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Molecular characterization and developmental expression patterns of thyroid hormone receptors (TRs) and their responsiveness to TR agonist and antagonist in *Rana nigromaculata*

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ABSTRACT

Considering some advantages of *Rana nigromaculata* as an experimental species, we propose that this species, like *Xenopus laevis*, could be used to assay thyroid hormone (TH) signaling disrupting actions. To validate the utilizability of *R. nigromaculata*, we investigated the responsiveness of *R. nigromaculata* to a TH receptor (TR) agonist (T3) and antagonist (amiodarone) by analyzing expression, based on characterizing TR cDNA and developmental expression patterns. With high levels of identity with the corresponding genes in *X. laevis*, both *TRα* and *TRβ* in *R. nigromaculata* exhibited roughly similar developmental expression patterns to those of *X. laevis*, in spite of some species-specific differences. Both *TRα* and *TRβ* expression had greater changes in the liver and intestine than in the tail and brain during metamorphosis. T3 exposure for 2 days induced more dramatic increases of *TRβ* expression in stage 27 than in stage 34 tadpoles but not in stage 42 tadpoles, showing that the responsiveness of *R. nigromaculata* to TH decreased with development and disappeared at the onset of metamorphic climax. Corresponding to greater changes of *TRβ* expression in the liver and intestine than in the tail and brain during metamorphosis, the liver and intestine had higher responsiveness to exogenous T3 than the tail and brain. Amiodarone inhibited T3-induced *TRβ* expression. Our results show that *R. nigromaculata* can be used as a model species for assaying TH signaling disrupting actions by analyzing *TRβ* expression, and intestine tissues at stage 27 are ideal test materials due to high responsiveness and easy accessibility.

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Introduction

Thyroid hormones (THs, T3 and T4) are involved in a wide range of biological processes associated with development, metabolism, growth and reproduction in vertebrates. THs are regulated by the hypothalamic–pituitary–thyroid (HPT) axis, and their actions are

mainly mediated through transcriptional regulation by TH receptors (TRs, *TRα* and *TRβ*) (Yen, 2001; Bernal et al., 2003). In the past decades, some chemicals have been found to disrupt the complex thyroid system via various pathways in animals (Brucker-Davis, 1998; Zoeller, 2007). Epidemiological data suggest that chemical pollutants may be associated with human thyroid imbalance

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(Langer et al., 2007). Because amphibian metamorphosis is controlled by THs, amphibian metamorphosis provides a unique biological model for studying thyroid disruptors, based on the potential utility of morphological and molecular markers of metamorphosis as endpoints of thyroid status in the developing tadpoles (Tata, 2006; Heimeier et al., 2010; Das et al., 2010). The Amphibian Metamorphosis Assay developed by OECD (2009) has been applied to screen thyroid disruptors that may interfere with the normal functioning of the HPT axis (Carr and Patino, 2011; Gutleb et al., 2007). In addition, TH-induced metamorphosis is also used to identify thyroid disruptors that may bind to TRs and alter TH actions and signaling (Heimeier et al., 2009; Helbing et al., 2006). TH-dependent gene expression and morphological changes induced by TH are regarded as good endpoints for evaluating TH signaling disrupting effects of chemicals (Heimeier et al., 2009; Helbing et al., 2006; Fini et al., 2012). In particular, TH-induced expression of TRs (especially TR β) has been used as a molecular biomarker for evaluating TH signaling disrupting effects in amphibians (Opitz et al., 2006; Veldhoen et al., 2006a, b).

In the literature, *Xenopus laevis* is the most-used model species for studying thyroid disrupting effects (Degitz et al., 2005; Heimeier et al., 2009). Given the obtainability of experimental animals, some native amphibian species, such as *Rana catesbeiana* and *Rana pipiens* (Helbing et al., 2006; Croteau et al., 2009), *Rana temporaria* (Arukwe and Jenssen, 2005; Gutleb et al., 2000) and *Rana rugosa* (Hanada et al., 2003; Oka et al., 2009), are used to assess effects of chemicals on the thyroid system. *Rana nigromaculata* (*Pelophylax nigromaculatus*) is a species of true frog distributed in East Asia. *R. nigromaculata* has been used in toxicological studies (Li et al., 2009; Wang and Jia, 2009) as well as biological studies (Park et al., 2001; Sumida et al., 2001, 2004; Takase et al., 2002). *R. nigromaculata* has some advantages as an experimental species. For example, *R. nigromaculata* tadpoles as well as frogs are maintained easily in the laboratory, and metamorphose into frogs approximately 7 weeks postfertilization. It is noteworthy that the natural death rate of *R. nigromaculata* tadpoles is very low, even close to zero. The relatively short generation time (one year) also provides a good reason for use of *R. nigromaculata* as an experimental species. Importantly, *R. nigromaculata* can be induced to breed by injecting luteinizing hormone releasing hormone (LHRH) and human chorionic gonadotropin (HCG) accompanied by simulating rainfall. Given the above advantages, we propose that *R. nigromaculata* could be a good experimental species for assaying thyroid disruptors.

To develop *R. nigromaculata* as a native model species for assaying TH signaling disrupting effects of chemicals, it is necessary to characterize TR cDNA, describe its developmental expression patterns and evaluate responsiveness to TR agonist/antagonists in *R. nigromaculata*. In this study, we aimed to obtain information on TR in *R. nigromaculata* to validate the utilizability of *R. nigromaculata* as a model species for assaying TH signaling disrupting effects.

1. Materials and methods

1.1. Animals and sampling

Initially, adult frogs were obtained from a *R. nigromaculata* farm in Bengbu, Anhui Province, China. Two generations were bred in our amphibian house. *R. nigromaculata* frogs were raised in glass tanks, in which dechlorinated water and a layer of scree constituted the amphibious environment, and fed with *Terzebrio molitor* daily. Breeding were induced by injecting 15 μ g LHRH (Ningbo second hormone factory, Zhejiang, China) and human chorionic gonadotropin (HCG, Yantai North Pharmaceutical Co. Ltd., Shandong, China; 300 IU for female

and 200 IU for male) dissolved in 0.6% NaCl, accompanied by simulating rainfall. Fertilized eggs were incubated in dechlorinated tap water and developed into tadpoles on the 5th day postfertilization. The tadpoles were transferred into tadpole tanks (100 cm $\times$ 60 cm $\times$ 30 cm) 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 5 tadpoles/L before Gosner stage 30 (1960); later, less than 1.5 tadpoles/L. The water was changed twice per week. The water quality for raising *R. nigromaculata* was as follows: without chlorine, pH 6.5–7, dissolved oxygen concentration >5 mg/L, and water hardness (CaCO₃) approximately 150 mg/L. In the amphibian house (23 $\pm$ 2°C), fluorescent lighting provided a photoperiod of 12 hr light:12 hr dark with a light intensity ranging from 600 to 1000 lx at the water surface for tadpoles and ranging 100 to 300 lx for frogs. The tadpoles completed metamorphosis within approximately 7 weeks postfertilization.

The developmental stages of *R. nigromaculata* were determined according to the Gosner system (Gosner, 1960). After anesthetization of the frogs (six months) by immersion in 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich; 1 g/L in water), brain tissues were collected to extract RNA for cloning and sequencing of TR cDNA. For TR mRNA expression analysis, the tissues of brain, liver, intestine, and tail were freshly sampled from the tadpoles at different stages after anesthetization by MS222. 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. Cloning and molecular characterization of TR genes in *R. nigromaculata*

1.2.1. RNA extraction and reverse transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Shanghai, China), following the manufacturer's instructions. Total RNA concentration was calculated from absorbance at 260 nm (Nanodrop ND-1000, Nanodrop, USA), and RNA quality was verified by electrophoresis on ethidium-bromide-stained 1% agarose gels and by A_{260 nm}/A_{280 nm} ratios in the range of 1.8–2.0. Then the RNA was reverse transcribed into cDNA using SuperScriptIII reverse transcriptase (Invitrogen, Shanghai, China), following the manufacturer's instructions.

1.2.2. Cloning and sequencing of TR genes

Full-length TR α and TR β cDNA sequences were generated by reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The partial fragments of TR α and TR β cDNA were obtained by RT-PCR. The primers of TR α and TR β were designed according to the corresponding sequences of *R. catesbeiana* (L06064.1 and L27344.1), *R. rugosa* (AB683466.1 and AB683467.1) and *R. pipiens* (DQ398026.1 and AY049025.1) in the NCBI GenBank database (Table 1). The PCR products were sequenced by Invitrogen Corporation (Shanghai, China). To obtain the 3' end and 5' end sequences of TR α and TR β genes, nested rapid amplification of cDNA end PCR was performed using the GeneRacer Kit (Invitrogen, Shanghai, China) according to the manufacturer's instructions. On the

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

Primer	Sequence
<i>Primers for partial fragment</i>	
TR α -F	5'-TTCAGCGAGTTTACCAAGATCATCAC-3'
TR α -R	5'-TTAGACTTCTCTGATCTCAAAGACCTC-3'
TR β -F	5'-AGCAGCATGTCAGGGTAC-3'
TR β -R	5'-TGAAGGCTTCTAAGTCCA-3'
<i>Primers for 3'-RACE PCR</i>	
TR α -3'GSP1	5'-CGAACGGGTTTGATCTGCACAGATAA-3'
TR α -3'GSP2	5'-AAATCATCGCAAACACAACATTCCCC-3'
TR β -3'GSP1	5'-GCGTGGTGTGTGGAGACAAGGC-3'
TR β -3'GSP2	5'-ACTGTGCGTGGTGTGTGGAGA-3'
<i>Primers for 5'-RACE PCR</i>	
TR α -5'GSP1	5'-GCCACTTCCGTGTCATCCAGATTGAAC-3'
TR α -5'GSP2	5'-TCCACCATTCTTCTAGTCTTCTCGCTT-3'
TR β -5'GSP1	5'-GCTGGCATGGCAGGCTCCAA-3'
TR β -5'GSP2	5'-AGTCCACTTTTCCACCTCCGGT-3'
<i>Primers for full-length PCR</i>	
TR α -F	5'-GACCAGAATCTTAGCGGGCTG-3'
TR α -R	5'-GGGAGCAAGAGAAGTTTCTAGTC-3'
TR β -F	5'-ATGCCTAGCAGCATGTCA-3'
TR β -R	5'-AAAGAAGCACAGTCTCTTC-3'
<i>Primers for quantitative real-time PCR</i>	
TR α -F	5'-TCTCCGGGCTGCTGTGCGTTA-3'
TR α -R	5'-GCATCTGAGACCACCCCAAGTCCA-3'
TR β -F	5'-GCTAGATGACTGTGCGTGGT-3'
TR β -R	5'-ACAGGAATAGCTTGGGTGAAGA-3'
rpl8-F	5'-GCTGTGCACTTCGCAGAAAGGCA-3'
rpl8-R	5'-ACCTGTAAGGGTCACGGAAGGCA-3'

basis of the partial fragment sequences of TR α and TR β , two pairs of gene specific primer (GSP) were designed for 3'-RACE and 5'-RACE of TR genes, respectively (Table 1). The RACE PCR products of TRs were purified by ethidium bromide gel electrophoresis. The gel-purified products were cloned into the pCR® 4-TOPO® plasmid using the TOPO TA Cloning® for Sequencing Kit (Invitrogen, Shanghai, China). The nucleotide sequences of TR α and TR β were determined by Sanger sequencing (Invitrogen, Shanghai, China). To obtain the complete coding region of TR α and TR β , two pairs of full-length primers were designed based on the RACE sequencing results (Table 1).

1.2.3. Sequence analysis and phylogenetic 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. Phylogenetic analysis was conducted with MEGA4 and the phylogenetic tree was drawn using the Neighbor-Joining method. The sequences of TR α and TR β in some species were from the NCBI GenBank database.

1.3. Expression patterns of TRs during *R. nigromaculata* development

For TR mRNA expression analysis in various tissues during *R. nigromaculata* development, we conducted quantitative real-time PCR (qRT-PCR) using SYBR Green I with the MX Real-time Polymerase Chain Reaction system (Stratagene

MX3005P, Agilent, USA). Specific primers of TR α and TR β were designed according to the corresponding cDNA sequences obtained by RACE (Table 1). Ribosomal protein L8 (rpl8) was used as a reference gene to normalize mRNA expression of TRs, because its stable expression during *R. nigromaculata* development and low tissue-specificity has been demonstrated in our previous study (Lou et al., 2014). Specific primers for rpl8 are shown in Table 1. PCR conditions were as follows: 95°C for 2 min, 40 cycles at 95°C for 20 sec, 60°C (TR α and rpl8) or 63°C (TR β) for 30 sec, and 68°C for 30 sec, followed by a melting curve analysis.

1.4. Responsiveness of *R. nigromaculata* to exogenous TR agonist and antagonist

R. nigromaculata tadpoles at Gosner stages 27 (pre-metamorphic stage), 34 (early pro-metamorphic stage), and 42 (metamorphic climax stage) were exposed to TR agonist T3 (1 nmol/L) in glass tanks (500 mL) with 400 mL of dechlorinated water. A blank control for each stage was set. Two replicate test tanks (5 tadpoles per tank) were employed for each treatment group. T3 replacement was performed every 24 hr following water changes. The tadpoles were not fed during the exposure period. The housing conditions were as described above. After exposure for 48 hr, the liver, brain, intestine and tail tissues of tadpoles were collected for RNA extraction. TR α and TR β expression levels were examined by qRT-PCR according to the methods described above.

By comparing the responsiveness of tadpole tissues to T3, we chose tissues from the most sensitive stage as test materials. The tadpoles were exposed to a series of concentrations of TR antagonist amiodarone (AM) in the presence of 0.1 nmol/L T3 or in the absence of T3. Two replicate test tanks (5 tadpoles per tank) were employed for each treatment group. Exposure conditions were as described above. After exposure for 48 hr, the liver, brain, intestine and tail tissues of tadpoles were collected for RNA isolation. TR α and TR β expression levels were examined by qRT-PCR according to the methods described above.

1.5. Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS, IBM, Chicago, USA). Quantitative data were shown as mean $\pm$ SD. The fold change of TRs expression in a specific stage or tissue was determined by $2^{-\Delta\Delta Ct}$ (cycle threshold, Ct). Statistical difference in mRNA expression between tissues was assessed by one-way analysis of variance and independent-sample T test. A $p < 0.05$ was regarded as significant.

2. Results

2.1. Molecular characterization of TR α and TR β cDNA in *R. nigromaculata*

The cDNA sequences of TR α and TR β were obtained using RT-PCR and 5'-/3'-RACE strategies. Two sequences were submitted to the GenBank (GenBank accession numbers:

KC139354, KC139355). The TR α cDNA was a transcript of 1760 bp that contained an open reading frame (ORF) of 1256 bp from 16 to 1272 bp, which encoded a peptide of 418

amino acids. The TR β cDNA was a transcript of 1152 bp that contained an ORF of 1122 bp from 1 to 1122 bp, which encoded a peptide of 373 amino acids. The homologies of nucleotide

Fig. 1 – Alignments of the amino acid sequences of TRs between *Rana nigromaculata* and other amphibian species. The relevant amino acid sequences were obtained from the NCBI GenBank database: *Rana catesbeiana* (Q02777.1, Q02965.1), *Xenopus laevis* (NP_001081595.1, BAL70322.1, NP_001090182.1, NP_001081250.1), *Xenopus tropicalis* (NP_001039261.1, NP_001039270.1). The red vertical lines indicate the borders of four domains: the N-terminal hypervariable region (A/B domain), DNA-binding domain (C domain), hinge region (D domain) and hormone-binding domain (E/F domain). Hyphens denote that the bases are gaps with those in the top line. Red asterisk marks the diversity of TR α and TR β between *Rana nigromaculata* and *Rana catesbeiana* and *Xenopus laevis*.

sequences and deduced amino acid sequences between TR α and TR β in *R. nigromaculata* were 72% and 86%, respectively.

A multiple alignment of TR-deduced amino acid sequences was performed in amphibians (Fig. 1). The deduced amino acids of both TR α and TR β were composed of the N-terminal hypervariable region (A/B domain), DNA-binding domain (C domain), hinge region (D domain) and hormone-binding domain (E/F domain). TR α and TR β in *R. nigromaculata* had high similarity in the DNA-binding domain, hinge region and hormone-binding domain, whereas there was a deletion of 42 amino acids in TR β compared with TR α in the A/B domain. In addition, 51 amino acids were different between TR α and TR β in C, D and E/F domains (Fig. 1). These differences between TR α and TR β in *R. nigromaculata* corresponded to the differences found in *X. laevis*, *Xenopus tropicalis* and *R. catesbeiana*.

TR α and TR β in *R. nigromaculata* resembled their counterparts in reptiles, birds, mammals and other amphibian species (Table 2). Phylogenetic analysis by the Neighbor-Joining tree revealed the phylogenetic relationship of TR α and TR β between *R. nigromaculata* and other species (Fig. 2). The amino acid sequences of *R. nigromaculata* TR α had high identity with *X. laevis* TR α A (98%, 10 different sites out of 418 amino acids) and *X. laevis* TR α B (98% homology, 10 different sites out of 418 amino acids). The amino acid sequence identity of TR α between *R. nigromaculata* and *R. catesbeiana* even reached 99%, with only 2 different sites among 418 amino acids. The amino acid sequence of *R. nigromaculata* TR β had 98% and 96% identity values with *X. laevis* TR β A and TR β B, respectively. In accordance with the high homology of TR α , only 4 of 373 amino acids of TR β were different between *R. nigromaculata* and *R. catesbeiana*.

2.2. TR expression patterns during metamorphic development

Expression of TR α and TR β was detectable in the liver, intestine, tail and brain of *R. nigromaculata* at Gosner stage 27–46 (Fig. 3). In the liver, the mRNA levels of TR α increased gradually from stage 27, peaked at stage 42 (forelimb breaking through operculum), and then reduced dramatically toward stage 46 (the end of metamorphosis). In the intestine and tail, however, TR α expression remained at relatively low and constant levels before stage 40, whereas it started to increase dramatically at stage 40, and peaked rapidly at stage 42, followed by a dramatic decrease toward the end of metamorphosis. Notably different from the above tissues, TR α expression was maintained at a high level in the brain and only exhibited a weak increasing trend from stage 27 to stage 41, followed by a slight decrease toward the end of metamorphosis.

In all tissues examined, TR β expression was low and relatively constant before stage 40, began to increase at stage 40, and peaked rapidly at stage 42. Then, the TR β mRNA levels reduced rapidly toward the end of metamorphosis, especially dramatically in the intestine and tail. At the end of metamorphosis, TR β expression in the intestine and tail almost declined to the levels observed before stage 40, whereas TR β expression in the liver and brain maintained higher levels than those before stage 40.

Overall, TR β mRNA expression had more obvious changes compared to TR α mRNA expression in all these tissues during *R. nigromaculata* development. The TR α levels in the liver, intestine and tail at stage 42 were approximately 10 times of

those at stage 27, while a smaller increase in TR α expression was observed in the brain from stage 27 to stage 42. However, the TR β mRNA levels in the liver, intestine, tail and brain had 205-, 370-, 35- and 19-fold increases from stage 27 to stage 42, respectively. In terms of the tissue difference, the magnitude of the change in TR β expression in the liver was comparable or slightly lower than that in the intestine, which was larger than those in the tail and the brain.

2.3. Responsiveness of *R. nigromaculata* TR expression to exogenous TR agonist and antagonist

T3 induced TR β mRNA expression in all tissues from stage 27 and 34 tadpoles, but not from stage 42 tadpoles. Moreover, the T3-induced increase in TR β expression in stage 27 tadpoles was more dramatic than in stage 34 tadpoles. The observation suggests that the responsiveness of TR β expression to T3 is dependent on developmental stage, i.e. the responsiveness decreases with the metamorphic development. At the same developmental stage, T3 had the greatest TR β induction in the liver and intestine and the weakest in the brain, showing tissue-specific TR β expression, i.e. liver $\approx$ intestine > tail > brain (Fig. 4).

Similarly, 1 nmol/L T3 did not induce TR α expression in stage 42 tadpoles. In the liver, T3 had a larger TR β induction in stage 27 than in 34 tadpoles, whereas no significant difference between stage 27 and stage 34 was observed in both intestine and tail. T3 did not promote TR α expression in the brain at any stage. The observations show that the responsiveness of TR α expression to T3 is also stage-dependent and tissue-specific. In these tissues, T3-induced changes in TR α expression were slight compared to TR β expression.

Considering their high responsiveness to TH and convenience for sampling, we chose intestinal tissues of stage 27 tadpoles as test materials to examine the responsiveness of *R. nigromaculata* to TR antagonists. The tadpoles were exposed to series of concentrations of AM in the presence of 0.1 nmol/L T3 or in the absence of T3. We found a significant increase (more than 3-fold) of TR β expression in the intestine following exposure to T3 compared to the control. AM at the range from 10 to 100 nmol/L dramatically inhibited T3-induced TR β expression, while exposure to AM alone did not affect TR β expression (Fig. 5). The result shows that the intestinal tissues of *R. nigromaculata* tadpoles are sensitive to TR antagonists, and TH signaling disrupting effects of TR antagonists could be assayed by evaluating effects of TR antagonists on T3-induced TR β expression.

3. Discussion

3.1. Molecular characterization of TR α and TR β in *R. nigromaculata*

Although *R. nigromaculata* has been used in biological and toxicological studies, its TRs have never been molecularly characterized. In this study, we described the cDNA of TR α and TR β in *R. nigromaculata*. TR α consisted of 1760-bp and encoded 418 amino acids, while TR β consisted of 1152-bp and encoded 373 amino acids, which accords with molecular characteristics

Table 2 – Nucleotide and amino acid sequence homology of thyroid hormone receptors (TRs) between *Rana nigromaculata* and other amphibian species.

TR α	<i>Rana nigromaculata</i>		TR β	<i>Rana nigromaculata</i>	
	Nucleotide	Amino acid		Nucleotide	Amino acid
<i>Rana catesbeiana</i>	98%	99%	<i>R. catesbeiana</i>	98%	99%
<i>Xenopus laevis</i> TR α A	87%	98%	<i>X. laevis</i> TR β A	90%	98%
<i>Xenopus laevis</i> TR α B	87%	98%	<i>X. laevis</i> TR β B	90%	96%
<i>Xenopus tropicalis</i>	87%	98%	<i>X. tropicalis</i>	89%	98%
<i>Alligator mississippiensis</i>	83%	94%	<i>X. mississippiensis</i>	84%	95%
<i>Gallus gallus</i>	82%	90%	<i>G. gallus</i>	84%	95%
<i>Equus caballus</i>	80%	84%	<i>E. caballus</i>	82%	95%
<i>Homo sapiens</i>	80%	86%	<i>H. sapiens</i>	81%	94%

of TRs in other amphibian species in the literature. The homologies of nucleotide sequences and deduced amino acid sequences between TR α and TR β in *R. nigromaculata* were 72% and 86%, respectively. The homology of TR α to TR β in *R. nigromaculata* is similar to *X. laevis*, in which 74% and 85% homologies for nucleotide sequences and amino acid sequences were reported between TR α and TR β , respectively (Yaoita et al., 1990).

TR α and TR β have been demonstrated to have high homology across vertebrates (Oka et al., 2013). In this study, the amino acid sequence homologies of TR α and TR β between *R. nigromaculata* and *X. laevis* were approximately 97% and 98%, respectively. However, TR α and TR β in *R. nigromaculata* exhibited higher homologies to the corresponding genes in *R. catesbeiana* than in *X. laevis*, which accords with the fact that *R. nigromaculata* has a closer evolutionary relationship with *R. catesbeiana* than with *X. laevis* (Frost et al., 2006).

3.2. TR expression patterns during metamorphic development

In previous studies, TR expression patterns in the intestine, brain, tail and limbs of *X. laevis* and *X. tropicalis* during metamorphic development were well characterized (Kawahara

et al., 1991; Opitz et al., 2006; Wang et al., 2008; Yaoita and Brown, 1990). However, the data concerning TR expression patterns in the *Rana* genus has been limited, although Hogan et al. (2007) described TR expression in the brain of *R. pipiens* at four developmental stages. To our knowledge, this is the first detailed description of the developmental expression patterns of TR α and TR β in multiple tissues of *Rana*. We found that the expression patterns of TR α and TR β were roughly similar in all examined tissues (liver, intestine, brain and tail) of *R. nigromaculata* tadpoles, i.e. TR expression remained at relatively low levels before stage 40, followed by an increase and peak at the metamorphic climax (stage 42), and then decreased dramatically or gradually. The developmental expression patterns in *R. nigromaculata* are consistent with previous description for the brain and intestine of *X. laevis* and *X. tropicalis* (Yaoita and Brown, 1990; Opitz et al., 2006; Wang et al., 2008). In *Xenopus*, TR α and TR β expression in the brain and intestine peaked at the climax of metamorphosis around stage 61/62 of the Nieuwkoop and Faber system (Nieuwkoop and Faber, 1956), which corresponds to stage 42 according to the Gosner system (Gosner, 1960) in *R. nigromaculata*. In the tail tissue, however, an obvious difference in TR α and TR β expression was observed between *R. nigromaculata* and *Xenopus*. Wang et al. (2008) reported that TR α and TR β

Fig. 2 – Phylogenetic relationships of TRs between *Rana nigromaculata* and other species. (a) TR α , (b) TR β . The numbers at each branch are the bootstrap values. The relevant amino acid sequences were obtained from the NCBI GenBank database: *Alligator mississippiensis* (ABD48806.1, ABD48808.1), *Gallus gallus* (NP_990644.1, NP_990778.1), *Anolis carolinensis* (XP_003222498.1, XP_003226266.1), *Eublepharis macularius* (BAF03080.1, BAF03081.1), *Rana catesbeiana* (Q02777.1, Q02965.1), *Xenopus laevis* (NP_001081595.1, NP_001090182.1), *Xenopus tropicalis* (NP_001039261.1, NP_001039270.1), *Monodelphis domestica* (NP_001184135.1, XP_001369854.1), *Homo sapiens* (CAA38899.1, NP_000452.2), *Sus scrofa* (NP_999355.1, XP_001928500.3), *Ovis aries* (NP_001094389.1, NP_001177320.1), *Mus musculus* (NP_835161.1, BAE21770.1), *Oncorhynchus kisutch* (BAE98175.1), and *Danio rerio* (NP_571415.1).

Fig. 3 – Relative expression levels of TRs in different tissues during *Rana nigromaculata* development. (a) liver, (b) intestine, (c) tail, (d) brain. Relative expression levels of TR α and TR β in four tissues were quantified by normalizing to the TR β level at stage 27 in the corresponding tissue. Data were shown as means $\pm$ SD (n: 8–10). The numbers on the dotted line (TR α ; TR β) indicate the fold changes of the TRs expression levels at stage 34 and stage 42 compared to those at stage 27.

expression increased dramatically from stage 58 to stage 64 in *X. tropicalis* tail. Opitz et al. (2006) also described a continuous increase in TR β expression and no change in TR α expression from stage 50 to stage 64 in *X. laevis* tail. In contrast to *Xenopus* tail tissue, we found that TR α and TR β expression in *R. nigromaculata* tail reached peak values at stage 42, followed by a marked decrease to the levels observed before stage 40. Our finding of the decrease in TR α expression after peaking at Gosner stage 42 in *R. nigromaculata* tail is consistent with a previous report concerning *R. catesbeiana* (Veldhoen et al., 2006b). In contrast to TR α expression, however, TR β expression in *R. catesbeiana* tail tissue was reported to increase continuously after this stage. Tail absorption in *Xenopus* occurs from NF stage 62 to stage 66, whereas in *R. nigromaculata* and *R. catesbeiana* it occurs from Gosner stage 42 to stage 46. We cannot explain the biological significance of this species difference in TR expression pattern in the tail tissue.

In spite of a rough similarity in the expression pattern, there were obvious differences in mRNA expression between TR α and TR β during *R. nigromaculata* development. In all tissues examined, TR β mRNA expression remained at a low level before stage 40, followed by a dramatic increase and peak at stage 42, and then decreased. In contrast to TR β , TR α expression exhibited large differences among these tissues, although TR α expression levels were relatively high in all tissues before stage 40 compared to TR β . In the brain, TR α expression maintained at a high level during the whole tadpole period, and only exhibited a weak increasing trend from stage 27 to stage 41/42, followed by a slight decrease toward the end of metamorphosis. In the liver, TR α expression increased progressively from stage 27 to stage 41/42. In the intestine and tail, TR α expression, like TR β expression, remained at a low level before stage 40, followed by a dramatic increase and peak at stage 42, and then decreased. To our knowledge, developmental expression

patterns of TR in tadpole livers have never been reported in previous studies. It is possible that the developmental expression patterns of TR α in the brain, liver, and intestine/tail represent respectively three typical expression patterns of TR α during *R. nigromaculata* development. Differences in the developmental expression pattern between TR α and TR β were also reported in *X. laevis* and *X. tropicalis* (Kawahara et al., 1991; Tata, 2006; Wang et al., 2008; Yaoita and Brown, 1990). In *Xenopus*, TR β expression is believed to be correlated to the TH levels rather than TR α expression because TR β expression parallels the change in the TH level (Kawahara et al., 1991; Yaoita et al., 1990). In a recent study, we described changes in the TH concentration in *R. nigromaculata* development, i.e. it maintains at a low level before stage 40, increases slightly at stage 40/41, followed by a dramatic increase and a peak around stage 42, and then decreases gradually (Ge et al., 2014). Thus, TR β expression in *R. nigromaculata*, like in *Xenopus*, is more correlated to the TH levels than TR α expression.

Previous studies have described TR expression patterns in several tissues (including the brain, limb, tail and intestine) during *X. laevis* and *X. tropicalis* development (Kawahara et al., 1991; Opitz et al., 2006; Wang et al., 2008; Yaoita and Brown, 1990), but the tissue-specific differences in the magnitude of TR expression change have never been noted, and the relationship between the magnitude of TR expression change and the responsiveness of TR expression to endogenous TH has not been discussed. In this study, we observed large tissue differences in the magnitude of the change in TR expression, in particular TR β expression, during *R. nigromaculata* development. TR β expression exhibited larger changes in the liver and intestine than in the tail and the brain. Considering the correlation of TR expression with TH levels, we suggest that in terms of TR expression, the intestine and liver might have higher responsiveness to rising TH levels than the tail and brain.

Fig. 4 – Effects of exposure to 1 nmol/L T3 for 48 hr on $TR\beta$ and $TR\alpha$ expression in the liver (A, a), intestine (B, b), tail (C, c), and brain (D, d) of *R. nigromaculata* at different stages. Data were shown as means $\pm$ SD (n : 10). Significant differences between T3 treatment groups and the controls were indicated by * (independent-samples T test, $p < 0.05$). All experiments were repeated twice with similar results.

3.3. Responsiveness of TR expression to exogenous TR agonist and antagonist

$TR\alpha$ and $TR\beta$ are involved in cell proliferation, differentiation and apoptosis to different degrees. The former is believed to play key roles in establishing tissue competence to respond to THs and in cell proliferation, whereas the latter functions in cell differentiation and apoptosis (Denver et al., 2009; Furlow and Neff, 2006). Expression of TRs (in particular $TR\beta$) has been used as a molecular biomarker for assaying TH signaling disrupting

actions in *X. laevis* because of their response to TH (Opitz et al., 2006; Veldhoen et al., 2006a). To validate *R. nigromaculata* as a native model species for assaying TH signaling disrupting effects in future study, in this study, we investigated the responsiveness of TR expression to an exogenous TR agonist (T3) and antagonist (AM) in *R. nigromaculata* tadpoles. In accordance with other amphibian species (Baker and Tata, 1992; Iwamuro et al., 2006; Jagnytsch et al., 2006; Helbing et al., 2006), $TR\beta$ expression had higher responsiveness than $TR\alpha$ expression to TH in *R. nigromaculata* tadpoles, showing that

Fig. 5 – Effects of exposure to amiodarone (AM) for 48 hr on TR β expression in the presence of 0.1 nmol/L T3 or absence of T3 in the intestine at stage 27 *Rana nigromaculata*. Data were shown as means $\pm$ SD (n: 10). Significant differences between AM treatment groups and the controls were indicated by * (one-way ANOVA, $p < 0.05$). All experiments were repeated twice with similar results.

TR β expression is a better endpoint than TR α expression for assaying TH signaling disrupting actions.

In addition, it was found that the responsiveness of TR expression to TH decreased with *R. nigromaculata* development. TR β expression had higher responsiveness to T3 in tadpole tissues at stage 27 than at stage 34, whereas the response disappeared in the tadpoles at the onset of metamorphic climax. The results are consistent with a previous *X. laevis* study by Opitz et al. (2006), who found that TR expression in head and tail tissues had a high responsiveness to TH in pre-metamorphic tadpoles, whereas the responsiveness decreased in pro-metamorphic tadpoles and strongly diminished in the tadpoles at early metamorphic climax. Opitz et al. (2006) explained that the decreases in the responsiveness to TH in *X. laevis* were likely to result from increases in endogenous TH plasma levels masking the effects of the exogenously added TH as well as from the increase in basal TR mRNA expression during metamorphic development. Further, we infer that the auto-induction of TR may be regulated by a feedback mechanism. When TR expression levels have peaked in tadpole tissues at stage 42, the tissues will no longer respond to TH induction. Before the TR levels in tadpole tissues reach the highest values, the tissues will respond to TH induction according to their needs for TR, i.e. pre-metamorphic tissues with lower TR expression have higher responsiveness, whereas pro-metamorphic tissues with higher TR expression have lower responsiveness. As shown in Fig. 3, both TR α and TR β expression levels in all tissues were higher in stage 34 tadpoles than in stage 27 tadpoles. Further studies are needed to clarify the molecular mechanism of developmental stage-dependent responsiveness of TR expression to TH. Considering the responsiveness, *R. nigromaculata* tadpoles at stage 27 are more suitable than stage 34 tadpoles as test materials for assaying TH signaling disrupting effects.

In previous studies, tissue-specific responsiveness to TH was observed in *X. laevis* (Kawahara et al., 1999; Iwamuro et al., 2003). Kawahara et al. (1999) reported that in terms of TR expression, the intestine was more sensitive to TH than tail, liver and limb tissues. In this study, we also found

tissue-specific responsiveness to TH in *R. nigromaculata*. At the same developmental stage, the responsiveness of TR β expression to TH ranked in the order of liver $\approx$ intestine > tail > brain (Fig. 4). The order of the responsiveness to TH corresponds to the order of the magnitude of the change in TR expression during *R. nigromaculata* development. In other words, the responsiveness of TH-response genes to exogenous TH might be coincident with that to endogenous TH. By comparing tissue-specific differences in the responsiveness to TH, we suggest that liver and intestine tissues are more suitable than tail and brain tissues as test materials for *in vivo* assay of TH signaling disrupting effects. Certainly, tail tissue can be used in *in vitro* study due to its advantage of easy culture *in vitro* (Eto et al., 2012; Tseng et al., 2007). During *R. nigromaculata* development, the intestine undergoes a dramatic morphological change, but the liver hardly changes in morphology. Moreover, the liver tissues at stage 27 are so small that they are difficult to collect. Thus, the intestines of *R. nigromaculata* are more ideal tissues for assaying TH signaling disrupting effects because their morphological changes could provide more information concerning thyroid disruption.

4. Conclusions

R. nigromaculata can be used as a supplement to *X. laevis* to assay TH signaling disruption due to its high responsiveness to TR agonist/antagonists and easy accessibility, in particular in East Asia. Given their developmental stage-specific and tissue-specific responsiveness, intestinal tissues of *R. nigromaculata* at stage 27 are ideal test materials for assaying TH signaling disruption with comprehensive information.

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