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

ARTICLE *in* FISH PHYSIOLOGY AND BIOCHEMISTRY · AUGUST 2012

Impact Factor: 1.62 · DOI: 10.1007/s10695-012-9701-6 · Source: PubMed

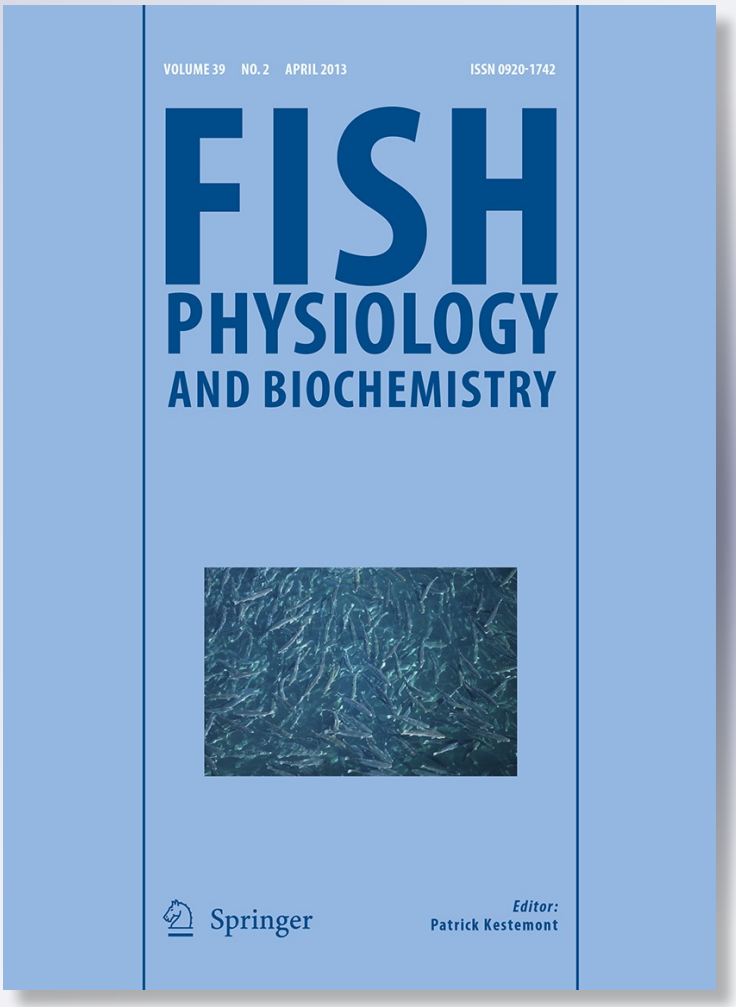
Molecular cloning, characterization, and gene expression of the androgen receptor in the large yellow croaker, Larimichthys crocea

Lulu Pu, Kunhuang Han, Fangjing Xie, Zhihua Zou, David Close, Ziping Zhang & Yilei Wang

Fish Physiology and Biochemistry

ISSN 0920-1742
Volume 39
Number 2

Fish Physiol Biochem (2013) 39:309-324
DOI 10.1007/s10695-012-9701-6



Springer

Fish Physiol Biochem (2013) 39:309–324
DOI 10.1007/s10695-012-9701-6

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

Lulu Pu · Kunhuang Han · Fangjing Xie · Zhihua Zou · David Close · Ziping Zhang · Yilei Wang

Received: 2 April 2012 / Accepted: 20 July 2012 / Published online: 4 August 2012

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 the important role that *AR* plays in gametogenesis, gonadal development, and the early stages of embryonic development.

Keywords *Larimichthys crocea* · *AR* · Gametogenesis · Gonadal development · Embryonic development

L. Pu · K. Han · F. Xie · Z. Zou · Y. Wang (✉)
Key Laboratory of Healthy Mariculture for East China Sea, Ministry of Agriculture, Fisheries College, Jimei University, Xiamen 361021, China
e-mail: ylwang@jmu.edu.cn

D. Close
Department of Zoology, University of British Columbia, Vancouver V6T 1Z4, Canada

Z. Zhang (✉)
Department of Biological Science, Seton Hall University, South Orange, NJ 07079, USA
e-mail: ziping.zhang@shu.edu

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, *ARα* and *ARβ*, 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 *ARα* and *ARβ* 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_2 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

Table 1 Oligonucleotide primers 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′
β-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/InterProScan/) 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 *β-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 *β-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 *β-actin* (ΔCt) and comparing the relative expression level of the target gene in different tissues and different embryonic stages according to ΔCt 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 ± 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 μm) and mounted onto 3-aminopropyltriethoxysilane-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 DIG-labeled RNA probe and 50 μg/ml (w/v) heparin in prehybridization buffer.

After hybridization, the slides were washed with prehybridization buffer, 2× SSCT, 0.2× SSCT, and 1× 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

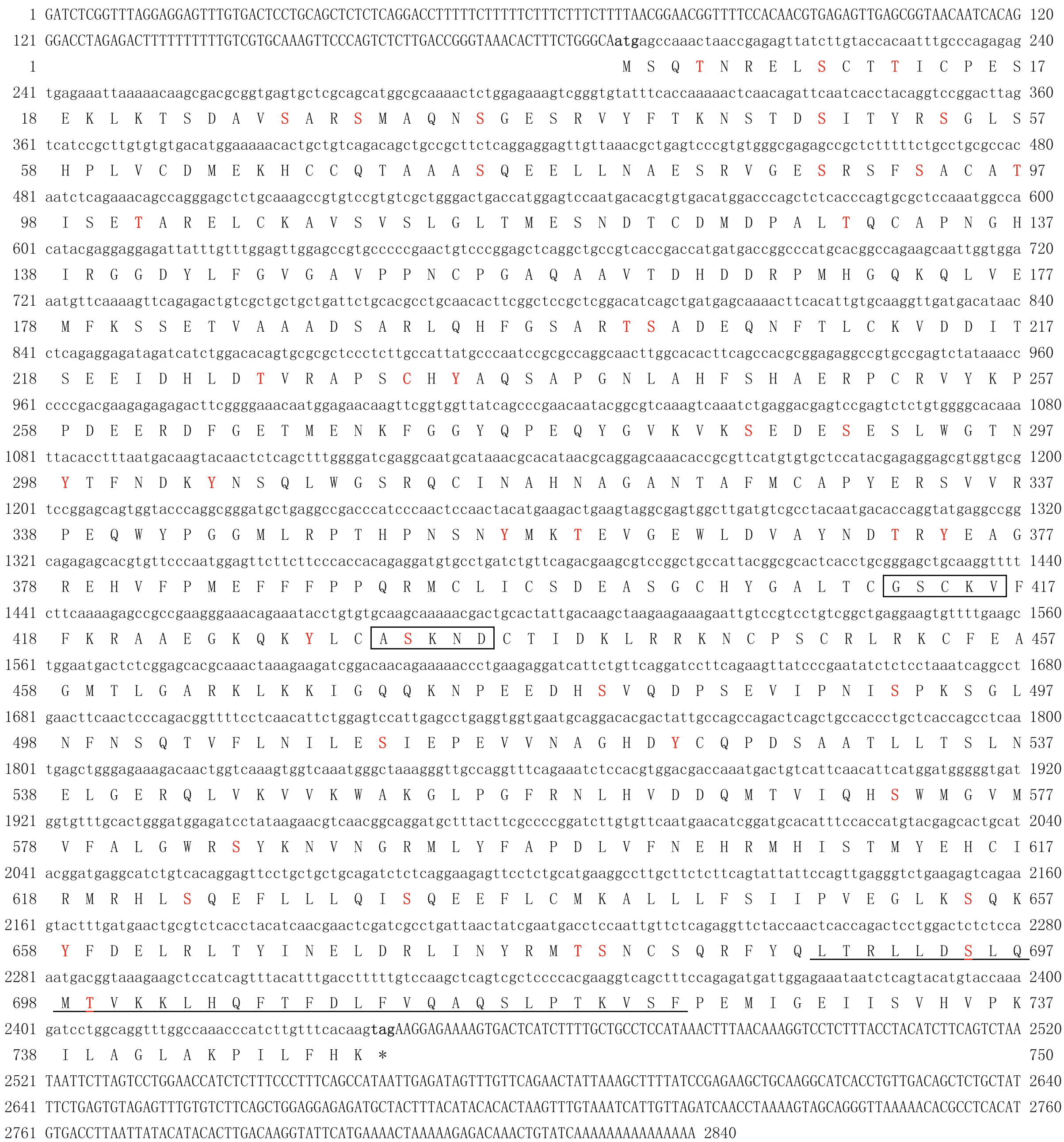


Fig. 1 Full-length cDNA sequence and deduced amino acid sequence of the large yellow croaker androgen receptor. Nucleotides and amino acid residues are numbered on the *left*. The initiation codon (ATG) and the stop codon (TAG) are characterized in *bold*. Potential protein kinase C phosphorylation sites 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: ARα and ARβ. The large yellow croaker AR, together with the ARs from Atlantic croaker and sea bass, was clustered in the fish ARβ 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).

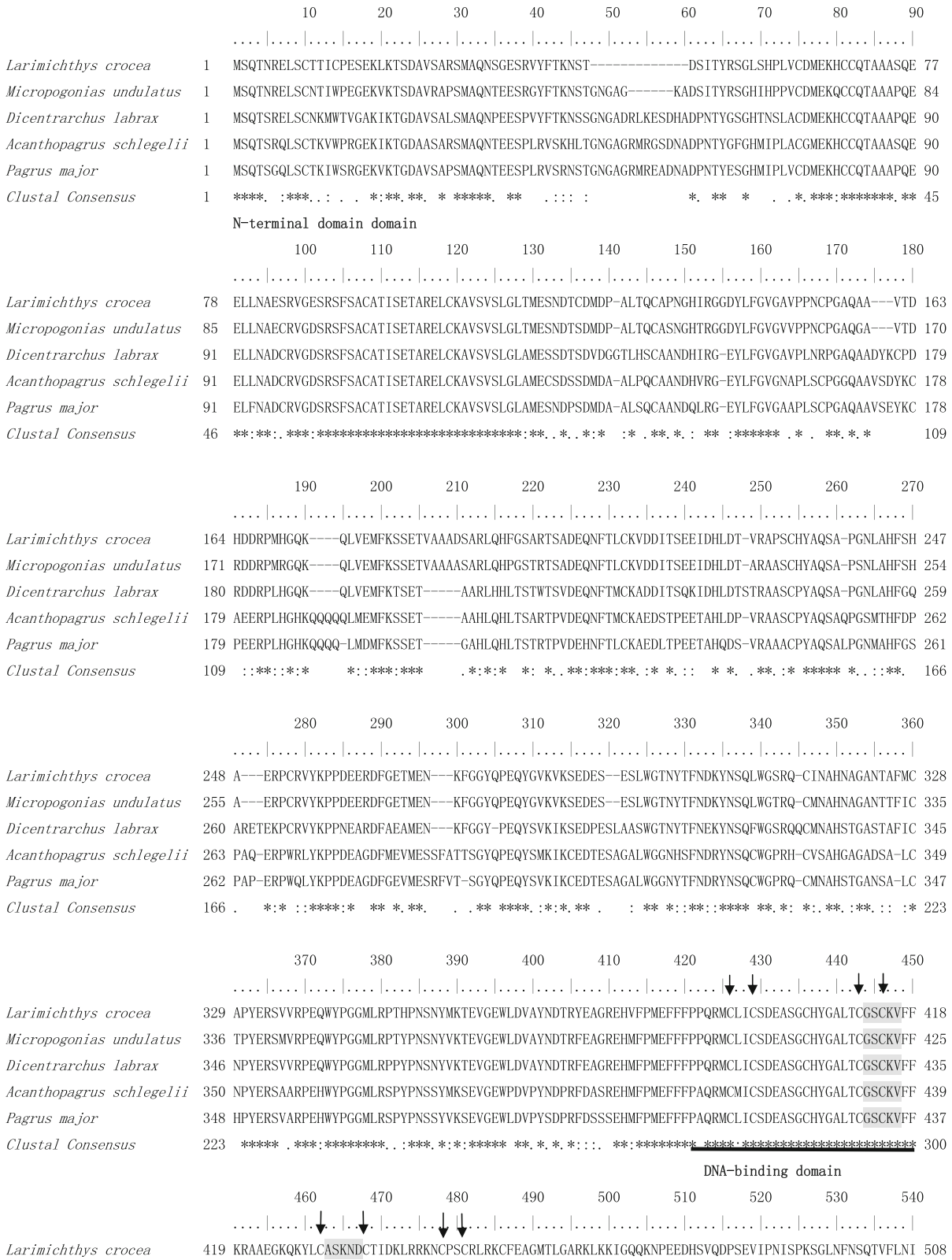
Localization of large yellow croaker *AR* mRNA in gametogenesis

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

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)

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



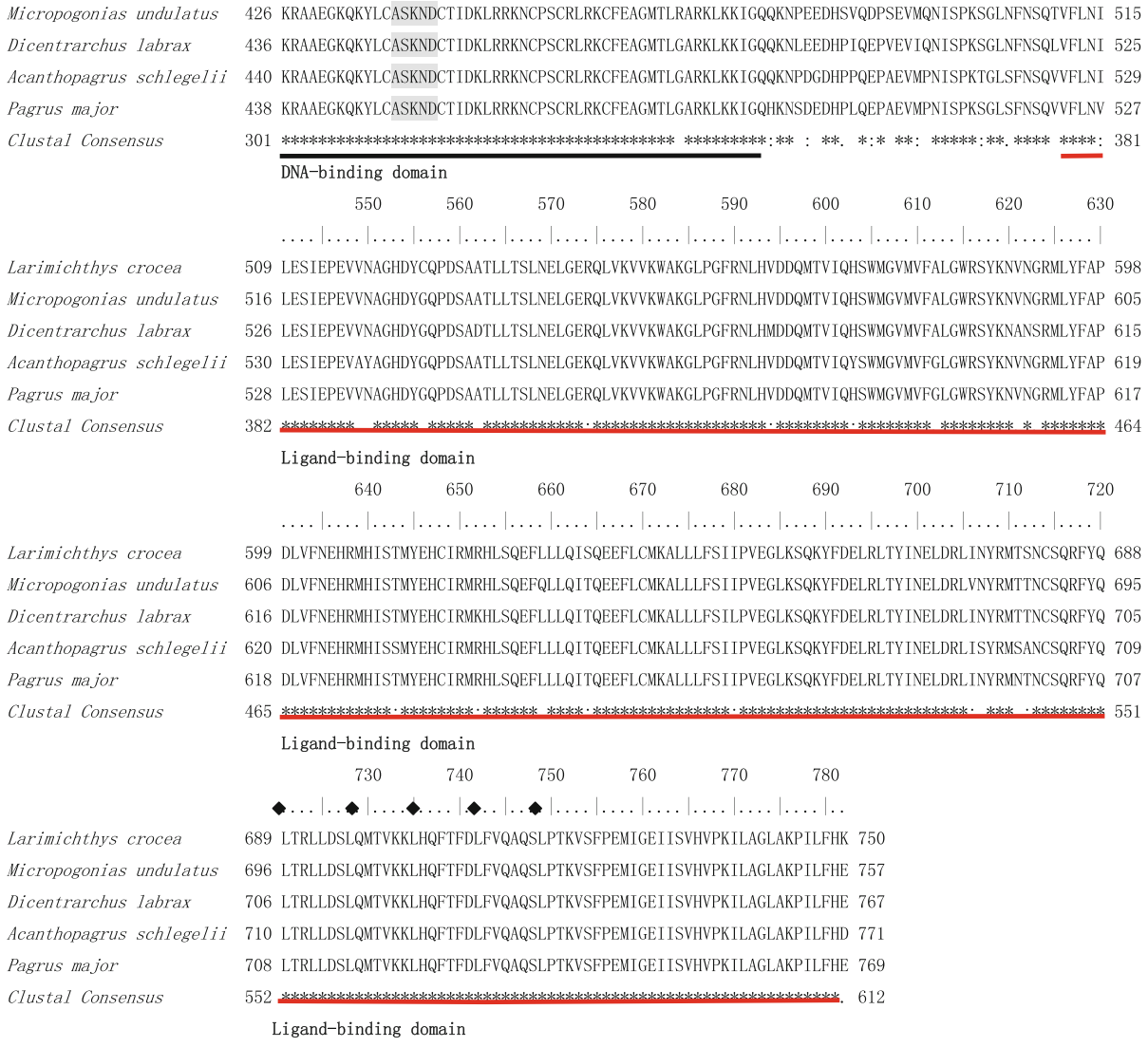


Fig. 2 continued

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, ARα DBD is 85–100 % identical to ARβ 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

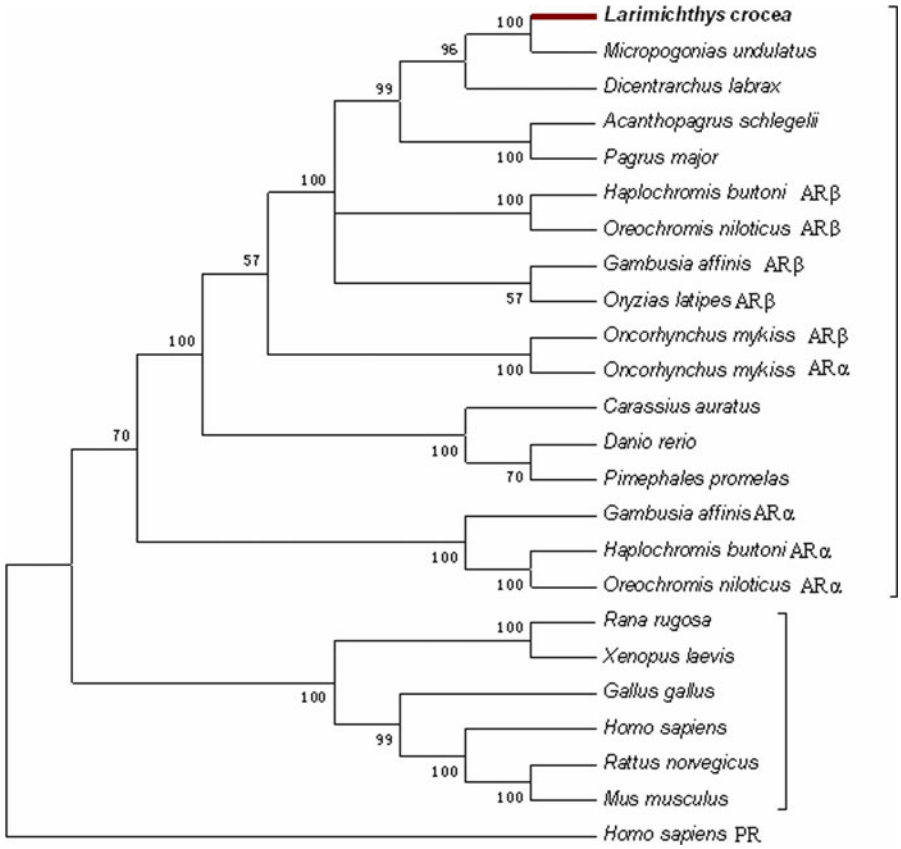


Fig. 3 Phylogenetic tree of the AR amino acid sequence between *Larimichthys crocea* and other species. *Homo sapiens*-PR protein was used as the out-group. Numbers indicate the bootstrap confidence values of 1,000 replicates. The GenBank accession numbers of the selected AR sequences are as follows: *Micropogonias undulatus* (AAU09477), *Dicentrarchus labrax* (AAT76433), *Acanthopagrus schlegelii* (AAO61694), *Pagrus major* (BAA33451), *Haplochromis burtoni* ARα (AAD25074), *Haplochromis burtoni* AR*β* (AAL92878), *Oncorhynchus mykiss* ARα (NP_001117656), *Oncorhynchus mykiss* AR*β* (NP_001117657), *Oreochromis niloticus* AR*β* (BAB20082), *Oreochromis niloticus* ARα (BAB20081), *Gambusia affinis* AR*β* (BAD81046), *Gambusia affinis* ARα(BAD52085), *Oryzias latipes* AR*β* (NP_001098151), *Oryzias latipes* ARα (NP_001164304), *Carassius auratus* (AAM09278), *Danio rerio* (NP_001076592), *Pimephales promelas* (AAF88138), *Rana catesbeiana*, (AAP85538), *Xenopus laevis* (AAC97386), G*allus gallus* (NP_001035179), *Homo sapiens* (AAA51729), *Rattus norvegicus* (AAA40733), *Mus musculus domesticus* (AAA37234), *Homo sapiens*-PR (AAA60081)

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 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*β* 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

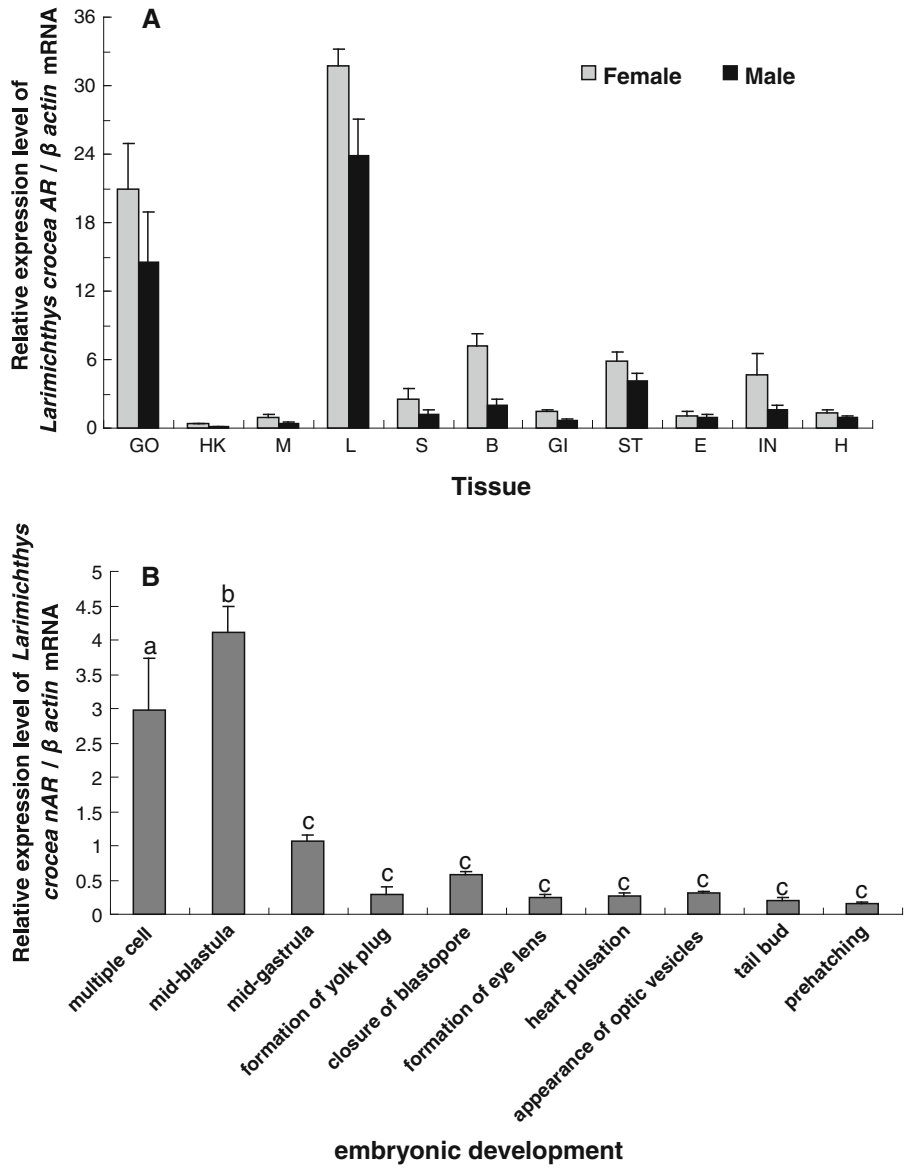
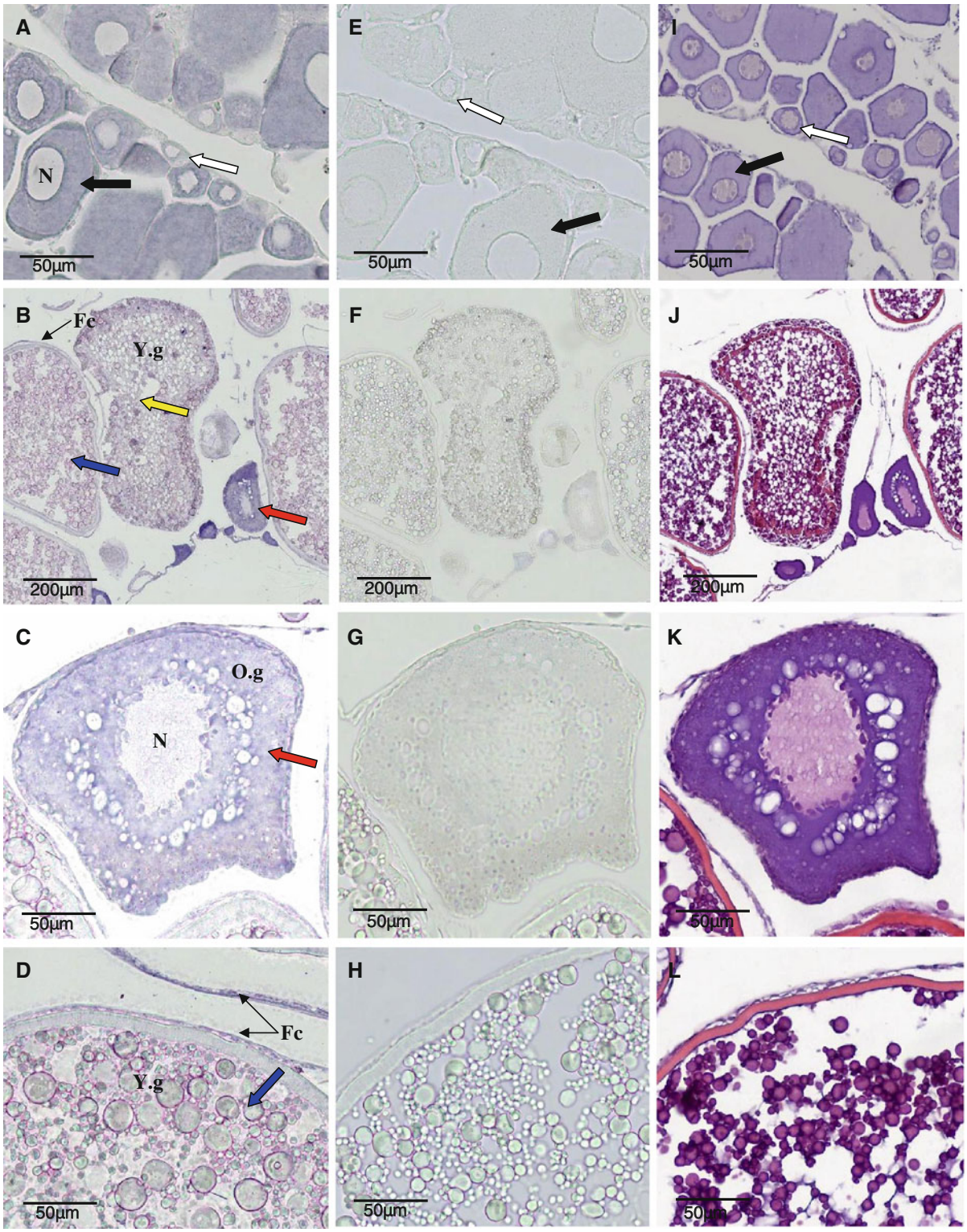


Fig. 4 Expression of AR analyzed by real-time PCR mRNA in different tissues of both sexes (**A**) and in all stages of embryonic development (**B**). *GO* gonad, *HK* head kidney, *M* muscle, *L* liver, *S* spleen, *B* brain, *GI* gill, *ST* stomach, *E* eye, *IN* intestine, *H* heart. Each *bar* represents mean ± SEM ($n = 5$). *a*, *b*, and *c* groups sharing the *same letter codes* were not significantly different ($p > 0.05$); groups with *different letter codes* were significantly different ($p < 0.05$). *β-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 ARβ types of the two AR subtypes. Phylogenetic analysis seems to indicate that the teleost ARβ type is the canonical form, whereas ARα 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 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) ▶



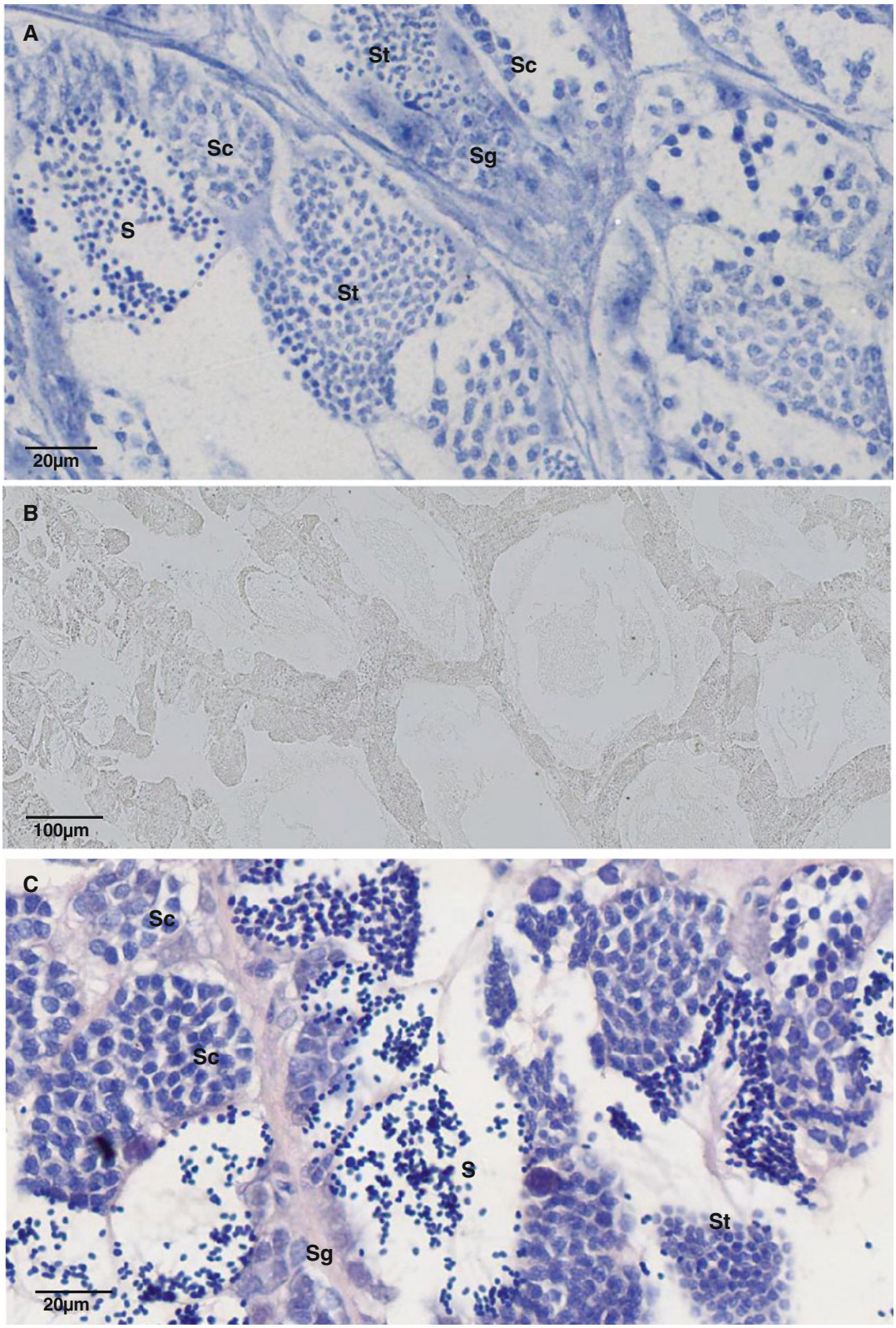
distribution and relative expression of *ARs* are quite different in fishes that contain two AR subtypes, such as rainbow trout *ARα*, which was predominantly expressed in the ovary and testis, and *ARβ*, which showed highest expression in brain (Takeo and Yamashita 1999). In cichlid fish, *ARα* showed significantly higher expression than *ARβ* in the pituitary, and *ARβ* expressed at a higher level than *ARα* in the anterior and middle brain (Harbott et al. 2007). In the male Japanese eel, *ARβ* mRNA showed preferential expression in testis, muscle, and spleen, but *ARα* 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 *AR* are detected in some tissues, but not in all tissues analyzed. The difference in expression patterns between *AR* and *ARα/β* suggests that the single AR type in some fishes has a dual role as two AR types (ARα and ARβ) 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 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.

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

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 *ARα* mRNA levels in the Japanese eel were high from the late oil droplet stage to the late vitellogenic stage, whereas *ARβ* mRNA levels were



high from the late oil droplet stage to the mid-vitellogenic stage (Tosaka et al. 2010). In the short-finned 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 multiple-cell 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.

Acknowledgments This project was supported by the National Natural Science Foundation of China (No. 30600467) and the Innovation Team Foundation of Jimei University (2010A001).

References

Bremner WJ, Millar MR, Sharpe RM, Saunders P (1994) Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. Endocrinology 135:1227–1234

Chaffin CL, Stouffer RL, Duffy DM (1999) Gonadotropin and steroid regulation of steroid receptor and aryl hydrocarbon receptor messenger ribonucleic acid in macaque granulosa cells during the periovulatory interval. Endocrinology 140:4753–4760

Chang JA, Nguyen HT, Lue TF (2002) Androgens in penile development, penile erection, and erectile dysfunction. In: Chang C (ed) Androgens and androgen receptor: mechanisms, functions and clinical applications. Kluwer Academic Publishers, Norwell, pp 289–298

Chen H, Chen W, Lin GW, Liu ZK, Xie YQ, Gao ZG (2007) The morphological characteristics and growth pattern of cage-cultured large yellow croaker (*Larimichthys crocea*) in Guanjing-Yang population. Hai Yang Ke Xue 29:331–336 (In Chinese)

Cheng G, Weihua Z, Mäkinen S, Mäkelä S, Saji S, Warner M, Gustafsson JA, Hovatta O (2002) A role for the androgen receptor in follicular atresia of estrogen receptor beta knockout mouse ovary. Biol Reprod 66:77–84

Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J (1991) Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. J Biol Chem 266:3107–3112

De Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J (2008) Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. Reproduction 136:225–234

Fang YQ, Weng YZ, Zhou J, Liu JF, Xie FJ (2000) Study on earlier gonadal maturation in cultured large yellow croaker, *Pseudosciaena crocea* I. Status of gonadal development in culture of net cages. Tai Wan Hai Xia 19:354–359 (In Chinese)

Gelmann EP (2002) Molecular biology of the androgen receptor. J Clin Oncol 20:3001–3015

Harbott LK, Burmeister SS, White RB, Vagell M, Fernald RD (2007) Androgen receptors in a cichlid fish, *Astatotilapia*

burtoni: structure, localization, and expression levels. J Comp Neurol 504:57–73

Hild-Petito S, Fazleabas AT (1997) Expression of steroid receptors and steroidogenic enzymes in the baboon (*Papio anubis*) corpus luteum during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab 82:955–962

Hillier SG, Tetsuka M (1997) Role of androgens in follicle maturation and atresia. Baillieres Clin Obstet Gynaecol 11:249–260

Hossain MS, Larsson A, Scherbak N, Olsson PE, Orban L (2008) Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. Biol Reprod 78:361–369

Huang BF, Sun YL, Wu FR, Liu ZH, Wang ZJ, Luo LF, Zhang YG, Wang DS (2011) Isolation, sequence analysis, and characterization of androgen receptor in Southern catfish, *Silurus meridionalis*. Fish Physiol Biochem 37:593–601

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y (1999) cDNA cloning of a novel androgen receptor subtype. J Biol Chem 274:25205–25209

Janssen PJ, Brinkmann AO, Boersma WJ, Van der Kwast TH (1994) Immunohistochemical detection of the androgen receptor with monoclonal antibody F39.4 in routinely processed, paraffin-embedded human tissues after microwave pre-treatment. J Histochem Cytochem 42:1169–1175

Johnson PF, McKnight SL (1989) Eukaryotic transcriptional regulatory proteins. Annu Rev Biochem 58:799–839

Kuo CM, Ting YY, Yeh SL (1988) Induced sex reversal and spawning of blue-spotted grouper, *Epinephelus fario*. Aquaculture 74:113–126

Kwon HC, Choi SH, Kim YU, Son SO, Kwon JY (2005) Androgen action on hepatic vitellogenin synthesis in the eel, *Anguilla japonica* is suppressed by an androgen receptor antagonist. J Steroid Biochem Mol Biol 96:175–178

Lin D, Zhan J, Luo J, Zheng ZY, Shi XW (1992) Studies on the gonadal development and the annual reproductive cycle of the cultured large yellow croaker, *Pseudosciaena crocea* (Richardson). J Fujian Normal Univ (Nat Sci) 8:81–87 (In Chinese)

Liu X, Su H, Zhu P, Zhang Y, Huang J, Lin H (2009) Molecular cloning, characterization and expression pattern of androgen receptor in *Spinibarbus denticulatus*. Gen Comp Endocrinol 160:93–101

Lokman PM, George KA, Divers SL, Algie M, Young G (2007) 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. Reproduction 133:955–967

Lutz LB, Cole LM, Gupta MK, Kwist KW, Auchus RJ, Hammes SR (2001) Evidence that androgens are the primary steroids produced by *Xenopus laevis* ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc Natl Acad Sci USA 98:13728–13733

Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci USA 88:5774–5778

Olsson PE, Berg AH, Von Hofsten J, Grahn B, Hellqvist A, Larsson A, Karlsson J, Modig C, Borg B, Thomas P (2005) Molecular cloning and characterization of a nuclear androgen receptor activated by 11-ketotestosterone. Reprod Biol Endocrinol 3:37–53

Pasmanik M, Callard G (1985) Identification and characterization of an androgen receptor in the brain of goldfish (*Carassius auratus*). Am Zool 25:115A

Quigley C (1998) The androgen receptor: physiology and pathophysiology. In: Nieschlag E, Behre HM (eds) Testosterone: action, deficiency, substitution. Springer, Heidelberg, pp 33–106

Schoenmakers E, Alen P, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, Claessens F (1999) Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochem J 341:515–521

Shan LX, Bardin CW, Hardy MP (1997) Immunohistochemical analysis of androgen effects on androgen receptor expression in developing leydig and sertoli cells. Endocrinology 138:1259–1266

Shen B, Zhang Z, Wang Y, Wang G, Chen Y, Lin P, Wang S, Zou Z (2009) Differential expression of ubiquitin-conjugating enzyme E2r in the developing ovary and testis of penaeid shrimp *Marsupenaeus japonicus*. Mol Biol Rep 36:1149–1157

Shu H, Zhang Y, Liu XC, Li GL, Lin HR (2006) Effects of ADSD implantation on endocrine and gonadal development in red-spotted grouper *Epinephelus akaara*. Curr Zool 52:316–327

Smolinsky AN, Doughman JM, Kratzke LT, Lassiter CS (2010) Zebrafish (*Danio rerio*) androgen receptor: sequence homology and up-regulation by the fungicide vinclozolin. Comp Biochem Physiol C: Toxicol Pharmacol 151:161–166

Sone K, Hinago M, Itamoto M, Katsu Y, Watanabe H, Urushitani H, Tooi O, Guillette LJ, Iguchi T (2005) Effects of an androgenic growth promoter 17beta-trenbolone on masculinization of Mosquitofish (*Gambusia affinis affinis*). Gen Comp Endocrinol 143:151–160

Takeo J, Yamashita S (1999) Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. J Biol Chem 274:5674–5680

Tosaka R, Todo T, Kazeto Y, Mark Lokman P, Ijiri S, Adachi S, Yamauchi K (2010) Expression of androgen receptor mRNA in the ovary of Japanese eel, *Anguilla japonica*, during artificially induced ovarian development. Gen Comp Endocrinol 168:424–430

Walters KA, Allan CM, Handelsman DJ (2008) Androgen actions and the ovary. Biol Reprod 78:380–389

Wang RS, Yeh S, Tzeng CR, Chang C (2009) Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev 30:119–132

Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Okafor J, Bondy CA (1998) Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J Clin Endocrinol Metab 83:2479–2485

Wen HS, Zhang XR, Chen CF, Jin GX, Chen XY, Shen B, He F (2009) cloning and expression analysis of male half-smooth tongue solt (*Cynoglossus semilaevis*)'s androgen receptor gene. J Ccean Univ China 39:387–391 (In Chinesis)

Wilson VS, Cardon MC, Thornton J, Korte JJ, Ankley GT, Welch J, Gray LE, Hartig PC (2004) Cloning and in vitro

expression and characterization of the androgen receptor and isolation of estrogen receptor α from the fathead minnow (*Pimephales promelas*). Environ Sci Technol 38:6314–6321

Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X, Xing L, Boyce BF, Hung MC, Zhang S, Gan L, Chang C (2002) Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc Natl Acad Sci USA 99:13498–13503

Yeh SL, Kuo CM, Ting YY, Chang CF (2003) Androgens stimulate sex change in protogynous grouper, *Epinephelus coioides*: spawning performance in sex-changed males. Comp Biochem Physiol C Toxicol Pharmacol 135: 375–382

You YL, Lin DJ (1997) The ultrastructure of the spermatozoon of the teleost, *Pseudosciaena crocea* (richardson). Dong Wu Xue Bao 43:119–126 (In Chinese)

You YL, Lin DJ, Chen LY (2001) Spermatogenesis of teleosts, *Pseudosciaena crocea*. Dongwuxue Yanjiu 22:461–466 (In Chinese)

Zhou P, Zhang ZP, Wang YL, Zou ZH, Xie FJ (2010) EST analysis and identification of gonad-related genes from the normalized cDNA library of large yellow croaker, *Larimichthys crocea*. Comp Biochem Physiol Part D Genomics Proteomics 5:89–97