Genetics and Molecular Research

Wnt7b gene expression and functional analysis in the mussel *Mytilus coruscus*

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ABSTRACT. To understand the potential functions of Wnt7b in different developmental stages and adult tissues of the mussel *Mytilus coruscus*, the Wnt7b gene was cloned using the rapid amplification of cDNA ends technique. The full-length Wnt7b gene was 1910 bp long, with a 1059-bp open reading frame encoding 352 amino acids. The amino acid sequence of the *M. coruscus* Wnt7b gene shared high homology with that of *Homo sapiens* (59%), *Mus musculus* (61%), *Danio rerio* (61% similarity), *Biomphalaria glabrata* (62% similarity), *Aplysia californica* (66% similarity), and *Crassostrea gigas* (74%). Wnt7b mRNA expression was detected by reverse transcription PCR in all tissues studied (mantle, adductor muscle, gill, foot, digestive gland, and male and female gonads), with the highest expression found in the gill, and in the male and female gonads. This indicates that Wnt7b may play an important role in gonadal maturation and in the functions of gills in the mussel *M. coruscus*. Expression of the Wnt7b gene during

larval development stages, including the trochophore, D-shaped veliger, umbo veliger, pediveliger, and juvenile stages, was also detected. Wnt7b mRNA was highly expressed in the D-shaped veliger, umbo veliger, and pediveliger larvae stages, suggesting that Wnt7b may participate in larval development and in the process of metamorphosis in the mussel *M. coruscus*. Taken together, these findings provide new insights into the functions of the Wnt gene family during mussel larval development and settlement and metamorphosis.

Key words: Mussel; *Mytilus coruscus*; Wnt7b gene; Gene cloning; Expression analysis

INTRODUCTION

Members of the Wnt gene family are proposed to mediate survival, proliferation, and differentiation of biological cells by encoding secretory glycoprotein signaling molecules, which are required for normal embryonic development and organogenesis (Nusse and Varmus, 1992; Cadigan and Nusse, 1997). Homologues of the Wnt gene family are present in both invertebrates and vertebrates, and this family has been well conserved throughout evolution (McMahon, 1992; Sidow, 1992). Wnt proteins have been identified in many species, such as nematodes, annelid, arthropods, echinoderms, and vertebrates, and multiple members of this family exist (Nusse and Varmus, 1992; Siegfried and Perrimon, 1994; Prud'homme et al., 2002; Kubota et al., 2009). Wnt proteins are known to participate in many biological processes including stem cell maintenance, embryonic development and growth, gonad development, bone formation, and metabolism (Reya and Clevers, 2005; Johnson and Rajamannan, 2006; Kubota et al., 2009). In humans, gene mutation in the Wnt pathway has been linked to a developmental disorder (Grzeschik et al., 2007). Wnt3a signaling in mice provides proliferative stimuli and also affects the fate of hematopoietic stem cells during hematopoiesis (Luis et al., 2009).

Expression and functional analysis of Wnt7b, a member of the Wnt family, have been extensively studied in human (*Homo sapiens*) (Lippmann et al., 2012), mouse (*Mus musculus*) (Parr et al., 2001), *Xenopus laevis* (Moon, 1993), zebrafish (*Danio rerio*) (Beretta et al., 2011), medaka (*Oryzias latipes*) (Yokoi et al., 2003), amphioxus (*Branchiostoma floridae*) (Schubert et al., 2000), sea anemones (*Nematostella vectensis*) (Sullivan et al., 2007), and coral (*Acropora millepora*) (Meyer et al., 2009). In humans, Wnt7b is required for endothelial cells derived from pluripotent stem cells to acquire blood-brain barrier properties (Lippmann et al., 2012), and may play an important role in the development of myopia in humans (Miyake et al., 2015). In mice, Wnt7b is required for normal blood vessel angiogenesis in the central nervous system (Daneman et al., 2009). The zebrafish genome contains four Wnt7 genes, which are involved in brain patterning events and neural specification (Beretta et al., 2011). In arthropods, the Wnt7b gene has only been detected in the Asian tiger mosquito (*Aedes albopictus*) (Chen et al., 2015). However, there have been no reports of the Wnt7b gene in mollusks.

The mussel, *Mytilus coruscus*, is an important economic species in China (Chang and Wu, 2007). Previous studies have focused on clarifying the mechanism of settlement and metamorphosis by pharmacology (Yang et al., 2013a, 2014), neurobiology (Yang et al., 2013b), and molecular microbiology (Yang et al., 2013c; Li et al., 2014b). However, the effect of Wnt signaling on settlement and metamorphosis, and on ontogeny are largely unknown.

Here, we describe the Wnt7b gene in the mussel *M. coruscus* for the first time, and investigate its gene expression pattern in different larval development stages and adult tissues. The aim of this study was to provide evidence for further genetic research into the Wnt family in mollusks, especially in the bivalve.

MATERIAL AND METHODS

Sample preparation

Adult mussels (*M. coruscus*) were obtained from Gouqi Island, Zhoushan, Zhejiang Province. Various tissues including mantle, adductor muscle, gill, foot, digestive gland, and male and female gonads were collected. In addition, the different stages of mussel larvae including trochophore, D-shaped, umbo, pediveliger, and juvenile stages were prepared by artificial fertilization. All samples were immediately frozen in liquid nitrogen and stored at -80°C. All animal handling procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Ocean University.

Total RNA extraction and first-strand cDNA synthesis

Samples of gill were homogenized and total RNA was extracted using the Molluse RNA kit (OMEGA, USA) following the manufacturer instructions, and quantified using the Nanodrop 2000 (Thermo Scientific, USA). RNA quality was assessed by 1% agarose gel electrophoresis. First-strand cDNA was synthesized using the SMARTer™ rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech, Japan) following the manufacturer instructions.

Cloning of full-length Wnt7b

Primers (Wnt7-F1, Wnt7-R1) specific for Wnt7b were designed based on the transcriptome annotation data of mussel *M. coruscus* using the Primer Premier 5.0 software (Table 1). PCR was performed in 25-μL reaction mixtures. The conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 1 min 30 s at 72°C, following by elongation for 10 min at 72°C. The PCR products were checked, the target bands were retrieved using a Gel Extraction Mini Kit, ligated into the pMD 19-T vector (TaKaRa, Dalian, China), transformed into competent *Escherichia coli* DH5α cells, plated onto LB-agar medium, and incubated overnight at 37°C. Positive clones were examined by sequencing (Sangon Biotech, Shanghai, China).

To obtain the full-length cDNA sequence of Wnt7b, gene-specific primers were designed for 3' RACE and 5' RACE (Table 1). RACE-cloning methods were performed according to simple modular architecture research tool (SMART)-RACE protocol (Clontech, Japan). The PCR products were examined and the target bands were collected, purified, and subcloned. The positive clones were sequenced by Sangon Biotech.

Bioinformatic analysis

The NCBI open reading frame (ORF) finder was used to analyze ORFs and predict amino acid sequences. The isoelectric point and molecular weight of each protein was calculated using the EXPASY database.

Table 1. Oligonucleotide primers used to amplify Wnt7b genes.

Primer	Sequence (5'-3')	Application
Wnt7b-F1	TGAGACTGAGAATGGGAG	cDNA amplification
Wnt7b-R1	AATTGACATAACAACACC	cDNA amplification
Wnt7b-3' RACE-F1	CGAGCGGGTAGAAAGGCAGTAAAA	3' RACE
Wnt7b-3' RACE-F2	ATACAAAGCCCAGGAGATCAGACG	3' RACE
Wnt7b-5' RACE-R1	ATCAGCACTACACCCTCCCCACTT	5' RACE
Wnt7b-5' RACE-R2	CCGTCTCCGATAGTTACTATGGCG	5' RACE
Wnt7b-RT-F	GTCGGGAGAACATGCAATC	RT-qPCR
Wnt7b-RT-R	GTTCGTTCGCTGCACTTATT	RT-qPCR
18S rRNA-F	GACCTCGGTTCTATTTTG	RT-qPCR (control)
18S rRNA-R	GGTATCTGATCGTCTTCG	RT-qPCR (control)

RACE = rapid amplification of cDNA ends. RT-qPCR = real-time quantitative PCR.

Conserved domains of amino acid sequences were predicted by the SMART. Protein phosphorylation and glycosylation sites were predicted by NetPhos 2.0 and NetNGlyc 1.0, respectively. Multiple sequence alignments were performed using the ClustalX program. The neighbor-joining algorithm was used to construct the phylogenetic tree (MEGA version 5.1).

Real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from various tissues including the mantle, adductor muscle, gill, foot, digestive gland, and male and female gonads, and from five development stages including the trochophore, D-shaped, umbo, pediveliger, and juvenile stages. cDNA was obtained by reverse transcription. Primers were designed based on the Wnt7b cDNA sequences (Table 1). 18S rRNA was used as a housekeeping gene and served as an internal control for Wnt7b expression analysis. Three biological replicates were used for RT-qPCR and each reaction was performed in triplicate on a LightCycler 96 System (Roche, Switzerland). The reactions were performed following the manufacturer instructions. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Significant differences in Wnt7b expression were assessed by one-way ANOVA and analyses were performed in the SPSS 19.0 software.

RESULTS

Characterization of Wnt7b cDNA and phylogenetic analysis

Full-length cDNA encoding *M. coruscus* Wnt7b was obtained using the RACE method (GenBank accession No. KX082976). Wnt7b of *M. coruscus* consisted of 1910 nucleotides, which encoded a protein of 352 amino acids with a calculated molecular mass of about 39 kDa (Figure 1). Wnt7b contained a 1059-bp ORF, a 146-bp 5'-untranslated region (UTR), and a 705-bp 3'-UTR. The Wnt7b protein contained 24 cysteine residues conserved in the Wnt family and two predicted N-glycosylation sites, [^{84}N] and [^{298}N]. The N-terminus of this protein contains a 23-amino acid transmembrane domain and a Wnt1 domain from amino acid 41 to 352 (Figure 2).

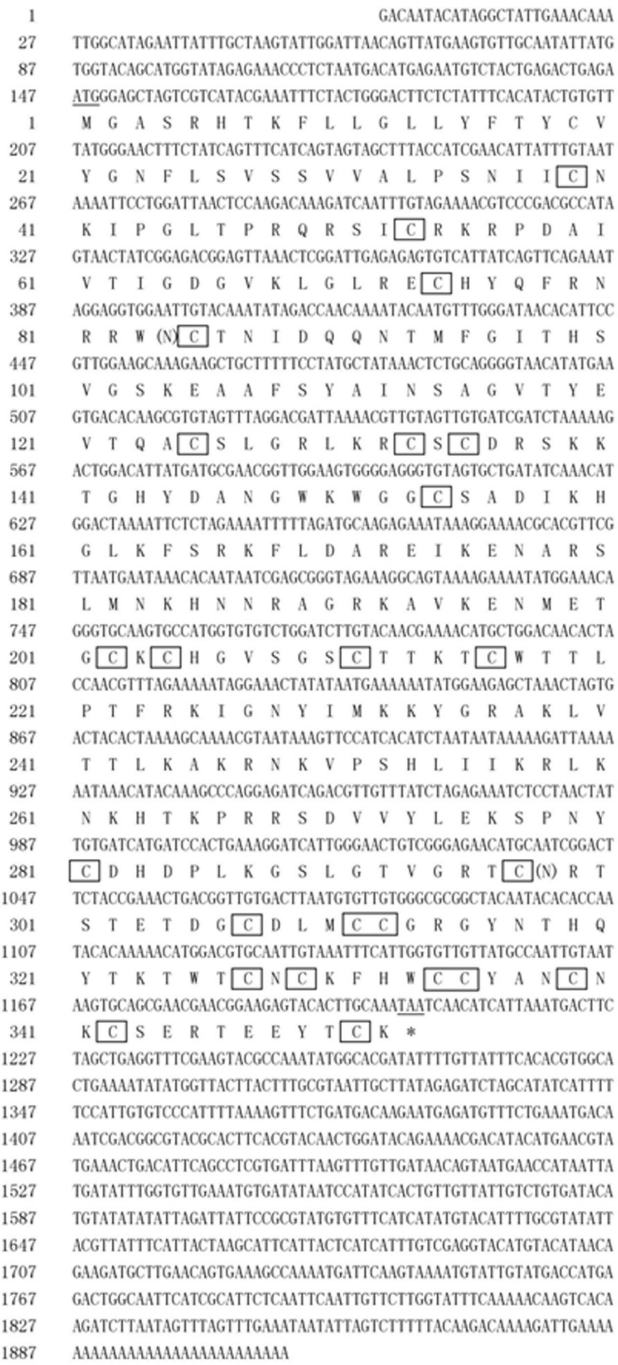

Figure 1. Nucleotide and deduced amino acid sequences of *Mytilus coruscus* Wnt7b. Start (ATG) and stop (TAA) codons are underlined. Potential N-glycosylation sites are in parentheses and well-conserved cysteine residues are in boxes.

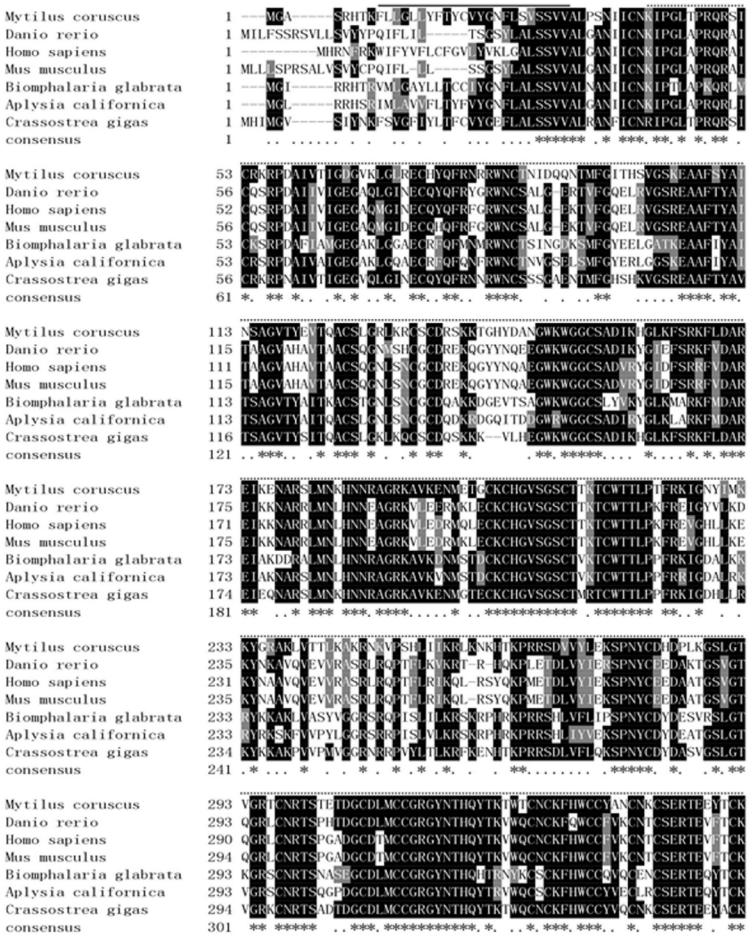

Figure 2. Alignment of Wnt7b amino acid sequences from multiple species. Black background: identical sequence; gray background: similar sequence; *same amino acid; dot indicates an amino acid with similarity. The putative transmembrane domain is indicated by a solid line above the sequence, and the WNT1 gene family domain is indicated by a dotted line above the sequence. GenBank accession Nos. of the Wnt7b sequences used for alignment are as follows: *Danio rerio* (XP_001920219.1), *Homo sapiens* (NP_478679.1), *Mus musculus* (NP_001157106.1), *Biomphalaria glabrata* (XP_013064023.1), *Aplysia californica* (XP_012942721.1), *Crassostrea gigas* (XP_011440896.1).

The deduced amino acid sequence of Wnt7b showed high homology with that of *H. sapiens* (59% similarity), *M. musculus* (61% similarity), *D. rerio* (61% similarity), *Biomphalaria glabrata* (62% similarity), *A. californica* (66% similarity), and *C. gigas* (74% similarity).

Phylogenetic analysis showed that *M. coruscus* Wnt7b first clustered with that of Mollusca oyster (*C. gigas*), then with that of squid (*Euprymna scolopes*), sea snail (*A. californica*), and snail (*B. glabrata*), and finally with that of sea urchin (*Strongylocentrotus purpuratus*) and vertebrates, including mouse (*M. musculus*) and human (*H. sapiens*) (Figure 3). The phylogenetic relationship of the Wnt7b gene between species was consistent with the evolutionary history.

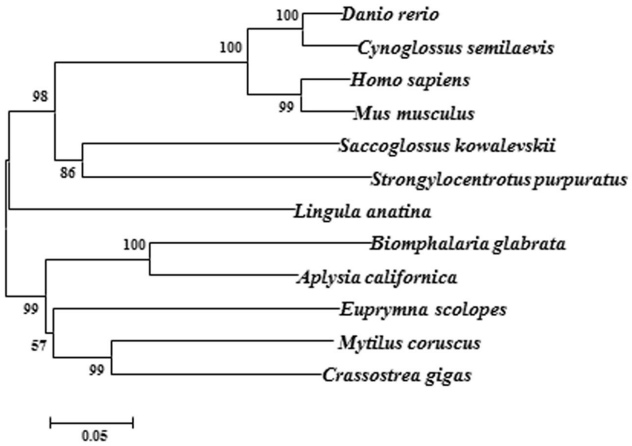

Figure 3. Phylogenetic tree showing the relationship of *Mytilus coruscus* Wnt7b with homologs of other species. GenBank accession Nos. are as follows: *Danio rerio* (XP_001920219.1), *Cynoglossus semilaevis* (XP_008310445.1), *Homo sapiens* (NP_478679.1), *Mus musculus* (NP_001157106.1), *Saccoglossus kowalevskii* (NP_001161677.1), *Strongylocentrotus purpuratus* (XP_787051.3), *Lingula anatina* (XP_013395212.1), *Biomphalaria glabrata* (XP_013064023.1), *Aplysia californica* (XP_012942721.1), *Euprymna scolopes* (ABD16199.1), *Mytilus coruscus*, and *Crassostrea gigas* (XP_011440896.1).

Expression of Wnt7b mRNA in different tissues

Wnt7b mRNA expression in seven tissues from adult mussel (*M. coruscus*) was examined using RT-qPCR, and was detected in all adult tissues (Figure 4). Wnt7b mRNA expression was significantly higher in the gill, and in the male and female gonads than in other tissues ($P < 0.05$). Lower expression of Wnt7b mRNA was found in muscle, digestive gland, foot, and mantle tissue.

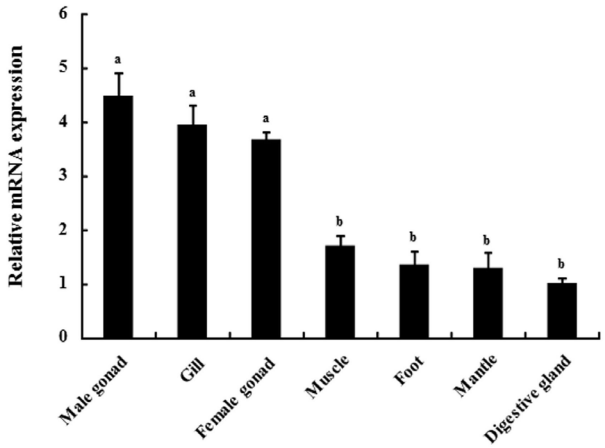

Figure 4. Tissue expression profiles of the *Mytilus coruscus* Wnt7b gene. mRNA levels were measured by RT-qPCR. Data from RT-qPCR are reported as means ± SE (N = 3) and different superscript letters represent significantly different values ($P < 0.05$).

Wnt7b mRNA expression at different development stages

Wnt7b mRNA expression in mussel *M. coruscus* at five development stages (Figure 5B) was also examined (Figure 5A). Wnt7b mRNA was expressed at variable levels across all development stages. The highest expression was observed in the D-shaped veliger larvae (9.9-fold), followed by the umbo veliger larvae (6.4-fold) and pediveliger larvae (5.5-fold) stages. The lowest Wnt7b mRNA expression was found in the trochophore stage (1.1-fold).

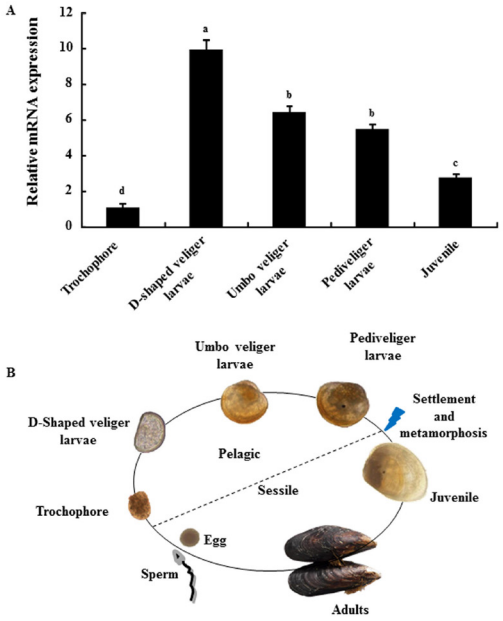

Figure 5. Expression of the Wnt7b gene in five developmental stages of *Mytilus coruscus* (**A**) and summary of the mussel (*M. coruscus*) life cycle (**B**). mRNA levels as measured by RT-qPCR. Data from RT-qPCR are reported as means ± SE (N = 3) and different superscript letters represent significantly different values ($P < 0.05$).

DISCUSSION

The Wnt signaling pathway is a conserved signal transduction pathway, and is crucial for mediating and controlling many biological processes *in vivo*. Previous studies investigating Wnt7b gene cloning and functional analysis have been performed in vertebrates (Van Camp et al., 2014; Yeo et al., 2014); however, there have been no reports in bivalve. The present study is the first to identify and characterize the Wnt7b gene in mussel (*M. coruscus*). The results showed that Wnt7b cDNA consists of a 1059-bp ORF encoding 352 amino acids. It contains two predicted N-glycosylation sites and 24 cysteine residues conserved in the Wnt family, which are consistent with the unique characteristics of the Wnt family (Nusse and Varmus, 1992). These data confirmed that the Wnt7b protein identified in the present study belongs to the Wnt family. Comparison of amino acid sequences showed that Wnt7b from mussel shares high similarity with that from *C. gigas* (74%), *H. sapiens* (59%), and *M. musculus* (61%). This suggests that the phylogeny of the Wnt7b gene is consistent with the evolutionary history of these species.

Previous studies have shown that the Wnt signaling pathway regulates gonadal development in mammals (Vainio et al., 1999). In mouse, high expression of the Wnt7b gene results in changes in ovarian steroidogenesis, resulting in mammary tumorigenesis (Peltoketo et al., 2010). In rainbow trout, Wnt signaling genes are highly expressed in early spermatogenesis, and Wnt7b has been shown to be involved in gonadal differentiation and gametogenesis (Nicol and Guiguen, 2011). In the present study, the male and female gonads were sampled in April, which represents the period when *M. coruscus* gonads are mature in the East China Sea, and the high expression of Wnt7b in the male and female gonads may be correlated with gonadal maturation. Thus, it would be interesting to determine whether the Wnt signaling genes are responsible for gonadal maturation in *M. coruscus*.

Wnt7b expression has been examined in mammals, and Wnt7b signaling is required for proliferation of the mesenchyme and vascular development in the lung (Shu et al., 2002). In bivalves, the gill is a tissue for gas exchange, ion transportation, and filter-feeding. The results of the present study showed that Wnt7b was highly expressed in the gills of *M. coruscus*, indicating that Wnt7b may be involved in regulating fundamental mechanisms in the gills of this species.

Metamorphosis is an important event during invertebrate larval development. The Wnt signaling pathway has been found to play an important role in signal transduction during larval competency and metamorphosis of the polychaete *Pseudopolydora vexillosa* (Chandramouli et al., 2013) and the bryozoan *Bugula neritina* (Wong et al., 2012). Similarly, the results of the present study showed that high expression of the Wnt7b gene between the D-shaped veliger larvae and the pediveliger larvae stages may result from the transition from a pelagic to a sessile phase. The D-shaped veliger larvae represent the stage at which the original shell (prodissoconch) begins to form (Gao et al., 2016). In addition, the morphological structure of the shell changed between the D-shaped veliger larvae and the umbo veliger larvae stages (Figure 5B). It takes more than a week for mussels (*M. coruscus*) to develop from pediveliger larvae with an eyespot to competent larvae, which have the ability to settle and metamorphose (data not shown). Mussel larval metamorphosis includes juvenile/adult shell dissoconch growth, loss of feeding organ velum, and gill development (Yang et al., 2013c). The Wnt signaling pathway is a multifunctional regulatory pathway. Therefore, high expression of the Wnt7b gene may have a profound effect on the mussel (*M. coruscus*) during larval development and metamorphosis. The settlement and metamorphosis of mussel larvae can be stimulated by chemical, biological, and physical cues (Li et al., 2014a; Yang et al., 2007, 2013a, 2014). However, whether the Wnt signaling pathway is involved in these processes, and how the signals are transmitted to certain receptors in order to activate those behaviors remain unknown. From the point of view of both aquaculture and biofouling, it would be worthwhile to gain a deeper understanding of the function of the Wnt signaling pathway during early embryonic development, larval development, and in the processes of settlement and metamorphosis in mussel (*M. coruscus*).

In conclusion, in the present study, the full-length cDNA of Wnt7b was successfully cloned from the mussel *M. coruscus*, and was used to investigate phylogeny, and to analyze the expression pattern in adult tissues and at different larval stages. The findings of the present study suggest that the Wnt7b gene may play an important role in larval development and metamorphosis of the mussel *M. coruscus*. Taken together, these findings provide insights into the function of the Wnt gene family during mussel larval development and in the processes of settlement and metamorphosis.

Conflicts of interest

The authors declare no conflicts of interest.

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