ERα

Forward: CCTAATTGGGCTGAGGGAGAACGAGAG

Reverse: CAGAATCCTGTTGAAATGCAGACTCTC

Skink ERα

Forward: GGATACCTGTAGCACCGGCGGGGGTAG

Reverse: GACTTAGCACTCACTTGGGATCTGAAG

Gecko ERα

Forward: ATGACCATGACCCTTCACACAAAAAC

Reverse: TCATATTGCTTGTTGCTCATTCTCATCTTC

Chameleon ERβ

Forward: GCCACGTCGAAGGAAAACCCCAAACTC

Reverse: TTTTGAGGGGATTTTCCACTCCATTGC

Skink ERβ

Forward: ATCAGGCAAAGGCTAGGGAACACGTAG

Reverse: CTGCTTTCTTTGCACTTCCCTGGATCC

Supplementary Table S2. Species name and GenBank accession number.

Scientific name	**GenBank Accession Numbers**	
	ERα	**ERβ**
Oryzias latipes	BAA86925	NP_001098172, NP_001121984
Danio rerio	BAB16893	NP_851297, NP_777287
Rutilus rutilus	BAD91035	BAR64352, BAD91036
Gambusia affinis	BAF76770	BAF76771, BAF76772
Atractosteus tropicus	BAG82653	BAG82654
Acipenser schrenckii	BAG82650, BAG82651	BAG82652
Protopterus dolloi	BAG82648	BAG82649
Cynops pyrrhogaster	BAJ05025	BAJ05026
Ambystoma mexicanum	BAJ05029	BAJ05030
Hynobius tokyoensis	BAJ05027	BAJ05028
Amietophrynus rangeri	BAJ05031	BAJ05032
Rugosa rugosa	BAJ04337	ACZ51368
Xenopus tropicalis	AB244211	NP001035101
Struthio camelus australis	KFV78144	KFV81671
Columba livia	AFU48567	AFU48568
Gallus gallus	ADQ38960	BAA88667
Gyps africanus	BAH01723	N/A
Sus scrofa	ABM88718	ABM88717
Rattus norvegicus	NP_036821	NP_036886
Homo sapiens	NP_000116	NP_001428
Equus caballus	AAD17316	NP_001296408
Mus musculus	AAA37580	NP_997590
Bradypodion pumilum	LC119088	LC119089
Plestiodon finitimus	LC119090	LC119091
Protobothrops flavoviridis	BAJ15425	BAJ15427
Elaphe quadrivirgata	BAJ15426	BAJ15428

Gekko japonicus	LC119092	N/A
Alligator mississippiensis	BAD08348	BAJ15429
Pseudemys nelsoni	BAF91191	BAJ15430

Supplementary Table S3.

Gene transcriptional activities of estrogens mediated by lizard ERβ

Chameleon ERβ	EC_{50} (M)	95% CI (M)	RP (%)
E1	1.4×10^{-9}	1.0×10^{-9} - 1.8×10^{-9}	0.6
E2	8.7×10^{-12}	5.6×10^{-12} - 1.3×10^{-11}	100
E3	2.7×10^{-10}	1.7×10^{-10} - 4.3×10^{-10}	3.3
EE2	2.5×10^{-11}	1.6×10^{-11} - 4.0×10^{-11}	34.3
DES	1.8×10^{-11}	1.2×10^{-11} - 2.5×10^{-11}	49.2
Skink ERβ	EC_{50} (M)	95% CI (M)	RP (%)
E1	6.8×10^{-10}	5.2×10^{-10} - 9.0×10^{-10}	0.6
E2	4.3×10^{-12}	2.7×10^{-12} - 7.0×10^{-12}	100
E3	1.0×10^{-10}	6.9×10^{-11} - 1.6×10^{-10}	4.2
EE2	1.5×10^{-11}	1.0×10^{-11} - 2.1×10^{-11}	29.5
DES	9.1×10^{-12}	6.3×10^{-12} - 1.3×10^{-11}	47.8