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### Molecular cloning, characterization, and gene expression of the androgen receptor in the large yellow croaker, *Larimichthys crocea*

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Abstract Androgens mediate a wide range of physiological responses and developmental processes in vertebrates, involving both reproductive and nonreproductive systems. The activity of androgens is mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily. In this study, an AR gene was cloned from the large yellow croaker (Larimichthys crocea) for the first time. qRT-PCR revealed ubiquitous expression of AR in all adult tissues examined, with higher expression in the gonad and liver of both sexes and highest expression in the blastula stage of embryonic development. Using in situ hybridization, we detected positive signals of AR in the spermatogonium, spermatocyte, spermatid, and spermatozoon during spermatogenesis, in the cytoplasm of all oocytes during oogenesis and in the follicle cells of stage IV oocytes. Our findings support

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Department of Biological Science, Seton Hall University, South Orange, NJ 07079, USA e-mail: ziping.zhang@shu.edu the important role that AR plays in gametogenesis, gonadal development, and the early stages of embry-onic development.

**Keywords** Larimichthys crocea  $\cdot AR \cdot$ Gametogenesis  $\cdot$  Gonadal development  $\cdot$ Embryonic development

#### Introduction

Androgens are intimately associated with the development, differentiation, maturation, and maintenance of the vertebrate reproductive system. In the male vertebrate, androgens mediate a wide range of physiological responses and developmental processes, involving both reproductive and nonreproductive systems (Chang et al. 2002). The biological activity of androgens via the hypothalamic-pituitary-testis axis plays various roles in the developmental stages of males, especially during spermatogenesis (Quigley 1998). In addition, androgens can reduce the ability of immature oocytes to become mature cells and thus undergo normal embryonic development (Cheng et al. 2002). Studies of sexual differentiation in fish have revealed that androgens could stimulate sex change in Epinephelus coioides (Yeh et al. 2003), E. fario (Kuo et al. 1988), and E. akaara (Shu et al. 2006).

The activity of androgens is mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily. AR is a ligand-dependent transcription protein that mediates many androgen activities during various developmental and physiological processes in vertebrates.

The AR gene contains eight exons and seven introns in vertebrates (Smolinsky et al. 2010). Like other nuclear receptors, AR is composed of three major functional domains: The N-terminal domain (NTD), the DNA binding domain (DBD), and the ligand binding domain (LBD). The first exon encodes NTD, also known as the transcriptional activation domain (TAD). This domain contains a transcriptional activation region (AF1) that affects transcription activity. The amino acid sequence of NTD has less sequence similarity (from 20 to 50 %) among teleosts. The DBD, encoded by the second and third exons, is responsible for binding to the androgen response element of the target gene and plays a role in the formation of receptor dimerization. The DBD contains eight conserved cysteine residues, with every four cysteine residues forming one zinc finger. The first zinc finger binds to specific sequences of target genes, and the second zinc finger then stabilizes the binding complex by hydrophobic interactions with the first finger (Gelmann 2002). The fourth to eighth exons encode the LBD protein domain, which is located in the C-terminal. This domain plays an important role in specific ligand binding and induces conformational changes associated with the formation of receptor dimers. The other transcriptional activation region in the LBD is AF2. The DBD and the LBD are highly conserved with homologies of 70 and 100 %, respectively, among man, mouse, and rat. The DBD and LBD conserved among fishes are also highly homologies (>90 and >77 % identity, respectively).

The full-length cDNA of *AR* genes was cloned in Cypriniformes, Perciformes, Siluriformes, and other teleost fish after the first *AR* gene was obtained from the Japanese eel (*Anguilla japonica*). Only one *AR* gene has been isolated from amphibians, birds, mammals, and some teleost fish. However, in some other teleost fishes, two distinct types of *AR* gene, *AR* $\alpha$  and *AR* $\beta$ , have been cloned, the result of a gene duplication event that occurred within the teleosts. Only one *AR* subtype has been isolated from more than ten fishes, such as goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*), and southern catfish (*Silurus meridionalis*) (Huang et al. 2011). Both *AR* $\alpha$  and *AR* $\beta$  have been cloned in

rainbow trout (*Oncorhynchus mykiss*) (Takeo and Yamashita 1999), Japanese eel (Ikeuchi et al. 1999), European eel (*A. anguilla*), mosquitofish (*Gambusia affinis affinis*) (Sone et al. 2005), medaka (*Oryzias latipes*), cichlid fish (*Haplochromis burtoni*) (Harbott et al. 2007), Nile tilapia (*Oreochromis niloticus*), and three-spined stickleback (*Gasterosteus aculeatus*) (Olsson et al. 2005).

The large yellow croaker (Larimichthys crocea) is one of the most economically important marine fish species in China, distributed mainly in coastal regions of East Asia. Their individual sizes and growth rates differed considerably; in particular, males had a lower growth rate than females (Fang et al. 2000). Monosex cultivation of the large yellow croaker became an effective method for eliminating this problem (Zhou et al. 2010). Although previous studies have focused on gonadal development at the cellular level and on the structure of the sperm and ovum (Lin et al. 1992; You and Lin 1997; You et al. 2001; Chen et al. 2007), the molecular mechanisms of gonadal development and sex differentiation in the large yellow croaker remain poorly studied. In the present study, we examined the temporal and spatial expression patterns of AR in female and male fishes as well as the cellular localization of AR mRNAs in the gonads of the large yellow croaker.

#### Materials and methods

#### Fish and sample collection

Sexually mature large yellow croakers (300–600 g) were purchased from the Fishery Technology Extension Station of Ningde, Fujian, China. Embryos were obtained during the reproductive season in 2009. Tissues (testis, ovary, liver, head kidney, intestine, gill, brain, muscle, eye, heart, stomach, spleen) and embryos at different stages were immediately frozen in liquid N<sub>2</sub> and stored at -80 °C until RNA extraction. Testis and ovary were collected for in situ hybridization and maintained in 4 % paraformaldehyde overnight at 4 °C.

#### RNA extraction and preparation of cDNA

Total RNA was extracted from the tissues and various embryonic stages, as described in a previous study

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<b>Table 1</b> Oligonucleotideprimers used in this study	Gene	Primers	Sequence
	AR	5' RACE outer primer	5'-CATTGGGAACACGTGCTCTCTGC-3'
		5' RACE inner primer	5'-CGGCCTCATACCTGGTGTCATTG-3'
		qRT- PCR sense primer	5'-AAATGGGCGAAAGGGTTG-3'
		qRT- PCR antisense primer	5'-AAGTAAAGCATCCTGCCGTTG-3'
		In situ hybridization sense primer	5'-CGGTGGGTTTGAGATGGC-3'
		In situ hybridization antisense primer	5'-CGTCGTTTGTCACGTCGC-3'
	$\beta$ -Actin	Real-time quantitative PCR sense primer	5'-TTATGAAGGCTATGCCCTGCC-3'
		Real-time quantitative PCR antisense primer	5'-TGAAGGAGTAGCCACGCTCTGT-3'

(Shen et al. 2009). Three micrograms of total RNA was used for first-strand cDNA synthesis, followed by RT-PCR amplification. The double-strand cDNA obtained was diluted and stored at -80 °C until use in molecular cloning and qRT-PCR.

#### Cloning of large yellow croaker AR cDNA

A cDNA fragment of AR was used from a previously constructed normalized gonad cDNA library of the large yellow croaker (Zhou et al. 2010). The partial sequence was homologous to known AR 3' sequences and was denoted as AR. Subsequently, gene-specific primers were used to generate the 5' regions with the universal primers UPM and NUP. The primers for 5'anchored rapid amplification of cDNA ends (5'-RACE) are listed in Table 1.

First-strand cDNA synthesis for the RACE reaction was performed with the SMART RACE cDNA Amplification Kit (Clontech, CA, USA) according to the manufacturer's instructions. The purified RACE PCR products were inserted into the pMD19-T vector (Takara, Dalian, China) and transformed in JM109 competent cells. Positive clones were subjected to DNA sequencing.

#### Sequence and phylogenic analysis

Homology analysis of nucleotide sequences was performed with BLAST software (http://blast.ncbi. nlm.nih.gov/Blast.cgi). The vector segments were identified by using the VecScreen system (http://www. ncbi.nlm.nih.gov/VecScreen/VecScreen.html). In order to obtain full-length sequences of *AR*, the PCR product fragments were spliced by using bl2seq. The Compute PI/Mw tool (http://cn.expasy.org/tools/pi\_tool.html) was used to compute the theoretical isoelectric point and molecular weight (Mw) for the full-length amino acid sequence of AR. Signal peptide cleavage sites were predicted with the SignaIP3.0 Server (http:// www.cbs.dtu.dk/services/SignalP/); *N*-glycosylation sites with the NetNGlyc1.0 Server (http://www.cbs. dtu.dk/services/NetNGlyc/); Ser, Thr, and Tyr phosphorylation sites with the NetPhos 2.0 Server (http:// www.cbs.dtu.dk/services/NetPhos/); and transmembrane helices in the protein with the TMHMM 2.0 Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/). InterProScan software (http://www.ebi.ac.uk/InterPro Scan/) was used to analyze the protein domains. The phylogenetic tree was constructed by using the bootstrap neighbor-joining method of MEGA 4.0. The bootstrap values were replicated 1,000 times to obtain the confidence value for the analysis. Multiple sequence alignments were generated by using the default settings of BioEdit (http://www.mbio.ncsu.edu/ bioedit/bioedit.html).

#### Quantitative real-time PCR

To determine the expression profile of *AR* in different tissues of the large yellow croaker, we collected RNA samples from four adult males and four adult females. In addition, RNA samples were isolated from at least 10 individuals for each of the ten embryonic development stages. Primers for *AR* and  $\beta$ -actin (endogenous control gene) were designed with Primer Express 5.0 (ABI) software (Applied Biosystems, Carlsbad, CA, USA) (Table 1) and tested to ensure amplification of single discrete bands with no primer–dimers. An aliquot of 3 µg of total RNA pretreated with DNase I was used as a template for cDNA synthesis in 20 µl reactions with random hexamers using the M-MLV

reverse transcriptase first-strand synthesis system for RT-PCR (Promega, Shanghai, China). For real-time PCR, an amount of cDNA corresponding to 25 ng of input RNA was used in each reaction. Reactions were performed with the SYBR Green PCR Master Mix (Applied Biosystems) and analyzed in the ABI 7500 real-time system. PCR products for AR and  $\beta$ -actin were ligated into pMD 18-T vector (TAKARA) and transformed in JM109 competent cells. Positive clones were subjected to DNA sequencing to check the sequence of the qRT-PCR products. The comparative threshold (CT) cycle method was used to calculate the relative concentrations. This method involves obtaining CT values for the target genes, normalizing to  $\beta$ actin ( $\Delta$ Ct) and comparing the relative expression level of the target gene in different tissues and different embryonic stages according to  $\Delta$ Ct values.

Data from all experiments were analyzed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). The samples from the four male and four female individuals and from the ten different embryonic development stages were tested, each assayed in triplicate. Data were expressed as the arithmetic mean  $\pm$  standard error of the mean. Significance of differences in different tissues of both sexes and in all stages of embryonic development was determined by the independent-samples *t* test. Differences were considered statistically significant at *p* < 0.05.

#### In situ hybridization

An *AR* cDNA sequence of the large yellow croaker was amplified by PCR and ligated into the pGEM-T Easy Vector (Promega) as a template to generate an antisense riboprobe. *ARs* forward and reverse primers are listed in Table 1. The *AR* cDNA-harboring vectors were linearized, and then, sense and antisense digoxigenin (DIG)-labeled cRNA probes were generated with an RNA labeling kit (Roche, Indianapolis, IN, USA).

Testis and ovary samples were fixed in 4 % paraformaldehyde overnight, embedded in paraffin, sectioned (5  $\mu$ m) and mounted onto 3-aminopropyl-triethoxysilane-coated glass slides. Paraffin sections were dewaxed by immersion in xylene (2 × 10 min) and then rehydrated through serial ethanol dilutions (100, 90, 80, 75, 50, and 30 % at 5 min each) followed by immersion in diethyl pyrocarbonate-treated 1× phosphate-buffered saline for 5 min and 0.1 mol/

L glycine for 5 min. Sections were digested with proteinase K (1 µg/ml) for 30 min at 37 °C and acetylated in 0.25 % (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0). Sections were prehybridized in prehybridization buffer containing 50 % (v/v) deionized formamide, 0.1 % (v/v) Tween 20, 0.5 mg/ml (w/v) yeast tRNA and 1 M citric acid (pH 6.0) at 58 °C for 3 h, followed by hybridization at 65 °C for 16 h in a humidified chamber with 0.5 mg/ml (w/v) heparin in prehybridization buffer.

After hybridization, the slides were washed with prehybridization buffer,  $2 \times SSCT$ ,  $0.2 \times SSCT$ , and  $1 \times PBST$  at 60 °C and then treated with blocking buffer at room temperature for 3 h. Hybridized probes were detected with anti-DIG antibody (Roche) overnight at 4 °C and then stained with NBT/BCIP/TMNT (Roche).

#### Results

Molecular cloning and characterization of large yellow croaker *AR* cDNA

From our established gonad cDNA library of large yellow croaker, we obtained a partial sequence that has the highest identity with the 3' AR fish sequences. A full-length large yellow croaker AR cDNA sequence was obtained by using a 5' RACE strategy. Both the nucleotide sequence and the deduced amino acid sequence of the large yellow croaker AR cDNA are shown in Fig. 1 (GenBank Accession No. ADD39720).

The AR cDNA contains a 2840-bp sequence that includes an open reading frame (nt 191 to 2443). It encodes a protein containing 750 amino acids with an estimated molecular mass of 84.5 kDa and a theoretical pI of 6.22. The AR contains 44 predicted phosphorylation sites (Ser:24, Thr:11, Tyr: 9), but no signal peptide cleavage site or transmembrane helix. Conserved domain analysis indicated that the AR consists of three functional and structural domains: an NTD (AA 1-388), a DBD (AA 389-470), and a C-terminal LBD (AA 505-749). The DBD has two zinc finger motifs with eight cysteine residues located at AA 394, 397, 411, 414, 430, 436, 446, and 449. The DBD also has a proximal box (P-box; AA 412-GSCKV-416) for binding specifically to the response element and a distal box (D-box; AA 431-ASKND-435) involved in the

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1	${\tt GATCTCGGTTTAGGAGGAGTTTGTGACTCCTGCAGCTCTCTCAGGACCTTTTTCTTTTCTTTC$	120
121	$GGACCTAGAGACTTTTTTTTTTTTGTCGTGCAAAGTTCCCAGTCTCTTGACCGGGTAAACACTTTCTGGGCA \\ \texttt{atg} ag ccaaacta \\ \texttt{accg} ag ag ttatcttg \\ \texttt{taccaca} \\ \texttt{attgccca} \\ \texttt{agag} \\ \texttt{cca} \\ \texttt{acc} \\ \texttt{accg} \\ \texttt{agg} \\ \texttt{cca} \\ \texttt{acc} \\ \texttt{accg} \\ \texttt{agg} \\ \texttt{tatcttg} \\ \texttt{taccaca} \\ \texttt{attgccca} \\ \texttt{agg} \\ \texttt{agg} \\ \texttt{acc} \\ \texttt{accg} \\ \texttt{agg} \\ \texttt{acc} \\ \texttt{accg} \\ \texttt{agg} \\ \texttt{acccaca} \\ \texttt{attgccca} \\ \texttt{agg} \\ \texttt{acccaca} \\ \texttt{accg} \\ \texttt{agg} \\ \texttt{acccaca} \\ \texttt{acccacaca} \\ \texttt{acccaca} \\ \texttt{acccacaca} \\ \texttt{acccaca} \\ \texttt{acccaca} \\ \texttt{acccacaca} \\ \texttt{acccacaca} \\ \texttt{acccacaca} \\ \texttt{acccacacaca} \\ acccacacacacaccacacacacacacacacacacaca$	240
1	M S Q T N R E L <mark>S</mark> C T T I C P E S	17
241	tgagaaattaaaaacaagcgacggtgagtgctcgcagcatggcgcaaaactctggagaaagtcgggtgtatttcaccaaaaactcaacagattcaatcacctacaggtccggacttagtagtggtgtatttcaccaaaaactcaacagattcaatcacctacaggtccggacttagtagtggtgtgtgt	360
18	EKLKTSDAV <mark>S</mark> AR <mark>S</mark> MAQN <mark>S</mark> GESRVYFTKNSTD <mark>S</mark> ITYR <mark>S</mark> GLS	57
361	t cat ccgcttgtgtgtgtacatggaaaaaacactgctgtcagacagctgccgcttctcaggaggagttgttaaacgctgagtcccgtgtgggcgagagccgctctttttctgcctgc	480
58	H P L V C D M E K H C C Q T A A A <mark>S</mark> Q E E L L N A E S R V G E <mark>S</mark> R S F <mark>S</mark> A C A T	97
481	a at ctcaga aa cag ccag gg ag ctctg caa ag ccg tg tccg tg tcg ctg gg act ga ccat gg ag tcca at ga cacg tg tg acat gg ac ccag ct ct cacccag tg cg ct cca aat gg cca at gg ac cag tg tg acat gg ac cag tg ac cag tg tg acat gg ac cag tg tg acat gg ac cag tg	600
98	I S E T A R E L C K A V S V S L G L T M E S N D T C D M D P A L T Q C A P N G H	137
601	catacgaggaggagattatttgtttggagttggagccgtgcccccgaactgtcccggagctcaggctgccgtcaccgaccatgatgaccggcccatgcacggccagaagcaattggtggagagaga	720
138	I R G G D Y L F G V G A V P P N C P G A Q A A V T D H D D R P M H G Q K Q L V E	177
721	a atgtt caa a agtt cag aga ctg tcg ctg ctg ctg ctg ctg cacg cctg caa cact tcg g ct ccg ctcg g a cat cag ctg atg ag caa a act tca cat tg tg caa g g ttg atg a cat a a cat a cat cat cat tg tg caa g g ttg atg a cat a c	840
178	M F K S S E T V A A A D S A R L Q H F G S A R <mark>T S</mark> A D E Q N F T L C K V D D I T	217
841	ctcagaggagatagatcatctggacacagtgcgcgctccctcttgccattatgcccaatccgcgccaggcaacttggcacacttcagccacgcggagaggccgtgccgagtctataaacccgcgcaggagaggccgtgccgagtctataaacccgcgcaggagaggccgtgccgagtctataaaccgcgcaggagaggccgtgccgagtctataacccgcgcaggcaacttggcacacttcagccacgcggagagggccgtgccgagtctataaaccgcgcaggagaggagaggccgtgccgagtctataaccgcgcaggcaacttggcacacttggcacacttcagccacgcggagaggccgtgccgagtctataaccgcgcaggagaggaggaggagggcggggcggggcggggcggggcggggccggggagggggg	960
218	SEEIDHLDTVRAPSCHYAQSAPGNLAHFSHAERPCRVYKP	257
961	ccccgacgaagagagagagacttcggggaaacaatggagaacaagttcggtggttatcagcccgaacaatacggcgtcaaagtcaaatctgaggacgagtccgagtctctgtggggcacaaaaatacggcgtcaaagtcaaatctgaggacgagtccgagtctctgtggggcacaaaaaaaa	1080
258	P D E E R D F G E T M E N K F G G Y Q P E Q Y G V K V K <mark>S</mark> E D E <mark>S</mark> E S L W G T N	297
1081	tta cacctt taat ga caag ta caactet cag ctt tgggg at cg ag g caat g cat a a a cg cag g ag caa a caccg cg t t cat g t g t g c t c cat a cg ag g g g g g g g g g g g g g g g g	1200
298	Y T F N D K Y N S Q L W G S R Q C I N A H N A G A N T A F M C A P Y E R S V V R	337
1201	tccggagcagtggtacccaggcgggatgctgaggccgacccatcccaactccaactacatgaagactgaagtaggcgagtggcttgatgtcgcctacaatgacaccaggtatgaggccgg	1320
338	PEQWYPGGMLRPTHPNSN <mark>Y</mark> MK <b>T</b> EVGEWLDVAYND <b>T</b> R <b>Y</b> EAG	377
1321	cagagagacacgtgttcccaatggagttcttcttcccaccacagaggatgtgcctgatctgttcagacgaagcgtccggctgccattacggcgcactcacctgcgggagctgcaaggttttt	1440
378	R E H V F P M E F F F P P Q R M C L I C S D E A S G C H Y G A L T C G S C K V F	417
1441	ctt caa aag ag ac cg ccg aag gg aa a cag aa t ac ctg tg tg caa g caa aa a cg ac tg ca cta ttg a caa g cta ag aa ag aa t tg t ccg t cctg tcg gc tg ag ga ag tg tt ttg a cag caa ag a c cg ccg ag gg aag tg tt ttg a cag caa ag a c cg ccg ccg ag gg aag tg tt ttg a cag caa ag a c cg ccg ccg ccg ccg cc	1560
418	FKRAAEGKQK <mark>Y</mark> LC <mark>ASKND</mark> CTIDKLRRKNCPSCRLRKCFEA	457
1561	tggaatgactctcggagcacgcaaactaaagaagatcggacaacagaaaaaaaccctgaagaggatcattctgttcaggatccttcagaagttatcccgaatatctctcctaaatcaggcct	1680
458	G M T L G A R K L K K I G Q Q K N P E E D H <mark>S</mark> V Q D P S E V I P N I <mark>S</mark> P K S G L	497
1681	gaacttcaactcccagacggttttcctcaacattctggagtccattgagcctgaggtggtgaatgcaggacacgactattgccagcca	1800
498	N F N S Q T V F L N I L E <mark>S</mark> I E P E V V N A G H D <mark>Y</mark> C Q P D S A A T L L T S L N	537
1801	tgagctgggagaaagacaactggtcaaagtggtcaaatgggctaaagggttgccaggtttcagaaatctccacgtggacgaccaaatgactgtcattcaacattcatggatgg	1920
538	ELGERQLVKVVKWAKGLPGFRNLHVDDQMTVIQH <mark>S</mark> WMGVM	577
1921	ggtgtttgcactgggatggagatcctataagaacgtcaacggcaggatgctttacttcgccccggatcttgtgttcaatgaacatcggatgcacatttccaccatgtacgagcactgcatgatgcatgatgcatgatgcatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgcactgatgatgatgatgatgatgatgatgatgatgatgatgat	2040
578	V F A L G W R <mark>S</mark> Y K N V N G R M L Y F A P D L V F N E H R M H I S T M Y E H C I	617
2041	acggatgaggcatctgtcacaggagttcctgctgctgcagatctctcaggaagagttcctctgcatgaaggccttgcttctcttcagtattattccagttgagggtctgaagagtcagaaaaaaaa	2160
618	RMRHL <mark>S</mark> QEFLLLQI <mark>S</mark> QEEFLCMKALLLFSIIPVEGLK <mark>S</mark> QK	657
2161	gtactttgatgaactgcgtctcacctacatcaacgaactcgatcgcctgattaactatcgaatgacctccaattgttctcagaggttctaccaactcaccagactcctggactctctcca	2280
658	Y F D E L R L T Y I N E L D R L I N Y R M T S N C S Q R F Y Q L T R L L D S L Q	697
2281	aatgacggtaaagaageteeatcagtttaeatttgacetttttgteeaageteagtegeteeecaegaaggteagettteeagagatgattggagaaataateteagtaeatgtaecaaa	2400
698	M T V K K L H Q F T F D L F V Q A Q S L P T K V S F P E M I G E I I S V H V P K	737
2401	gatectggcaggtttggccaaacccatettgtttcacaagtagAAGGAGAAAAGTGACTCATCTTTTGCTGCCTCCATAAACTTTAACAAAGGTCCTCTTTACCTACATCTTCAGTCTAA	2520
738	I L A G L A K P I L F H K *	750
2521	TAATTCTTAGTCCTGGAACCATCTCTTTCCCCTTTCAGCCATAATTGAGATAGTTTGTTCAGAACTATTAAAGCTTTTATCCGAGAAGCTGCAAGGCATCACCTGTTGACAGCTCTGCTAT	2640
2641	TTCTGAGTGTAGAGTTTGTGTCTTCAGCTGGAGGAGAGAGGATGCTACTTTACATACA	2760
2761	GTGACCTTAATTATACATACACTTGACAAGGTATTCATGAAAAACTAAAAAGAGACAAACTGTATCAAAAAAAA	

**Fig. 1** Full-length cDNA sequence and deduced amino acid sequence of the large yellow croaker androgen receptor. Nucleotides and amino acid residues are numbered on the *left*. The initiation codon (ATG) and the stop codon (TAG) are

characterized in *bold*. Potential protein kinase C phosphorylation sites are in *red*. The P-box (GSCKV) and D-box (ASKND) are shown as *boxes*. The leucine repeat in the putative leucine zipper is *underlined*. (Color figure online)

recognition of spacing between half-sites of the response element and in dimerization. The LBD folds into 12 helices and a leucine zipper consisting of leucine residues (Figs. 1, 2).

Sequence homology and phylogenetic analysis

Homology analysis of the encoded protein alignment was performed with BioEdit software. The amino acid

sequence of the large yellow croaker AR showed 93, 84, 80, and 79 % identity with Atlantic croaker AR, sea bass (*Dicentrarchus labrax*) AR, red sea bream AR, and black sea bream AR, respectively. The large yellow croaker AR showed that the homology in NTD is relatively low, whereas DBD and LBD are highly homologous (>90 % identity) among teleost fishes. Comparison of the amino acid sequences revealed that the P-box and D-box are completely conserved (100 % identity) among the different teleost fishes. The large yellow croaker AR is very similar to human AR, with identical sequences in the DBD and LBD and sequence identities of 91 and 71 %, respectively.

Phylogenetic analysis of AR sequences indicated that there are two major classes of ARs in vertebrates, teleost AR and tetrapod AR. The teleost AR can be divided into two small groups: AR $\alpha$  and AR $\beta$ . The large yellow croaker AR, together with the ARs from Atlantic croaker and sea bass, was clustered in the fish AR $\beta$  branch (Fig. 3).

Large yellow croaker *AR* cDNA expression analyses

Tissue distribution of large yellow croaker *AR* mRNA was evaluated by qRT-PCR. According to relative quantification analyses, *AR* mRNA expression was detectable in all adult tissues examined (Fig. 4A). In all tissues tested, females exhibited higher expression levels than did males. The highest expression of *AR* appeared in the liver and gonad of each sex. A moderately high expression of *AR* occurred in brain, stomach, spleen, and intestine of both sexes. *AR* mRNA had the lowest level of expression in gill, head kidney, muscle, eyes, and heart.

Using qRT-PCR, we found that large yellow croaker AR mRNA was detectable in all stages of embryonic development. The expression levels increased gradually from the multiple-cell to the gastrula stage and then declined substantially at the gastrula stage. Starting at the yolk plug stage, the AR mRNA was maintained at a low level (Fig. 4B).

Localization of large yellow croaker *AR* mRNA in gametogenesis

To identify the localization of large yellow croaker AR mRNA in gametogenesis, we performed in situ hybridization on 5-µm-thick paraffin sections. The

Fig. 2 Multiple sequence alignment of the Larimichthys crocea AR amino acid sequence with Micropogonias undulatus, Dicentrarchus labrax, Acanthopagrus schlegelii and Pagrus major AR proteins. The DNA binding domain and the ligand binding domain are underlined. In the DNA binding domain, cysteine residues thought to be involved in two zinc finger domains are indicated with arrows (down arrow). In the ligand binding domain, the leucine repeat in the putative leucine zipper is indicated with diamonds (filled diamond). Gray shading indicates regions that are involved in recognition of the response element (P-box [GSCKV]) and in recognition of the space between response elements (D-box [ASRND]), respectively. The AR amino acid sequences were obtained from GenBank (see Fig. 4)

hybridization signal was observed in all germ cells examined using the antisense *AR* probe. No signal was observed when the sense *AR* probe was used, indicating the specificity of the antisense probe to the large yellow croaker *AR* mRNA. Significant differences were found in the localization pattern of large yellow croaker *AR* mRNA in oogenesis and spermatogenesis (Figs. 5, 6). Positive signals were observed in the cytoplasm of all oocytes in different developmental stages (from stage I to IV, and in involuting oocytes) (Fig. 5), in follicle cells of stage IV oocytes (Fig. 5D). Positive signals were predominantly observed in germ cells during spermatogenesis; these germ cells were identified as spermatogonium, spermatocyte, spermatid, and spermatozoon (Fig. 6).

#### Discussion

Only one AR subtype was isolated from the large yellow croaker in our study, similar to findings for goldfish, zebrafish, black sea bream, and southern catfish, although two AR gene subtypes have been cloned in other teleost fishes (Takeo and Yamashita, 1999; Ikeuchi et al. 1999; Sone et al. 2005; Olsson et al. 2005). Similar to all teleost AR proteins, there are three domains in the large yellow croaker AR protein: NTD, DBD, and LBD. The NTD is the first effector region of AR and is largely responsible for transactivation (Gelmann 2002). The NTD is weakly conserved among all fish species: there is only 23 % amino acid identity in the NTD between the large yellow croaker and the zebrafish. The NTD domains of teleost AR are variable in length, from 300 to 500 amino acids, and their tertiary structure is unknown. This high variability shows that the domain has some

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		10	20	30	40	50	60	70	80	90
					.	.				
Larimichthys crocea	1	MSQTNRELSCTTICF	PESEKLKTSDAV	/SARSMAQNS	GESRVYFTKN	ST	DSITY	RSGLSHPLVO	CDMEKHCCQTA	AASQE 77
Micropogonias undulatus	1	MSQTNRELSCNTIWF	PEGEKVKTSDAV	/RAPSMAQNT	EESRGYFTKN	STGNGAG	KADSITY	RSGHIHPPVO	CDMEKQCCQTA	AAPQE 84
Dicentrarchus labrax	1	MSQTSRELSCNKMWT	VGAKIKTGDAV	SALSMAQNP	EESPVYFTKN	SSGNGADRLKE	SDHADPNTY	GSGHTNSLAG	CDMEKHCCQTA	AAPQE 90
Acanthonagrus schlegelii	1	MSQTSRQLSCTKVWF	PRGEKIKTGDA	ASARSMAQNT	EESPLRVSKH	LTGNGAGRMRG	SDNADPNTY	GEGHMIPLAG	CGMEKHCCQTA	AASQE 90
Pagrus major	1	MSQTSGQLSCTKIWS	RGEKVKTGDAV	/SAPSMAQNT	EESPLRVSRN	STGNGAGRMRF	ADNADPNTY	ESGHMIPLV	COMEKHCCQTA	AAPQE 90
Clustal Consensus	1	**** .*** .	* *** **	* *****	** ···		* **	* *	* *****	** ** 45
erustar consensus	1	N-terminal doma	in domain			•				
		100	110	120	130	140	150	160	170	180
		100	110	120	150	140	150	100	110	100
Lonimiahthua anagaa	70		····  ····  ·		I CI TMESNDT		ADVCUTDCC			
Winner and the second states	10	ELLNAESKVGESKSF	CACATICETA	DEL CRAVENE	LGL I MEOND I	CDMDF ALTQU	AFNGHINGG	NI FOVORVI	TINCEGAQAA-	VID 105
Micropogonias undulatus	80	ELLNAECKVGDSRSF	SACATISETA	ELCKAVOVO	LGLIMESNDI:	SDMDP-ALTQU	ASNGHIKGG		PNCPGAQGA-	VID 170
Dicentrarchus labrax	91	ELLNADCRVGDSRSF	SACATISETAN	CELCKAVSVS	LGLAMESSDT:	SDVDGGTLHSC	AANDHIKG	SILFGVGAVI	'LNKPGAQAAD	INCPD 179
Acanthopagrus schlegelii	91	ELLNADCRVGDSRSF	SACATISETA	RELCKAVSVS	LGLAMECSDS	SDMDA-ALPQC	AANDHVRG-I	SYLFGVGNAF	'LSCPGGQAAV	SDYKC 178
Pagrus major	91	ELFNADCRVGDSRSF	SACATISETA	RELCKAVSVS	LGLAMESNDP	SDMDA-ALSQC	AANDQLRG-	EYLFGVGAAI	2LSCPGAQAAV	SEYKC 178
Clustal Consensus	46	**:**:. ***:****	********	******	***:***.	.*:* :* .*	*.*.: **	*****	· . **.*.*	109
		190	200	210	220	230	240	250	260	270
			.			.				
Larimichthys crocea	164	HDDRPMHGQKG	LVEMFKSSETV	AAADSARLQ	HFGSARTSAD	EQNFTLCKVDD	ITSEEIDHL	DT-VRAPSCH	IYAQSA-PGNL	AHFSH 247
Micropogonias undulatus	171	RDDRPMRGQKG	LVEMFKSSETV	AAAASARLQ	HPGSTRTSAD	EQNFTLCKVDD	ITSEEIDHL	DT-ARAASCH	IYAQSA-PSNL	AHFSH 254
Dicentrarchus labrax	180	RDDRPLHGQKG	LVEMFKTSET-	AARLH	HLTSTWTSVD	EQNFTMCKADD	ITSQKIDHL	DTSTRAASCI	YAQSA-PGNL	AHFGQ 259
Acanthopagrus schlegelii	179	AEERPLHGHKQQQQQ	LMEMFKSSET-	AAHLQ	HLTSARTPVD	EQNFTMCKAED	STPEETAHL	OP-VRAASCH	YAQSAQPGSM	THFDP 262
Pagrus major	179	PEERPLHGHKQQQQ-	LMDMFKSSET-	GAHLQ	HLTSTRTPVD	EHNFTLCKAED	LTPEETAHQ	DS-VRAAACI	PYAQSALPGNM	AHFGS 261
Clustal Consensus	109	::**::*:*	*::***:***	.*:*:	* *: **	*:***:**.:*	**.:: **	k **. :*	***** *:	:**. 166
		280	290	300	310	320	330	340	350	360
						.				
Larimichthys crocea	248	AERPCRVYKPPD	EERDFGETMEN	VKFGGYQ	PEQYGVKVKS	EDESESLWG	TNYTFNDKY	NSQLWGSRQ-	-CINAHNAGAN	TAFMC 328
Micropogonias undulatus	255	AERPCRVYKPPD	EERDFGETMEN	NKFGGYQ	PEQYGVKVKS	EDESESLWG	TNYTFNDKY	NSQLWGTRQ-	-CMNAHNAGAN	TTFIC 335
Dicentrarchus labrax	260	ARETEKPCRVYKPPN	EARDFAEAMEN	NKFGGY-	PEQYSVKIKS	EDPESLAASWG	TNYTFNEKY	NSQFWGSRQQ	QCMNAHSTGAS	TAFIC 345
Acanthopagrus schlegelii	263	PAQ-ERPWRLYKPPD	EAGDFMEVMES	SSFATTSGYQ	PEQYSMKIKC	EDTESAGALWG	GNHSFNDRY	SQCWGPRH-	-CVSAHGAGAD	SA-LC 349
Pagrus major	262	PAP-ERPWQLYKPPD	EAGDFGEVMES	SRFVT-SGYQ	PEQYSVKIKC	EDTESAGALWG	GNYTFNDRY	NSQCWGPRQ-	-CMNAHSTGAN	SA-LC 347
Clustal Consensus	166	. *:* ::****:	* ** *. **.	**	****. :*:*.:	** . : **	*::**::*	*** **.*:	*:.**.:**.	:: :* 223
		370	380	390	400	410	420	430	440	450
						.			↓.	<b>*</b>
Larimichthys crocea	329	APYERSVVRPEQWYF	GGMLRPTHPNS	SNYMKTEVGE	WLDVAYNDTR'	YEAGREHVFPM	EFFFPPQRM	CLICSDEAS	GCHYGALTCGS	CKVFF 418
Micropogonias undulatus	336	TPYERSMVRPEQWYF	GGMLRPTYPNS	SNYVKTEVGE	WLDVAYNDTR	FEAGREHMEPN	EFFFPPORM	CLICSDEAS	CHYGALTCGS	CKVFF 425
Dicentrarchus lahray	346	NPVFRSVVRPFOWVF	GGMI RPPYPNS	NVVKTEVGE	WI DVAVNDTRI	FFAGREHMEPM	FFFFPPORM	CLICSDEAS	CHYGAL TOGS	CKVFF 435
Acanthonagrus schlegelii	350	NPVFRSAARPFHWVF	CGMLRSPVPN	SVMKSEVGE	WPDVPVNDPR	FDASRFHMFPM	FFFFPAORM	MICSDEAS	CHYGAL TOGS	CKVFF 439
Pagrue major	3/8		CCMI RSPVPNS	SVVKSEVCE		EDSSSEHMEPM	EEEEDVOBW		CHVCALTCOS	CKVEF 437
lustal Concensus	940 999	TH TENOVARI ENWIP	COMERCITING	01110E10E	игры 190LU	HMFFM مىلەرمەر ،،،		5. *********	1011ULLUUD	
GIUSTAI CONSENSUS	440	- የሚያምም የ የ የ የ የ የ የ የ የ የ የ የ የ የ የ የ የ የ	······································	·· ·· ·· · · · · · · · · · · · · · · ·	······································	•••• • <del>•</del> ••		hinding	domoir	<u></u> 500
		460	470	190	400	500	510	PIUTING (	520	540
			470	400	490	500	010	520	000	540
T • • 1.1	410	····  ····  ·★··						····  ····		····
<i>Larimicninys crocea</i>	419	KAAEGKQKYLCASK	INDCITDKLRRF	ANGPSCRERK	ufeagMilgA	ralka i GQQKN	PEEDHSVQD	-SEVIPNISI	NSGLNFNSQT	VFLNI 508

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Micropogonias undulatus	426 KRAAEGKQKYLCASKNDCTIDKLRRKNCPSCRLRKCFEAGMTLRARKLKKIGQQKNPEEDHSVQDPSEVMQNISPKSGLNFNSQTVFLNI 518						
Dicentrarchus labrax	436 KRAAEGKQKYLCASKNDCTIDKLRRKNCPSCRLRKCFEAGMTLGARKLKKIGQQKNLEEDHPIQEPVEVIQNISPKSGLNFNSQLVFLNI 528						
Acanthopagrus schlegelii	440 KRAAEGKQKYLCASKNDCTIDKLRRKNCPSCRLRKCFEAGMTLGARKLKKIGQQKNPDGDHPPQEPAEVMPNISPKTGLSFNSQVVFLNI 529						
Pagrus major	438 KRAAEGKQKYLCASKNDCTIDKLRRKNCPSCRLRKCFEAGMTLGARKLKKIGQHKNSDEDHPLQEPAEVMPNISPKSGLSFNSQVVFLNV 52						
Clustal Consensus	301 ************************************						
	DNA-binding domain						
	550 560 570 580 590 600 610 620 630						
Larimichthys crocea	$509\ LESIEPEVVNAGHDYCQPDSAATLLTSLNELGERQLVKVVKWAKGLPGFRNLHVDDQMTVIQHSWMGVMVFALGWRSYKNVNGRMLYFAP\ 598000000000000000000000000000000000000$						
Micropogonias undulatus	$516\ LESIEPEVVNAGHDYGQPDSAATLLTSLNELGERQLVKVVKWAKGLPGFRNLHVDDQMTVIQHSWMGVMVFALGWRSYKNVNGRMLYFAP\ 60300000000000000000000000000000000000$						
Dicentrarchus labrax	526 LESIEPEVVNAGHDYGQPDSADTLLTSLNELGERQLVKVVKWAKGLPGFRNLHMDDQMTVIQHSWMGVMVFALGWRSYKNANSRMLYFAP 61						
Acanthopagrus schlegelii	530 LESIEPEVAYAGHDYGQPDSAATLLTSLNELGEKQLVKVVKWAKGLPGFRNLHVDDQWTVIQYSWMGVMVFGLGWRSYKNVNGRMLYFAP 619						
Pagrus major	528 LESIEPEVVNAGHDYGQPDSAATLLTSLNELGERQLVKVVKWAKGLPGFRNLHVDDQMTVIQHSWMGVMVFGLGWRSYKNVNGRMLYFAP 617						
Clustal Consensus	382 ************************************						
	Ligand-binding domain						
	640 650 660 670 680 690 700 710 720						
Larimichthys crocea	599 DLVFNEHRMHISTMYEHCIRMRHLSQEFLLLQISQEEFLCMKALLLFSIIPVEGLKSQKYFDELRLTYINELDRLINYRMTSNCSQRFYQ 688						
Micropogonias undulatus	606 DLVFNEHRMHISTMYEHCIRMRHLSQEFQLLQITQEEFLCMKALLLFSIIPVEGLKSQKYFDELRLTYINELDRLVNYRMTTNCSQRFYQ 698						
Dicentrarchus labrax	616 DLVFNEHRMHISTMYEHCIRMKHLSQEFLLLQITQEEFLCMKALLLFSILPVEGLKSQKYFDELRLTYINELDRLINYRMTTNCSQRFYQ 70						
Acanthopagrus schlegelii	620 DLVFNEHRMHISSMYEHCIRMRHLSQEFLLLQITQEEFLCMKALLLFSIIPVEGLKSQKYFDELRLTYINELDRLISYRMSANCSQRFYQ 705						
Pagrus major	618 DLVFNEHRMHISTMYEHCIRMRHLSQEFLLLQITQEEFLCMKALLLFSIIPVEGLKSQKYFDELRLTYINELDRLINYRMNTNCSQRFYQ 703						
Clustal Consensus	465 ************************************						
	Ligand-binding domain						
	730 740 750 760 770 780						
	$\blacklozenge \dots   \dots \blacklozenge   \dots , \blacklozenge \dots   \blacklozenge \dots   \dots \diamondsuit   \dots   \dots   \dots   \dots   \dots   \dots   \dots   \dots$						
Larimichthys crocea	689 LTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPKILAGLAKPILFHK 750						
Micropogonias undulatus	696 LTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPKILAGLAKPILFHE 757						
Dicentrarchus labrax	706 LTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPKILAGLAKPILFHE 767						
Acanthopagrus schlegelii	710 LTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPKILAGLAKPILFHD 771						
Pagrus major	708 LTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPKILAGLAKPILFHE 769						
Clustal Consensus	552 ***********************************						
	Ligand-binding domain						

#### Fig. 2 continued

functional characteristics that are unique compared with steroid hormone receptors in other families. The DBD is highly conserved from human to fish, with the DBD amino acid sequence of the large yellow croaker AR being 90 % identical to that of human AR. In teleost fishes, AR $\alpha$  DBD is 85–100 % identical to AR $\beta$  DBD. Conservation of DBD of the AR gene throughout evolution implicates this region as being critical for the activity of the molecule. The DBD includes two zinc fingers that are composed of eight cysteine residues. When AR binds to the target DNA, the second finger is necessary for receptor dimerization (Dahlman-Wright et al. 1991). The second zinc finger also contributes to the specificity of receptor DNA binding and stabilizes the binding complex by hydrophobic interactions with the first zinc finger (Schoenmakers et al. 1999). In the DBD, the P-box (GSCKV) and the D-box (ASKND) contribute to AR binding to the androgen response element of the target gene; these boxes are responsible for recognizing the space between the half-sites of the response elements (Schoenmakers et al. 1999). The LBD of AR contains 12 helices that form a ligand binding pocket. Helix 12 can be induced by ligand binding to overlie the pocket, which discloses a groove (Gelmann 2002). The LBD of AR has five leucine residues to form a "leucine



Fig. 3 Phylogenetic tree of the AR amino acid sequence between *Larimichthys crocea* and other species. *Homo sapiens*-PR protein was used as the out-group. Numbers indicate the bootstrap confidence values of 1,000 replicates. The GenBank accession numbers of the selected AR sequences are as follows: *Micropogonias undulatus* (AAU09477), *Dicentrarchus labrax* (AAT76433), *Acanthopagrus schlegelii* (AAO61694), *Pagrus major* (BAA33451), *Haplochromis burtoni* AR $\alpha$  (AAD25074), *Haplochromis burtoni* AR $\beta$  (AAL92878), *Oncorhynchus mykiss* AR $\alpha$  (NP\_001117656), *Oncorhynchus mykiss* AR $\beta$ 

zipper" that is used to stabilize the dimerization of AR (Johnson and McKnight 1989). The LBD of the large yellow croaker is highly homologous (>80 %) to that of other teleost fishes, such as zebrafish, Atlantic croaker, kelp bass, and rainbow trout. The LBD from the large yellow croaker AR shares 71 % sequence identity with the LBD of human, mouse, and rat. Despite some differences in the primary amino acid sequence of LBD between the large yellow croaker AR and human AR, the three-dimensional structures of the LBDs of these molecules are quite similar. Sequence analysis showed that the LBD is a highly conserved domain among different fishes, suggesting

(NP\_001117657), Oreochromis niloticus AR $\beta$  (BAB20082), Oreochromis niloticus AR $\alpha$  (BAB20081), Gambusia affinis AR $\beta$  (BAD81046), Gambusia affinis AR $\alpha$ (BAD52085), Oryzias latipes AR $\beta$  (NP\_001098151), Oryzias latipes AR $\alpha$  (NP\_0011 64304), Carassius auratus (AAM09278), Danio rerio (NP\_001076592), Pimephales promelas (AAF88138), Rana catesbeiana, (AAP85538), Xenopus laevis (AAC97386), Gallus gallus (NP\_001035179), Homo sapiens (AAA51729), Rattus norvegicus (AAA40733), Mus musculus domesticus (AAA37 234), Homo sapiens-PR (AAA60081)

that the AR has a conserved function in the ligand binding of fishes.

Phylogenetic analysis shows that the AR of the large yellow croaker is more closely related to that of other teleost fishes than to mammals, amphibians, and birds. Moreover, analysis has also revealed that the large yellow croaker AR has higher homology with the AR $\beta$  subtype of other teleost fishes. Similarly, most cyprinid family and perciformes species analyzed to date have been shown to contain only one type of AR protein (Wilson et al. 2004; Pasmanik and Callard 1985; Hossain et al. 2008; Huang et al. 2011). Researchers have suggested the loss of the second

Fig. 4 Expression of AR analyzed by real-time PCR mRNA in different tissues of both sexes (A) and in all stages of embryonic development (B). GO gonad, HK head kidney, M muscle, L liver, S spleen, B brain, GI gill, ST stomach, E eye, IN intestine, H heart. Each bar represents mean  $\pm$  SEM (n = 5). a, b, band c groups sharing the same letter codes were not significantly different (p > 0.05); groups with different letter codes were significantly different (p < 0.05).  $\beta$ -actin served as a reference gene



*AR* from both the cyprinids and the perciformes before genome duplication as one possible explanation (Hossain et al. 2008). The phylogenetic tree showed that all single AR subtype sequences cluster together with the AR $\beta$  types of the two AR subtypes. Phylogenetic analysis seems to indicate that the teleost AR $\beta$ type is the canonical form, whereas AR $\alpha$  may be a duplicated copy generated later and lost from some fish species (Hossain et al. 2008).

Our results show that large yellow croaker AR is distributed ubiquitously in tissues of both sexes. Likewise, in zebrafish, male *Cynoglossus semilaevis* and southern catfish, which also contain only one type embryonic development

of AR, AR mRNA is expressed ubiquitously in all analyzed tissues (Hossain et al. 2008; Huang et al. 2011; Wen et al. 2009). On the other hand, the tissue

Fig. 5 Localization of AR transcripts in oogenesis. In situ hybridization with the corresponding DIG-labeled antisense RNA probe (A–D) and sense probe as a negative control (E– H) for AR transcripts. Regular histological sections were stained with HE (I–L). Fc follicle cell, Og oil globule, Yg yolk globule, N nucleus. Stages I, II, III, and IV and involuting stage oocytes are indicated with white, black, red, blue and yellow arrows, respectively. The positive signals were observed in the cytoplasm of all oocytes in different developmental stages and in follicle cells of stage IV oocytes. Scale bars are shown in each panel. (Color figure online)



distribution and relative expression of ARs are quite different in fishes that contain two AR subtypes, such as rainbow trout  $AR\alpha$ , which was predominantly expressed in the ovary and testis, and  $AR\beta$ , which showed highest expression in brain (Takeo and Yamashita 1999). In cichlid fish,  $AR\alpha$  showed significantly higher expression than  $AR\beta$  in the pituitary, and  $AR\beta$  expressed at a higher level than  $AR\alpha$  in the anterior and middle brain (Harbott et al. 2007). In the male Japanese eel,  $AR\beta$  mRNA showed preferential expression in testis, muscle, and spleen, but  $AR\alpha$ mRNA was observed in gill, muscle, heart, spleen, head kidney, liver, brain, testis, and ovary (Ikeuchi et al. 1999), demonstrating that the two subtypes of ARare detected in some tissues, but not in all tissues analyzed. The difference in expression patterns between AR and AR $\alpha/\beta$  suggests that the single AR type in some fishes has a dual role as two AR types  $(AR\alpha \text{ and } AR\beta)$  in other fishes.

The differences in AR expression in different tissues suggest that it plays different roles in these tissues. In the present study, the large yellow croaker AR mRNA was found to be overwhelmingly expressed in the livers of females and males. Vitellogenin (VTG), the synthesis of which is crucial for proper oocyte development, is synthesized in the liver, a process that numerous studies have demonstrated to be mediated through the AR. In Spinibarbus denticulatus, for example, AR is expressed in the liver and was shown to be important in mediating VTG synthesis (Liu et al. 2009). In addition, androgen action on VTG synthesis in the liver of the Japanese eel was found to be suppressed by an AR antagonist (Kwon et al. 2005). It has thus been suggested that androgen controls the synthesis of VTG in fish via the AR.

Androgen and *AR* have also been confirmed to play an important role in normal spermatogenesis and male fertility in mice (Wang et al. 2009). *AR* has been described in germ cells, Leydig cells, peritubular myoid cells and Sertoli cells of mammals and shown to be important in mouse spermatogenesis and fertility (Bremner et al. 1994; Janssen et al. 1994; Shan et al. 1997). A study of zebrafish testis indicated that Sertoli cells in contact with early spermatogonia express the highest levels of *AR* mRNA (De Waal et al. 2008). Furthermore, the expression of *AR* mRNA in zebrafish increased significantly in the testis during the course of gonad development (Hossain et al. 2008). In the Japanese eel, a high expression of *AR* mRNA in Sertoli Fig. 6 Localization of AR transcripts in spermatogenesis. In situ hybridization with the corresponding DIG-labeled antisense RNA probe (A) and sense probe as a negative control (B) for AR transcripts. A regular histological section was stained with HE (C). Positive signals were predominantly observed in germ cells during spermatogenesis. Sg spermatogonium, Sc spermatocyte, St spermatid, S spermatozoon. Scale bars are shown in each panel

cells in close proximity to early spermatogonia suggested that it may stimulate spermatogonial proliferation and differentiation (Miura et al. 1991). In zebrafish, the signals of AR in spermatogonium, spermatids, and sperm were significantly stronger than in primary spermatocyte and secondary spermatocyte (De Waal et al. 2008). Likewise, in our study, we found a higher level of AR mRNA expression in testis, and a positive AR in situ hybridization signal was detected in all germ cell types of spermatogenesis. The high level of AR expression found in testis suggests the direct action of androgens on mediating spermatogenesis in the male large yellow croaker.

Previous studies indicated that AR was important for granulosa cell development and female reproduction (Weil et al. 1998; Chaffin et al. 1999; Hild-Petito and Fazleabas, 1997). Reduced fertility in AR knockout female mice is due to defective folliculogenesis (Yeh et al. 2002). AR expression has also been studied in granulosa cells of ovaries from humans, nonhuman primates, and a number of mammals (Weil et al. 1998; Chaffin et al. 1999; Hild-Petito and Fazleabas, 1997). In non-mammals, such as chicken (Hillier and Tetsuka 1997), frog (Lutz et al. 2001), and Japanese eel (Tosaka et al. 2010), AR is necessary for stimulating the growth of ovaries. In the primate ovary, AR is most abundant in granulosa cells of follicles and to a lesser degree in theca cells (Weil et al. 1998). In addition to theca and granulosa cells, AR mRNA is also present within the oocytes of rodent, ovine, and porcine ovaries (Walters et al. 2008). In the female Japanese eel, both AR mRNAs were largely observed in the follicle cells at all stages of ovarian development. The predominant localization of AR mRNA in the follicle cells suggested that androgens are important in oocyte growth by acting on these cells (Tosaka et al. 2010). Furthermore, a study that used in situ hybridization found that  $AR\alpha$  mRNA levels in the Japanese eel were high from the late oil droplet stage to the late vitellogenic stage, whereas  $AR\beta$  mRNA levels were



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high from the late oil droplet stage to the midvitellogenic stage (Tosaka et al. 2010). In the shortfinned eel (A. australis), AR mediates 11-KT levels to regulate the development of previtellogenic oocytes, thus increasing their size (Lokman et al. 2007). In our study, higher levels of AR mRNA expression were also found in the ovary, and positive signals were observed in the cytoplasm of all oocytes throughout oogenesis; however, AR mRNA was detectable in the follicular layer of stage IV oocytes only. Although it is possible that endogenous AR may reside largely in the nucleus, many studies have suggested that before ligand binding, the receptor resides in the cytoplasm (Gelmann 2002). We further confirm this view at the genetic level. All of these in situ hybridization results support the hypothesis that the AR in fish is involved in oogenesis.

In S. denticulatus, AR mRNA was first observed with a very weak signal at the multiple-cell and blastula stages. Little or no signal was detected in the gastrula and neurula stages (Liu et al. 2009). In zebrafish, the AR transcript level was maintained until 50 % epiboly after which it was drastically reduced by the five-somite stage (Hossain et al. 2008). Using qRT-PCR, we found that the AR transcript was deposited into the large yellow croaker embryos and that the AR mRNA level increased from the multiplecell to the gastrula stage. The level of expression declined substantially afterward during the course of embryonic development. Our results for the large yellow croaker AR are similar to those for the S. denticulatus (Liu et al. 2009) and zebrafish AR (Hossain et al. 2008), all of them demonstrating a high-level expression at the early developmental stages of the embryo. Furthermore, the expression levels increased gradually from 24 h postfertilization onward in the embryonic development of zebrafish, indicating that the AR transcript was maternally deposited into the embryo and had a potential role during late embryonic development (Hossain et al. 2008). However, in our study, the AR mRNA was maintained at a low level during the stages of embryonic development after yolk plug formation. Our results revealed that AR may have a potential role during the early embryonic development of the large yellow croaker.

In summary, an AR in the large yellow croaker was cloned and its gene expression was characterized in selected tissues and at different stages of embryonic development. Our results show that positive signals of *AR* mRNA were observed in all germ cell types during spermatogenesis, in the cytoplasm of all oocytes and in the follicles of stage IV oocytes and involuting stage oocytes. These findings indicate that *AR* not only has important roles in the embryonic development of the large yellow croaker, but also in the gametogenesis of both sexes.

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