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Molecular cloning, characterization, tissue distribution and mRNA expression changes during the hibernation and reproductive periods of estrogen receptor alpha (ESR1) in Chinese alligator, *Alligator sinensis*

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ABSTRACT

Chinese alligator, *Alligator sinensis*, is a critically endangered reptile species unique to China. Little is known about the mechanism of growth- and reproduction-related hormones gene expression in Chinese alligator. Estrogens play important roles in regulating multiple reproduction- and non-reproduction-related functions by binding to their corresponding receptors. Here, the full-length cDNA of estrogen receptor alpha (*ERα/ESR1*) was cloned and sequenced from Chinese alligator for the first time, which comprises 1764 bp nucleotides and encodes a predicted protein of 587 amino acids. Phylogenetic analysis of ESR1 showed that crocodilians and turtles were the sister-group of birds. The results of real-time quantitative PCR indicated that the *ESR1* mRNA was widely expressed in the brain and peripheral tissues. In the brain and pituitary gland, *ESR1* was most highly transcribed in the cerebellum. But in other peripheral tissues, *ESR1* mRNA expression level was the highest in the ovary. Compared with hibernation period, *ESR1* mRNA expression levels were increased significantly in the reproductive period ($P < 0.05$) in cerebellum, pituitary gland, liver, spleen, lung, kidney and ovary, while no significant change in other examined tissues ($P > 0.05$). The *ESR1* mRNA expression levels changes during the two periods of different tissues suggested that ESR1 might play an important role in mediation of estrogenic multiple reproductive effects in Chinese alligator. Furthermore, it was the first time to quantify *ESR1* mRNA level in the brain of crocodilians, and the distribution and expression of *ESR1* mRNA in the midbrain, cerebellum and medulla oblongata was also reported for the first time in reptiles.

1. Introduction

Chinese alligator, *Alligator sinensis*, one of the critically endangered species of 23 existent crocodiles, is an endemic species in China (Chen et al., 2003). It is listed as Critically Endangered on the IUCN Red List and has a high research value. In China, Chinese alligator is listed as national level to protect animals that its wild population is endangered. Due to the wild population decrease and narrow distribution, it has received wide attention from researchers both at home and abroad. In recent years, people focus on the reproductive biology of this species to solve the problem of the continuation of populations. At present, studies of Chinese alligator reproductive biology mainly focus on reproductive behavioral ecology, and we know very little about the mechanism of the regulation of reproductive hormones. Chinese alligator is a typical seasonal breeder and its reproductive cycle is controlled hormonally. The reproductive cycle of *A. sinensis* can be divided into different periods, including post-reproductive period (August–October), hibernation period (November–December–January–February–March), pre-reproductive period (April–May) and reproductive period (June–July). Chinese alligators suffer from the low temperature and lack of food in hibernation period and have aggressive and mating behaviors in reproductive period (Chen et al., 2003). Reproductive cycle is affected by hormonal and local factors, including estrogens (Ogawa et al., 1998; Hamlin et al., 2014).

In vertebrates, estrogens have a pivotal role in development and differentiation of numerous tissues, such as the gonadal system and central nervous system (McEwen and Alves, 1999; Acharya et al., 2015; Luzio et al., 2015). They play various roles in embryonic development, growth and reproduction that have been reported many times in recent years (Acharya et al., 2015; Gárriz et al., 2015; Li et al., 2015). The signaling actions of estrogens are mediated through two forms of estrogen nuclear receptors, estrogen receptor alpha (ERα/ESR1) and estrogen receptor beta (ERβ/ESR2), which belong to the nuclear receptor superfamily and may have different biological effects (Walter et al., 1985; Laudet et al., 1992; Beato et al., 1995; Enmark et al., 1997; Gustafsson, 1999). ESRs have traditionally been thought of as ligand-dependent transcription

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factors (Tetel et al., 2009; Tetel and Pfaff, 2010). Thirty years ago, the first ESR was cloned and sequenced from human (Walter et al., 1985; Green et al., 1986). Now, both forms of the ESRs have been found in fishes, amphibians, reptiles, birds and mammals (Katsu et al., 2006a, 2006b and 2010; Liu et al., 2014; Zhang et al., 2014a). Although reptiles and amphibians occupy an indispensable position in vertebrates, fewer researches about full-length cDNAs of ESR were reported compared with other vertebrates. In crocodilians, including our present study, the full-length cDNA sequence of *ESR1* has been cloned in four species, *Alligator mississippiensis* (NM_001287274) (Katsu et al., 2004), *Caiman crocodilus* (AB055220) (Sumida et al., 2001), *Crocodylus niloticus* (AB209933) (Katsu et al., 2006b), *A. sinensis* (KU356777).

Estradiol is the principal ovarian estrogen, which is essential for the normal development and differentiation of ovary in many vertebrates (Mahmoud et al., 1985; Aoki et al., 2011; Luzio et al., 2015). Especially in reptiles, developmental exposure to bisphenol A disrupts sexual differentiation in painted turtles (Jandegian et al., 2015) and the estradiol showed significant seasonal variation coincident with the reproductive activity (Hamlin et al., 2014). However, the mechanisms of estradiol action on ovarian development and differentiation were still unknown. In the brains of birds and mammals, estrogens regulate multiple reproductive and non-reproductive functions including influence neurogenesis, neuroprotection, learning and memory, energy homeostasis, auditory processing, social communication, cognition, sexual differentiation, aggressive behaviour and reproductive behaviour (McEwen and Alves, 1999; Barha and Galea, 2010; Cornil et al., 2012; Remage-Healey et al., 2012; Bailey et al., 2013; McCarthy and Nugent, 2013; Bless et al., 2014; Arevalo et al., 2015; Shiga et al., 2016). In contrast, researches on the functions of estrogen in the reptile brain have only just started.

In order to better understand the molecular basis of the reptilian reproductive endocrine system and to provide additional data to the study of evolution of vertebrate steroid hormone receptors, we have cloned the full-length cDNA encoding *ESR1* from Chinese alligator. We performed multiple protein sequence alignment of ESR1 with other vertebrate species, and found that alligator ESR1 coding sequence (CDS) showed high homology to ESR1 from other reptilian and avian species. To evaluate the evolutionary relationships of Chinese alligator with the above vertebrate species, we constructed a neighbor-joining approach for phylogenetic tree based on full-length amino acid sequences of ESR1. In this study, we also report *ESR1* mRNA tissue distribution as well as the mRNA expression levels changes during the hibernation and reproductive periods.

2. Materials and methods

2.1. Animals

Six sexually mature female alligators (*A. sinensis*), about 15 years old, around 170 cm in total length and 32 to 35 kg weight, were collected from the Anhui Research Center for Chinese Alligator Reproduction. Alligators were killed with a lethal dose of Nembutal, and various tissues were isolated and immediately preserved in RNAstore (Tiangen Biotech, Beijing, China) and then stored at −80 °C until analysis. The tissues, including the heart, liver, spleen, lung, kidney, stomach, pancreas, ovary, pituitary gland and brain (brain was also dissected into five portions, including cerebrum, midbrain, cerebellum, medulla oblongata, and hypothalamus), were collected from December 2014 (hibernation period) and July 2015 (reproductive period), respectively. All experiments were approved by the authorization of the forestry authorities of China and were organized to minimize the number of animals used.

2.2. Molecular cloning of cDNA encoding ESR1

2.2.1. RNA isolation

Total RNA was extracted from the above-tissues using a RNAprep Pure Tissue Kit (Tiangen Biotech, Beijing, China) according to the protocol supplied by the manufacturer. The quality of RNA samples were assessed by 2% agarose gel electrophoresis. RNA concentration was determined by measuring absorbance at 260 nm. Then RNA samples were dissolved in RNase-free water at a concentration of 1 μg/μl and preserved in a −80 °C refrigerator for further experiments.

2.2.2. First strand cDNA synthesis

cDNA was synthesized using a PrimeScript™ RT reagent Kit (Takara Bio Inc., Dalian, China) according to the manufacturer's instructions. Briefly, each 20 μl reaction mixture comprised 10.0 μl RNase Free dH_2O, 4.0 μl 5× PrimeScript Buffer 2, 2.0 μl 5× gDNA Eraser Buffer, 1.0 μl gDNA Eraser, 1.0 μl RT Primer Mix, 1.0 μl PrimeScript RT Enzyme Mix I and 1.0 μl total RNA. The reverse transcription reaction was performed for 15 min at 37 °C and then for 5 s at 85 °C. The cDNA was assayed by 2% agarose gel electrophoresis and then stored at −20 °C.

The first strand cDNA of the alligator ovary was used to clone the full-length cDNA of *ESR1*. However, the first strand cDNA of other tissues was used for gene expression analysis.

2.2.3. Primers, amplification and sequencing

According to the published *ESR1* cDNA sequence high conserved regions from other crocodilians (*A. mississippiensis*, *C. niloticus and C. crocodilus*) (Table 1), we designed one primer pair, ESR1F and ESR1R (Table 2). This pair of primers was designed using Primer 5.0 software and synthesized by Sangon biological engineering (Shanghai) co., LTD. With the primers, a cDNA fragment was amplified by RT-PCR using the first strand cDNA as template. The 30 μL reaction system contained 3.0 μL of 10× PCR Buffer, 2.0 μL of Mgcl2, 2.0 μL of dNTP, 1.0 μL of each primer, 1.0 μL of Taq DNA polymerase (TaKaRa, Dalian, China), 1.0 μL of cDNA and 19.0 μL of ddH_2O. PCR conditions were as follows: initial activation at 94 °C for 3 min, amplification was for 35 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 56 °C, extension for 1 min at 72 °C, and a final cycle at 72 °C for 10 min, and then cooled to 4 °C. PCR products were analyzed by 1.0% agarose gel electrophoresis and purified utilizing a TIANgel Midi Purification Kit (Tiangen Biotech, Beijing, China). The recovered products were cloned into pMD19-T vector and sequenced by Sangon Biological Engineering (Shanghai) co., LTD.

Table 1
Species used in this study.

Organism	GenBank ID
Alligator mississippiensis	NP_001274203
Caiman crocodilus	BAB79436
Crocodylus niloticus	BAE45626
Alligator sinensis	**KU356777**
Anolis carolinensis	NP_001277446
Gekko japonicus	BAU36960
Chrysemys picta	NP_001269175
Lepidochelys olivacea	ACF28457
Pseudemys nelsoni	BAF91191
Elaphe quadrivirgata	BAJ15426
Gallus gallus	NP_990514
Columba livia	NP_001269754
Zonotrichia albicollis	ADK26789
Taeniopygia guttata	NP_001070169
Homo sapiens	AAI28575
Bos taurus	NP_001001443
Mus musculus	BAJ65337
Rattus norvegicus	BAI48013
Xenopus laevis	NP_001083086
Xenopus tropicalis	AAQ84780
Danio rerio	AAI62466
Poecilia reticulata	NP_001284416
Salmo salar	NP_001117064
Larimichthys crocea	NP_001290305

The species of ESR1 cDNA clone in this study is in bold.