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Molecular characterization and mRNA expression of ribosomal protein L8 in *Rana nigromaculata* during development and under exposure to hormones

Qinqin Lou^{1,2}, Shan Cao^{1,2}, Wei Xu^{1,2}, Yinfeng Zhang¹, Zhanfen Qin^{1,*}, Wuji Wei²

1. State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: louqinqindy@163.com

2. College of Environment, Nanjing University of Technology, Nanjing 210009, China

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ABSTRACT

Like *Xenopus laevis*, some species of the *Rana* genus are also used to study endocrine disrupting chemicals (EDCs). Although ribosomal protein L8 (*rpl8*) is the most-used reference gene for analyzing gene expression by quantitative reverse transcription polymerase chain reaction in *Rana*, its suitability as the reference gene has never been validated in any species of the *Rana* genus. We characterized *rpl8* cDNA in *Rana nigromaculata*, a promising native species in East Asia for assaying endocrine disrupting effects. We found that the *rpl8* cDNA consisted of 919 bp and encoded 257 amino acids, exhibiting high identities of amino acid sequence with known *rpl8* in other *Rana* species. Then, we examined the stability of mRNA expression during development. Compared with elongation factor 1 alpha 1, another common housekeeping gene, neither stage-specific nor tissue-specific expression of the *rpl8* gene was found in all tissues examined (brain, liver, intestine, tail, testis and ovary) during *R. nigromaculata* development. Finally, we investigated *rpl8* expression under exposure to hormones. No change in *rpl8* mRNA expression was found under exposure to thyroid hormone (T4) and estrogen (estradiol), whereas expression of the corresponding biomarker genes was induced. Our results show that *rpl8* is an appropriate reference gene for analyzing gene expression by quantitative reverse transcription polymerase chain reaction for assaying EDCs using *R. nigromaculata*, and might also provide support for using *rpl8* as a reference gene in other *Rana* species due to the high conservation of *rpl8* among the *Rana* genus.

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Introduction

Amphibians are an important class of experimental animals for biological and toxicological studies (Burggren and Warburton, 2007; Song et al., 2010). With the growing concern about endocrine disrupting chemicals (EDCs) in recent decades, in particular, amphibians are widely used to evaluate endocrine disrupting effects by environmental chemicals (Kloas and Lutz, 2006). Mostly,

they are used to evaluate reproductive endocrine disruption due to the high sensitivity of the reproductive system to sex hormones. Some EDCs have been demonstrated to induce the feminization of gonads and secondary sexual characteristics, transcription of estrogenic biomarkers, and sex hormone imbalance in amphibians (Hayes et al., 2006; Hogan et al., 2008; Qin et al., 2007). In addition, amphibians have become a unique biological model for studying thyroid-disrupting effects of EDCs because amphibian

* Corresponding author. E-mail: qinzhanfen@rcees.ac.cn (Zhanfen Qin).

metamorphosis is controlled by thyroid hormone (TH) (Berg et al., 2009; Takase et al., 2007). Some endpoints have been well-developed in the amphibian model for studying thyroid disrupting effects, including the time to metamorphosis, limb growth, intestinal remodeling, TH level, and mRNA expression of TH-related genes (Finì et al., 2012; Heimeier and Shi, 2010).

In the literature, *Xenopus laevis* is the most-used model amphibian species for endocrine disruption research (Degitz et al., 2005; Hayes et al., 2006). In addition, some native amphibian species, especially some species of the *Rana* genus, such as *Rana catesbeiana* (Veldhoen et al., 2006), *Rana pipiens* (Croteau et al., 2009), *Rana temporaria* (Mortensen et al., 2006) and *Rana rugosa* (Oka et al., 2009), have also been used as experimental species for assaying the endocrine-disrupting effects of chemicals. *Rana nigromaculata* (also called as *Pelophylax nigromaculatus*) is a species of true frog distributed widely in East Asia. *R. nigromaculata* has been used by biological and toxicological researchers in East Asia due to some of its advantages as an experimental species such as a shorter generation time (one year) and larval period (6–7 weeks) and easier raising in laboratory with lower natural mortality rate (Li et al., 2009; Park et al., 2001; Yang et al., 2005). In particular, *R. nigromaculata* can be induced to breed by injecting luteinizing hormone releasing hormone and human chorionic gonadotropin accompanied by simulating rainfall in the laboratory. Moreover, our preliminary work has shown that *R. nigromaculata* is sensitive to estrogenic/androgenic chemicals and thyroid-disrupting chemicals. Given its availability and these advantages, *R. nigromaculata* is suggested to be a promising native amphibian species in East Asia for evaluating reproductive endocrine disruption as well as thyroid-disrupting effects of chemicals.

As mentioned above, mRNA expression of some sex- and thyroid-related genes is one of the most-used endpoints for evaluating endocrine disrupting effects of EDCs in amphibians (Lehigh Shirey et al., 2006; Tan and Zoeller, 2007). Quantitative analysis of gene expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR) typically requires the use of a constitutively expressed reference gene, which is adequately expressed in certain tissues with minimal variability in expression among samples under the experimental conditions used, as an internal control to normalize for differences in starting the cDNA template among samples (Arukwe, 2006; Huggett et al., 2005; Morse et al., 2005). Ribosomal protein L8 (*rpl8*) is a frequently used reference gene for normalizing gene expression in amphibians using qRT-PCR in previous studies (Gunderson et al., 2011; Hogan et al., 2007). However, nucleotide sequences of *rpl8* were characterized in only three species of the *Rana* genus, i.e., *R. catesbeiana*, *Rana clamitans*, and *Rana sylvatica*, in NCBI (www.ncbi.nlm.nih.gov). In particular, the suitability of *rpl8* as a reference gene has never been validated in any species of the *Rana* genus. No information on the *R. nigromaculata rpl8* gene is available in the literature, despite the fact that some researchers have used *rpl8* as the reference gene in *R. nigromaculata* research (Zhang et al., 2013).

In this study, we aimed to characterize *rpl8* cDNA and the stability of mRNA expression during development and under exposure to hormones, in order to validate *rpl8* as an appropriate reference gene for qRT-PCR for assaying EDCs using *R. nigromaculata* and other species of the *Rana* genus. Also, we investigated the stability of mRNA expression of elongation factor 1 alpha 1 (*ef1a1*), another frequently used housekeeping gene in amphibian research, for comparison.

1. Materials and methods

1.1. Animals and sampling

Adult frogs (originally from Anhui Province) were raised in amphibious glass tanks with sand and dechlorinated water in our amphibian house and fed with *Terzebrion molitor* daily. Several pairs of adult frogs were induced to breed by injecting 15 µg luteinizing hormone releasing hormone (Ningbo Second Hormone Factory, China) and human chorionic gonadotropin (Yantai North Pharmaceutical Co. Ltd., China; 300 IU for female, 200 IU for male) dissolved in 0.6% NaCl, accompanied by simulating rainfall. Fertilized eggs were incubated in dechlorinated tap water and developed into stage 26 tadpoles (Gosner, 1960) on the 5th day postfertilization. The tadpoles were transferred into tadpole tanks with a water depth of 15 cm and fed with commercial diets (Totoro Supplies, Hong Kong, China) twice daily. The density of tadpoles in water was less than 1.5 tadpoles/L, and the water was changed twice per week. The tadpoles completed metamorphosis within 6–7 weeks postfertilization. The raising conditions for juvenile frogs were the same as those for adult frogs. In the amphibian house, fluorescent lighting provided a photoperiod of 12 hr light:12 hr dark with a light intensity ranging from 600 to 1000 lx on the water surface. Water temperature was maintained at (23 ± 2)°C.

To obtain the full length cDNA sequence of *rpl8*, we sampled the brain tissues from juvenile *R. nigromaculata* frogs (three months after metamorphosis) after anesthetizing with a 0.1% solution of 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich, Poole, UK). To compare the mRNA expression stability of *rpl8* with *ef1a1* during development, we sampled the tissues of brain, liver, intestine, tail, testis and ovary from the tadpoles at Gosner stages 26, 34, 39, 42 and 46 (Gosner, 1960) after anesthetization. The tissue samples were frozen immediately in liquid nitrogen, followed by storage at –80°C until RNA extraction. All animal procedures were conducted according to Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission of the People's Republic of China, 1988).

1.2. Full length cDNA sequence of *rpl8*

1.2.1. RNA extraction and reverse transcription

Total RNA was extracted from the brain tissues using TRIzol reagent (TIANGEN, Beijing, China). RNA concentration was measured with NanoDrop 2000 (Thermo Scientific, USA). RNA quality was verified by $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratio in the range of 1.8–2.0 and by electrophoresis on ethidium bromide-stained 1.2% agarose gels. Then the RNA (1000 ng) was reverse transcribed into cDNA using a TIANScript RT Kit (TIANGEN, Beijing, China), following the manufacturer's instructions.

1.2.2. RT-PCR and rapid amplification of cDNA ends

The full-length cDNA sequence of *rpl8* was generated by RT-PCR and rapid amplification of cDNA ends (RACE). The *rpl8* cDNA fragments of *R. nigromaculata* were obtained by RT-PCR. Pairs of PCR primers of *rpl8* were designed according to the known sequence of *R. catesbeiana* in the NCBI GenBank

database (Table 1). To obtain the 3' end and 5' end sequences of *rpl8*, nested rapid amplification of cDNA ends PCR was performed using a GeneRacer Kit (Invitrogen, USA) according to the manufacturer's instructions. On the basis of the partial fragment sequences of *rpl8*, two pairs of gene specific primer (GSP) were designed for 3'-RACE and 5'-RACE (Table 1). The sequence of *rpl8* was determined by Sanger sequencing (Invitrogen, Shanghai, China). Full-length cDNA of *rpl8* was assembled by its internal core fragment, 5'-RACE fragment and 3'-RACE fragment.

1.2.3. Sequence analysis

The cDNA sequence and deduced amino acid sequence were analyzed using BLASTn and BLASTp, respectively. The open reading frame was found by ORF Finder in NCBI (www.ncbi.nlm.nih.gov). The sequence alignments were carried out using CLUSTAL X.

1.3. mRNA expressions of *rpl8* and *ef1a1* during development

Total RNA extraction and the first-strand cDNA synthesis were as described above. qRT-PCR was performed using a Real-time Polymerase Chain Reaction system (Mx3005P, Strategene, USA) and SYBR Green RealMasterMix (TIANGEN, Beijing, China). Specific primers for *rpl8* and *ef1a1* are shown in Table 1. The primers for *ef1a1* were designed according to the known sequence of *X. laevis* (NM_001087442.1). The PCR conditions were as follows: 95°C for 2 min, 40 cycles at 95°C for 20 sec, 60°C for 30 sec, and 68°C for 30 sec.

1.4. Exposure to TH and the response of *rpl8*, *ef1a1* and TH receptors

In amphibian research assaying thyroid-disrupting effects of chemicals, THs (T3 and T4) are often used as positive

reference chemicals, with mRNA expression of TH receptors (TRs, especially TR β) as molecular biomarkers (Fini et al., 2012; Hinthner et al., 2011). To evaluate the stability of *rpl8* and *ef1a1* mRNA expression under exposure to thyroid-disrupting chemicals, we performed a 2-day experiment, in which *R. nigromaculata* tadpoles at stage 27 were exposed to a series of concentrations of T4 (0.01, 0.1, 1, 10, 100, 1000 nmol/L) in tanks. T4 was dissolved in distilled water to prepare a stock solution concentration. Each tank contained 400 mL dechlorinated water and 6 tadpoles. The experimental water was completely replaced daily. The raising conditions for the tadpoles were as described above. At the end of the exposure, intestine tissues of the tadpoles were sampled and RNA in the intestine was extracted. qRT-PCR for *rpl8* and *ef1a1* were conducted as described above. The primers for TR β of *R. nigromaculata* followed our previous study (GenBank accession number: KC139355), as shown in Table 1. The PCR conditions for TR β were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 20 sec, 63°C for 30 sec and 68°C for 30 sec.

1.5. Exposure to estradiol and the response of *rpl8*, *ef1a1* and estrogen receptor

Because estrogen can induce mRNA expression of its receptor (estrogen receptor, ER) (Tata et al., 1993), ER expression is regarded as a molecular biomarker for estrogenic effects in amphibian research, with estradiol (E2) as a positive reference chemical (Kloas et al., 2009; Lutz et al., 2005). To examine the stability of *rpl8* and *ef1a1* mRNA expression under exposure to estrogenic chemicals, we conducted a 4-day E2-exposure experiment of *R. nigromaculata* tadpoles. The tadpoles at Gosner stage 42 were exposed to E2 (1, 10, 100 nmol/L, DMSO as the solvent) for 4 days in glass tanks. Each tank contained 8 L dechlorinated water and 10 tadpoles. The experimental water was completely replaced daily. The raising conditions for the tadpoles were as described above. At the end of the exposure, the gonads of the tadpoles were sampled and fixed for sex identification by hematoxylin and eosin stain. The liver tissues of the tadpoles were sampled, and RNA was extracted following the methods mentioned above. The qRT-PCR for *rpl8* and *ef1a1* was conducted as described above. Specific primers for ER α were designed according to the sequence of *Rana rugosa* (AB491673.1) and shown in Table 1. The PCR conditions for ER α were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 20 sec, 65°C for 30 sec and 68°C for 30 sec.

1.6. Statistical analysis

Statistical analyses were performed using SPSS software version 16.0 (SPSS, USA). Experimental data were expressed as mean $\pm$ SD. The expression of *rpl8* during development was shown by the Ct (cycle threshold) value and analyzed by one-way analysis of variance. The fold changes of ER α and TR β expression were quantified by normalizing to *rpl8* and determined by $2^{-\Delta\Delta C_t}$. Statistical difference was assessed by one-way analysis of variance. Post-hoc comparison was made using Dunnett's test. Statistical significance was defined as $p \leq 0.05$.

Table 1 – Primers used for rapid amplification of cDNA ends (RACE) and quantitative RT-PCR (qRT-PCR).

Primer	Sequence	
Primers for partial fragment		
rpl8-F	5'-TTCAGCGAGTTTACCAAGATCATCAC-3'	
rpl8-R	5'-TTAGACTTCCTGATCCTCAAAGACCTC-3'	
Primers for 3'-RACE PCR		
3'GSP1	5'-CCAAGAAGGTCATCTCATC-3'	
3'GSP2	5'-CTCCAAGAAGGTCATCTCATC-3'	
Primers for 5'-RACE PCR		
5'GSP1	5'-CAGATAGACGAGCTTTAGTTC-3'	
5'GSP2	5'-GAAGTTATCAGATAGACGAGCT-3'	
Primers for qRT-PCR		
Gene	PCR efficiency	Sequence
rpl8-F	99.9%	5'-GCTGTGCGACTTCGCGAGAAAGGCA-3'
rpl8-R		5'-ACCTGTAAGGGTCACGGAAGGCA-3'
ef1a1-F	99.7%	5'-CTTGCTATGCACCTGTGCTG-3'
ef1a1-R		5'-CACATGGGCTTGCCAGGGAT-3'
ER α -F	108.0%	5'-ACAAGAACCAGGGGCGTTG-3'
ER α -R		5'-CATCATCCGAAACGTGTAGC-3'
TR β -F	99.7%	5'-GCTAGATGACTGTGCGTGGT-3'
TR β -R		5'-ACAGGAATAGCTTGGGTGAAGA-3'

Rana-nigromaculata	MGRVIRGQRKGAGSVFRAHVKHKRGPAPLRAVDFAERHGYIKGIVKDIHDPGRGAPLAKVAFRDPYRFKKRTELFIAAE	80
Rana-catesbeiana	-----GSVFRAHVKHKRGPAPLRAVDFAERHGYIKGIVKDIHDPGRGAPLAKVAFRDPYRFKKRTELFIAAE	68
Rana-clamitans	-----GSVFRAHVKHKRGPAPLRAVDFAERHGYIKGIVKDIHDPGRGAPLAKVAFRDPYRFKKRTELFIAAE	68
Rana-sylvatica	-----QRKGAGSVFRAHVKHKRGPAPLRAVDFAERHGYIKGIVKDIHDPGRGAPLAKVAFRDPYRFKKRTELFIAAE	73
Xenopus-tropicalis	MGRVIRGQRKGAGSVFKAHVKHKRGAAPLRAVDFAERHGYIKGIVKDIHDPGRGAPLAKVAFRDPYRFKKRTELFVAAE	80
Xenopus-laevis	MGRVIRGQRKGAGSVFKAHVKHKRGAAPLRAVDFAERNGYIKGIVKDIHDPGRGAPLAKVAFRDPYRFKKRTELFVAAE	80
ruler	1.....10.....20.....30.....40.....50.....60.....70.....80	
Rana-nigromaculata	GIHTGQFVYCGKKAQLNIGNVLPVGTMPGTIVCCVEEKPGRGKLARASGNYATVISHPETKKTRVKLPSSGSKKVISS	160
Rana-catesbeiana	GIHTGQFVYCGKKAQLNIGNVLPVGTMPGTIVCCVEEKPGRGKLARASGNYATVISHPETKKTRVKLPSSGSKKVISS	148
Rana-clamitans	GIHTGQFVYCGKKAQLNIGNVLPVGTMPGTIVCCVEEKPGRGKLARASGNYATVISHPETKKTRVKLPSSGSKKVISS	148
Rana-sylvatica	GIHTGQFVYCGKKAQLNIGNVLPVGTMPGTIVCCVEEKPGRGKLARASGNYATVISHPETKRTRVKLPSSGSKKVISS	153
Xenopus-tropicalis	GIHTGQFVYCGKKAQLNIGNVLPVGTMPGTIVCCVEEKPGRGKLARASGNYATVISHPETKKTRVKLPSSGSKKVISS	160
Xenopus-laevis	GIHTGQFVYCGKKAQLNIGNVLPVGTMPGTIVCCVEEKPGRGKLARASGNYATVISHPETKKTRVKLPSSGSKKVISS	160
ruler	90.....100.....110.....120.....130.....140.....150.....160	
Rana-nigromaculata	ANRAIVGVVAGGGRIDKPIKAGRAYHKYKAKRNCWPRVRGVAMNPVEHPFGGNGHQHIGKPSTIRRDAPAGRKVGIIAA	240
Rana-catesbeiana	ANRAIVGVVAGGGRIDKPIKAGRAYHKYKAKRNCWPRVRGVAMNPVEHPFGGNGHQHIGKPSTIRRDAPAGRKI----	223
Rana-clamitans	ANRAIVGVVAGGGRIDKPIKAGRAYHKYKAKRNCWLRVRGVAMNPVEHPFGGNGHQHIGKPSTIRRDAPAGRKV----	223
Rana-sylvatica	ANRAIVGVVAGGGRIDKPIKAGRAYHKYKAKRNCWPRVRGVAMNPVEHPFGGNGHQHIGKPSTIRRDAPAGRKVGIIAA	233
Xenopus-tropicalis	ANRAIVGVVAGGGRIDKPIKAGRAYHKYKAKRNCWPRVRGVAMNPVEHPFGGNGHQHIGKPSTIRRDAPAGRKVGIIAA	240
Xenopus-laevis	ANRAIVGVVAGGGRIDKPIKAGRAYHKYKAKRNCWPRVRGVAMNPVEHPFGGNGHQHIGKPSTIRRDAPAGRKVGIIAA	240
ruler	170.....180.....190.....200.....210.....220.....230.....240	
Rana-nigromaculata	RRTGRLGGTKTVQEKEN	257
Rana-catesbeiana	-----	223
Rana-clamitans	-----	223
Rana-sylvatica	RRTGRLRGTKTVQE---	247
Xenopus-tropicalis	RRTGRLRGTKTVQEKEN	257
Xenopus-laevis	RRTGRLRGTKTVQEKEN	257
ruler	250.....	

Fig. 1 – Alignments of the deduced amino acid sequences of *rpl8* between *Rana nigromaculata* and other amphibian species. The relevant amino acid sequences were obtained from the NCBI GenBank database: *Rana catesbeiana* (AAS09885.1), *Rana clamitans* (AET97634.1), *Rana sylvatica* (AEA10369.1), *Xenopus laevis* (NP_001080465.1), *Xenopus tropicalis* (NP_988925.1). Hyphens denote that the bases are gaps with those in the top line. The number on the right and bottom means the length of the amino acid sequences.

Table 2 – Nucleotide and amino acid sequence identity of *rpl8* between *Rana nigromaculata* and other amphibian species.

Amphibian species	<i>Rana nigromaculata</i>	
	Nucleotide	Amino acid
<i>Rana catesbeiana</i>	98%	99%
<i>Rana clamitans</i>	98%	99%
<i>Rana sylvatica</i>	97%	99%
<i>Xenopus laevis</i>	89%	98%
<i>Xenopus tropicalis</i>	88%	98%

2. Results

2.1. Full length cDNA sequence of *rpl8* of *R. nigromaculata* and homologic analysis

To obtain the full-length sequence of *rpl8* cDNA of *R. nigromaculata*, we conducted RT-PCR and RACE. The sequence was submitted to the GenBank (GenBank accession number: KF585213). The *rpl8* cDNA of *R. nigromaculata* was a transcript of 919 bp that contained an open reading frame (ORF)

of 774 bp from 59 to 832 bp, which encoded a peptide of 257 amino acids (Fig. 1). *R. nigromaculata* had higher identity of nucleotide sequence with three *Rana* species (97%–98%) than with *X. laevis* (89%) and *X. tropicalis* (88%). Correspondingly, the identity of amino acid sequences between *R. nigromaculata* and three species of the *Rana* genus was 99%. The identity of amino acid sequences between *R. nigromaculata* and *X. laevis* also reached 98% (Table 2).

2.2. Expression of *rpl8* and *ef1a1* during *R. nigromaculata* development

Using SYBR Green-based qRT-PCR, we investigated mRNA expression of *rpl8* and *ef1a1* in the brain, liver, intestine, tail, testis and ovary during *R. nigromaculata* development. No stage-specific difference in *rpl8* mRNA expression was found among all tissues examined during *R. nigromaculata* development from Gosner stage 26 to stage 46 (Fig. 2). Also, there was no tissue-specific difference in *rpl8* mRNA expression in these tissues at each developmental stage (Fig. 2). By contrast, *ef1a1* expression was changeable in different tissues during development. Compared with *ef1a1*, *rpl8* exhibited a more stable expression in different tissues during *R. nigromaculata* development.

Fig. 2 – The mRNA expression of *rpl8* (a) and *ef1a1* (b) in different tissues during *Rana nigromaculata* development. There was no data on the tails at stage 46 due to their loss at this stage. There was no data on the gonads before stage 39 due to the difficulty in distinguishing between the testis and the ovary.

2.3. Response of *rpl8*, *ef1a1* and *TRβ* expression to TH

In the 2-day T4-exposure experiment of *R. nigromaculata* tadpoles at stage 27, we found that T4 had no effect on *rpl8* mRNA expression (Fig. 3a), but raised slightly the *ef1a1* mRNA expression at 0.01 and 10 nmol/L (Fig. 3b) and induced an increase in *TRβ* mRNA expression in the intestine in a concentration-dependent manner compared with the control (Fig. 3c).

Fig. 3 – Effects of T4 on mRNA expression of *rpl8* (a), *ef1a1* (b) and *TRβ* (c) in intestine tissues of *Rana nigromaculata* tadpoles. Data were shown as means $\pm$ SD ($n = 6$). Significant differences between T4 treatment groups and the control were indicated by * (one-way analysis of variance, $p \leq 0.05$).

2.4. Response of *rpl8*, *ef1a1* and *ERα* expression to E2

In the 4-day E2-exposure experiment of *R. nigromaculata* tadpoles at stage 42, we found that E2 had no effect on *rpl8* expression, but up-regulated *ef1a1* expression in 1 nmol/L and promoted *ERα* expression in liver tissues of the females in a

Fig. 4 – Effects of E2 on mRNA expression of *rpl8* (a), *ef1a1* (b) and *ERα* (c) in liver tissues of female *Rana nigromaculata* tadpoles. Data were shown as means $\pm$ SD ($n = 5-8$). Significant differences between E2 treatment groups and the control were indicated by * (one-way analysis of variance, $p \leq 0.05$).

concentration-dependent manner compared with the control (Fig. 4).

3. Discussion

Although *rpl8* as the reference gene has been used to analyze gene expression in some frog species, the *rpl8* nucleotide sequences of many species including *R. nigromaculata* have been unknown. In this study, we for the first time obtained the ORF of *rpl8* in *R. nigromaculata* (774 bp, encoding a peptide of 257 amino acids). *R. nigromaculata* had high identities of nucleotide sequence and amino acid sequence with the known sequences of *rpl8* from three frogs (*R. catesbeiana*, *R. clamitans*, and *R. sylvatica*) of the *Rana* genus. Although the nucleotide sequence identity of *rpl8* between *R. nigromaculata* and *Xenopus* species was only 88%–89%, their identity of amino acid sequence reached 98% (Table 2). These results show that *rpl8* has very high identity among amphibian species. Furthermore, the data demonstrate that *R. nigromaculata* has higher identity with the *Rana* genus than with *X. laevis* and *X. tropicalis*, which accords with the fact that *R. nigromaculata* has a closer evolutionary relationship with the *Rana* genus than with *Xenopus* species (Frost et al., 2006).

The stable expression in various tissues during development is a requirement for the reference gene in analyzing gene expression, which is especially important for amphibians because they undergo a metamorphic process. Shi and Liang (1994) demonstrated ubiquitous expression of *rpl8* gene at fairly constant levels during tadpole development as well as postmetamorphic *X. laevis*. Thus, *rpl8* is used as the reference gene for analyzing gene expression in *X. laevis* research. So far, however, the expression stability of *rpl8* has not been validated in *Rana*, although *rpl8* has been used as the reference gene for analyzing gene expression in some species of the *Rana* genus including *R. nigromaculata* (Gunderson et al., 2011; Hogan et al., 2007). For example, Zhang et al. (2013) used *rpl8* to normalize mRNA expression of sex-related genes to investigate toxic effects of microcystin-LR on *R. nigromaculata* using qRT-PCR, without validation of the suitability of *rpl8* as the reference gene. In the present study, we for the first time described *rpl8* expression during tadpole development (from stage 26 to stage 46) and demonstrated that *rpl8* had little tissue-specific difference among various tissues in *R. nigromaculata*. Compared with *ef1a1*, *rpl8* was more stable and suitable as a reference gene in research on *R. nigromaculata*. It needs to be emphasized that we only demonstrated the expression stability of *rpl8* during tadpole development, because tadpoles, but not embryos or frogs, are used to assay EDCs. It is unclear whether *rpl8* expression is stable during embryonic development of *R. nigromaculata*. Recently, several studies reported that *rpl8* expression was not stable in *X. laevis* and *X. tropicalis* during embryogenesis, suggesting that *rpl8* is unsuitable as a reference gene for analyzing gene expression in this developmental period of this amphibian species by qRT-PCR (Dhorne-Pollet et al., 2013; Sindelka et al., 2006). In the literature, in fact, many researchers used *rpl8* as the reference gene for analyzing gene expression in *X. laevis* and *X. tropicalis* during embryonic development (Langlois et al., 2010; Morvan-Dubois et al., 2008). The cases of *X. laevis* and *X. tropicalis* highlight the fact that it is

necessary to validate the suitability of a candidate gene as the reference gene prior to use. Thus, if the study involves the gene expression of *R. nigromaculata* during embryonic development, it is necessary to validate the expression suitability of *rpl8* in this period.

Although housekeeping genes are expressed constitutively in every cell during development, it does not mean that their expression is not regulated in the cells of various tissues under certain experimental conditions (Bustin and Nolan, 2004; Huggett et al., 2005). In particular, the expression of housekeeping genes might be altered following exposure to some chemicals (Arukwe, 2006; Filby and Tyler, 2007). So, it is essential to validate the suitability of a housekeeping gene as the reference gene in a specific toxicological experiment (Arukwe, 2006). Our primary study has shown that *R. nigromaculata* is a promising native species for evaluating endocrine-disrupting effects of chemicals, especially on thyroid disruption and reproductive endocrine disruption (unpublished data). In the present study, we used T4 and E2 as positive reference chemicals to investigate whether thyroid disruptors and estrogenic chemicals had influences on *rpl8* and *ef1a1* expression in *R. nigromaculata*. Consequentially, we found that the expression of *ef1a1* was not very steady in our experimental systems, however *rpl8* mRNA expression was not altered by exposure to either T4 or E2, suggesting that *rpl8* is suitable as the reference gene for analyzing gene expression in *R. nigromaculata* studies on EDC. Given the high conservation of *rpl8* among the *Rana* genus, our results might provide support for using *rpl8* as the reference gene for analyzing gene expression in other species of the *Rana* genus.

4. Conclusions

Our results show that *rpl8* is an appropriate reference gene for analyzing gene expression by qRT-PCR for assaying EDCs using *R. nigromaculata*, and might also provide support for using *rpl8* as a reference gene in other *Rana* species due to the high conservation of *rpl8* among the *Rana* genus.

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