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Molecular identification and expression of the *Foxl2* gene during gonadal sex differentiation in northern snakehead *Channa argus*

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Abstract *Channa argus* is one of the most commercially important fish species in China. Studies show that males of *C. argus* grow faster than females at the same age. In order to explore the sex differentiation mechanism of *C. argus*, we isolated the full length of the sex-related gene *Foxl2* cDNA and analysed its expression patterns during gonadal sex differentiation. Alignment of known Foxl2 amino acid sequences from vertebrates confirmed the conservation of the Foxl2 open reading frame, especially the forkhead domain and C-terminal region. Quantitative RT-PCR revealed that *Foxl2* is predominantly expressed in brain, pituitary, gill and ovary, with its highest level in ovary but low levels in testis and other tissues, reflecting a potential role for *Foxl2* in the brain–pituitary–gonad axis in *C. argus*. Our ontogenetic stage data showed that *C. argus Foxl2* expression was significantly upregulated from 1 to 11 days posthatching (dph) and that the initiation of expression preceded the first anatomical ovarian differentiation (27 dph), suggesting that *Foxl2* might play a potential role in early gonadal sex differentiation in *C. argus*. In addition, the Foxl2 protein was primarily located in granulosa cells surrounding the oocytes of mature *C. argus*, implying that *Foxl2* may have a basic function in granulosa cell differentiation and the maintenance of oocytes.

Keywords *Foxl2* · Molecular identification · Gene expression · Early gonadal development · *Channa argus*

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Introduction

Sex determination and differentiation are two important events in the development of gonads in vertebrates (Hughes 2001). Sex determination is a complex process that may be affected by direct environmental

induction or genetic regulation (Devlin and Nagahama 2002; Raghuveer et al. 2011). For example, offspring of some fishes and reptiles can become male or female in response to an environmental factor(s) such as temperature (e.g., Janzen 1994), whereas the sex of many birds and mammals is determined by genotype at conception (Bull 1983; Conover and Heins 1987). In vertebrates, sex determination is characterised by a lack of conservation (Ijiri et al. 2008; Raghuveer et al. 2011). Sex differentiation of gonads after sex determination is a developmental process of most species and is considered as the differentiation from an undifferentiated gonadal primordium towards a testis or an ovary (Baron et al. 2005). At least some of the genes involved in the process of gonadal sex differentiation have now been identified, e.g., *Sry*, *Sf-1*, *Wt1*, *Sox9*, *Dmrt1*, *Amh*, *Gata4*, *Dax1*, *Wnt4*, *Cyp19a1a* and *Foxl2* (Devlin and Nagahama 2002; Koopman 2001; Matsuda et al. 2002; Wilhelm et al. 2007). However, various genes that regulate sex differentiation are relatively conserved in most vertebrates (Angelopoulou et al. 2012; Baron et al. 2005; Devlin and Nagahama 2002; Raymond et al. 1998; Smith et al. 2013). For example, some mammalian sex differentiation-related genes have been identified in teleosts, including the *Dmy*/*Dmrt1*, *Sox9* and *Sry* genes that promote testicular differentiation, while *Cyp19a1a* and *Foxl2* are implicated in ovarian differentiation in most teleosts (Devlin and Nagahama 2002; Sandra and Norma 2010). In teleosts, the proportion of androgens and oestrogens is of great importance for gonadal differentiation, and this hormonal balance is maintained by aromatase which is a key enzyme catalysing the conversion from androgens to oestrogens (Guiguen et al. 2010). The expression of aromatase is regulated by several factors such as Foxl2 (forkhead box L2) and Sf-1 (steroidogenic factor-1) (Pannetier et al. 2006; Wang et al. 2007). While extensive studies have been carried out on aromatases during sexual differentiation (Guiguen et al. 1999, 2010; Zhang et al. 2014), there has been limited research conducted on the role of *Foxl2* during sexual differentiation in teleosts (Wang et al. 2004; Sridevi and Senthilkumaran 2011). In this context, it is of interest to clone the *Foxl2* gene to quantify the expression pattern of *Foxl2* during gonadal differentiation in a teleost. This analysis might provide further valuable evidence for the significance of *Foxl2* in the regulation of aromatases.

Forkhead transcription factors play critical roles in the regulation of cellular differentiation and proliferation (Cunningham et al. 2003; Vaquerizas et al. 2009). They are also involved in several other biological processes, including tissue development, establishment of the body axis and metabolic processes (Carlsson and Mahlapuu 2002). Forkhead box L2 (Foxl2) is a member of the forkhead family of transcription factors involved in ovarian development, granulosa cell differentiation and the maintenance of ovarian function (Cocquet et al. 2003; Nakamoto et al. 2006). In humans, *Foxl2* gene mutations cause blepharophimosis ptosis epicanthus inversus syndrome (BPES), characterised by eyelid malformations and premature ovarian failure (POF) (Crisponi et al. 2001). The same phenomenon is observed in the disruption of *Foxl2* function of mice (Uda et al. 2004). The genetic programme for somatic testis determination is activated in an XX gonads mouse lacking *Foxl2* from meiotic prophase oocytes, implying the pivotal function of *Foxl2* to repress the male gene pathway at several stages of female gonadal differentiation (Ottolenghi et al. 2005). Moreover, genes involved in testis differentiation such as *Sox9* are sharply upregulated after birth in *Foxl2* null mice (Ottolenghi et al. 2005). As far as is presently known, *Foxl2* is the first gene in the developmental pathway showing differential expression between genders for gonadal differentiation in vertebrates, including teleosts (Cocquet et al. 2003; Ijiri et al. 2008; Leet et al. 2011; Loffler et al. 2003). In goats, rainbow trout and tilapia, the expression of *Foxl2* is specifically initiated before morphological sex differentiation in female gonads and can persist until adulthood (Baron et al. 2004; Wang et al. 2004; Pailhoux et al. 2001). These results suggest that *Foxl2* is evolutionarily conserved among vertebrates and may be involved in the early stages of female sex differentiation. On the other hand, *Foxl2* is also involved in the transcriptional regulation of aromatase. Goats with polled intersex syndrome (PIS), whose *Foxl2* function has been disrupted, display a reduction in the expression of aromatase (Pailhoux et al. 2001, 2002). Studies of chicken and rainbow trout demonstrate that the spatiotemporal expression profile of *Foxl2* is strongly correlated with the expression of aromatase during sex differentiation and later follicular development (Baron et al. 2004; Govoroun et al. 2004). *Foxl2* is also involved in the transcriptional regulation of aromatase in teleosts such

as Japanese flounder (Yamaguchi et al. 2007) and medaka (Nakamoto et al. 2006). These results suggest that *Foxl2* is involved in regulation of oestrogen synthesis via transcriptional regulation of aromatase during ovarian development.

Snakeheads (Teleostei: Channidae) are a group of freshwater fishes native to Asia and Africa (Ng and Lim 1990). The northern snakehead (*Channa argus*), which is mainly distributed in the Yangtze River and the Amur River basin, is one of the most commercially important fish species in China. Total production of the snakehead reached 480,594 tonnes in 2012, ranking it ninth in all freshwater fish species production in China (Fisheries Bureau of the Agriculture Ministry of China 2013). Aquaculture practices and studies show that males of *C. argus* grow faster than females and the average weight of males is 30 % larger than females at the same age (Jiang et al. 2013). It may therefore be possible to improve aquaculture production of *C. argus* through genetic manipulation of the sex ratio by obtaining all-male snakehead broods. However, there are few studies of sex differentiation of *C. argus*, except for the screening of sex-linked molecular markers (Jiang et al. 2013). In order to explore whether *Foxl2* plays a role in the sexual development of *C. argus*, we investigated early gonadal development and sex differentiation by histological analysis and also identified the full length of *Foxl2* cDNA. We analysed its expression patterns by qPCR during gonadal sex differentiation and the localisation of the Foxl2 protein by immunohistochemistry in mature gonads of *C. argus*. Our data provide valuable information for further studies of the sex differentiation mechanism of *C. argus* and will contribute to ongoing work focusing on the development of this species as a major aquaculture species in China.

Materials and methods

Animals and sampling

Ten *C. argus* specimens with an average weight 500 g were obtained from Liangzi Lake, Hubei Province. The fish were transported to Huazhong Agricultural University (HZAU) and reared for 1 week in a circulating water system at the College of Fisheries. Six individuals (three males and three females) were anesthetised with 1000 mg/L MS-222 before dissection. Various tissues (spleen, kidney, liver, gonad, gill, brain, pituitary, intestines, heart and muscle) were collected for gene cloning and tissue distribution analysis of gene expression.

To study the expression of the *Foxl2* gene in gonads during early development, fertilised eggs of *C. argus* were collected from Wusi Lake in Ezhou City, Hubei Province. The eggs were incubated at 25 ± 1 °C in indoor tanks with an air pump for sufficient dissolved oxygen. Fry hatched on the third day after fertilisation. After hatching, larvae were transferred to rectangular plastic boxes for rearing and were fed with yolk and zooplankton after mouth-opening formation. The zooplankton was collected daily from South Lake next to the HZAU campus. During the period of artificial cultivation, the fish were kept at a water temperature of 25 ± 1 °C and with a 14:10-h light–dark cycle. Samples were collected at 1, 4, 7, 11, 17, 23, 30, 34, 41 and 48 days posthatching (dph). Six individuals were sampled each time at 1–23 dph and three individuals at 30–48 dph. Sampling was repeated five times on each occasion. For smaller fry at 1–30 dph stages, the whole body was collected for subsequent experiments. For bigger larvae at 34–48 dph stages, the fish head and posterior segment (from cloaca to tail end) were removed after anesthetising with 100 mg/L MS-222, and the abdomen segment was collected for subsequent experiments.

All adult tissues and larval samples were immediately immersed in liquid nitrogen and stored at −80 °C until RNA extraction. This experiment was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees (IACUC) of HZAU, Wuhan, P. R. China.

Cloning and sequencing of *Foxl2* cDNA

Total RNA was isolated from ovary tissue of adult *C. argus* individuals using TRIzol Reagent (Invitrogen, USA) following the manufacturer's instructions. The cDNA synthesis was carried out using a Molony murine leukaemia virus (M-MLV) Reverse Transcriptase kit (Promega, USA) according to the manufacturer's protocol. To obtain partial sequence of *C. argus Foxl2* cDNA, a pair of primers (*Foxl2*-F, *Foxl2*-R) was designed based on the conserved domains of the known *Foxl2* sequences (Nile tilapia *Oreochromis niloticus*, NM_001279778; honeycomb grouper

Epinephelus merra, EU555180; and three-spot wrasse *Halichoeres trimaculatus*, AB547448). To obtain the full-length cDNA sequence of *Foxl2*, rapid amplification of cDNA end (RACE) was performed by the standard method (Sambrook et al. 2001) using the gene-specific primers and adaptor primers (Table 1). The primers were designed using the software Primer Premier 5.0.

PCR amplifications were performed in a total volume of 10 μL, including 1.0 μL 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.4 U *Taq* DNA polymerase (Fermentas, Canada), 0.2 mM dNTP, 0.4 μM of each primer and 1.0 μL cDNA template. The PCR conditions were as follows: 3 min at 95 °C for predenaturation; 35 cycles of 30 s at 94 °C for denaturation, 30 s at 62 °C for annealing and 45 s at 72 °C for extension; 10 min at 72 °C for final extension.

All amplified products were checked by electrophoresis on a 1.5 % agarose gel. The target DNA fragments were purified using an AxyPrep™ gel extraction kit (Axygen, USA) and ligated into a pMD18-T vector (TakaRa, Japan). Following transfection into *Escherichia coli* DH5α competent cells, recombinants were identified by blue and white spot selection. Putative clones were further screened by PCR amplification and were sequenced by the Sangon Biotech Company (Shanghai, China).

Sequence analysis

Open reading frame (ORF) and protein predictions were performed using the ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced protein sequence was analysed with the BLAST programme on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/). Clustal X 1.83 was used to perform multiple alignment of amino acid sequence. A phylogenetic tree was constructed by the neighbour-joining (NJ) method using MEGA 5.03 software.

Real-time quantitative PCR

Gene expression was determined by real-time quantitative PCR (qPCR) using a Rotor-Gene 6500 Thermocycler (Corbett Research, Australia). The gene-specific primers were designed based on the full-length *Foxl2* cDNA sequence (Table 1). The primer sequences of β-actin were obtained from Jia and Guo (2008). The reaction mixture of the qPCR consisted of 10 μL 2× GoTaq® qPCR Master Mix (Promega, USA), 0.2 μL 100× CXR reference dye, 0.4 μL of each gene-specific primer (10 μM), 2.0 μL cDNA template and 7 μL nuclease-free water in a total volume of 20 μL. The qPCR cycling parameters were 95 °C hold for 2 min, then 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s. Melt curve analysis was carried out over a range from 55 to 99 °C at the end of each PCR run. All qPCRs were performed in triplicate biological replicates using standard reagents. The standard curve quantification method was adopted to analyse the data. Standard curves for each gene were obtained through a tenfold dilution series of plasmids. The β-actin gene was used as an internal control. The relative expression levels of the target

Table 1 Primers designed for cloning and expression analysis of *Foxl2*

Name of primer	Sequence (5′ → 3′)	Application
Foxl2-F	CTATGTCGCTCTCATTGCC	cDNA fragment PCR
Foxl2-R	AGTGTTTGGTCTCGTGTTCC	
F-3′ RACE outer	CTGTCAGATGGCGGGTGGCA	3′ RACE cloning
F-3′ RACE inner	CTTCAGTTCGCCTGCTCCCG	
F-5′ RACE outer	CGTTGCCACCCGCCATCTG	5′ RACE cloning
F-5′ RACE inner	CGTGTAGGACATTGGATTGGGCG	
Oligo-dT adaptor	GACTCGAGTCGACATCGA$(T)_{17}$	Adaptor primer for RACE
Adaptor	GACTCGAGTCGACATCG	
Foxl2-RT-F	AGATGGCGGGTGGCAACG	Real-time PCR for *Foxl2*
Foxl2-RT-R	CGGGATGGTGATGGTGGCTC	
β-actin-F	CACTGTGCCCATCTACGAG	Real-time PCR for β-actin
β-actin-R	CCATCTCCTGCTCGAAGTC	

genes were expressed as ratios of the copy numbers of target gene to copy numbers of β-actin gene.

Expression patterns of *Foxl2* mRNA

Total RNA was extracted from various adult tissues and fry using TRIzol Reagent as described above. To avoid genomic DNA interference on qPCR, the total RNA was treated with RNase-free DNase I (TaKaRa, Japan). Approximately 1 μg of DNase I-treated total RNA was used for the synthesis of first-strand cDNA. Then, the cDNA was stored at −20 °C for qPCR and expression analysis as described above.

Histology of early gonad development

Five to ten fry were collected at 6, 15, 21, 27, 30, 34 and 48 dph. The samples were immersed in Bouin's solution at room temperature for at least 48 h and stored in 70 % ethanol until histological processing. Tissues were dehydrated in a series of alcohol, clarified in benzene and embedded in paraffin. Cross sections were cut into 6-μm slices and were stained with haematoxylin–eosin. A thorough observation of gonads was performed by cutting the whole body or abdomen segment into serial sections (Gao et al. 2009) to observe the differentiation of the gonads. The sections were observed and photographed using a Nikon 80i microscope (Japan).

Localisation of Foxl2 protein in the mature gonads by immunohistochemistry

Live, wild adult individuals of *C. argus* were collected from markets in Wuhan. The fish were kept in a circulating water system at the College of Fisheries for 1 week. Two individuals (one male and one female) were anesthetised with 1000 mg/L MS-222 before dissection. A small segment of gonad was collected from both fishes and was fixed in Bouin's solution for immunohistochemical analysis. An anti-Foxl2 polyclonal antibody (PA1-802, Thermo, USA) was used to determine the cellular localisation of the Foxl2 protein in the gonads. Histological sections were prepared as described above. After paraffin removal and dehydration, the sections were washed with citric acid buffer (0.1 M citric acid and sodium citrate, pH 6.0), incubated in 3 % (v/v) H_2O_2 and 10 % (v/v) normal goat serum to block nonspecific binding and then were incubated overnight at 4 °C with the primary antibody (diluted 1:50), which was a synthetic peptide corresponding to residues M(1)MASYPEPEDTAGT(14) of mouse Foxl2, derived from a rabbit host. After incubation with the secondary antibody which was labelled by peroxidase (anti-mouse/rabbit, DAKO K5007), the sections were exposed to 3,3′-diaminobenzidine (DAB) and stained with haematoxylin to visualise the nuclei in the gonadal tissues. As a negative control, the sections were treated in the same way but with Tris-buffered saline instead of the primary antibody. Finally, the sections were observed and photographed using a Motic BA310 microscope (China).

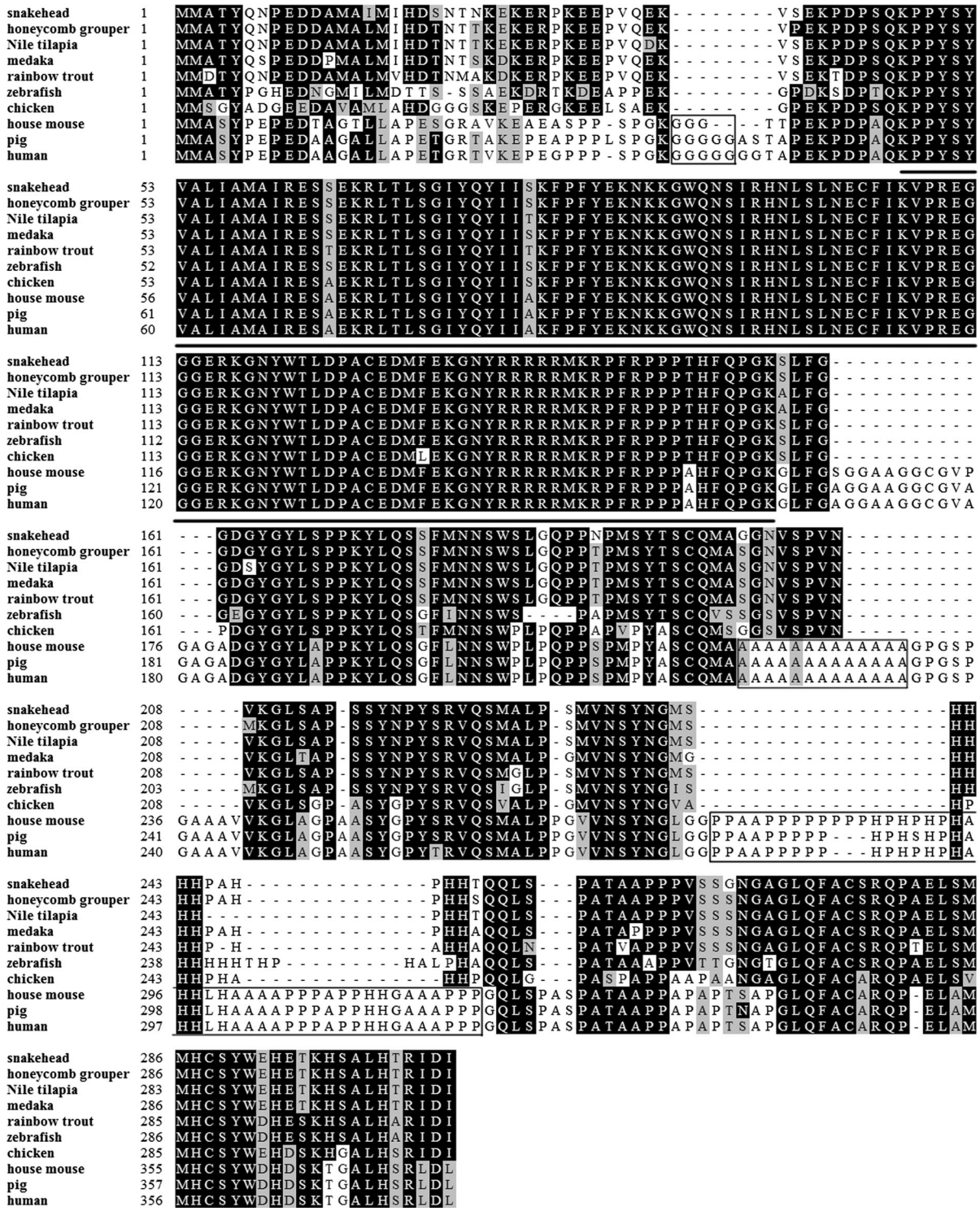
Statistical analysis

All data from qPCRs were expressed as the mean ± SE. Statistical differences were tested by one-way ANOVA using STATISTICA 6.0. $P < 0.05$ was considered to be statistically significant.

Results

Cloning and phylogenetic analysis of *Foxl2* gene

From RACE PCR, a 1966-bp full-length *Foxl2* cDNA (GenBank accession no. KF746072) was obtained, including a 213-bp 5′-untranslated region (5′ UTR), an 832-bp 3′ UTR and a 921-bp open reading frame (ORF), encoding a putative 306 amino acids. The putative *Foxl2* amino acid sequence contained the characteristic forkhead (FH) domain ranging from residue 47 to 157 (Fig. 1). The *C. argus Foxl2* amino acid sequence showed a high level of homology to those of other vertebrates. As expected, it was very similar to *Foxl2* orthologs in teleost fishes such as *Epinephelus merra* (97 %), *Oreochromis niloticus* (95 %), *Oryzias latipes* (95 %), *Oncorhynchus mykiss* (92 %) and *Danio rerio* (81 %). It also showed 79 % similarity to *Mus musculus* and 61 % similarity to *Homo sapiens*. The forkhead domain of *Foxl2* was highly conserved among these vertebrate species, whereas the 14-polyalanine tracts (A), glycine (G) and proline (P) repeats were only present in mammalian orthologs (Fig. 1). Phylogenetic (NJ) analysis demonstrated that *C. argus Foxl2* clustered with other teleosts, and it was closest to the *Foxl2* homolog of


snakehead
honeycomb grouper
Nile tilapia
medaka
rainbow trout
zebrafish
chicken
house mouse
pig
human

◀**Fig. 1** Amino acid sequence comparison of *Channa argus Foxl2* with other known orthologs. The glycine-rich repeats (G), proline repeats (P) and polyalanine tracts (A) are *boxed*. The *underlined part* indicates the forkhead (FH) domain. GenBank accession numbers of the *Foxl2* amino acid sequences used are as follows: northern snakehead *Channa argus* (KF746072); honeycomb grouper *Epinephelus merra* (ACD62374); Nile tilapia *Oreochromis niloticus* (NP_001266707); medaka *Oryzias latipes* (NP_0010988358); rainbow trout *Oncorhynchus mykiss* (NP_001117957); zebrafish *Danio rerio* (NP_001038717); chicken *Gallus gallus* (AEE80502); pig *Sus scrofa* (NP_001231594); house mouse *Mus musculus* (NP_036150); and human *Homo sapiens* (AAY21823)

the honeycomb grouper (*E. merra*) and the Nile tilapia (*O. niloticus*). Teleostean *Foxl2* was separated from elasmobranch, amphibian, avian and mammalian *Foxl2* (Fig. 2).

Tissue distribution of *Foxl2* mRNA expression levels in adults

qPCR standard curves showed a significant linear relationship between the values of threshold cycle (CT) and the logarithm of gene copy number in both *Foxl2* gene ($R^2 = 0.9990, P < 0.0001$, Fig. 3a) and β-actin gene ($R^2 = 0.9985$, $P < 0.0001$, Fig. 3b), and the amplification efficiencies were both above 93 %. Therefore, the two standard curve equations can be used to reliably calculate gene copy number of the two genes.

Expression of *Foxl2* mRNA in females had the highest tissue-specific level in ovary, followed by a high level in pituitary and gill, and a very low level in other tissues (Fig. 4a). The expression level of *Foxl2* mRNA in males was the highest in gill, followed by a high level in pituitary, a moderate level in brain, whereas the expression levels were very low in testis and other tissues (Fig. 4b).

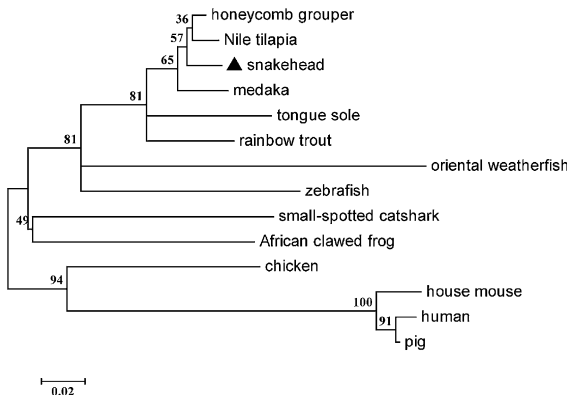


Fig. 2 Phylogenetic relationships of *Channa argus Foxl2* with other species based on deduced amino acid sequences. The *numbers in each branch* represent the bootstrap values obtained using the neighbour-joining (NJ) method. GenBank accession numbers of the sequences used are as follows: northern snakehead *Channa argus* (KF746072); honeycomb grouper *Epinephelus merra* (ACD62374); Nile tilapia *Oreochromis niloticus* (NP_001266707); medaka *Oryzias latipes* (NP_0010988358); rainbow trout *Oncorhynchus mykiss* (NP_001117957); zebrafish *Danio rerio* (NP_001038717); half-smooth tongue sole *Cynoglossus semilaevis* (ACY05959); oriental weather fish *Misgurnus anguillicaudatus* (BAJ19137); chicken *Gallus gallus* (AEE80502); pig *Sus scrofa* (NP_001231594); house mouse *Mus musculus* (NP_036150); human *Homo sapiens* (AAY21823); small-spotted catshark *Scyliorhinus canicula* (ABP63571); and African clawed frog *Xenopus laevis* (BAH22852)

Expression of *Foxl2* gene during the early development stages

The mRNA expression levels of *Foxl2* gene were detected during the early developmental stages from 1- to 48-day posthatching (dph) larvae. The expression of *Foxl2* mRNA increased significantly from a moderate level at 1 dph to the highest level at 4 dph and then decreased significantly to a moderate level (as

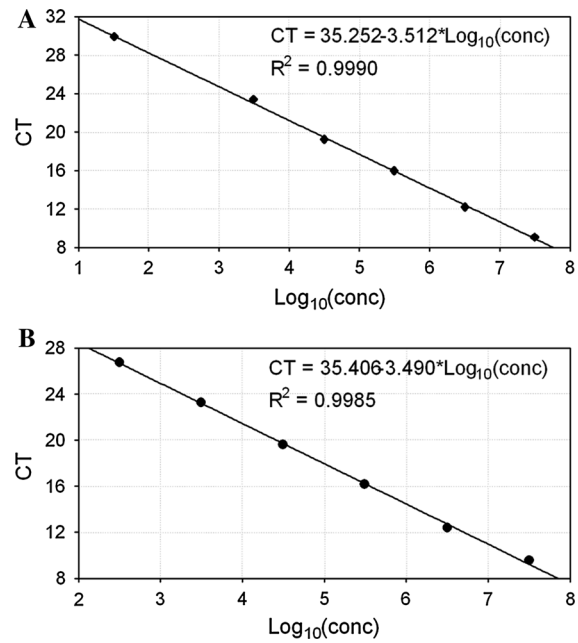


Fig. 3 Standard curves of *Foxl2* gene (**a**) and β-actin gene (**b**) showing a linear relationship between the values of threshold cycle (CT) and the logarithm of gene copy number. *conc* concentration of gene copy number (copies/μL)

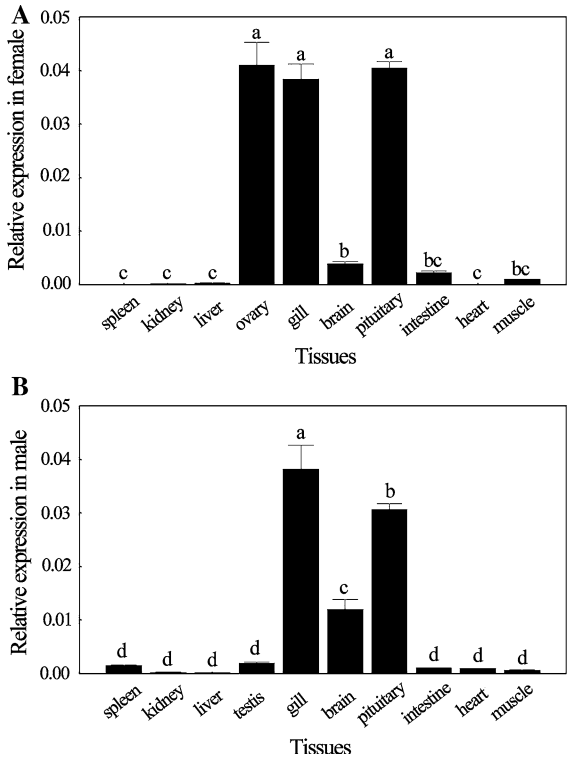


Fig. 4 Tissue distributions of relative *Foxl2* mRNA expression levels in female (**a**) and male (**b**) of *Channa argus* by qPCR. The relative expression level was measured as a ratio of copy numbers of *Foxl2* gene to copy numbers of β-actin gene. *Columns* represent the means of three replicates for each treatment. *Error bars* represent standard error of the means. *Different letters above the bars* indicate significant difference at $P < 0.05$ level

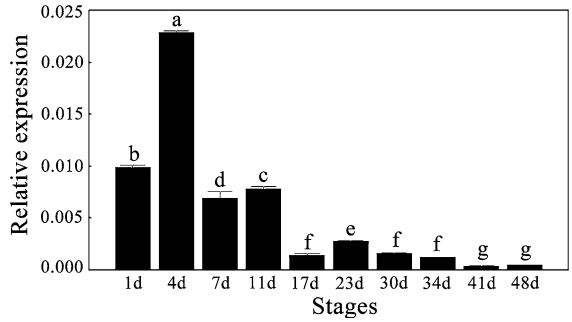


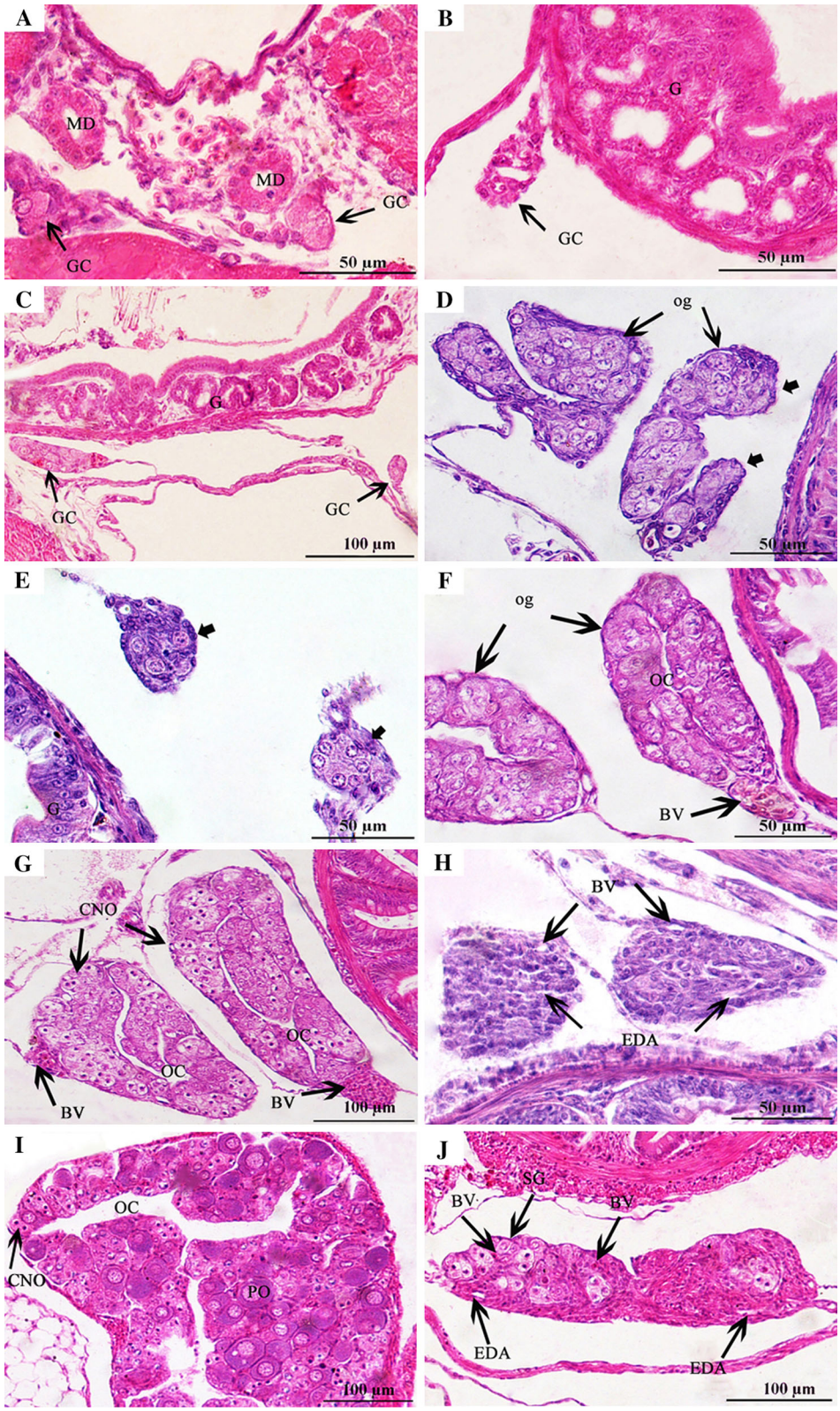
Fig. 5 Expression of *Foxl2* gene during early development stage of *Channa argus*. The relative expression level was expressed as a ratio of copy numbers of *Foxl2* gene to copy numbers of β-actin gene. 1–48 are days posthatching (dph) at early development stages. *Columns* and *bars* represent the means and standard errors, respectively. *Different letters above the bars* indicate significant difference ($P < 0.05$)

Fig. 6 Histological sections of larval and juvenile *Channa argus* gonad showing the early gonad development and sex differentiation. **a** Primordial gonads at 6 dph. A pair of primordial gonads is indicated by *arrows*. **b** Primordial gonads at 15 dph. Germ cells are undergoing early mitosis. **c** Undifferentiated gonads at 21 dph, showing the multiplication of germ cells in number. **d** Presumptive initial ovary at 27 dph, showing somatic elongations. Two somatic elongations forming the initial ovarian cavity are indicated by the *thick arrows*. **e** Presumptive testis at 27 dph. The aggregations of stromal cells are indicated by the *thick arrows*. **f** Ovary at 30 dph, showing the ovary cavity (OC) and oogonium. **g** Ovary at 34 dph, showing oocytes at chromatin-nucleolus stage (CNO). **h** Testis at 34 dph, showing the efferent duct anlage and blood vessel. **i** Ovary at 48 dph, showing some oocytes at perinucleolus stage (PO). **j** Testis at 48 dph, showing evident efferent duct anlage and spermatogonia undergoing mitosis. *BV* blood vessel, *CNO* chromatin-nucleolus oocyte, *EDA* efferent duct anlage, *G* gut, *GC* germ cells, *MD* mesonephric duct, *OC* ovarian cavity, *og* oogonium, *PO* perinucleolus oocyte, *SG* spermatogonium

judged by relative expression) at which it was maintained until 11 dph. Afterwards, the expression level decreased significantly to a low level at 17 dph where it was maintained until 34 dph (but with a minor increase at 23 dph), and further decreased significantly to a very low level at 41 until 48 dph (Fig. 5).

Histology of early gonad development

Histological sections were employed to trace early gonad development and sex differentiation of *C. argus* from 6 to 48 dph. After hatching, gonadal primordial germ cells (GCs) were present under the mesonephric duct at 6 dph (Fig. 6a). The germ cells were morphologically distinguished from somatic cells by their relatively large diameter and their histological features. Primitive gonads appeared under the dorsal coelomic epithelium. At 15 dph, the gonads projected into the abdominal cavity and the germ cells had increased in number by active mitosis (Fig. 6b). A pair of pear-shaped gonads was present under the abdominal cavity and was linked to a cord-like tissue from the dorsal coelomic epithelium at 21 dph. The gonads did not exhibit any morphological characteristics indicative of a differentiating ovary or testis until 21 dph (Fig. 6c). Gonad size and number of germ cells increased dramatically between 21 and 27 dph. An apparent change in larval gonad histology occurred at 27 dph.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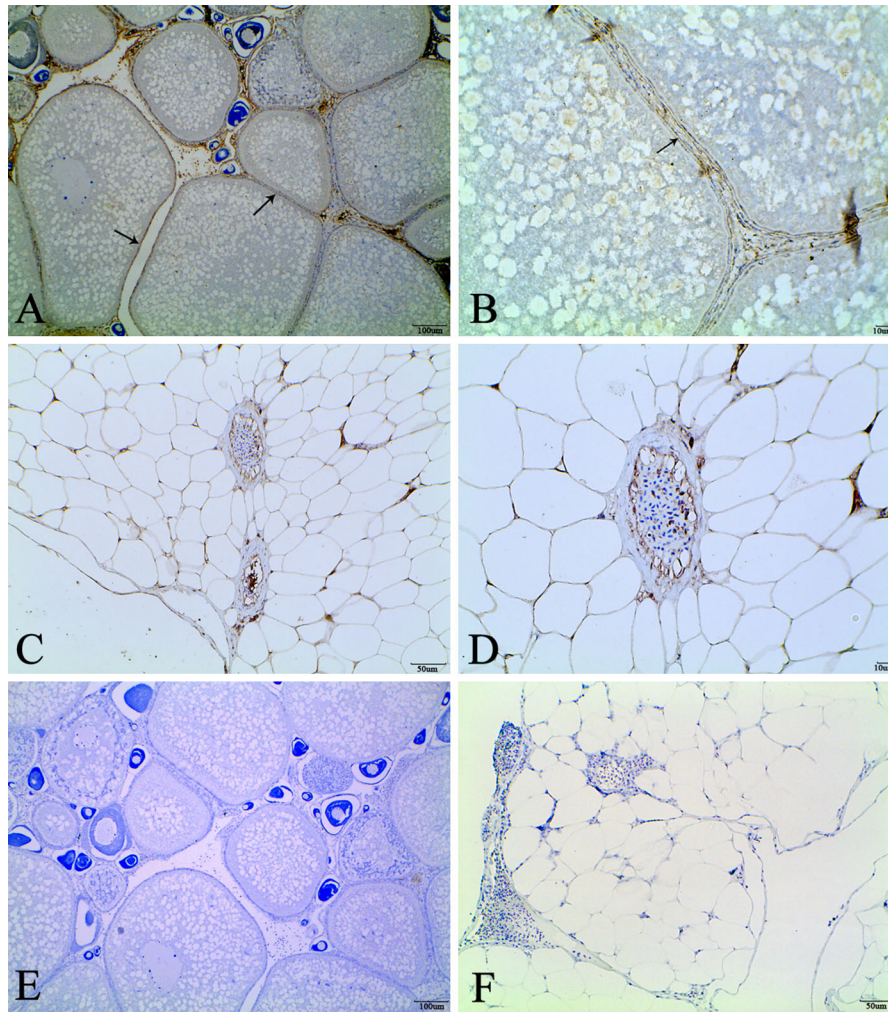

Fig. 7 Immunohistochemical analysis of Foxl2 in mature gonads of *Channa argus*. **a**–**d** Immunohistochemical analysis of Foxl2 in the ovaries (**a**, **b**) and testes (**c**, **d**). **b**, **d** Enlarged areas of **a** and **c**, respectively. The positive antigen was dyed brown with 3,3′-diaminobenzidine (DAB) (*arrows*). **e**, **f** Negative controls

The presumptive initial ovarian cavity formation was indicated by the presence of two elongated aggregations of germ cells in the gonads at 27 dph (Fig. 6d). In contrast to ovarian development, the presumptive testis had germinated to be two aggregations of stromal cells at 27 dph. The initial testes retained the original pear-like shape of undifferentiated gonads and were much smaller than the ovaries at the same developmental stage of 27 dph (Fig. 6e). At 30 dph, the ovarian cavity was completely formed and oogonia were undergoing active mitosis to become oocytes (Fig. 6f). At 34 dph, ovarian gonads were observed to contain numerous oocytes at chromatin-nucleolus phase, and the ovarian cavity was clear in the central part of the ovary (Fig. 6g). Some slit-like spaces in the central stromal tissue of testes formed the efferent duct anlages at 34 dph, and blood vessels were observed in the dorsal region of testes (Fig. 6h). At 48 dph, numerous perinucleolus oocytes were found in the ovarian gonad (Fig. 6i). Some spermatogonia were undergoing mitotic proliferation at 48 dph, suggesting the onset of meiosis in the development of testes. The two gonadal tissues of testes that were attached at both sides of the mesentery were observed to fuse together (Fig. 6j).

Localisation of the Foxl2 protein in the mature gonads

In the ovary, Foxl2 immunoreactivity was detected in the granulosa cells surrounding the oocytes, but not in the oocytes (Fig. 7a, b). In contrast, no specific signals were detectable in the testis (Fig. 7c, d). No positive signals were observed in the negative control of the ovary (Fig. 7e), nor in the testis (Fig. 7f).

Discussion

Two *Foxl2* paralogs named *Foxl2a* (*Foxl2*) and *Foxl2b* (*Foxl3*) have been reported in some teleost species (Baron et al. 2004; Crespo et al. 2013). They are identified by blast searches against the available genomic database in fish, which is in agreement with the fish genome duplication event (Jiang et al. 2011). In rainbow trout, *Foxl2a* and *Foxl2b* genes were specifically expressed in the ovary, but they displayed different temporal patterns of expression (Baron et al. 2004). In European sea bass, however, the expressions of *Foxl2* in ovary and *Foxl3* in testis showed a strong sexual dimorphism, and they varied significantly during the reproductive cycle (Crespo et al. 2013). In most mammalian species and teleosts, only one form of *Foxl2* gene with a conserved DNA-binding domain has been identified (Cocquet et al. 2003). In the present study, only one type of *Foxl2* gene cDNA was cloned from the *C. argus* ovary using the RACE strategy. Alignment of the putative *Foxl2* amino acid sequences indicated that the C-terminal region and the forkhead domain of the Foxl2 were highly conserved, but the N-terminal region was divergent among teleost fishes, birds, amphibians and mammals. The forkhead and C-terminal region might be conserved in their functions through evolution, whilst the N-terminal region might have evolved under weaker constraints (Cocquet et al. 2002). The forkhead domain of Foxl2 contributed to the nuclear localisation of this protein by nuclear localisation signal (NLS) at the C-terminal (Berry et al. 2002; Romanelli et al. 2003). Homopolymers of amino acids, such as polyalanine tracts (A), glycine-rich repeats (G) and proline repeats (P), were present in the mammalian orthologs but not in those of nonmammalian vertebrates including *C. argus*. The elongation of *Foxl2* in mammals during evolution may increase protein sizes and potentially promote acquisition of new functions (Mortlock et al. 2000). The NJ phylogenetic tree reveals that the *C. argus Foxl2* has greatest homology with its teleostean counterparts, the honeycomb grouper and the Nile tilapia. Molecular phylogenetic analysis agrees with the traditional taxonomy because these three fish are all members of the order Perciformes.

Tissue distribution analysis revealed that *C. argus Foxl2* in females was predominantly expressed in ovary, pituitary and gill, with a relatively lower level of expression in brain. In contrast, the expression levels of *Foxl2* were high in gill, pituitary and brain of male *C. argus*, but with low expression level in testis, which revealed an obvious sexually dimorphic pattern of expression in the gonads. The gonadotropin-releasing hormone receptor (GnRHR) is a composite regulatory element that can be activated by Smads, AP-1 and Foxl2 in mammals (Ellsworth et al. 2003). A detectable level of *Foxl2* transcript was found in *C. argus* brain of both sexes, implying that *Foxl2* might be involved in the transcriptional regulation of GnRHR in this species. In female *C. argus*, the transcript of *Foxl2* was much higher in gonad and pituitary than that in the brain, implying that Foxl2 probably executed its functions via the transcriptional regulation of the GnRH–GtH–sex steroids pathway. Similarly, it has been demonstrated that Foxl2 may be involved in the regulation of the hypothalamus–pituitary–gonadal axis in Nile tilapia (Wang et al. 2004), honeycomb grouper (Alam et al. 2008) and protogynous wrasse (Kobayashi et al. 2010). In mammals, birds and teleosts, *Foxl2* is reported to be highly expressed in ovary but barely detectable in testis (Cocquet et al. 2002; Govoroun et al. 2004; Wang et al. 2004; Loffler et al. 2003; Nakamoto et al. 2006). Similarly, *Foxl2* was highly expressed in the ovary and poorly expressed in the testis of *C. argus*. This expression pattern is consistent with the conserved functions of *Foxl2* across these different species. However, the expression of *Foxl2* in protogynous wrasse was abundant in the ovary and testis (Kobayashi et al. 2010). This situation is most likely attributed to the process of natural sex change in protogynous wrasse. It is interesting that *Foxl2* was also expressed in the gill of *C. argus*, an organ unique to aquatic fish, which may indicate a possible new function of *Foxl2* in vertebrate evolution.

Careful histological observations of the gonadal morphogenetic process are of primary importance for

a precise understanding of the mechanism of gonadal sex differentiation (Nakamura et al. 1998). Histological observations of gonadal differentiation in fish are often described as either anatomical or cytological. For differentiated gonochorists in teleost fish, the formation of the ovarian cavity in females and the efferent ducts in males is generally accepted as the criterion of anatomical sexual differentiation (Nakamura et al. 1998; Strüssmann and Nakamura 2002). Cytological differentiation of the testis involves primordial germ cells (PGCs) undergoing mitotic division to become spermatogonia and then primary spermatocytes. In the ovaries, PGCs differentiate into oogonia which subsequently become oocytes (Sacobie and Benfey 2005). Generally, the first sign of fish gonadal development is the appearance of a primordial germ cell (PGC) (Gao et al. 2009; Meijide et al. 2005; Sacobie and Benfey 2005). In this study, the gonadal development of *C. argus* had already begun before the first sampling date of 6 dph. By this time, the size of the gonads and the number of PGCs had increased dramatically, but the gonads remained undifferentiated between 6 and 21 dph. Anatomical gonadal differentiation of *C. argus* occurred at approximately 27 dph when the initial ovarian cavity began to form, but testicular differentiation was observed until 34 dph with the formation of the efferent duct anlages. Therefore, ovarian differentiation precedes testicular differentiation in *C. argus*, which is consistent with many other teleosts (Devlin and Nagahama 2002; Meijide et al. 2005). Cytological gonadal differentiation in *C. argus* was observed between 34 and 48 dph. Similar to cichlid fish (Meijide et al. 2005), *C. argus* ovaries were easily identified by their well-developed perinucleolar oocytes, whereas testes did not reach a clear state of distinction at the cytological level. At 48 dph, spermatogonia undergoing mitotic proliferation could be observed, but spermatocytes were not formed, meaning that cytological testicular differentiation had not occurred by the end of this study. These results indicate that anatomical differentiation of fish gonads preceded cytological differentiation and confirms the pattern described for other teleosts (Sacobie and Benfey 2005; Sandra and Norma 2010). A longer sampling duration is required to reveal the cytological differentiation of testis in *C. argus*.

During the early developmental stages, *Foxl2* transcription was at a moderate level at hatching day in *C. argus*, suggesting that the expression of *Foxl2* was initiated around this time. The expression of *Foxl2* increased significantly to the highest level at 4 dph and was maintained at a moderate level from 7 to 11 dph before further decreasing to a low level at 17 dph. These results indicate that the initiation of *Foxl2* expression preceded the first anatomical and morphological differentiation of female and male gonads. Similarly, *Foxl2* expression was strongest at 5 dph, the period preceding premeiotic proliferation in willow minnow (Ashida et al. 2013). In medaka, however, although *Foxl2* was expressed in somatic cells surrounding germ cells in XX specimens from hatching day, the initiation of expression followed the first morphological difference between male and female gonads and before folliculogenesis (Nakamoto et al. 2006). In mouse, chicken and turtle, *Foxl2* expression starts in female gonads and is upregulated shortly after the sex determination switch point (Loffler et al. 2003). Therefore, the start of expression of *Foxl2* before folliculogenesis is well conserved among vertebrates (Nakamoto et al. 2006). Although the role of *Foxl2* at this stage is unknown, the strong short-term upregulation of *Foxl2* expression after hatching in *C. argus* and other fishes implies that *Foxl2* might have a role in early gonadal differentiation and development. Changes in expression may promote or repress some cellular bioprocesses and then result in morphological change in the gonads (Cao et al. 2012).

Foxl2 is involved in the differentiation of granulosa cells and the maintenance of ovarian function in various vertebrates (Cocquet et al. 2003; Nakamoto et al. 2006). Previous research has demonstrated that the expression of *Foxl2* in the ovary is restricted to the granulosa (follicular) cells of the oocytes in Nile tilapia (Wang et al. 2004), medaka (Nakamoto et al. 2006), catfish (Sridevi and Senthilkumaran. 2011) and rice-field eel (Hu et al. 2014). However, no signals have been detected in the oocytes of these fishes, as is also the situation in mammals (Cocquet et al. 2002). In this study, an immunohistochemical analysis showed that the Foxl2 protein was observed in the granulosa cells around the oocytes but not in the mature oocytes of *C. argus*. These results indicate that expression of *Foxl2* is well conserved among vertebrates and that *Foxl2* may have a basic function in the differentiation of granulosa cells and the maintenance of oocytes of *C. argus*.

In conclusion, *Foxl2* was isolated from *C. argus*; it was expressed predominantly in the brain, pituitary,

gonads and gill, indicating that the brain–pituitary–gonad axis is the main target tissue of *C. argus*. *Foxl2* expressions were strongly upregulated from 1 to 11 dph, and the initiation preceded the first anatomical ovarian differentiation (27 dph), suggesting that *Foxl2* may play a role in early gonadal differentiation and development in *C. argus*. The Foxl2 protein was detected in the granulosa cells surrounding the oocytes but not in the mature oocytes, implying that *Foxl2* may have a basic function in granulosa cell differentiation and the maintenance of oocytes of *C. argus*.

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References

Alam MA, Kobayashi Y, Horiguchi R, Hirai T, Nakamura M (2008) Molecular cloning and quantitative expression of sexually dimorphic markers Dmrt1 and Foxl2 during female-to-male sex change in *Epinephelus merra*. Gen Comp Endocrinol 157:75–85

Angelopoulou R, Lavranos G, Manolakou P (2012) Sex determination strategies in 2012: towards a common regulatory model? Reprod Biol Endocrinol 10:13

Ashida H, Ueyama N, Kinoshita M, Kobayashi T (2013) Molecular identification and expression of FOXL2 and DMRT1 genes from willow minnow *Gnathopogon caerulescens*. Reprod Biol 13:317–324

Baron D, Cocquet J, Xia X, Fellous M, Guiguen Y, Veitia RA (2004) An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation. J Mol Endocrinol 33:705–715

Baron D, Houlgatte R, Fostier A, Guiguen Y (2005) Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73:959–966

Berry FB, Saleem RA, Walter MA (2002) FOXC1 transcriptional regulation is mediated by N- and C-terminal activation domains and contains a phosphorylated transcriptional inhibitory domain. J Biol Chem 277:10292–10297

Bull JJ (1983) Evolution of sex determining mechanisms. The Benjamin/Cummings Publishing Company, London

Cao M, Duan J, Cheng N, Zhong X, Wang Z, Hu W, Zhao H (2012) Sexually dimorphic and ontogenetic expression of *dmrt1*, *cyp19a1a* and *cyp19a1b* in *Gobiocypris rarus*. Comp Biochem Physiol A 162:303–309

Carlsson P, Mahlapuu M (2002) Forkhead transcription factors: key players in development and metabolism. Dev Biol 250:1–23

Cocquet J, Pailhoux E, Jaubert F, Servel N, Xia X, Pannetier M, De Baere E, Messiaen L, Cotinot C, Fellous M (2002) Evolution and expression of FOXL2. J Med Genet 39:916–921

Cocquet J, De Baere E, Gareil M, Pannetier M, Xia X, Fellous M, Veitia R (2003) Structure, evolution and expression of the FOXL2 transcription unit. Cytogenet Genome Res 101:206–211

Conover DO, Heins SW (1987) Adaptive variation in environmental and genetic sex determination in a fish. Nature 326:496–498

Crespo B, Lan-Chow-Wing O, Rocha A, Zanuy S, Gómez A (2013) *foxl2* and *foxl3* are two ancient paralogs that remain fully functional in teleosts. Gen Comp Endocrinol 194:81–93

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S (2001) The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27:159–166

Cunningham MA, Zhu Q, Unterman TG, Hammond JM (2003) Follicle-stimulating hormone promotes nuclear exclusion of the forkhead transcription factor FoxO1a via phosphatidylinositol 3-kinase in porcine granulosa cells. Endocrinology 144:5585–5594

Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191–364

Ellsworth BS, Burns AT, Escudero KW, Duval DL, Nelson SE, Clay CM (2003) The gonadotropin releasing hormone (GnRH) receptor activating sequence (GRAS) is a composite regulatory element that interacts with multiple classes of transcription factors including Smads, AP-1 and a forkhead DNA binding protein. Mol Cell Endocrinol 206:93–111

Fisheries Bureau of the Agriculture Ministry of China (2013) China fishery statistical yearbook. Chinese Agricultural Press, Beijing **(in Chinese)**

Gao Z, Wang HP, Rapp D, O'Bryant P, Wallat G, Wang W, Yao H, Tiu L, MacDonald R (2009) Gonadal sex differentiation in the bluegill sunfish *Lepomis macrochirus* and its relation to fish size and age. Aquaculture 294:138–146

Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, Couty I, Batellier F, Cotinot C (2004) Isolation of chicken homolog of the *FOXL2* gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev Dyn 231:859–870

Guiguen Y, Baroiller JF, Ricordel MJ, Iseki K, McMeel O, Martin S, Fostier A (1999) Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol Reprod Dev 54:154–162

Guiguen Y, Fostier A, Piferrer F, Chang CF (2010) Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol 165:352–366

Hu Q, Guo W, Gao Y, Tang R, Li D (2014) Molecular cloning and analysis of gonadal expression of Foxl2 in the rice-field eel *Monopterus albus*. Sci Rep 4:6884

Hughes IA (2001) Minireview: sex differentiation. Endocrinology 142:3281–3287

Ijiri S, Kaneko H, Kobayashi T, Wang DS, Sakai F, Paul-Prasanth B, Nakamura M, Nagahama Y (2008) Sexual

dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod 78:333–341

Janzen FJ (1994) Climate change and temperature-dependent sex determination in reptiles. Proc Natl Acad Sci USA 91:7487–7490

Jia W, Guo Q (2008) Gene structures and promoter characteristics of interferon regulatory factor 1 (IRF-1), IRF-2 and IRF-7 from snakehead *Channa argus*. Mol Immunol 45:2419–2428

Jiang W, Yang Y, Zhao D, Liu X, Duan J, Xie S, Zhao H (2011) Effects of sexual steroids on the expression of *foxl2* in *Gobiocypris rarus*. Comp Biochem Physiol B 160:187–193

Jiang L, Wang ZW, Zhou L, Gui JF (2013) Screening of sex-linked AFLP markers in one cultured population of *Channa argus*. Acta Hydrobiol Sin 37:1174–1178 **(in Chinese)**

Kobayashi Y, Horiguchi R, Nozu R, Nakamura M (2010) Expression and localization of forkhead transcriptional factor 2 (Foxl2) in the gonads of protogynous wrasse, *Halichoeres trimaculatus*. Biol Sex Differ 1:3

Koopman P (2001) The genetics and biology of vertebrate sex determination. Cell 105:843–847

Leet JK, Gall HE, Sepulveda MS (2011) A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation. J Appl Toxicol 31:379–398

Loffler KA, Zarkower D, Koopman P (2003) Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: *FOXL2* is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 144:3237–3243

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417:559–563

Meijide FJ, Nostro FLL, Guerrero GA (2005) Gonadal development and sex differentiation in the cichlid fish *Cichlasoma dimerus* (Teleostei, perciformes): a light- and electron-microscopic study. J Morphol 264:191–210

Mortlock DP, Sateesh P, Innis JW (2000) Evolution of N-terminal sequences of the vertebrate HOXA13 protein. Mamm Genome 11:151–158

Nakamoto M, Matsuda M, Wang DS, Nagahama Y, Shibata N (2006) Molecular cloning and analysis of gonadal expression of *Foxl2* in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun 344:353–361

Nakamura M, Kobayashi T, Chang XT, Nagahama Y (1998) Gonadal sex differentiation in teleost fish. J Exp Zool 281:362–372

Ng P, Lim K (1990) Snakeheads (Pisces: Channidae): Natural history, biology and economic importance. Essays in zoology. Papers Commemorating the 40th Anniversary of the Department of Zoology. National University of Singapore, Singapore, pp 127–152

Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D (2005) *Foxl2* is required for commitment to ovary differentiation. Hum Mol Genet 14:2053–2062

Pailhoux E, Vigier B, Chaffaux S, Servel N, Taourit S, Furet JP, Fellous M, Grosclaude F, Cribiu EP, Cotinot C (2001) A 11.7-kb deletion triggers intersexuality and polledness in goats. Nat Genet 29:453–458

Pailhoux E, Vigier B, Vaiman D, Servel N, Chaffaux S, Cribiu EP, Cotinot C (2002) Ontogenesis of female-to-male sex-reversal in XX polled goats. Dev Dyn 224:39–50

Pannetier M, Fabre S, Batista F, Kocer A, Renault L, Jolivet G, Mandon-Pepin B, Cotinot C, Veitia R, Pailhoux E (2006) FOXL2 activates *P450 aromatase* gene transcription: towards a better characterization of the early steps of mammalian ovarian development. J Mol Endocrinol 36:399–413

Raghuveer K, Senthilkumaran B, Sudhakumari C, Sridevi P, Rajakumar A, Singh R, Murugananthkumar R, Majumdar K (2011) Dimorphic expression of various transcription factor and steroidogenic enzyme genes during gonadal ontogeny in the air-breathing catfish, Clarias gariepinus. Sex Dev 5:213–223

Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hirsch B, Hodgkin J, Zarkower D (1998) Evidence for evolutionary conservation of sex-determining genes. Nature 391:691–695

Romanelli MG, Tato L, Lorenzi P, Morandi C (2003) Nuclear localization domains in human thyroid transcription factor 2. Biochim Biophys Acta 1643:55–64

Sacobie C, Benfey T (2005) Sex differentiation and early gonadal development in brook trout. North Am J Aquac 67:181–186

Sambrook J, Russell DW, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Sandra GE, Norma MM (2010) Sexual determination and differentiation in teleost fish. Rev Fish Biol Fish 20:101–121

Smith EK, Guzmán JM, Luckenbach JA (2013) Molecular cloning, characterization, and sexually dimorphic expression of five major sex differentiation-related genes in a Scorpaeniform fish, sablefish (*Anoplopoma fimbria*). Comp Biochem Physiol B 165:125–137

Sridevi P, Senthilkumaran B (2011) Cloning and differential expression of *FOXL2* during ovarian development and recrudescence of the catfish, *Clarias gariepinus*. Gen Comp Endocrinol 174:259–268

Strüssmann CA, Nakamura M (2002) Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol Biochem 26:13–29

Uda M, Ottolenghi C, Crisponi L, Garcia JE, Deiana M, Kimber W, Forabosco A, Cao A, Schlessinger D, Pilia G (2004) *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet 13:1171–1181

Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM (2009) A census of human transcription factors: function, expression and evolution. Nat Rev Genet 10:252–263

Wang DS, Kobayashi T, Zhou LY, Nagahama Y (2004) Molecular cloning and gene expression of Foxl2 in the Nile tilapia, *Oreochromis niloticus*. Biochem Biophys Res Commun 320:83–89

Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi K, Nagahama Y (2007) Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding protein/steroidogenic factor 1. Mol Endocrinol 21:712–725

Wilhelm D, Palmer S, Koopman P (2007) Sex determination and gonadal development in mammals. Physiol Rev 87:1–28

Yamaguchi T, Yamaguchi S, Hirai T, Kitano T (2007) Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophy Res Commun 359:935–940

Zhang Y, Zhang S, Lu H, Zhang L, Zhang W (2014) Genes encoding aromatases in teleosts: evolution and expression regulation. Gen Comp Endocrinol 205:151–158