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Molecular cloning and sexually dimorphic expression of *wnt4* in olive flounder (*Paralichthys olivaceus*)

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Abstracts WNT4 (wingless-type MMTV integration site family, member 4) is regarded as a key regulator of gonad differentiation in mammalians. However, the potential role of *wnt4* in teleosts during gonad differentiation and development is still unclear. The full-length cDNA sequence of *wnt4* in olive flounder (*Paralichthys olivaceus*) was obtained using RACE (rapid amplification of cDNA ends) technique. The *wnt4* ORF contains 1059 nucleotides, encoding a protein with a signal peptide domain and a *wnt* family domain. Expression in tissues of adult flounders was analyzed by real-time RT-PCR. The results showed that *wnt4* was widely expressed in multiple tissues of flounders, and the expression level was significantly higher in ovary than in testis. Then *wnt4* expression pattern was investigated during gonadal differentiation period and at gonadal development stages (I–V). The results showed the expression levels were significantly higher in testis than in ovary during gonadal differentiation. Notably, *wnt4* expression had a very significant increase before testis differentiation. At gonad different developmental stages, there was no expression signal at stage I or stage II, and the expression of *wnt4* was much stronger in ovary than in testis at stage III and stage IV, followed by a faint expression in stage V in both sexes. Our results imply that cloned *wnt4* could be *wnt4a.* It is a sex-related gene and its expression pattern in gonadal differentiation period of flounder is different from that in mammalians or other teleosts. Flounder *wnt4* might play more important role in testis than in ovary during gonadal differentiation.

Keywords Olive flounder · *wnt4* · Molecular cloning · Sexually dimorphic expression · Gonadal differentiation · Gonadal development

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Introduction

WNT4 (wingless-type MMTV integration site family, member 4) is a member of *wnt* family proteins which act as ligands to activate the Wnt pathways. *Wnt4* is

highly conserved in all species and its expression has been predominantly observed in the differentiating ovary in various mammalian species, including the goat (*Capra aegagrus hircus*) (Pailhoux et al. 2002), the tammar wallaby (*Macropus eugenii*) (Yu et al. 2006; Pask et al. 2010) and humans (Peltoketo et al. 2004; Jääskeläinena et al. 2010). *Wnt4* is regarded as a key regulator of gonadal differentiation in mammalians. In teleosts, Wnt/beta-catenin signaling regulates gonadal differentiation in zebrafish (*Danio rerio*) (Sreenivasan et al. 2014). Amberg et al. (2013) identified Wnt/beta-catenin signal pathway genes that are potentially important to gonadal differentiation in shovelnose sturgeons (*Scaphirhynchus platorynchus*). These results throw significant light on the importance of Wnt signaling in gonadal differentiation and development in fish. However, in teleosts, the potential roles of *wnt4* during gonadal differentiation and development have only been explored in a few species, such as protandrous black porgy (*Acanthopagrus schlegeli*) (Wu and Chang 2009), rainbow trout (*Oncorhynchus mykiss*) (Nicol et al. 2012) and half-smooth tongue sole (*Cynoglossus semilaevis*) (Hu et al. 2014). Contrary to the expression pattern in mammalians, *wnt4* has no sexually dimorphic expression during gonadal differentiation in black porgy. Further research showed that *wnt4* still has important roles in late ovarian growth. Nevertheless, *wnt4a* expression showed a slight sexual dimorphism in favor of males in rainbow trout (Nicol et al. 2012). In half-smooth tongue sole, *wnt4a* expression has no difference between female gonads and male gonads from 7 to 160 days, which includes most of the gonadal differentiation period (Ma et al. 2006). However, the *wnt4a* expression level was significantly higher in the testis than in ovary from 1 to 2 year in this fish (Hu et al. 2014). Thus, the expression patterns of *wnt4*, especially during gonadal differentiation in other teleost fish, need more researches.

Olive flounder (*Paralichthys olivaceus*) is a commercially important flatfish species cultured in East Asia. Females grow much faster than males, so it is a proposed way to increase production by controlling the sex ratio. The sex of flounder is determined by genotype and temperature effects (Ospina-álvarez and Piferrer 2008). Though some sex-related genes such as *cyp19a* (Kitano et al. 2001), *mis* (Yoshinaga et al. 2004), *dmrt1* (Jo et al. 2007), *foxl2* (Yamaguchi et al. 2007), *sox9* (Wen et al. 2011), *nr0b1* and *nr5a2* (Wang et al. 2015) have been reported to be involved in flounder gonadal differentiation, the sex determination and differentiation mechanism are still unknown. Identification of more sex-related genes and investigation of their expression profiles would be helpful to understand the sex differentiation mechanism of this fish. To date, *wnt4* expression pattern has not been investigated in this fish.

In consideration of the roles of *wnt4* in gonadal differentiation in mammalians and the importance of Wnt signaling in gonadal differentiation and development in fish, we cloned the full-length of *wnt4* cDNA and detected the expression patterns of *wnt4* in adult tissues, gonadal development stages and the differentiating gonads. The results will be helpful to understand the *wnt4* roles in teleosts.

Materials and methods

Fish samples, determination of gender and developmental stages

Samples for wnt4 cDNA cloning and investigation of wnt4 expression in tissues of adult fish by real-time RT-PCR

Adult flounder (total length 370–450 mm) were purchased from the Nanshan fish market (Qingdao, China) and temporarily reared at the institute aquarium. Various tissues (gonad, brain, heart, liver, intestinal, stomach, head kidney, muscle, kidney, eye, spleen and gill) were taken from males and females ($n = 3$), respectively.

Gonad samples for analysis of wnt4 expression levels at five development stages by RT-PCR

Flounder (TL from 100 to 470 mm) were purchased from the Nanshan fish market (Qingdao, China) and temporarily reared at the institute aquarium. Gonads were taken at each development stage ($n = 3$).

Gonad samples for analysis of wnt4 expression pattern during gonadal differentiation by real-time RT-PCR

To obtain phenotypic females and males, artificial gynogenesis of the flounder was performed firstly (You et al. 2001); then, the larvae at total length (TL) 12 mm were divided into two groups and reared at

control (18 ± 0.5 °C) and high (28 ± 0.5 °C) water temperatures until TL 150 mm, respectively. The gonads of fish were taken under MZ125 dissecting microscope (Leica, German) at 20, 30, 38, 50, 63 and 70 mm TL ($n = 3$) at different time point.

Fifty individuals were randomly selected at 150 mm TL from the above two groups, respectively, for gender determination to analyze the sex ratio.

Determination of gender and development stage

Before sampling, all fish were anaesthetized by 40 mg ml^{-1} 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, Yufubao, China; http://www.shuichan.cc). Each gonad sample above was divided into two parts. One was fixed to determine the gender and development stage via histological analysis as described by Radonic and Macchi (2009) and Sun et al. (2009), and the other was stored immediately in liquid nitrogen for RNA isolation.

All animal work has been conducted according to relevant national and international guidelines. Animal protocols were approved by Institute of Oceanology, Chinese Academy of Science.

RNA extraction and cDNA synthesis

Total RNA was extracted by using E.Z.N.A®. MicroElute® Total RNA Kit (OMEGA, Norcross, USA). The quality and quantity of total RNA were verified by gel electrophoresis and optical density readings with NanoDrop© ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE, USA), respectively. First strand cDNA was synthesized from 1 μg of total RNA using PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. The cDNA samples were used as templates in the following RT-PCR and real-time PCR experiments.

cDNA cloning of flounder *wnt4* gene

Using RNA from ovary of adult flounder as template, *wnt4* cDNA was obtained by 3′-/5′-RACE method using SMARTer RACE cDNA amplification kit (Clontech, Tokyo, Japan) in accordance with the manufacturer's protocol. Briefly, the degenerate primers for flounder *wnt4* were obtained by sequence alignment of other fishes and the partial flounder *wnt4* was amplified by using Nest RT-PCR. The complete coding sequence was obtained by 3′-RACE and 5′-RACE. All these PCR products were subcloned in pEasy-T1 (TransGen Biotech, Beijing, China) and sequenced with an ABI 3730 automated sequencer. Primers are listed in Table 1.

Alignment and phylogenetic analysis of WNT4

The multiple sequence alignment was conducted by DNAMAN software. The evolutionary history was

Table 1 Primers used to amplify the flounder *wnt4* and investigate *wnt4* expression patterns

Primer name	Nucleotide sequence (5′–3′)	Function
wnt4-cF1	AACTGGCTVTACCTGGCNAARCTG	Nest RT-PCR
wnt4-cR1	CTGYCDTCRGTGGGSAGYAT	
wnt4-cF2	CAYTTGACRDAGCAGCACCAGTGGAA	
wnt4-cR2	CCRCAGCACAKNAGCTCRCAGCC	
wnt4-RACE-sen1	AGTCCAGATTTCTGCGACTACGACC	3′-RACE
wnt4-RACE-sen2	GCCAGTGTAACAGAACGTCAAAGG	
wnt4-RACE-ant1	ACGCACCGCATCCATCACCTCCAC	5′-RACE
wnt4-RACE-ant2	ATCCATCACCTCCACACTGCGCTTAC	
RT-*wnt4*-F1	CCATCTCAGCAGCCAGTGT	RT-PCR
RT-*wnt4*-R1	GTTACGAGGCACCAGGACTT	
qRT-*wnt4*-f1	AGTCAGTCCAGAAGGGTTCCAG	Real-time RT-PCR
qRT-*wnt4*-r1	CTCCTCTCTCTCACACCCACAA	
β-actin-f1	ACTACCTCATGAAGATCCTG	RT-PCR
β-actin-r1	TTGCTGATCCACATCTGCTG	
qRT-β-actin-f1	AACCGCTGCCTCCTCCTCAT	Real-time RT-PCR
qRT-β-actin-r1	TCGGGACAACGGAACCTCTC	

inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 1.12531541 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved in 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 223 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Analysis of *wnt4* expression levels in gonads at different development stages (stage I–V) by RT-PCR

Based on the *wnt4* cDNA sequence, RT-PCR primers were designed (Table 1). The cDNA from gonad at different development stage was used as the template in the mixture [0.2 mM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3) and 1 unit Taq DNA polymerase (Invitrogen, Carlsbad, USA)]. The PCR conditions were as follows: preheating at 94°C for 5 min, 35 cycles of PCR at 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 5 min.

Real-time RT-PCR and data analysis

To quantify *wnt4* expression during gonadal differentiation and in different tissues of adult flounder, real-time RT-PCR was carried out in an Eppendorf Real-time Detection System. The total reaction volume was 20 µl containing 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, USA), 1 µl diluted cDNA and 0.4 µl of each primer (Table 1). The real-time RT-PCR program consisted of one cycle of 95 °C for 20 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s and 72 °C for 30 s. A single-cycle melting curve analysis of amplified products was performed following each real-time RT-PCR to confirm that only one PCR product was amplified and detected. For all samples, real-time RT-PCR amplification of β-actin was used as reference in the same condition.

The comparative CT method was used to analyze flounder *wnt4* expression level as described by Wen et al. (2009). Briefly, differences in the CT for the target and the internal control, denoted the ΔCT, were calculated to normalize the differences in the amount of template of each reaction and the RT-PCR efficiency. The certain sample regarded as the reference was called the calibrator. The ΔCT for each sample was subtracted from the ΔCT of the calibrator, and the difference was called ΔΔCT. The expression level of *wnt4* could be calculated by $2^{-\Delta\Delta CT}$, and the value stands for an n-fold difference relative to the calibrator. All data were given in terms of relative mRNA expression as mean ± SEM. The results were subjected to *t* test analysis, and the *P* values of less than 0.05 were considered statistically significant.

Fig. 1 Alignment of the amino acid sequence of flounder WNT4 orthologues from various species. The *solid black box* indicates signal peptide (transmembrane domain) of the *wnt4* protein, while the rest represents the WNT superfamily. The *light gray* represents the conservation is 75 %; the *dark gray* represents the conservation is 100 %

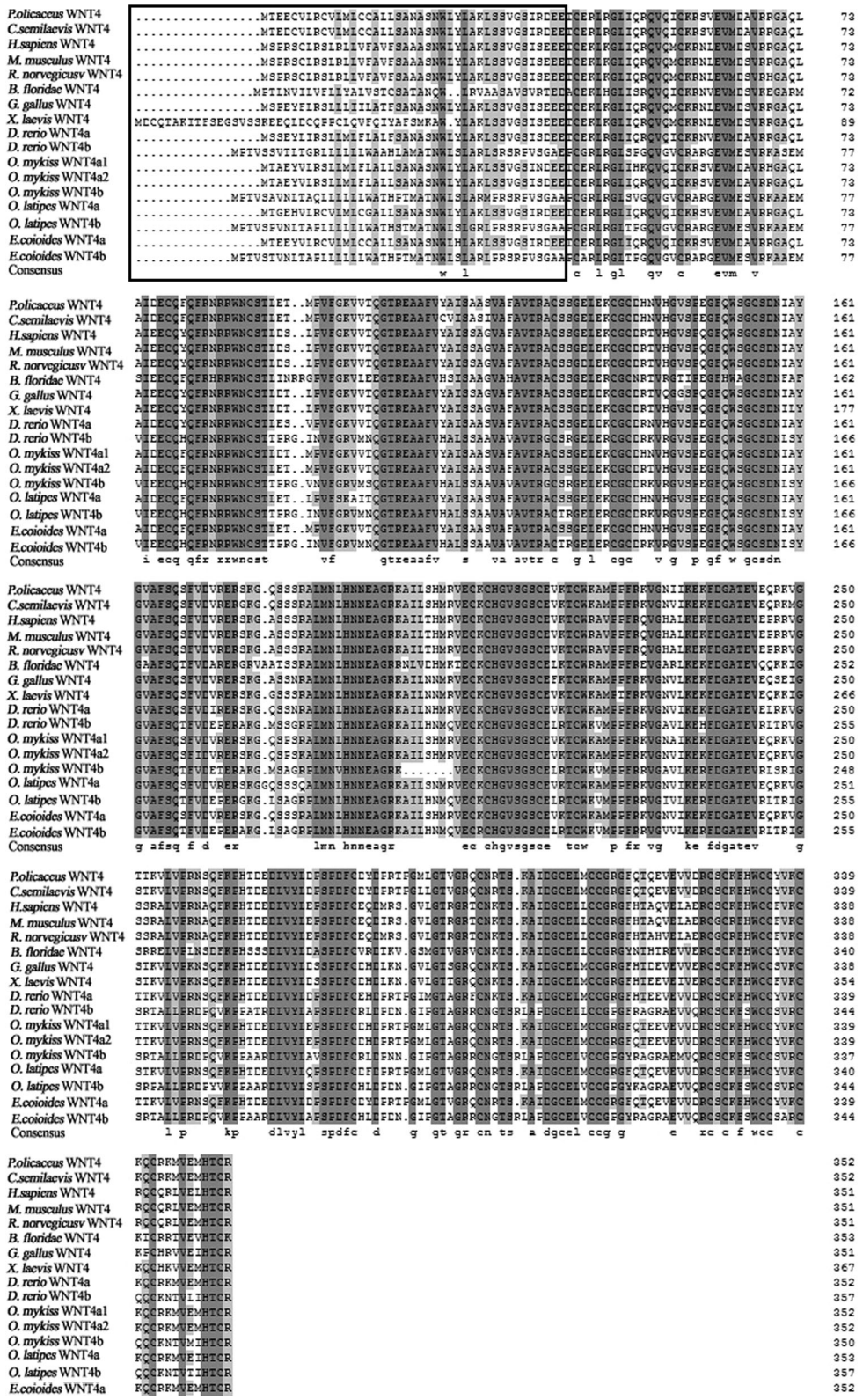
Results

WNT4 is highly conserved across species

The *wnt4* cDNA isolated from flounder was 1584 bp long with 129 bp 5′UTR, 396 bp 3′UTR and 1059 bp ORF (GenBank accession number: JX999942) which encodes a potential protein of 352 amino acid residues. WNT4 are cysteine rich and highly conserved in species. The N-terminus contains the transmembrane domain of about 45 amino acids (Fig. 1). The flounder WNT4 shares 80.0 % amino acid identity with human, 66.0 % with amphioxus (*Branchiostoma belcheri*), 90.0 % with zebrafish, 96.0 % with medaka (*Oryzias latipes*), 97 % with half-smooth tongue sole. Phylogenetic analysis showed that flounder *wnt4* was most close to *wnt4a* (Fig. 2).

The *wnt4* expression in flounder gonads and non-gonadal tissues

After morphological observation and histological analysis, the gonads of the purchased adult flounder


P.olicaccus WNT4
C.semilaevis WNT4
H.sapiens WNT4
M. musculus WNT4
R. norvegicusv WNT4
B. floridae WNT4
G. gallus WNT4
X. laevis WNT4
D. rerio WNT4a
D. rerio WNT4b
O. mykiss WNT4a1
O. mykiss WNT4a2
O. mykiss WNT4b
O. latipes WNT4a
O. latipes WNT4b
E.coioides WNT4a
E.coioides WNT4b
Consensus

were confirmed to be at stage IV (data not shown). The *wnt4* expression level in various tissues was examined by real-time RT-PCR using *β-actin* gene as a reference. The female liver tissue sample which has the highest ΔCT value was regarded as the reference sample, called the calibrator. As shown in Fig. 3, *wnt4* is widely expressed in all tissues, and there is obvious sexual dimorphism in gonad, brain, stomach, kidney, head kidney, eye, gill and spleen. The expression level in ovary is 1.5 times higher than that in testis.

The *wnt4* expression at five development stages of flounder gonads

The ovarian development stage was determined on the basis of ovarian follicle growth and maturation, while testicular stage determination was according to the relative proportions of spermatocyte, spermatid and spermatozoa. To investigate *wnt4* expression levels during flounder gonadal development stages, paraffin section was used to determine the gonad stage firstly (Fig. 4). Then *wnt4* primers (Table 1) were used in RT-PCR to detect *wnt4* expression levels in gonads from stage I to stage V using *β-actin* as a positive control (Fig. 5). The results showed that *wnt4* was expressed at stage III and stage IV gonads, followed by a very faint expression at stage V gonads in both sexes. There was no expression detected at stage I or stage II. In testis, there is no difference between stage III and stage IV; however, in ovary, the expression level in stage III is higher than that in stage IV. Notably, the expression of *wnt4* appeared much stronger in ovary than in testis at stage III, suggesting that this is the stage where differences in *wnt4* expression between ovaries and testis are the greatest.

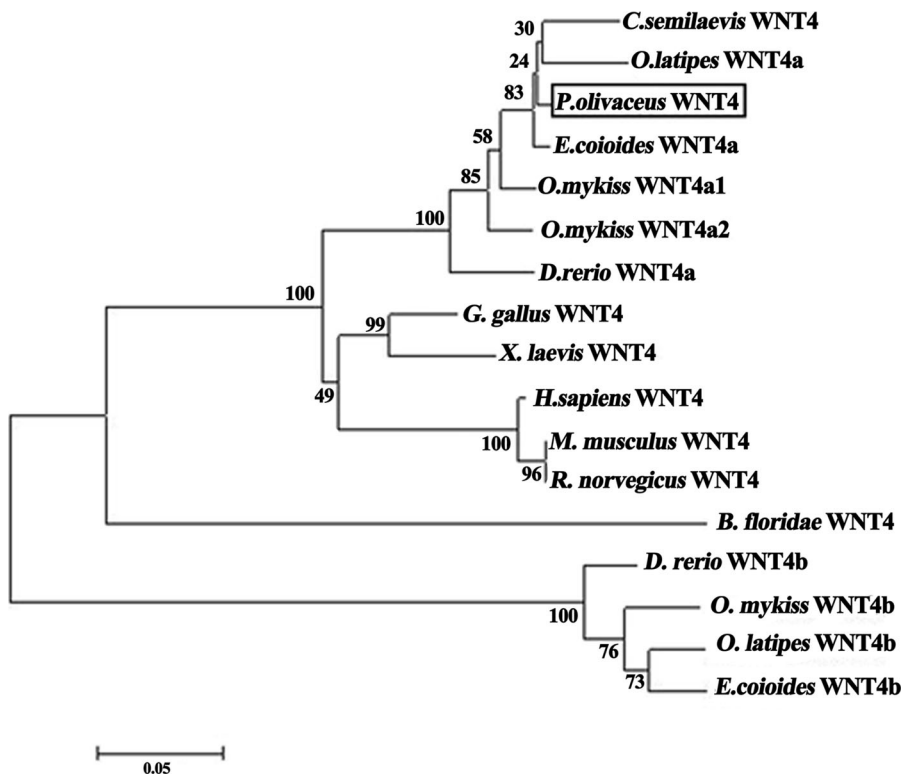


Fig. 2 Neighbor Joining tree of WNT4 orthologues *C. semilaevis*, *Cynoglossus semilaevis* (KJ825677); *H. sapiens, Homo sapiens* (NP_110388.2); *M. musculus, Mus musculus* (NP_033549.1); *R. norvegicus, Rattus norvegicus,* (NP_445854.1); *B. floridae, Branchiostoma floridae, (*AAC80431.1*); G. gallus, Gallus gallus,* (NP_990114.1); *X. laevis, Xenopus laevis,* (NP_001081197.1); *D. rerio, Danio rerio,* (NP_001035477.1); *O. mykiss, Oncorhynchus mykiss,* (AER52059.1, AER52060.1, AER52061.1); *O. latipes, Oryzias latipes, (*ACM50931.1, ACM 50932.1*); E. coioides, Epinephelus coioides, (*AGF91873.1, AGF91874.1*)*

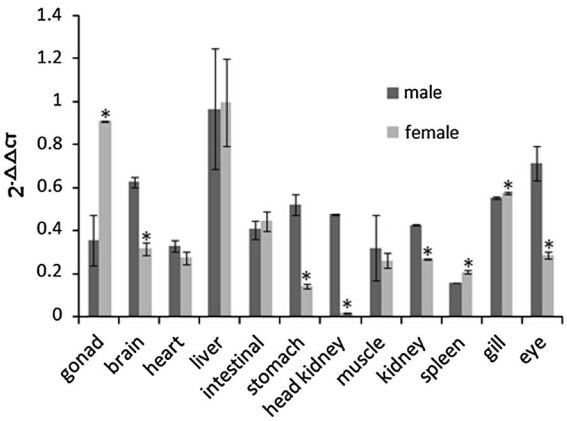


Fig. 3 Real-time RT-PCR analysis of *wnt4* expression in various tissues of male and female adult flounder. The female liver tissue sample with which has the highest △CT value was regarded as the reference, called the calibrator. Error bars indicate standard deviations. Significant differences between male and female are indicated $*P < 0.05$

Sexual ratio of flounder juveniles reared at 18 °C or 28 °C

To investigate the sexual ratio of artificial gynogenetic flounder which have been reared at 18 and 28 °C, respectively, we observed the gonads via histological methods. Fifty fish at 150 mm TL reared at 18 °C were dissected, and the proportion of males is 16 % (8/50); then, the fish reared at 18 °C were named female-enriched group. At the same time, 50 fish at 150 mm TL reared at 28 °C were dissected and the proportion

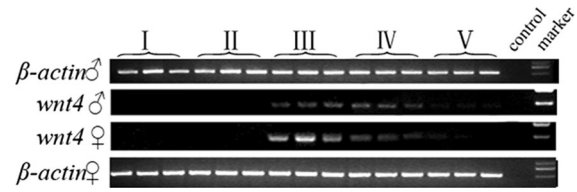


Fig. 5 *wnt4* expression patterns in gonads at different gonadal developmental stages in *P. olivaceus*. I: stage I; II, stage II; III, stage III; IV, stage IV; V, stage V

of males is 98 % (49/50); then, the fish reared at 28 °C were called male-enriched group.

The *wnt4* quantificative expression in gonads during sex differentiation

Gonadal tissues were taken out when the juveniles were 20, 30, 38, 50, 63 or 70 mm in TL ($n = 3$ at each length). The TL 20 mm sample of male-enriched group was regarded as the reference, called the calibrator. The transcription of *wnt4* had a same trend during gonad differentiation in male-enriched group and female-enriched group: *wnt4* expression level increased before TL 50 mm, then decreased until TL 63 mm. The expression levels were significantly higher in male-enriched group than in female-enriched group except that the juveniles were 20 mm in TL. Notably, there was a very significant increase while juveniles of male-enriched group were 50 mm in TL, and the expression level was 14 times higher than juveniles of female-enriched group (Fig. 6).

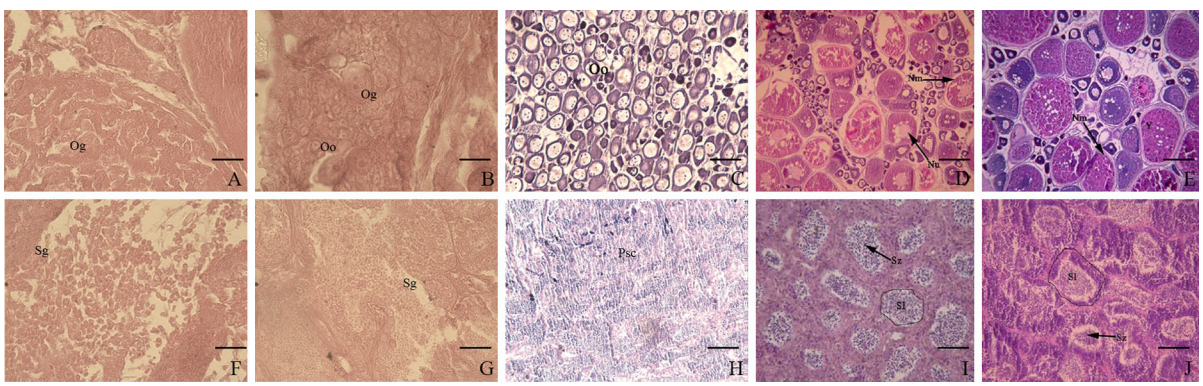


Fig. 4 Paraffin sections of *P. olivaceus* gonads at different development stages **A** ovary at stage I; **B** ovary at stage II; **C** ovary at stage III; **D** ovary at stage IV; **E** ovary at stage V; **F** testis at stage I; **G** testis at stage II; **H** testis at stage III; **I** testis at stage IV; **J** testis at stage V. *Og* oogonium, *Oo* oocytes, *Nm* nucleus membrane, *Nu* nucleolus, *Y* yolk, *Sg* spermatogonium, *Psc* primary spermatocyte, *Sz* spermatozoa, *Sl* seminal lobule. *Scale bar* (**A**, **B**, **F**, **G**), 100 μm; (**C**, **D**, **E**, **H**, **I**, **J**), 25 *μm*

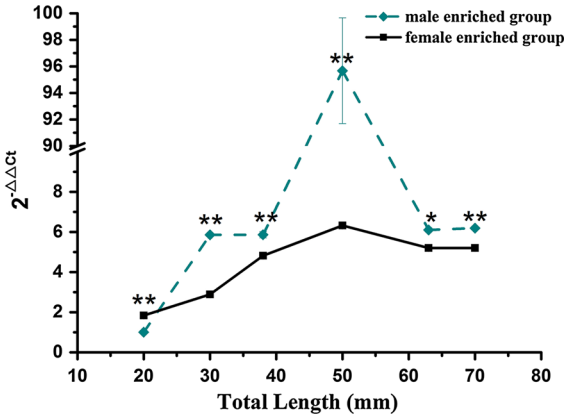


Fig. 6 mRNA expression level analysis of *wnt4* in gonads during gonads differentiation by real-time RT-PCR. The fish reared at 18 °C were named female-enriched group; the fish reared at 28 °C were called male-enriched group. The TL 20 mm sample of male-enriched group was regarded as the reference sample, called the calibrator. Error bars indicate standard deviations. Significant differences between male-enriched group and female-enriched group are indicated $*P < 0.05$, $**P < 0.01$

Discussion

In contrast to the single copy in mammalians, most teleost fish have two *wnt4* genes, *wnt4a* and *wnt4b*, which result from the teleost fish whole-genome duplications 320 million years ago. *Wnt4a* is highly conserved in vertebrates, whereas *wnt4b* is not (Nicol et al. 2012). In this study, from flounder gonad we obtained one *wnt4* cDNA sequence which was most close to *wnt4a* as shown by the phylogenetic analysis. According to the present knowledge, *wnt4b* expression is restricted to the nervous system (Nicol et al. 2012), which may be the reason that *wnt4b* cDNA was not obtained from gonad in this study. However, *wnt4b* gene does exist in flounder after analyzing the data of flounder genome sequence provided by Prof. Chen SL (unpublished data, personal communication).

The male proportions of artificial gynogenetic flounder which had been reared at 18 °C and 28 °C, respectively, were 8:50 and 49:50, which are consistent with the result reported by Yamamoto (1999). According to a previous study conducted by our laboratory (Sun et al. 2009), the gonadal differentiation period in flounder is closely related to its total length. The ovarian cavity could be observed in the differentiating ovary when the TL of juveniles reached 38.0 ± 1.7 mm. When juveniles reached 86.5 ± 5.9 mm TL, oocytes could be observed, which means the completion of the ovarian differentiation. The onset of testis differentiation was detected in juveniles at 63.5 ± 3.4 mm TL, when spermatogenic cells proliferated quickly and the sperm duct was formed. The seminal lobule could be observed when juveniles reached approximately 76.0 mm TL. In this study, we obtained female-enriched juveniles and male-enriched juveniles by artificial gynogenesis and then temperature treatment. Then we investigated the *wnt4* sex-specific expression in undifferentiated gonads (TL 20 mm) and in gonad differentiating period (TL 30 mm to 70 mm). Gonads of fish, respectively, reared at 18 °C and 28 °C were collected at 20, 30, 38, 50, 63 and 70 mm TL ($n = 3$) at different time point. For the juveniles reared at 18 °C, gonads of fish at TL 30 mm were ready to onset. TL 38 mm represented the onset of ovarian differentiation, after which (TL 50 mm and TL 63 mm) was the ovarian differentiation period. TL 70 mm was close to the end of the ovarian differentiation. For juveniles reared at 28 °C, gonads at TL 50 mm were ready to onset, TL 63 mm represented the onset of testis differentiation, and TL 70 mm was close to the end of the testis differentiation (Suppl. Fig. 1). We found the expression levels of flounder *wnt4* in gonads increased from 20 mm TL to 63 mm TL during gonadal differentiation, with a significant sexual dimorphism in favor of males. This result is different from *wnt4* expression patterns during gonadal differentiation process in mammalians and other teleosts. In mammalians, *wnt4* is mainly expressed in differentiating ovaries. In teleosts, black porgy *wnt4* expression level had no sexual dimorphism and did not increase during gonadal sex differentiation. Rainbow trout *wnt4a1/2* expression had a slight sexual dimorphism in favor of males during early gonadal differentiation (Nicol et al. 2012), and half-smooth tongue sole *wnt4a* expression level had no sexual dimorphism during gonadal differentiation and increased from 70 days to 1 year (Hu et al. 2014). Both olive flounder and half-smooth tongue sole belong to Pleuronectiformes, and *wnt4a* amino acid sequences of the two fish have 97 % identities. However, the *wnt4a* of the two fish species had different expression patterns during gonad differentiation, which may suggest that *wnt4* plays a different role in the two fish species. Notably, the expression level was quite high in juvenile gonads of male-enriched group in TL 50 mm. At that time, the

ovary has already started differentiation, whereas the testis differentiation is ready to onset. The *wnt4* high expression at that time implies that *wnt4* might be important for triggering testis histological differentiation in flounder; however, the exact role needs further investigation.

For gonads at five development stages, the expression of *wnt4* was much stronger in ovary than in testis at stage III of adult flounder using RT-PCR, suggesting that this is the stage where differences in *wnt4* expression between ovaries and testis are the greatest. Since the gonads of flounder which were used to investigate *wnt4* expression in various tissues were at stage IV using real-time RT-PCR and the expression level in ovary is 1.5 times higher than that in testis, we could draw a conclusion that *wnt4* expression was stronger in ovary than in testis at both stage III and stage IV gonads. It is known that the gonads of flounder enter the cycle of reproduction at stage III (Yamamoto, 1999), and the highest expression could be detected at stage III in female flounder in this study. This result indicates that *wnt4* might play important roles in female flounder reproduction. However, it is different in half-smooth tongue sole, in which *wnt4* expression was highest in stage II (Hu et al. 2014). In addition, *wnt4* was expressed in various tissues of adult flounder, which is similar to the results in rainbow trout (Nicol et al. 2012) and half-smooth tongue sole (Hu et al. 2014). The widely expression pattern can be attributed to the various physiological ways that *wnt4* is involved in (Ungar et al. 1995).

In summary, we obtained the sequence of *wnt4* and showed that the expression level was significantly higher in ovary than in testis for stage III and IV gonads. However, during the gonads differentiation, the expression levels were significant higher in testis than in ovary. In addition, *wnt4* expression level had a significant increase before testis differentiation in juveniles of male-enriched group. The results would be helpful to understand the roles of *wnt4* during gonadal differentiation and development.

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