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Identification, characterization and mRNA transcript abundance profiles of estrogen related receptor (*ERR*) in *Sepiella japonica* imply its possible involvement in female reproduction

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ABSTRACT

Estrogen related receptors (ERRs) are widely detected in vertebrates and apparently have functions in reproduction. The functions of ERRs in reproduction of invertebrates, especially in mollusk cephalopods, are largely unknown. In the present study, An homologue of vertebrate *ERR* gene was first cloned from female *Sepiella japonica,* an important Cephalopod species in coastal water of China. Results indicate the *S. japonica ERR* (*sjERR*) gene is comprised of 1513 nucleotides, containing a 1389 bp open reading frame, which encode for 463 amino acid (aa) residues. The deduced sjERR protein possessed six typical nuclear receptors (NR) domains (A–F), with a DNA-binding domain (DBD) and a highly conserved ligand-binding domain (LBD), compared to the other molluscan ERRs. Results from tissue analyses indicated that *sjERR* mRNA transcript abundance was in largest amounts in tissues of the brain, liver, ovary that are possibly involved in reproduction. The *sjERR* mRNA transcript abundance was temporally regulated during the different sexual maturation phases of female *S. japonica* and was affected by *in vivo* administrations of vertebrate steroid estradiol-17β (E2). An *in vivo* knockdown of *sjERR* gene expression resulted in a marked down-regulation in expression of genes involved in ovarian development, such as *Vitellogenin*, *CDK1,* and *Cyclin B*, indicating there is a possible involvement of *sjERR* in reproduction. Both fusion protein transient transfections and immunohistochemical analyses indicated a presence of sjERR in the nucleus, implying a possible mechanism of action of the sjERR in the nucleus through activation of specific gene transcriptions.

1. Introduction

Estrogens are closely associated with the development, gonadal differentiation, maturation, and maintenance of the reproductive system in vertebrates (Lü et al., 2016a). The pleiotropic effects of estrogens are mainly mediated through transcriptional regulation of target genes by binding to two types of nuclear hormone receptors, estrogen receptor α (ER-α) and estrogen receptor β (ER-β), which are members of the nuclear receptor super-family (Mitsunagaa et al., 2004). In the nuclear receptor super-family, however, there are

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other estrogen-related putative receptors, for which no known ligands are identified and these are classified as orphan nuclear receptors, also confirmed to be essential for reproduction (Blumberg and Evans, 1998). Estrogen-related receptors (ERRs) belong to such orphan nuclear receptors and comprise three members, ERR-α, ERR-β, and ERR-γ (Tremblay and Giguère, 2007). Though quite closely related to the ERs in both amino acid sequences and structure, ERRs do not bind to E2 and have been reported to be either constitutively active (Greschik et al., 2002) or activated by unidentified ligands (Tarrant et al., 2006). These ERR proteins, however, can bind to DNA as monomers and in doing so activate estrogen receptor-related receptor response elements (ERREs) with the extended half-site consensus of TnAAGGTCA (Park et al., 2017), or bind to estrogen-response elements (EREs) containing the recognition motif of AGGTCA as monomers, homodimers or heterodimers (Lu et al., 2001), thus may actually share an estrogen signaling pathway with ERs by having the common DNA binding sites, and regulating identical target genes in a synergistic or antagonistic manner (Ouyang et al., 2009). Possible involvement of ERRs in physiological and developmental processes of reproduction has been confirmed in various laboratories for vertebrates (Kraus et al., 2002; Xi et al., 2012). For example, in mice, the activation of ERRs was associated with an increase in the production of testosterone, as well as an increase in abundance of P450c17 mRNA transcripts and protein in primary Leydig cells (Park et al., 2017). In contrast, the loss of ERRs resulted in reduction of the number of germ cell number in mouse embryos, indicating there is an involvement of ERRs in the proliferation of gonadal germ cells (Mitsunagaa et al., 2004). Although accumulating evidence has indicated that ERRs may also be involved in reproduction of invertebrates, such as Arthropoda (He et al., 2010), Crustacea (Liu, 2018) and Mollusks (Nagasawa et al., 2015), the precise functions of ERRs in the reproduction of invertebrates remains largely unknown.

The common Chinese cuttlefish, *Sepiella japonica*, is a typical marine invertebrate, distributed in the Indo-West Pacific coastal waters of China, Japan, Indonesia, and India. It was once one of the four major marine fishery species in China in the 1970s (Wu et al., 2010). Recently, artificial breeding techniques have been developed, and successful aquaculture techniques have been utilized for this species in China. Results from a preliminary study indicated there was an abundance of *ER* mRNA transcript during the sexual maturation process of *S. japonica*, indicating there is a function of *ER* in reproduction (Lü et al., 2016a, 2016b). The possible functions of *ERR* in *S. japonica* reproduction and the mechanism of action remains to be ascertained. In the present study, cDNA clones encoding for *S. japonica* homolog of *ERR* were isolated and the subcellular location of the receptor protein was analyzed by using both the fusion protein transient transfections and immunohistochemical assays. Furthermore, the tissue-specific profile of mRNA transcript abundance for the receptor during gonad development was also investigated using quantitative real-time PCR. The possible functions of *sjERR* in the estradiol (E2) signaling and reproduction in *S. japonica* was also investigated using E2 administration and siRNA knockdown assays in the present study. The results expand on the knowledge of the molecular mechanism and functions of *ERRs* in invertebrate reproduction.

2. Materials and methods

2.1. Sample collections

For *ERR* cDNA cloning, subcellular localization, and tissue distribution analyses, eight mature adult female *S. japonica* with a bodyweight 100–150 g, were collected from a research station in Xishan island, affiliated with the Marine Fisheries Research Institute of Zhejiang. The ovaries were in the interstitial growth developmental period, based on previous criteria classifications for this variable (Lü et al., 2016a). Eight tissues of the whole brain, liver, intestine, pancreas, muscle, heart, gill, and ovary from female *S. japonica* were sampled after the specimens were killed using anesthesia (anesthetized with seawater containing 17.0 g/L magnesium chloride).

To evaluate the profile of *sjERR* mRNA transcript abundance during the reproductive cycle of female *S. japonica*, four gonadal development stages were categorized in females using previously developed criteria: oogonium production period, protoplasmic growth period, interstitial growth period, and trophoplasmic growth period (Lü et al., 2016a). Tissues of the whole brain, liver, and ovary from eight females were sampled at each of these stages.

For E2 administration and *ERR* knockdown analysis, adult female *S. japonica* in the interstitial growth developmental period were maintained in seawater tanks (around 25 °C) and fed daily with miscellaneous shrimp at the same research station. The *in vivo* administration and sampling were implemented according to the detailed procedure subsequently described in this manuscript. All samples were collected and stored at −80 °C prior to use.

2.2. Isolation and sequence analysis of sjERR

Total RNA extraction and synthesis of first-strand cDNA were conducted using the procedures previously described by Lü et al (2016a). Partial cDNA fragments of *sjERR* were amplified using specific primers (Table 1), designed based on the nucleotide sequence obtained from previous ovarian transcriptome analyses of *S. japonica* (Lü et al., 2016b). The 3′ and 5′-regions of *sjERR* were then amplified using the RACE method with the specific primers designed based on the partial sequence that was ascertained. The PCR reactions were conducted with the parameters described previously (Lü et al., 2016a). The PCR products were purified and cloned into the pMD18-T simple vector (TaKaRa, Kusatsu, Japan) and sequenced in both directions.

The open reading frame (ORF) was identified using the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Potential functional motifs such as N-glycosylation sites, phosphorylation sites, zinc finger domains, DNA-binding domain (DBD) and ligand-binding domain (LBD) were analyzed using the PROSITE database (Expert Protein Analysis System; Swiss Institute of Bioinformatics; http://myhits.isb-sib.ch/cgi-bin/motif_scan). The nuclear location signal (NLS) was analyzed using the NetNES 1.1

Table 1
Nucleotide sequences of primers and siRNAs used.

Primer name	Sequence (5'-3')	Application
ERR-F	TCATTATGGTGTGTCGTCAT	cDNA fragment cloning
ERR-R	CATTGCTATCAGGAGTAACATC	
5'R-*ERR*-F	CACGGTCTGCAAGGTCTGAAACAA	5'-RACE
5'R-*ERR*-R	CACCTTCTCGTAGCATGCCTACACT	
3'R-*ERR*-F	ACAGCATTCATGGCTTGAGA	3'-RACE
3'R-*ERR*-R	AATCACCAGTCTTGGCGTAA	
q-*ERR*-F	CAGACCTTGCAGACCGTGAACT	Real-time PCR
q-*ERR*-R	GGTTGTCCATCTCAGGTGAGCA	
Vg-F	CACCTGCGACTGAACCTAAA	qPCR for Vg
Vg-R	CAAGACGCTCAAGCAACATG	
CDK1-F	AACCTCACTACCAGACTACA	qPCR for CKD1
CDK1-R	GTCCAAGACAGGAATCAGTT	
Cyclin-B-F	CTAATGGTGACTGGACTGAT	qPCR for Cyclin-B
Cyclin-B-R	GTATGCTGCTTATCTTGTAG	
siRNA_*ERR*- sense	CCUACAGCAUUCAUGGCUUTT	RNA interference
siRNA_*ERR*- antisense	AAGCCAUGAAUGCUGUAGGTT	
negative control- sense	UUCUUCGAACGUGUCACGUTT	RNA interference
negative control- antisense	ACGUGACACGUUCGGAGAATT	
β-actin F	GCCAGTTGCTCGTTACAG	Real-time PCR
β-actin R	GCCAACAATAGATGGGAAT	

Server (http://www.cbs.dtu.dk/services/NetNES/). The secondary structure of the ERR protein was predicted using SOPM(https://npsa-prabi. ibcp.fr/cgi-bin/npsa_automat.pl? page = npsa_sopm. server. Html). Sequence alignments based on the amino acid sequences of known ERRs were determined using Clustal X 1.81. Phylogenetic trees were constructed using the Neighbor-Joining method with a bootstrap test of 1000 replicates implemented in the MEGA version 5.2 (Kumar et al., 2008).

2.3. Subcellular localization of sjERR

To investigate the subcellular localization of sjERR, the full-length *ERR* gene was amplified from a pMD18-T/*sjERR* vector with specific primers containing Hind III and BamH I as adapters. The resulted *ERR* PCR products were digested at Hind III and BamH I sites, and cloned into pEGFP-N1 vector to generate *sjERR*-pEGFP. The recombinant plasmids (*sjERR*-pEGFP) as well as pEGFP-N1 (negative control) were transfected into HEK293 T cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were incubated at 37 °C for 4 h, and cultured with fresh medium. After fixing with 4 % paraformaldehyde for 20 min, cyto-membrane staining was conducted with 1:1000 diluted Dil for 20 min and nuclei were stained using DAPI for 10 min, at room temperature. The cells were visualized using a digital confocal microscope (Leica TCSSP5, Germany). Separate images at 405, 488, and 549 nm were obtained and presented as merged images.

An immunohistochemical assay was also conducted to investigate the subcellular localization of sjERR. Fresh cuttlefish tissues of the liver and ovary were collected, and immersion fixed overnight in 4 % paraformaldehyde. Fixed tissues were sequentially dehydrated in the ascending ethanol series (70 %, 95 %, 100 %) and were subsequently cleared using xylene. The samples were embedded in paraffin, sectioned at a 5 μm thickness, and mounted on glass slides. The slides were incubated with 3 % H_2O_2 for 10 min and were incubated with a rabbit antibody of anti-sjERR (diluted 1:1000 in 3 % BSA; Pufei Biotechnology Inc., China) for 2 h at room temperature. Samples were then incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc., USA) for 30 min. Sections were washed and stained with the ABC reagents (Pufei Biotechnology Inc., China) for 20 min and observed using the microscope.

2.4. Tissue distributions and patterns of sjERR mRNA transcript relative abundance during ovarian development

The profiles of *sjERR* mRNA transcript abundance in different tissues and different sexual maturation stages of females were determined using quantitative Real-Time PCR (qRT-PCR). Total RNA extraction and cDNA synthesis were performed as previously described. Specific qRT-PCR primers used for *sjERR* are also listed in Table 1. The qRT-PCR was conducted using the parameters, as described previously (Lü et al., 2016a). For standardization of the data, β-actin was used as the internal reference gene in all of the experiments. The relative mRNA transcript abundance was calculated using the comparative Ct method $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The relative mRNA transcript abundance in one randomly selected tissue was set at 100 % and the relative abundances in all the other tissues were expressed as a percentage relative to that of the selected tissues.

2.5. E2 regulation of sjERR mRNA transcript abundance

To investigate the effect of E2 hormone regulation on *sjERR* mRNA transcript abundance of *S. japonica,* 50 μl, and 100 μl 1×10^{-5} M vertebrate E2, dissolved in 0.9 % saline were intravenously injected using the procedure described by Gore et al (2005), into female cuttlefish of two groups maintained in separate tanks, with 12 females in each group; and 100 μl 0.9 % saline was injected into one control group, with 12 females in the group. The liver, brain and ovarian tissues from individuals in each group were sampled 48 h post-hormone administrations. Total RNA extraction and cDNA synthesis were performed, and the qRT-PCR was conducted using the procedure that was previously described. The *sjERR* mRNA transcript profile after E2 administration to cuttlefish was compared to that of the control.

2.6. siRNA knockdown analysis of sjERR

To investigate the possible functions of *sjERR* in *S. japonica* reproduction, a highly effective dsRNA (sequences shown in Table 1) was selected to knockdown the *ERR* in females to examine its impact on mRNA transcript profiles of *Vitellogenin* (VG), cyclin-dependent kinase 1 (*CDK1*) and *Cyclin B* genes known essential for ovary maturation. There were 100 μl 2×10^{-3} M dsRNA, dissolved in DEPC water intravenously injected into 12 females (Group I); 100 μl 2×10^{-3} M negative control-dsRNA (NC-dsRNA), dissolved in DEPC water was intravenously injected into 12 females (Group II); and 100 μl 0.9 % saline were injected into 12 females (Control Group). Total RNA extraction, cDNA synthesis, and qRT-PCR were conducted using the procedure previously described in this manuscript. The abundance profiles of *VG*, *CDK1* and *Cyclin B* mRNA transcripts, as well as *sjERR* mRNA transcript, in *sjERR* knockdown cuttlefish in Groups I, was compared to that of Groups II as well as to that of the Control group.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 14.0 software, and data are presented as the mean ± standard deviations. Data obtained from the qRT-PCR analysis were analyzed using a one-way analysis of variance (ANOVA). Differences among groups were considered significant at $P < 0.05$.

3. Results

3.1. Isolation and characterization of sjERR cDNA

The full length of the *sjERR* cDNA sequence was 1513 bp. The ORF was 1389 bp, encoding 463 amino acids (Fig. 1; GenBank accession number: MG518633). The deduced sjERR protein contained two typical N-glycosylation sites, seven protein kinase C (PKC) phosphorylation sites and two nuclear localization signals (NLS), as depicted in Fig. 1. Six typical domain organizations, characteristic of the nuclear receptor gene family were also identified, initiating from the N-terminal A/B domain, subsequently followed by the DNA-binding C domain, hinge D domain, and ending with the ligand-binding E/F domain (Fig. 2). The DNA-binding C domain of sjERR contained the classical eight cysteine residues, which were integral in the formation of one C4 type (two domains) zinc finger. Following the zinc finger domain, there was a T and an A box domain, which have been verified to be essential for monomer binding between ERR and ERREs or EREs sites of vertebrate DNA. At the C terminals of E/F domains, a ligand depended transactivation function motif (AF-2) was present. A comparison of sjERR amino acid sequences with that reported for other species indicated there was a large amount of homology between sjERR and ERRs from mollusk species, with the greatest identity (98.3 %) between sjERR and that of *Sepia latimanus*, a species that belongs to the same cephalopod class. There was a much lesser homology between sjERR and ERRs of non-mollusk species with only 41.2 % identity between the sjERR and human ERR-γ. The sequence homology of ERRs between species, however, varied between domains, with A/B, D and E domains having the most divergence between species. In contrast, comparatively, there was greater conservation in the C domains between species, with sjERR having an 86.8 % identity with human ERR-α in the C domains. There were 11 putative α-helices (proposed by analogy with mammalian ERRγ; Greschik et al., 2002) dispersed along the E domain (LBD) in sjERR, which is consistent with that typically reported to be present in vertebrate ERRs (Fig. 2).

3.2. Phylogenetic analysis of sjERR

A phylogenetic tree was constructed with Mega5.2 by using amino acid sequences of sjERR and other ERRs from diverse taxa of Insecta, Crustacea, Gastropoda, Cephalopod, Bivalve, Teleost fish, birds, and mammals, already deposited in GeneBank (accession number shown in Fig. 3). The results indicate there were basically three groups of ERRs: Arthropoda ERRs, Mollusk ERRs, and Vertebrate ERRs, with Arthropoda ERRs more closely related to vertebrate ERRs. The vertebrate ERR clade was further divided into three sister groups, represented as ERR-α, ERR-β, and ERR-γ, with ERR-α being a more basal lineage and ERR-β and ERR-γ isoforms more closely related. In Mollusc, *sjERR* clustered with other identified molluscan ERRs and was more closely related to the cephalopod cuttlefish and octopus ERRs, compared to bivalve and gastropod ERRs, reflecting the slight ERR divergence among mollusks.

3.3. Subcellular localization of sjERR

Both fusion protein transient transfection and immunohistochemical analyses were used to analyze the possible subcellular localization of sjERR. With use of the transient transfection assay, there was a large amount of green fluorescence signal in a substantial proportion of transfected cells after 24 h of transfection (Fig. 4). As expected, green fluorescence was exclusively detected in the nucleus, indicating the nuclear receptor localization of sjERR. The nuclear receptor localization was further verified with

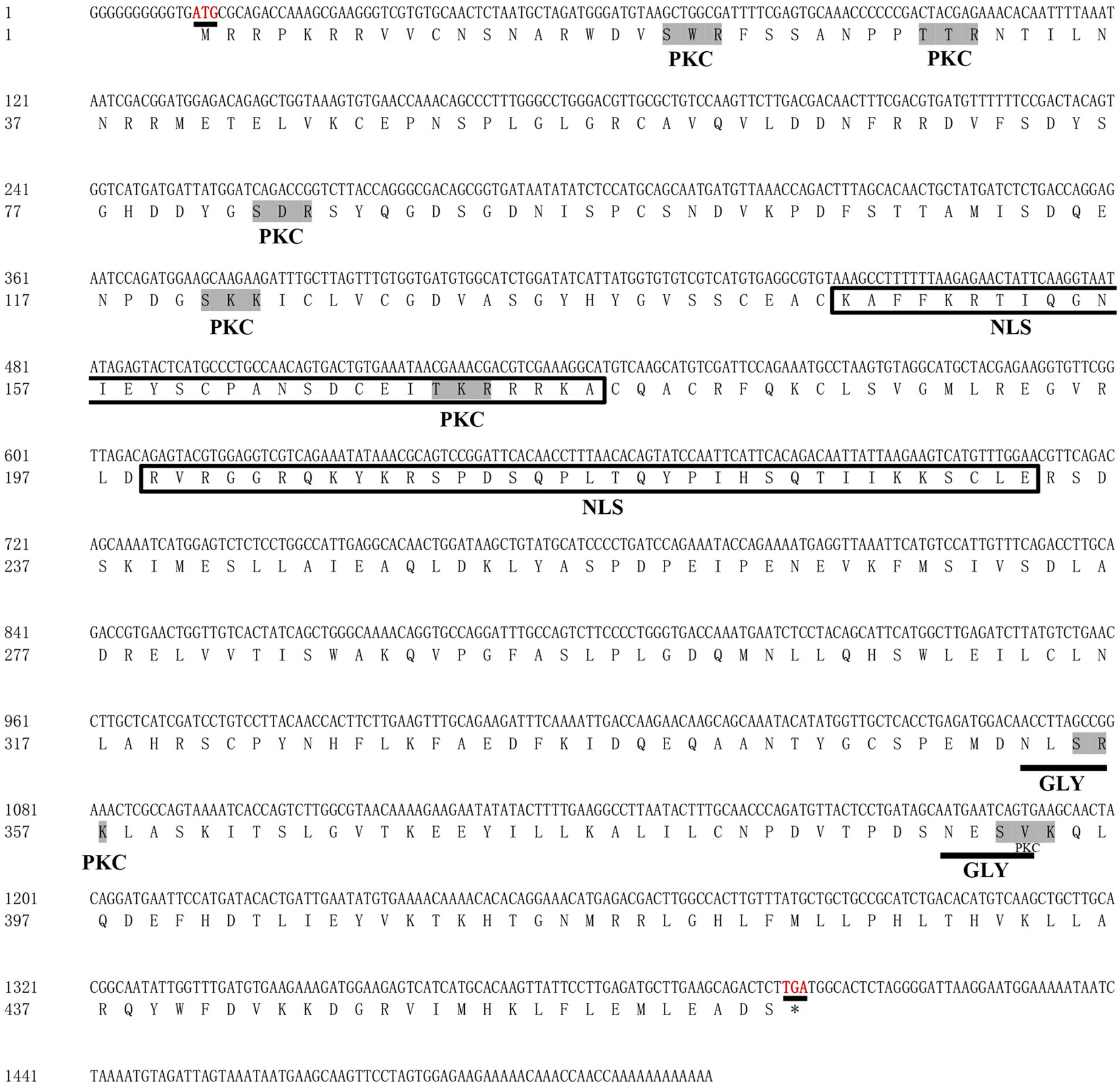

Fig. 1. Full-length cDNA and deduced amino acid sequence of *sjERR*; Initiation codon (ATG), the stop codon (TAG) and the N-glycosylation site (GLY) are underlined; nuclear localization sites (NLS) and protein kinase C phosphorylation sites (PKC) are indicated by boxes and open gray boxes; Three amino acid residues corresponding to Glu^{353}, Arg^{394}, and His^{524} in human ER-α essential for recognition of estradiol are indicated with asterisks (*); Numbers on the left refer to the position of the nucleotides and amino acids.

immunohistochemical analysis, using primary and secondary polyclonal antibodies incubated with ovarian and liver tissue sections of *S. japonica*, with the immunostaining signal mainly detected in the nucleus (Fig. 5).

3.4. *sjERR* mRNA transcript abundance in different tissues at different ovarian developmental stages

The relative abundance of *sjERR* transcripts was analyzed in various tissues of female *S. japonica*. The results indicate the *sjERR* mRNA transcripts were present in all tissues examined (Fig. 6). Relatively greater abundances of *sjERR* transcripts were detected in the brain, liver, and ovary that are tissues recognized as being important in reproductive functions. There were temporal variations of the *sjERR* mRNA transcript relative abundances during the different sexual maturation phases of female *S. japonica,* with there being relatively smaller abundances of the *sjERR* transcripts at the oogonia phase (Fig. 7), whereas there were markedly greater ($P < 0.05$) relative abundances of the *sjERR* transcripts during protoplasmic growth and interstitial growth phases, which is consistent with the periods during which there is rapid ovarian development, and there was a subsequent decrease during the trophoplasmic growth phase when the ovary is fully mature. The fluctuation of *sjERR* relative abundances of mRNA transcript correlated with the ovarian

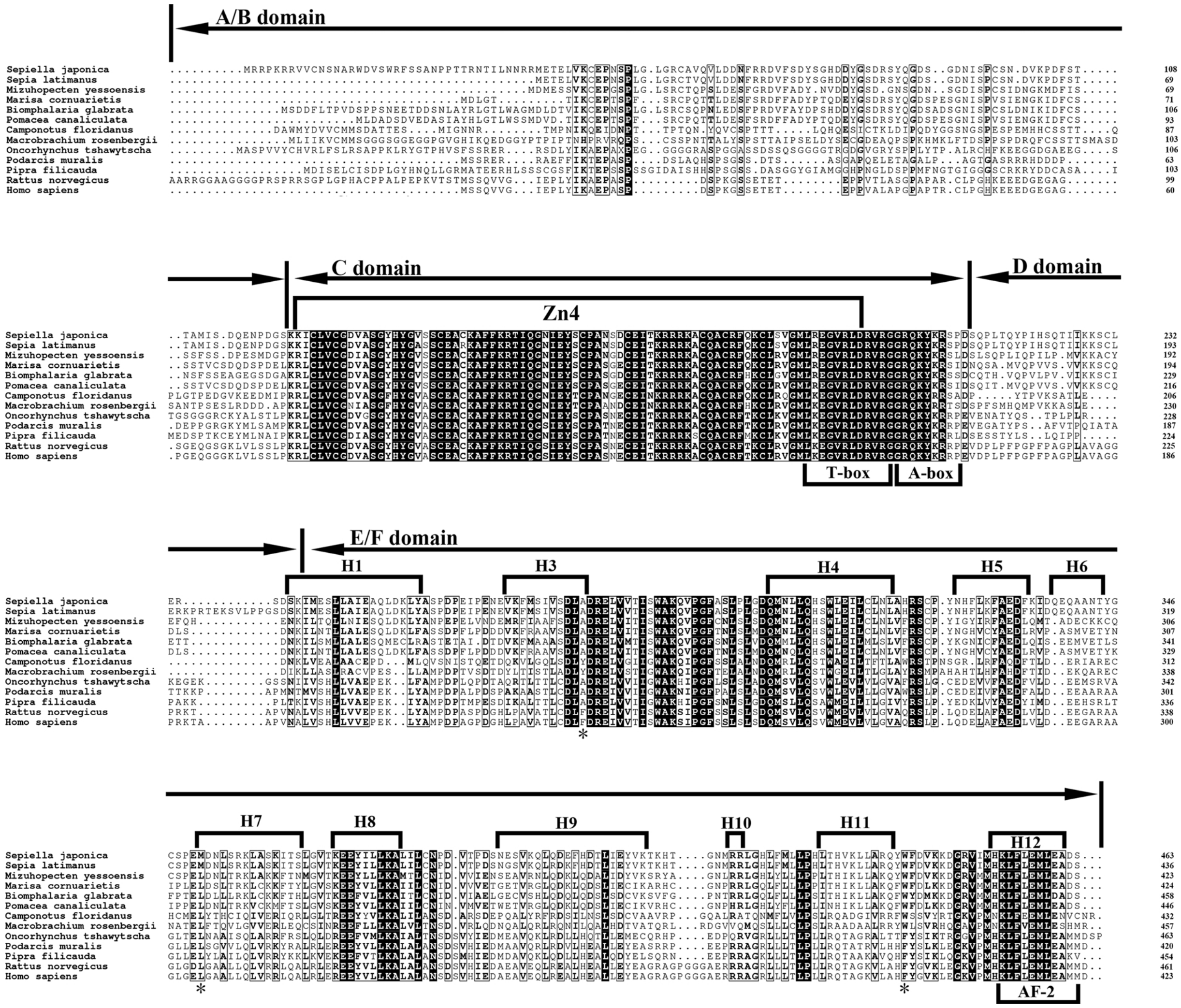

Fig. 2. Aligned deduced amino acid sequence of ERR in *S. japonica* with that of cuttlefish (*S. latimanus*; MH508244), scallop (*M.yessoensis*; XP_021379968.1), ramshorn snail (*M.cornuarietis*; ABI97120.1), planorbid snail (*B. glabrata*; XP_013080349.1), golden apple snail (*P.canaliculata*; XP_025103397.1), Florida carpenter ant (*Camponotus floridanus*; XP_011267688.1), shrimp (*Macrobrachium rosenbergii*, AOY10609.1), salmon (*Oncorhynchus tshawytscha*; XP_024252699.1), lizard (*Podarcis muralis*; XP_028565958.1), Manakin (*Pipra filicauda*; XM_027739497.1), human (*Homo sapiens*; XP_016872802.1); Gaps (-) were introduced to optimize the sequence alignment; Important regions/residues are indicated as follows: the six domains of ERR (A–F domains) by bracketing arrows, AF-2 site, T-box, A-box, and eight cysteine residues of zinc fingers and the helix domain by the black bracket.

developmental phases may indicate a possible involvement of *sjERR* in the reproduction of female *S. japonica.*

3.5. E2 regulation of sjERR mRNA transcript relative abundances

To analyze whether *sjERR* gene regulation, as determined by mRNA transcript abundance, is involved in E2 signaling, the response of *sjERR* gene expression to *in vivo* E2 administration was investigated in brain, ovarian, and liver tissues of female *S. japonica*. The results indicate both amounts (50 and 100 μl) of E2 administration induced an increase in *sjERR* mRNA transcript relative abundance in all three tissues of female *S. japonica*, however, only in the ovary was the effect statistically significant ($P < 0.05$; Fig. 8). These results indicate *sjERR* is possibly involved in E2 signaling in cephalopods which occurs in many vertebrate species.

3.6. Effect of sjERR knockdown on ovarian gene expression

To assess whether the *sjERR* protein functions in the reproduction of female *S. japonica,* a *sjERR* knockdown study was conducted *in vivo* using the RNAi technologies. The results indicate *sjERR* knockdown *in vivo* led to a decrease in the relative abundance of *sjERR* mRNA transcript in the brain, liver, and ovary of female *S. japonica* ($P < 0.05$; Fig. 9). Meanwhile, this experimental approach also

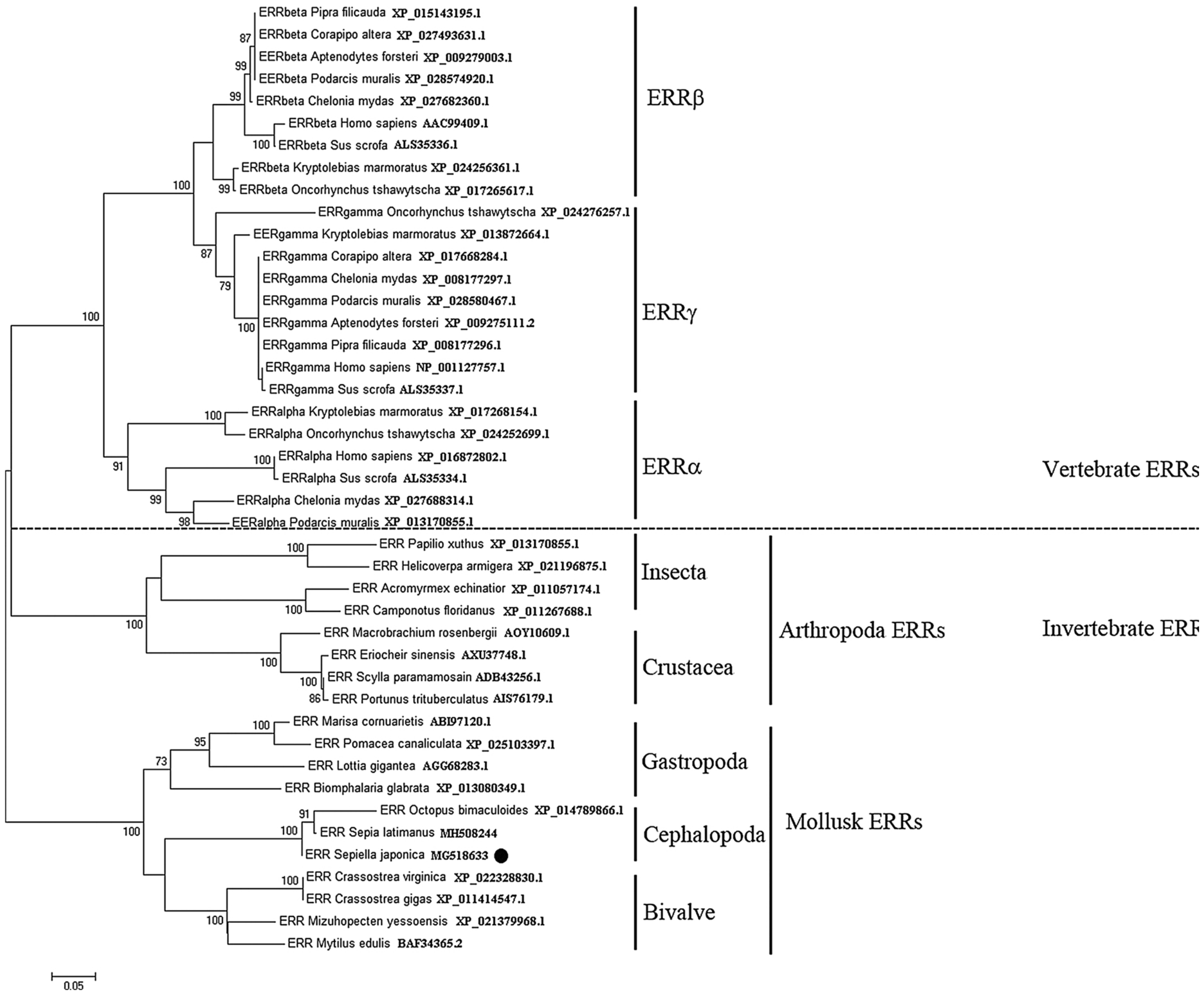

Fig. 3. Phylogenetic tree based on amino acid sequences of *ERR*s from 28 species deposited in GeneBank; a.
Accession number of each amino acid sequence used is provided following the latin name of the species; The tree was constructed using the MEGA 5.2 program by neighbor-joining method with a bootstrap of 1000 replicates.

resulted in down-regulation of the vitellogenin, CDK1, and cyclin B gene expressions ($P < 0.05$; Fig. 10), that are integral for vitellogenesis and oocyte maturation, further indicating the function of *sjERR* proteins in ovarian development and reproduction of *S. japonica*.

4. Discussion

Three paralogous genes, *ERR*-α, *ERR*-β, and *ERR*-γ, have been identified in vertebrates, except for fish, in which there is an additional diversity of *ERR* genes (Bardet et al., 2004; Tarrant et al., 2006). A genome duplication (Tarrant et al., 2006; Postlethwait et al., 2004) has been proposed to account for additional *ERR* diversity within vertebrate lineages (Bertrand et al., 2004). In mollusks, however, only one type of *ERR* has been defined (Bannister et al., 2007, 2013; Nagasawa et al., 2015). In the present study, cDNA clones encoding for the homolog of *ERR* were isolated from *S. japonica* using degenerate primers. Again, there was only one type of *ERR* detected. This, however, is the first complete *ERR* gene identified in cephalopods.

The deduced sjERR protein had six nuclear receptor domains (A/B, C, D, E/F domains), which were typical for ERRs. The highly conserved C domain (also known as DNA-binding domain, DBDs), included two zinc-finger motifs, a T and an A box, which are essential for monomer binding (Wilson et al., 1993), and the highly conserved E domain (also known as ligand-binding domain, LBDs), including AF-2, which is essential for transcriptional activity of ERRs. These findings lead to the suggestion that there is an orthodox working mechanism of sjERR by binding to DNA as monomers, homodimers or heterodimers to activate specific gene transcriptions. Any mutation in these regions, therefore, may lead to failure of binding to target sites of DNA or have negative effects of gene transcript activation as compared with wild-type ERRs (Bardet et al., 2004; Huppunen et al., 2004). Such an orthodox working mechanism was further evidenced by the presence of NLS in the sjERR and the latter fusion protein transient transfection and

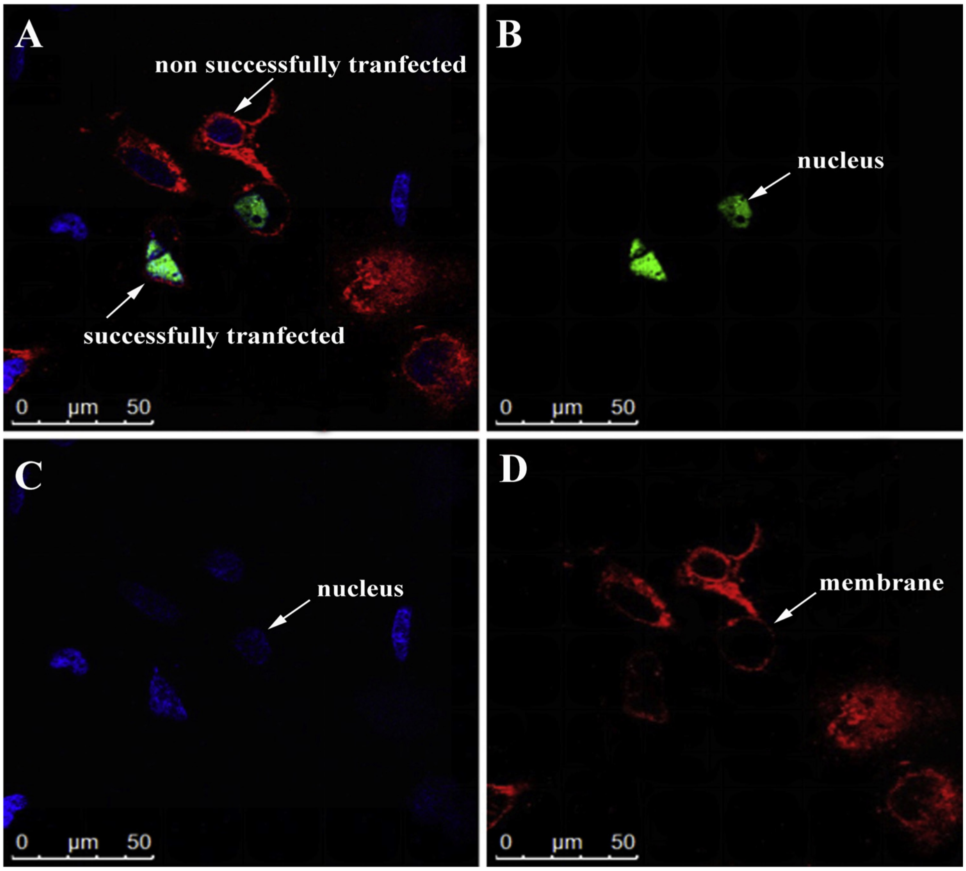

Fig. 4. *sjERR*-EGFP fusion protein in HEK293 cells; (A) ERR was localized in the nuclei of HEK293 cells; (B) Nucleus of cells stained green with *ERR*-EGFP; (C) Nucleus of cells stained blue with DAPI; (D) Plasma membranes of cells stained red with DiI. Nucleus and the plasma membrane indicated with white arrows in B-D (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

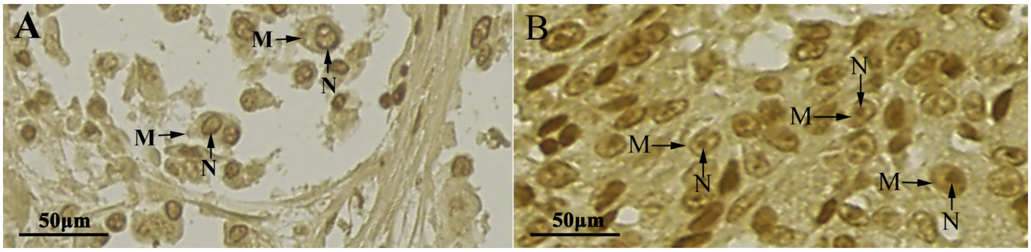

Fig. 5. Immunohistochemical analysis by incubating *sjERR* polyclonal antibody against tissue sections of *S. japonica*; A. Immunohistochemical analysis in ovarian tissues; B. Immunohistochemical analysis in liver tissues; Nucleus (N) and the plasma membrane (M) indicated by black arrows.

immunohistochemical analyses, indicating there is a nuclear receptor feature of sjERR and a possible function that is mediated through DNA binding and gene transcription activity in the nucleus. Such an sjERR function, however, is not likely mediated through a ligand-dependent function, because there are three amino acid residues that are essential for recognition of estradiol (Glu^{353}, Arg^{394}, and His^{524}) by ERs (Kallen et al., 2004) that have been replaced by Ala^{276} in helices 3, Met^{351} in helices 7, and Trp^{440} in helices 11 in sjERR. The His^{524} is absolutely essential for E2 binding as a result of forming a hydrogen bond with the 17-hydroxyl moiety of E2, and a replacement of much bulkier amino acid residues, such as Trp that would preclude the binding of E2, which has long been recognized in *ERs* (Baker and Chandsawangbhuwana, 2007). The replacement of Glu^{353} by Ala instead of Phe, an amino acid residue essential for constitutive activation of ERRs, would abolish its constitutive activity, as has been verified in the human ERR-α (Chen et al., 2001). Whereas, Kallen et al (2004) speculated that Ala residues were also present at the corresponding positions of ERR-β and ERR-γ, indicating that either a different structural configuration would have apparent constitutive activity or a different mode of regulation for these other ERR isotypes. Considering the divergence of sjERR sequence from vertebrate ERRs*,* as manifested from the constructed phylogenetic tree, a different mode of constitutive activity or regulation of sjERR is likely.

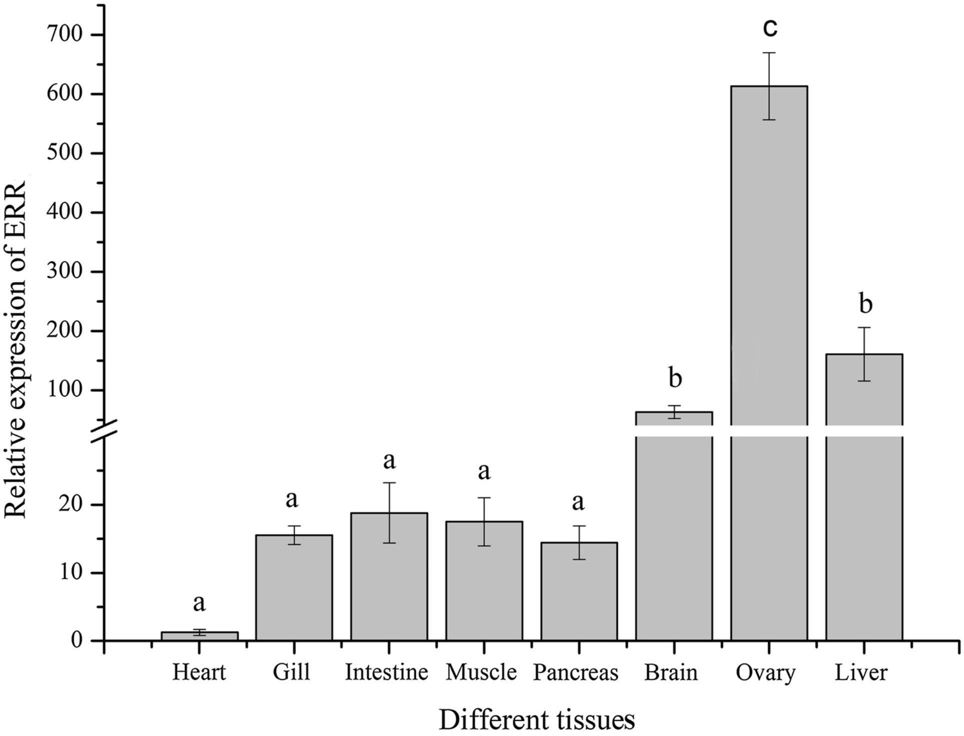

Fig. 6. qRT-PCR analysis of *sjERR* mRNA in different tissues in the late-vitellogenic stage of females; β-actin served as a reference gene; Each bar represents means ± standard deviation ($n = 8$); Bars with different letters are different ($P < 0.05$).

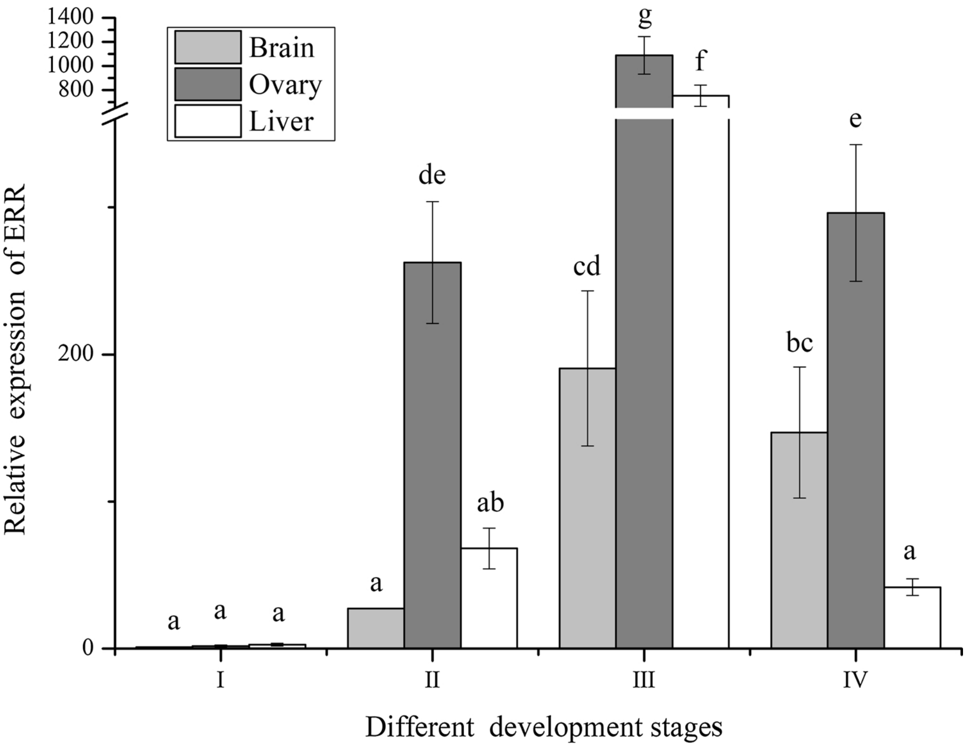

Fig. 7. Relative abundance of *sjERR* mRNA in brain, liver, and ovary during the reproductive cycle of females; I-IV represents oogonia phase, protoplasmic growth phase, interstitial growth phase, and trophoplasmic growth phase, respectively; Each bar represents means ± standard deviation ($n = 8$); Bars with different. letters are different ($P < 0.05$).

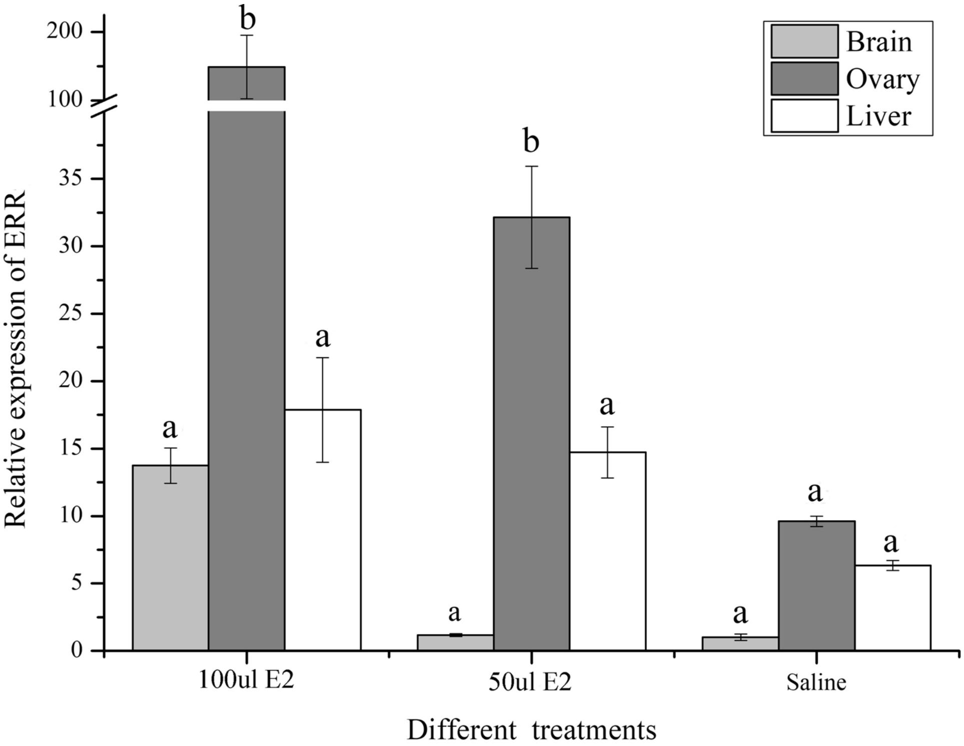

Fig. 8. Effect of E2 administration on relatively abundance of *sjERR* mRNA transcript in brain, ovary, and liver of the females; Each bar represents means ± standard deviation ($n = 12$); Bars with different letters are different ($P < 0.05$).

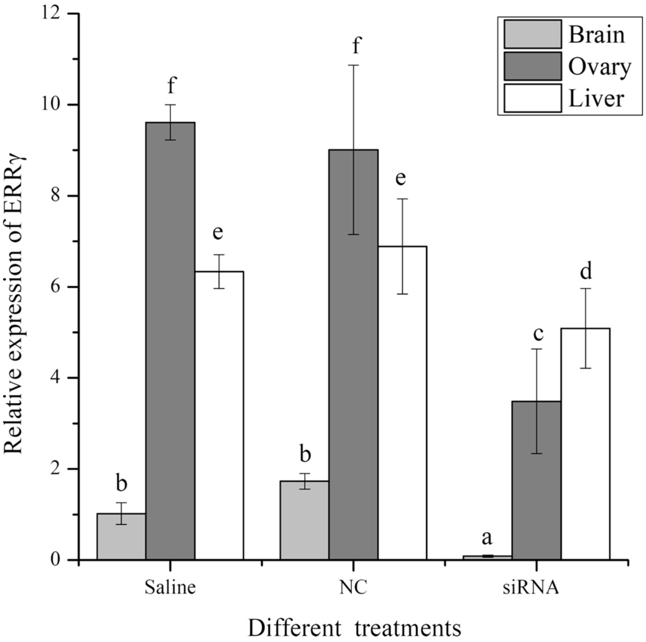

Fig. 9. Effect of *sjERR* knockdown on relative abundance of *sjERR* mRNA transcript in the brain, ovary, and liver of the females; Each bar represents means ± standard deviation ($n = 12$); Bars with different… letters are different ($P < 0.05$).

The possible function of *sjERR* in reproduction is evident because the *sjERR* mRNA transcripts were mainly detected in reproduction-related tissues, such as the ovary, liver, and brain, which is consistent to what exists in many other taxa (Tarrant et al., 2006; Ranhotra, 2009). The relatively greater *sjERR* gene expression, as indicated by the greater abundance of mRNA transcript for this gene, in the ovary is consistent with the *ERR* functions in ovarian development, as has been confirmed to occur in vertebrates

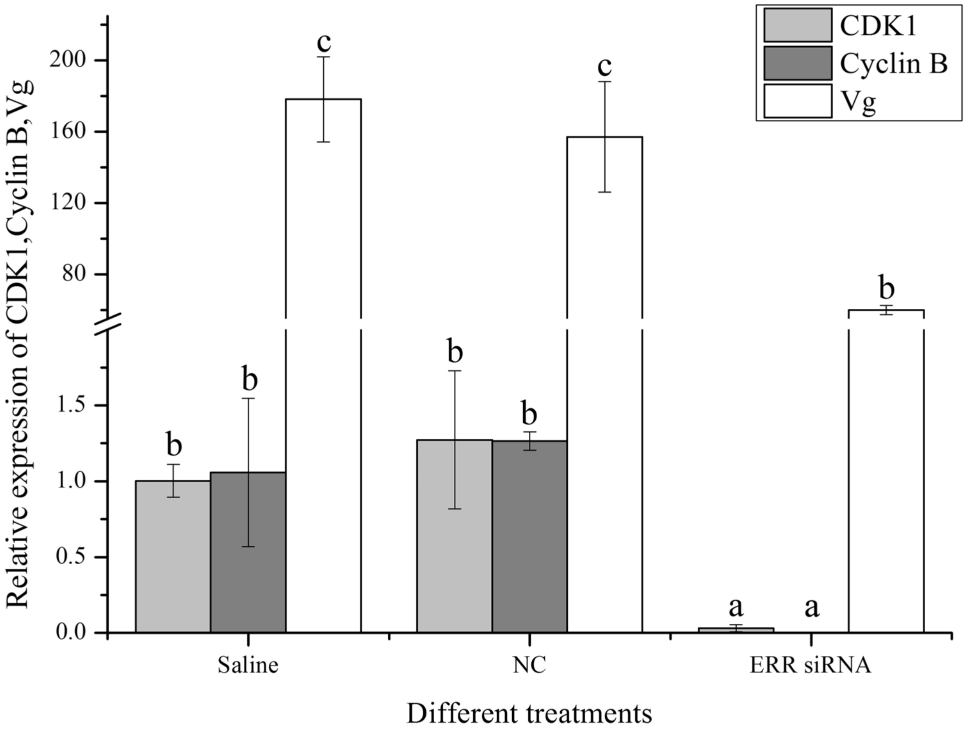

Fig. 10. Effect of *sjERR* knockdown on the relative abundance of *Vg, CDK1* and *Cyclin* B mRNA transcripts in the ovary; Each bar represents means ± standard deviation ($n = 12$); Bars with different letters are different ($P < 0.05$).

(Park et al., 2017). The relatively greater abundance of *sjERR* mRNA transcript in the liver may indicate a possible function of *ERR* in the endocrine regulation of liver metabolism (B'chir et al., 2018; Kim and Choi, 2019), or otherwise, suggest a vitellogenesis function of the *ERR* in the liver, as has been implicated to exist in many other taxa (Liu et al., 2003; Liu, 2016). The relatively greater abundance of *sjERR* mRNA transcript in the brain may indicate a possible relationship between *ERRs* and neuroendocrine functional control in the brain to regulate a variety of behaviors and physiological functions, which include reproduction (Saito and Cui, 2018). The possible function of *sjERR* in reproduction was also indicated by a fluctuation of *sjERR* mRNA transcript abundance correlated with the ovarian developmental phases in *S. japonica* (Fig. 7). Such a correlation between the abundance of *ERR* mRNA and ovarian status, commonly found in other taxa (Tarrant et al., 2006; Ouyang et al., 2009; He et al., 2010), is consistent with E2 fluctuations in different ovarian developmental phases (Lü et al., 2016a) which is possibly due to a temporal regulation of *ERR* gene expression by E2 (Liu et al., 2003). Furthermore, this was verified by the results from the E2 administration experiments in the present study, in which, both quantities of E2 when administered *in vivo* induced *sjERR* gene expression, as indicated by increases in *sjERR* mRNA transcript, in the ovary of *S. japonica* (Fig. 8). This type of response to E2 on *ERR* abundance has also has been observed in several other taxa (Liu et al., 2003; Tarrant et al., 2006; Li et al., 2010) and could either be mediated by an E2 enhanced ER binding to multiple steroid hormone response element half-sites (MHREs) in *ERR* promoters for transcriptional activation (Liu et al., 2003), or mediated through a direct E2 binding to a G-protein-coupled receptor *GPR30/GPER* to activate signaling pathways to enhance *ERR* abundance by facilitating histone acetylation and transcription factor recruitment at distinct nucleosomes of the *ERR* promoter (Li et al., 2010). Whether *GPR30/GPER* or *ER* also mediate this process in *S. japonica* still remains to be ascertained. These responses to E2 administration may indicate the involvement of *sjERR* in E2 signaling that is conserved in the mollusk lineage.

The possible involvement of *sjERR* in female *S.japonica* reproduction was further indicated from results of the study of *sjERR* gene knockdown analysis, where *sjERR* interference down-regulated vitellogenin, CDK1 and Cyclin B gene expression, as well as *sjERR* gene expression *per se* in the ovary. The ERR regulated *vitellogenin* gene expression was also detected in the silkworm *Bombyx mori*, possibly due to an ERE element in the promoter region of the *Vitellogenin* gene for sjERR binding and subsequently gene activation (Shen et al., 2018). The ERR regulated expression of cell cycle control genes, such as *Cyclin B*, and *CDK*1 was also observed in several taxa, including shrimp (*Macrobrachium rosenbergii*) (Liu, 2018) and humans (Krishna et al., 2018), although the immediate promoter targets of the ERR are unknown. Vitellogenesis is an integral process of oogenesis (Tsukimura, 2001) and CDK1, Cyclin B are two basic components in maturation promoting factor (MPF), which are essential for final maturation of oocytes (Lohka et al., 1988). Possible *sjERR* regulation of ovarian development-related gene expression provided further evidence for *sjERR* involvement in female *S. japonica* reproduction.

In summary, in the present study, a typical *sjERR* was cloned and the mRNA transcript was detected in abundant amounts in tissues of the brain, liver, and ovary that were possibly involved in the reproduction of *S. japonica*. Sub-cellular location analyses indicated a nucleus localization of sjERR. Furthermore, the *sjERR* gene expression, as indicated by relative abundances of sjERR mRNA transcript, was temporally regulated during the different sexual maturation phases of females and was markedly affected by the *in vivo* administration of vertebrate E2. Inhibition of *sjERR* production markedly inhibited the expression of ovarian development-related genes including *Vitellogenin*, *CDK*1 and *Cyclin B*, which indicates there is a possible function of *sjERR* in reproduction. The results of the present study provide new insights into the regulation of ovarian development in cephalopods. Further studies focused on the network of ERR signaling will result in a greater understanding of the regulative mechanism of ERR in cephalopod reproduction.

Declaration of Competing Interest

The authors of this manuscript certify that they have no conflicts of interest to declare.

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