Pakistan J. Zool., vol. 44 (5), pp. 1215-1223, 2012.

Isolation and Characterization of Thyroid Hormone Receptors (*TRα* and *TRβ*) in Black Rock Fish, *Sebastes schlegelii*

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Abstract.- Thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) play a vital role for developmental and physiological functions in vertebrates. They are mediated via thyroid hormone receptors (TR). In the present paper we describe the cloning and characterization of two TR genes, thyroid hormone receptor alpha (*TRα*) and thyroid hormone receptor beta (*TRβ*) from ovary mRNA of a ovoviviparous fish *Sebastes schlegelii*. Results revealed that main domains and motifs of the two genes were highly conserved. Quantitative real time PCR showed that the mRNA expression levels of both TRα and TRβ were high at 5 dps and slightly lower and constant during 10, 20 and 33 dps. In 105 days old juveniles and three years mature fish, the expression level was higher in gonads, brain, liver and kidney than in other tissues. The highest expression was observed in the gonads. The developmental and tissue expression patterns indicated their involvement in the growth and gonadal development. TRα exhibited higher expression than TRβ at all developmental stages and in all detected tissues except female liver, which might indicate the functional differences of these two genes.

Key words: Thyroid hormone receptor, *Sebastes schlegelii.*

INTRODUCTION

In vertebrates many developmental and physiological functions are controlled by thyroid hormones (THs) which are pleiotropic factors and their effects are mediated through thyroid hormone receptors (TRs). The thyroid gland produces triiodothyronine (T_3) and thyroxine (T_4) which regulates the cellular function of growth, development, metabolism, differentiation and the maintenance of homeostasis (Dauncey, 1990; Brent 1996; Chin and Yen 1997; Power *et al*., 2001; Szisch *et al*., 2005). In newly born fish the THs have profound role in the growth and its survival (Yamano and Miwa, 1998; Masafumi *et al*., 2008; Kang and Chang, 2004; Lema and Nevitt, 2006; Matta *et al*., 2002; Swapna *et al*., 2006).

The THs show their actions by genomic pathway or through non-genomic pathway with a few of them are through TRs (Oetting and Yen, 2007; Davis *et al*., 2008). TRs include a part of superfamily of nuclear receptors which are identified by regions or domains for DNA binding and ligand-binding. They are separated by hinge, regions which may target the receptor to the nucleus (Evans, 1988; Lazar and Chin, 1990). TRs act as ligand-dependent transcription factors (Gronemeyer and Laudet, 1995). This superfamily also possesses steroid hormone receptors, vitamin D, retinoid receptors and orphan receptors. TRs generally act as homodimers or heterodimers with a retinoid X receptor (RXR) partner and control transcription of target genes (Marchand *et al*., 2001). Although heterodimers are generally considered as non-permissive in some particular cellular environments, RXR agonists can bind and activate transcription (Castillo *et al*., 2004; Li *et al*., 2004; Shulman *et al*., 2004).

Nuclear receptors have conserved domains which are important for their characterization. Particularly, the DNA-binding domain (DBD) and the ligand-binding domain (LBD) are highly conserved among vertebrate species (Nelson and Habibi, 2006). The DBD associates with nuclear proteins at the thyroid response elements (TREs) in order to regulate TR gene activation or repression (Harvey and Williams, 2002) and heterodimerization strongly increases binding to the TRE and transcriptional activity (Ikeda *et al*., 1994; Mangelsdorf and Evans, 1995).

Diverse isoforms of TR genes (*TRα*1, *TRα*2, *TRβ1*, *TRβ2, TRαa*, *TRαb*, *TRβa* and *TRβb*) have

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0030-9923/2012/0005-1215 $ 8.00/0
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been isolated from human (Weinberger *et al*., 1986), rat (Thompson *et al*., 1987), and fishes (Yamano *et al*., 1994; Liu *et al*., 2000; Marchand *et al*., 2001; Kawakami *et al*., 2003a, 2008; Nelson and Habibi, 2006, 2009; Masafumi *et al*., 2008; Galay-Burgos *et al*., 2008; Harada *et al*., 2008; Manchado *et al*., 2009; An *et al*., 2010). All these genes and isoforms can regulate various functions and thus it is significant to control their tissue-specific and larval development patterns (An *et al*., 2010).

Until now the *TRα* and *TRβ* of *Carassius auratus*, *Paralichthys olivaceus*, *Danio rerio* and *Salmo salar* have been cloned (Yamano and Inui, 1995; Liu *et al*., 2000 Marchand *et al*., 2001; Nelson and Habibi, 2006). Maitra and Bhattacharya (1989) have reported the presence of TRs in the piscine oocyte nuclei. It has also been reported by Nelson and Habibi (2006, 2009) that TR subtype expression level is variable during reproductive maturity; hence it is considered that THs are involved in reproduction. Similar findings have also been observed in fathead minnow that the expression level of TR is higher in mature comparative to immature gonads, which also suggest the involvement of TR in testicular and ovarian development (Filby and Tyler, 2007). The expression of TRα in *P. olivaceus* is reported to be found ubiquitous and constant during developmental stages while expression of TRβ is demonstrated in late stages or is tissue specific during metamorphosis (Yamano and Miwa, 1998).

The black rockfish, *S. schlegelii,* is a commercially important species in marine culture. Although artificial hatching and large scale aquaculture has been very successful for this ovoviviparous species, little is known about its thyroid hormone receptors for growth and developmental impact. In the present work we report the cloning of *TRα* and *TRβ* in the mature black rockfish from ovaries. We also describe their expression patterns in various tissues of immature and mature fish as well as in fry and juveniles in order to understand the function of these genes.

MATERIALS AND METHODS

Experimental fish

The experimental fish black rockfish *S. schlegelii* were brought from a commercial rockfish hatchery in Yantai city, China. Whole body samples of different developmental stages including fry at 5 days post spawn (dps), 10 dps, fingerlings at 20 dps and 33 dps were collected. Three individuals each of 3-year-old mature female and male as well as juveniles at 105 dps (sex undefined) were sacrificed, and different organs such as gonad, liver, spleen, kidney, brain, heart, muscle, gill, intestine tissues were dissected out and snapped frozen in liquid nitrogen. The samples were stored at −80°C until the total RNA was extracted for analysis.

RNA isolation and synthesis of the first strand cDNA

Total RNA from whole body of each different developmental stage as well as the sampled tissues of 105 dps juvenile and mature adult fish were isolated separately using Trizol (Invitrogen) reagent and treated with DNase I (TaKaRa) following the protocol of the manufactures. The purified samples were resuspended in RNase free water and stored at -80°C until use. The quality of the RNA was evaluated by 1.2% agarose gel electrophoresis and spectrophotometric measurement.

The first strand cDNA was synthesized from 2μg of total RNA by Moloney Murine Leukemia Virus reverse transcriptase (TaKaRa, Shiga, Japan) in a total volume of 25 μl at 30°C for 10 min with Oligo-dT15 primer (Tiangen) following the protocol of the manufacturer.

Gene cloning and sequencing

For cloning of TRα and TRβ core sequences, we designed a pair of degenerated primers based on the highly conserved regions of other known species (TRα *Acanthopagrus schlegelii* (ABQ96861), *Amphiprion melanopus* (ACH43023), *Pseudopleuronectes americanus* (AAV66919) while for TRβ following sequences were used, *Acanthopagrus schlegelli* (AB96862), *Amphiprion melanopus* (ACH43022), *Paralichthys olivaceus* (BAA08201) *Ephinephelus coioides* (ABP 62962)). The PCR was performed for 35 cycles at 94°C (30 s), 50°C (30s), and 72°C (30s) using a PTC-Peltier thermal cycler (Biorad) with primers TRα-F and TRα-R and TRβ-F and TRβ-R, respectively. The partial product size of TRα was 610bp and TRβ was 531bp. The obtained PCR products were separated by 1.2%

agarose gel electrophoresis and purified using PCR purification kit (Omega Bio-tech). The purified products were ligated to pEASY-T1 vector (TransGen Biotech, Beijing) using ligase (TaKaRa Dalian, China). Competent cells of *E. coli* Trans5a strain (TransGen Biotech) were transformed with the recombinant DNA. The recombinants were screened by colony PCR with M13 primers and sequenced with ABI 3730 DNA analyzer (Applied Biosystems). The obtained sequences were analyzed by the Blast program (ttp://blast. ncbi. nlm. nih. gov) with other known sequences.

5' and 3' RACE-PCR

The gene specific primers (GSP) were designed from the obtained partial sequences for the 3'RACE and 5'RACE. The remaining unknown regions of the cDNAs were obtained by 3'RACE reactions with primer TRα-3' (sense) and TRβ-3' (sense), and by 5'RACE reactions with primer TRα-5' (antisense) and TRβ-5' (antisense), respectively (Table I). The PCR conditions for the 3'RACE of TRα was as follows: 1 cycle of denaturation at 95°C for 5 min, denaturation at 95°C for 30s, annealing at 55□for 30s, and extension at 72°C for 30s, followed by 1 cycle of 7 min at 72°C for the final extension. The 3'RACE PCR for TRβ was performed following the above procedure with the annealing temperature being set to 60°C. The PCR conditions for the TRα 5'RACE are as followed1 cycle of denaturation at 95°C for 5 min, denaturation at 95°C for 30s, annealing at 57°C for 30s, and extension at 72°C for 50s, followed by 1 cycle of 7 min at 72°C for the final extension, while for the TRβ 5'RACE the annealing was at 60°C for 60s. The ligation and transformation methods conducted as mentioned above.

Phylogenetic analysis of TRα and TRβ

The amino acid sequence alignments of the deduced TRα and TRβ proteins were conducted using Clustal X1.81. The GenBank Accession No.s of sequences selected for alignment are as follows: *Homo sapiens* TRα (AAA66021), TRβ (P10828), *Mus musculus* TRα (36241), TRβ (AAB20226), *Rattus norvegicus*, TRα (AAA42238), TRβ (AAA40916), *Gallus gallus* TRα (CAA68792) TRβ (CAA35544), *Sebastes schlegelii,* TRα (00124), TRβ (78644), *Acanthopagrus schlegelii* TRα (ABQ96861), TRβ (ABQ96861), *Amphiprion melanopus* TRα (ACH43023), TRβ (ACH43022), *Solea senegalensis* TRαa (BAF91725), TRb (BAF91726), *Paralichthys olivaceus* TRαa (BAA03928), TRβ(BAA08201), *Epinephelus coioides* TRαa (ABP6296), TRβ (ABP62962), *Salmo salar* TRα (NP_001117100), TRβ (NP_001117172), *Carassius auratus* AAX84685, TRaa1, TRβ (AAX84684), *Danio rerio* TRα (XP_001921013), TRβ (XP_692982). The phylogenetic tree was constructed by neighbor-joining method with the MEGA. 4 based on the position corrected distances. One thousand bootstraps were performed for the NJ trees to estimate for the topological stability.

Table I.- Primers used in this study.

Name of primers	Sequence of primers
TRα-F	5'-GTGTGCGGRGAYAARGCSACB-3'
TRα-R	5' CTCMGAGAACATGGGYARYTT 3'
TRβ-F	5' GAAACCAGTGCCAGGAATGT 3'
TRβ-R	5'CTGGATCATAGCGAACAGCA3'
TRα-3'	5' TTGGTGAAGACGTTACAGACGAGG 3'
TRα-5'	5' GCTGCCTTGTGAAGACCA 3'
TRβ-3'	5'TAAGCT GGATGG AGG TTCTTC TGG 3'
TRβ-5'	5'CAA GTC GGT TGC CAT TCC 3'
UPM	5'CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT 3'
TRα-RT-F	5'GAA CCG ACA GAA GAG GAA G 3'
TRα-RT-R	5'TGG TGA ACT CGC TGA AGG C 3'
TRβ-RT-F	5'TGG GAA TGG CAA CCG ACT 3'
TRβ-RT-R	5'TCA CCA TAC GGA TGA GGA 3'
18S-F	5'GGT CTG TGA TGC CCT TAG ATG TC 3'
18S-R	5'AGT GGG GTT CAG CGG GTT AC 3'

Quantitative real-time PCR

Gene specific primers for real-time PCR TRα (TRα-RT-F and TRα-RT-R) and of TRβ (TRβ-RT-F and TRβ-RT-R) were designed across the putative exon-intron borders to avoid amplification of genomic DNA (Table I). A pair of primers for 18S rRNA gene, 18S-F and 18S-R (Table I), was designed and used as internal normalizer. All real-time quantitative RT-PCR were performed in triplicates on a Prism ABI7500 Sequence Detection System (Applied Biosystems, Forster City, CA). The reactions were performed in a total volume of 20 μl

containing 10 µl 2x SYBR premix Ex Taq (TaKaRa), 1 µl cDNA, 0. 8 µl each of forward and reverse primers, 0.4 µl Rox reference Dye II (50×) and 7 µl PCR graded water. The qRT-PCR program was 60°C for 5 mins; 95°C for 10 mints, followed by 35 cycles of 95°C for 30s, 60°C for 30s, 72°C for 30s. Negative controls (no-template reaction) were always included. As an internal control, 18S rRNA was selected as a normalizer to balance the differences in RNA abundance (Zhong *et al*., 2008). All data were compared as the change with respect to the corresponding 18s calculated threshold cycle (Ct) levels. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified.

Statistical analysis was performed by one-way analysis of variance (ANOVA) with SPSS13.0 software. A probability value of $P<0.05$ was considered to indicate statistical significance.

RESULTS

With RT-PCR and subsequent 5' and 3'RACE the full length cDNA sequences of *S. schlegelii TRα* and *TRβ* genes (namely *ssTRα* and *ssTRβ*) were obtained that are the genes sequence numbers JQ409563(alpha) and JQ409564(Beta). The deduced amino acid sequence of ssTRα showed high similarities with those of other fish species including *A. schlegelii* (91%), *P. americanus* (90.4%), *A. melanopus* (89. 2%), *L. petersii* (88.6%). *S. salar* (86.3%), *D. rerio* (85.8%), *H. sapiens* (78%). The ssTRα has two domains like other TRα proteins. The first was the DNA binding domain (DBD) and second ligand binding domain (LBD). DBD began from 60 residues and ended at 135 residues while LBD started from 163 to 383 residues (Fig. 1). TRα DBD showed conservation from 75-90%, while LBD exhibited 87-96 % similarities when they were compared with other vertebrates' TRα.

The ssTRβ showed very high similarities with TRβ sequences of *E. coioides* (97.9%), *A. melanopus* (97.2%), *P. olivaceus* (97.4%), *A. schlegelii* (96.9%), *S. senegalensis* (96.4%), *D. rerio (*94%), *S. salar* (92.6%), *C. auratus* (91.2%), *H. sapiens* (84.8%). Like TRα, two domains in TRβ are highly conserved. ssTRβ DBD started from 32 to 99 residues and showed the conservation of 89-97% with other vertebrates, while LBD starts form 183 to 362 and exhibited the conservation of 88-98% with other vertebrates (Fig. 2). TRβ showed higher conservation as compared to TRα.

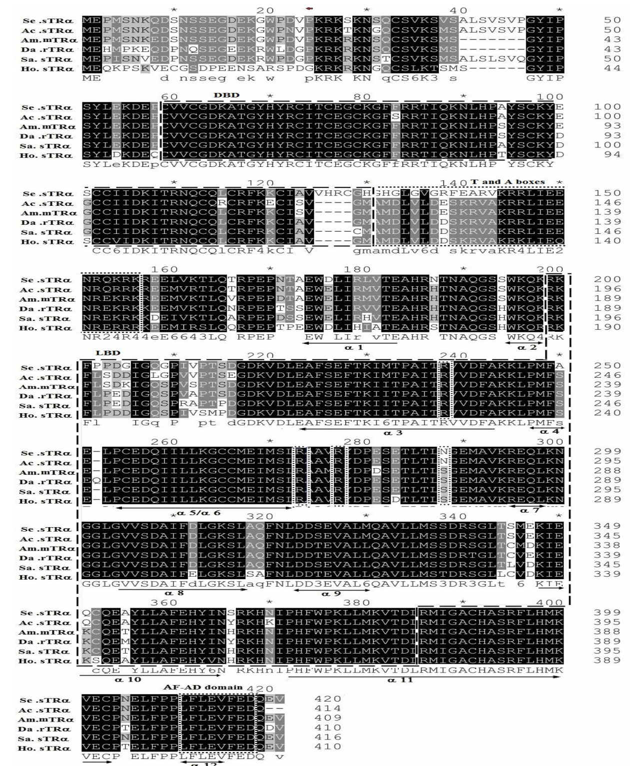

Fig. 1. Alginment of amino acid sequence of *S. schlegelii* TRα compared with *Acanthopagrus schlegelii* TRα (Ac sTRα), *Amphiprion melanopus* TRα (Am mTRα), *Danio rerio* TRa (Da rTRα), *Salmo salar* TRα (Sa sTRα), *Homo sapiens* TRα (Ho sTRα). The DBD, LBD, T and A boxes and AF-AD domains are separated by boxes. The 12 highly conserved helices are indicated by arrows (α1-α12).

Amino acids which are directly related to the THs were also conserved in *S. schlegelii.* These amino acids directly bind with ligand in three dimensional forms. In ssTRα these were Arg at the positon of 238, 272, 276 and Asn at the position of 287, respectively. In the ssTRβ these residues were at the position of Arg 216, 250, 254, and Asn at 265, respectively. All these residues in TRα and TRβ were well conserved in vertebrates.

The ssTRα and ssTRβ have well conserved

DBD, T and A boxes which play a vital roles in DNA dimerization and formation of a long -helix. ssTRα and ssTRβ LBD form a long helix. They are highly conserved in all investigated. Particularly helices 3, 5, 6, 11, 12 and two C-terminals are strongly conserved. At the end of C-terminal the AF2-AD domain is well conserved. Like in other fish TRβ the ssTRβ had an additional 9-amino acid insertion as compared to the human and amphibian TRβ.

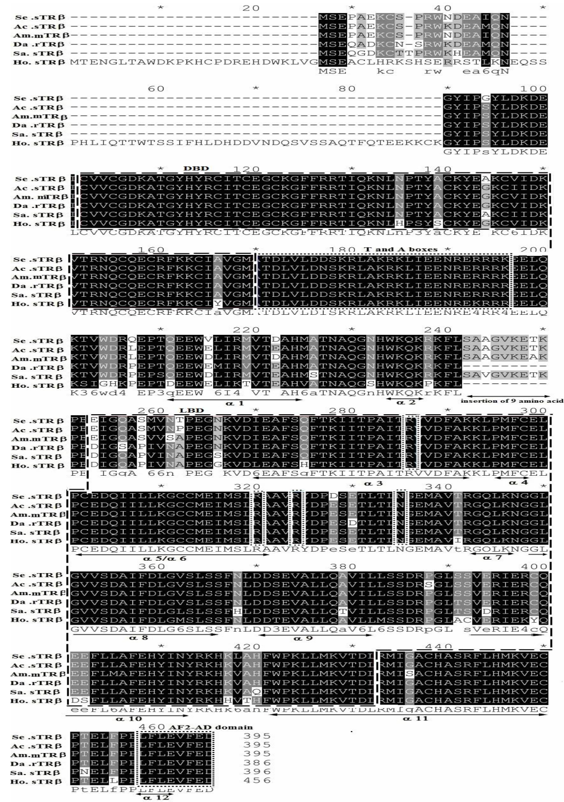

Fig. 2. Alignment of amino acid sequence's Sebastes schlegelii TRb were compared with *Acanthopagrus schlegelii* TRβ (Ac sTRβ), *Amphipirion melanopus* TRβ (Am mTRβ), *Danio rerio* TRβ (Da rTRβ), *Salmo salar* TRb (Sa sTRβ), *Homo sapiens* TRb (Ho sTRβ). The DBD, LBD, T/A boxes, and AF-AD domains are separated by boxes dashes lines and dots and residues also indicated by dots. The 12 highly conserved helices are indicated by arrows (α1-α12)

Phylogenetic analysis

The phylogenetic analysis showed that TRα and TRβ were the different genes because they were found on different sides of clusters. The phylogenetic tree revealed that both TRs of *S. schlegelii* were closely clustered first to teleost TR, and then linked to the other vertebrate cluster that was grouped together separately in phylogenetic tree (Fig. 3).

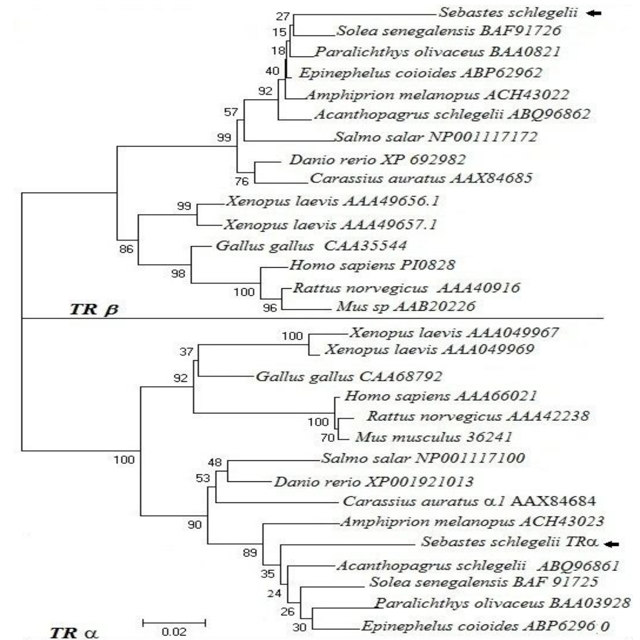

Fig. 3. The phylogenetic tree constructed by neighbor-joining method showing the relationship of *S. schlegelii* TRα and TRβ with other known vertebrate TRs. The sequences of *S. schlegelii* TRs are indicated by arrows. The names and the accession numbers of the amino acid sequences sleected from the GenBank are written in tree. Boostrap values (%) indicate 1000 replicates and the scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site.

Expression of ssTRα and ssTRβ

To analyze the expression of *ssTRα* and *ssTRβ*, quantitative real-time PCR was performed in early larval stages and in various tissues of immature and mature fish. During early developmental days, analysis using the mRNA of whole body exhibited that the expression of *ssTRα* and *ssTRβ* were detected in all stages of 5 dps, 10 dps, 20 dps and 33 dps at different levels. The expression of two genes was relatively high at the 5 dps but decreased significantly ($P<0.05$) at 10 dps, 20 dps and 33 dps. The expression level of *ssTRα* was always higher than *ssTRβ* at all detected stages (Fig. 4A). In the

juvenile of 105 dps the *ssTRα* and *ssTRβ* mRNA expression level varied in terms of folding among various tissues. The expressions were high in gonads, kidney, brain and liver while low in the rest of tissues. The difference was statistically significant (P<0.05). Like in the larvae stages *ssTRα* exhibited higher expressions than *ssTRβ* in all tissues assayed (Fig. 4B). In three year old males and females the expression of *ssTRα* showed some variations among tissuesGonads, kidney, brain and liver exhibited higher expression than the other tissues with the highest level being observed in female gonad and male brain (Fig. 4D). The *ssTRβ* also showed significantly higher expression in gonads, kidney, brain and liver than in other tissues with bigger variation among tissues than *ssTRα* in both the males and females. The highest expression was also observed in the ovary (Fig. 4C). Overall, the expression of *ssTRβ* was lower than *ssTRα* in almost all tissues detected. The only exception was in male liver where the expression of *ssTRβ* was slightly higher than *ssTRα* (Fig. 4).

DISCUSSION

TR genes have been investigated in number of other teleost species such as Japanese flounder (Yamano and Miwa, 1998), conger eel (Kawakami *et al*., 2003a), sea bream (Nowell *et al*., 2001), coho salmon (Harada *et al*., 2008); Atlantic halibut (Galay-Burgos *et al*., 2008), bluefin tuna (Kawakami *et al*., 2008) Senegalese sole (Manchado *et al*., 2009), black porgy (An *et al*., 2010). Here we cloned TRα and TRβ from gonad of *S. schlegelii* and described their expressions at various developmental stages and in various tissues in order to understand the potential role of thyroid hormone during the development, growth and maturation of this ovoviviparous rock fish. Our ssTRα showed 79% to 91% identity while TRβ denoted 80 % to 97% identities with aligned species. To the best of our knowledge this is the first charactorization of TR genes in Sebastinae subfamily.

TRα and TRβ in *S. schlegelii* denoted five domains which are conserved with other known teleots species. Marchand *et al.* (2001) named these domains as N-T (N-terminal), HD (Hinge domain), DBD (DNA binding domain), LBD (Lignad binding domain) and C-T (C-terminal), however Wu *et al.* (2007) described that N-T's TR specific motif are present in chordates in the variable A/B domain (Figs. 1, 2). *S. schlegelii* TRs exhibited high conservation when the domains were compared other vertebrates.

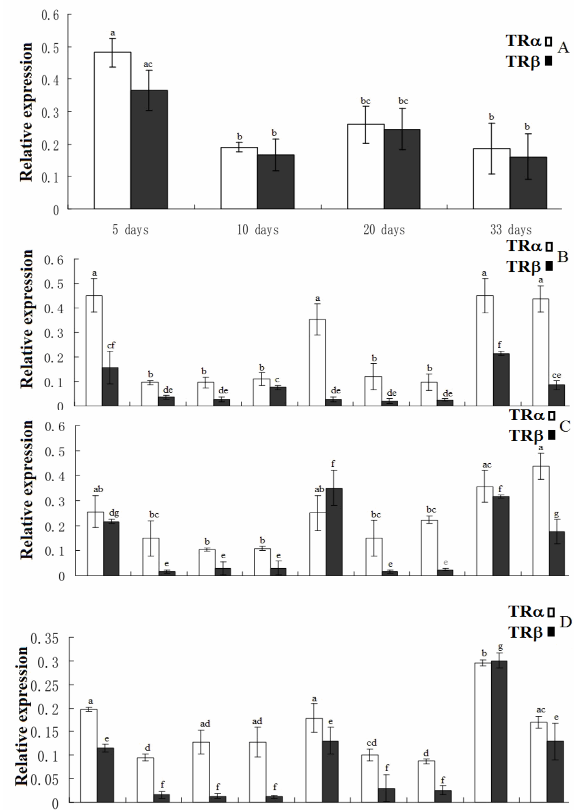

Fig. 4. Quantitative real-time PCR analysis of TRα and TRβ expression at different developmental stages (A), in various tissues of juveniles (B), and in tissues of mature female (C) and male individuals (D). Values sharing different letters are significantly different ($p<0.05$) from each other. Values are expressed as means ± SD (n = 3)

In *S. schlegelii* an insertion of 9 amino acids in TRβ. (SAAGVKETK) was noted at the hinge domain. This insertion has been nominated in most other teleosts such as *A. schlegelii (*SAAGVKETK) *A. melanopus* (SAAGVKEAK), *S. salar (*SAVGVKETK), and *D. rerio* has no this insertion (Fig.2). The TRβ transactivating activity is considered ligand-dependent and is repressed in

absence of T3 (Liu *et al*., 2000). The DNA binding and transactivation potential of this insertion on TRβ revealed a low impact on T3 binding affinity. Thus the function of the amino acids remains unclear (Marchand *et al*., 2001). It is note worthy that TRα has no signature of such amino acids insertion. The phylogenetic analysis distinctly showed two clusters, which demonstrates that TRα and TRβ respectively joined to their teleost counterparts. These two genes showed the linkage of early gene duplication occoured in early vertebrate evolution (Laudet, 1997).

In *S. schlegelii* TR genes expressions were found ubiquitously among investigated tissues and this has been reported in other teleost fishes (Nowell *et al*., 2001; Kawakami *et al*., 2003a). TR genes' expressions in *S. schlegelii* were noted high in kidneys, liver, gonads and brain. In *H. hippoglossus* TRβ was present in all tissue but varied among tissues investigated (Galay-Burgos *et al*., 2008), whereas in *S. aurata* the transcript level was reported similar in skeletal muscle, heart, intestine, brain, kidney, liver, and gill (Nowell *et al*., 2001). It is also noticeable that the expression of the two TRs in *S. schlegelii* were found at all developmental stages from 5 days to 33 days after birth, but at 5 days the level was significantly higher than the later stages. These results might suggest the functional differences of gene at deferent developmental stages. Having the developmental expression of TRα and TRβ in *S. Schlegelii* suggested that both of the genes are involved in the early development and larvae growth, while tissue specific expression patterns in juvenile and mature fish, especially the higher transcription levels in gonad (ovary and testis), brain and liver might suggest their probable involvement in the maturation, behavioral and metabolic functions in *S. schlegelii*. The fact that the comparative expression of TRα was always higher than TRβ at all developmental stages and in all tissues investigated suggested the possible difference between these two genes in this species. Keeping in view the present results we are here able to suggest future experimental focuses for specific tissue investigation by in situ hybridization, whole mount in situ hybridization and immuno-histochemical assays for the better understanding of their functions in *S. schlegelii*.

ACKNOWLEDGEMENTS

This work was supported by the National High-Tech Research and Development programme (2012AAI 00802) and the National Natural Science Foundation of China (31172385). The first author is thankful to China Government Scholarship Council for award of Ph.D. research scholarship.

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(Received 23 March 2012, revised 20 April 2012)