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Molecular cloning, characterization and expression pattern of androgen receptor in *Spinibarbus denticulatus*

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ABSTRACT

Androgens play key roles in sex differentiation, gonadal maturation and reproductive behaviors and their actions are generally mediated through androgen receptor (AR). In the present study, isolation, sequencing and characterization of cDNA encoding AR and its temporal and spatial expression profiles in both sexes of *Spinibarbus denticulate* were carried out. Androgen receptor of *Spinibarbus denticulate* (sdAR) was 3172 bp in length and encoded a 95.4 kDa protein of 865 amino acids. Phylogenetic analysis and multiple amino acids sequence alignment indicated the close relationship and high score similarity of sdAR with ARs of other cyprinid species. A single transcript of approximate 3.2 kb was identified in testis, liver and brain. RT-PCR assay characterized that sdAR mRNA was broadly distributed in both central nervous system (CNS) and most of peripheral tissues in male fish, while was confined to olfactory, telencephalon and hypothalamus of CNS and peripheral tissues including liver, spleen, head kidney, heart, and red muscle in females. During the embryonic development, sdAR mRNA was firstly detected at 16-cells stage and mid blastula stage with very weak signal. Little or no signal was detected in mid gastrula and neurula stages. The expression was occurred in the following developmental phases as well as in larvae of 4 days post hatching. During gonadal recrudescence process, liver of both sexes and testis were the most AR mRNA abundant tissues. In male fish, abundance of sdAR mRNA significantly varied in pituitary at fully recrudesced stage and brain at late recrudescing phase, respectively. No significant variation was found throughout the ovary recrudesce in each tissue checked. Our present work provided preliminary evidences that AR mediated androgen action on reproduction and development in both sexes of *S. denticulate*.

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1. Introduction

Androgens play important roles in development, maintenance, and regulation of male phenotype and reproductive physiology by exerting their effects via binding and activating androgen receptor (AR) (Quigley et al., 1995). AR belongs to nuclear receptor super-family, with signature composition of highly conserved DNA binding domain (DBD), ligand binding domain (LBD) and N-terminal localized variable transactivation domain (TAD). The receptor is induced by hydrophobic ligands of testosterone (T) and dihydro-testosterone (DHT) to assume a transcription-activated (or inhibited) configuration and allows transmission of extracellular signals into intracellular responses by targeting promoter response elements and recruiting related cofactors (Black and Pascha, 2004).

Until recently, AR has been identified and characterized in a number of teleosts, among which, two types of ARs have been found in species including Atlantic croaker (Sperry and Thomas, 1999b), Japanese eel (Ikeuchi et al., 1999), rainbow trout (Takeo and Yamashita, 1999) and so on. Generally, each type of AR displayed different expression pattern and binding characteristics. Sperry and Thomas (1999a) isolated two ARs (kbAR1 and kbAR2) in kelp bass and demonstrated that kbAR1 was only detected in the brain, but kbAR2 was distributed in both brain and ovary. The distinct binding affinities of the two kbARs to different ligands were further characterized as that kbAR1 could only bind T with high affinity, while kbAR2 was of high affinity with not only T, but also DHT, mobolerone, methyltestosterone (MT) and 11-ketoetstosterone (11-KT) using comparative binding test.

Two-whole genome expansion in ray-finned fishes was known as the cause for gene duplications (Wittbrodt et al., 1998; Thornton, 2001). However, two types of AR were not popularly found

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in all teleosts. Instead, only one AR has been identified in cyprinid species like goldfish (Pasmani and Callard, 1988) and zebrafish (Jørgensen et al., 2007) so far. Though a potentially duplicated gene with 99% identity to the zebrafish AR (zfAR) had been recently discovered on chromosome 14 by blastn of the zfAR nucleotide sequence in the zebrafish genome (Jørgensen et al., 2007), both cloning work and future functional studies of this deduced zfAR is further required.

Spinibarbus denticulatus belongs to Cyprinidae, Barbinae, *Spinibarbus*, and is a local economically important fish in China. We previously investigated the expression pattern of four estrogen receptors (ERs) during the male reproductive cycle in *S. denticulatus* (Zhu et al., 2008). In the present study, AR cDNA was isolated and its temporal and spatial expression patterns were characterized in both male and female fish so as to provide preliminary clues for the advanced studies on different function and mechanism of androgen in the reproductive physiology of each sex of *S. denticulatus*.

2. Materials and methods

2.1. Animals and sample collection

Spinibarbus denticulatus were obtained from Zhuhai Jinni Fishery Science and Technology Company, LTD, where fish were well-cultured in pond under natural conditions. Fertilized eggs, embryos and larval *S. denticulatus* were obtained from April to May 2005, during reproductive season. Six fish of each sex at three different stages of reproductive cycle were obtained, respectively, from January to October 2004 ($6 \times 3 = 18$ fish in total). Fish were anesthetized and sacrificed by decapitation. Tissues for gene cloning, distribution and quantitative real-time RT-PCR were sampled and immediately frozen in liquid nitrogen and stored at −80 °C. During each sampling, gonads were weighed to determine the gonadosomatic index (GSI = [gonad weight/body weight] × 100). Small pieces of middle portion of the gonads were excised and fixed in Bouin's solution for routine histological identification of reproductive stages. All the animal experiments were in accordance with the guidelines and approval of Sun Yat-Sen University Animal Care and Use Committee.

2.2. cDNA library construction

Brain mRNA of sexually mature *S. denticulatus* was isolated using FastTrack®2.0 Kit (Invitrogen, USA), 5 μg of which was used to construct a cDNA library by ZAP Express® cDNA Synthesis Kit and ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene, USA) according to the manufacturer's instructions.

2.3. PCR screening of brain cDNA library for sdAR

The full length sdAR was cloned from brain cDNA library by piecing together partial and 5′ and 3′-end sequences using a similar PCR program of 94 °C 3 min, 40 cycles 94 °C 15 s, 50 °C 15 s, 72 °C 1–3 min in the PTC-200 thermocycler (MJ Research, Watertown, MA, USA). As Fig. 1 displayed, two partial cDNAs of sdAR were obtained, respectively, using degenerate primer pairs, which were based on the conserved AR cDNA sequences from goldfish (AY090897) and zebrafish (NM 001083123). Then, the complete sdAR cDNA sequence was amplified by combining the gene-specific primers and 5′ or 3′-adaptor primers of the library. To avoid PCR amplification mismatches and confirm the authenticity of the pieced sequences, an independent PCR were carried out using the primers designed around start and stop codon of sdAR. All the primer pairs used were listed in Table 1. Products of the predicted size were gel-separated and purified with E.Z.N.A® Gel Extraction Kit (Omega BioTek, USA) and inserted into pTZ57R Vector (MBI Fermentas, USA). Positive clones containing the expected size inserts were sequenced using M13F and M13R primers.

2.4. Sequence analysis

All the sequenced partials were pieced together by DNAssist Version 2.0 (Patterton and Graves, 2000). The potential open reading frames were analyzed using DNAtools6.0 (Rasmussen, 2001) and translated to the corresponding amino acids. The cDNA sequence and the deduced amino acid sequence were compared with

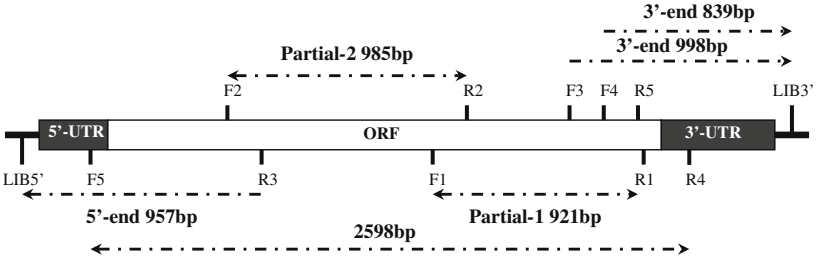

Fig. 1. Cloning strategy for isolating the sdAR cDNA using RT-PCR. Relative position of each primer and purposes of PCR products and the predicted lengths were shown. Primer pairs of F4 and R5 (265 bp) were used for both qualitative and quantitative RT-PCR and amplifying probe for Northern hybridization.

Table 1
Sequences of PCR and hybridization primers.

Primer	Sequences (from 5′ to 3′)	Primer	Sequences (from 5′ to 3′)
F1	GAGCTGCTGAGGGGAAGCAG	R1	TCATTTCYGGAAAGCTGACC
F2	GCCTTTGCTSRACTGCTCAGTGAG	R2	GGTGCAGTCATTCCTGCTAG
F3	AGGTCCTACAAGAACGCCAACG	R3	GTCACCTCACCTGCTAGCTG
F4	CTCTGCATGAAGGCCCTCCTC	R4	AGGCTGGTTTAGGCTGGATC
F5	CTCTGCGCTAGCAGGAATGACTG	R5	CAGGAAAGCTGACCTTCGTG
LIB5′	CAGGAAACAGCTATGACCTTG	LIB3′	GGGCGAATTGGGTACACTTA
18sF	CCTGAGAAACGGCTACCACATCC	18sR	AGCAACTTTAGTATACGCTATTGGAG

Mixed Bases: Y: C/T R: A/G M: A/C S: G/C H: A/C/T K: G/T.

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