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Identification of *Hc-β-catenin* in freshwater mussel *Hyriopsis cumingii* and its involvement in innate immunity and sex determination

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ABSTRACT

β-catenin is a multifunctional protein that participates in a variety of physiological activities, including immune regulation, sex determination, nervous system development and, cell differentiation. However, the function of *β-catenin in* freshwater mussel *Hyriopsis cumingii* remains unclear. Herein, the gene encoding *β-catenin* from *H. cumingii* (*Hc-β-catenin*) was cloned and characterised. The full-length 5544 bp gene includes an open reading frame (ORF) of 2463 bp encoding a putative protein of 820 amino acids residues containing 12 armadillo (ARM) repeats. After injecting *H. cumingii* with *Aeromonas hydrophila* or *lipopolysaccharides*, *Hc-β-catenin* transcription was induced in hemocytes and gills, and the greatest responses occurred at 24 h after bacterial challenge, confirming an important role in immune responses. Quantitative real-time PCR analysis showed that *Hc-β-catenin* mRNA was distributed in the gill, foot, liver, kidney, mantle, adductor muscle and gonad of male and female mussels. In gonad, *Hc-β-catenin* expression was markedly higher in females than males. During the embryonic period, *Hc-β-catenin* expression was highest at 3 day. In 1-, 2- and 3-year-old mature mussels, *Hc-β-catenin* expression in female gonad tissue was notably higher than in males. *In situ* hybridisation revealed a significant hybridisation signal in female gonads, indicating that *Hc-β-catenin* is a pro-ovarian, anti-testis gene. Our findings demonstrate that *Hc-β-catenin* is important in immune regulation and sex determination in freshwater mussel.

1. Introduction

Hyriopsis cumingii is the main freshwater pearl mussel species in China. Pearls produced by *H. cumingii* are smooth, bright in colour, round in shape, and have great economic value. China is the largest producer of pearls in the world, of which more than 80% of freshwater pearls are produced by *H. cumingii* [1], making this the most important pearl aquaculture species.

β-catenin was discovered as an adhesion molecule in 1980, and is a member of the catenin family. It is also an important transcription factor of the classical Wnt signalling pathway that regulates various developmental processes such as limb regeneration [2], nervous system development, body axis formation [3], and adrenal cortical development [4]. Signalling pathways in Wnt cells are mainly divided into three types, including the well-studied classical *Wnt/β-catenin* pathway, which relies on *β-catenin* to control various developmental processes such as gender fate determination, differentiation, and survival [5].

In addition to developmental processes, evidence suggests that the Wnt signalling pathway participates in immunity, particularly in dendritic cells (DCs). *β-catenin* is a key downstream mediator of canonical Wnt signalling in DCs. Although *β-catenin* is a core modulator of several intersecting signalling pathways, many immune responses can be activated by *β-catenin* independently [6]. *β-catenin* has been found to regulate stem cell differentiation and proliferation [7,8], affect interferon (IFN)-β synthesis, and participate in cancer development [9,10]. Changes in the localisation and expression levels of *β-catenin* often result in exacerbation of diseases and disorders associated with cancer and metastasis [11]. Whole-genome RNA interference (RNAi) screening revealed a novel role for the Wnt/*β-catenin* signalling pathway as a negative regulator of viral-induced innate immune responses [12]. The

Abbreviations: qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends; ISH, *In situ* hybridisation; ARM, Armadillo; AH, *Aeromonas hydrophila*; PBS, phosphate-buffered saline

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function of *β-catenin* in immunity was also demonstrated in *Chinese tongue sole* [13,14], and recent studies have shown that the Wnt signalling pathway plays a role in immunity against white spot syndrome virus (WSSV) in Pacific white shrimp *Litopenaeus vannamei* [15].

The sex mechanism of molluscs is complex and includes hermaphrodites and sexual reversal [16,17]. *H. cumingii* is dioecious, but there are significantly differences in the breeding performance of male and female mussels; adult male mussels produce better breeding pearls than females [18]. Various sex-related genes have been studies in mammals, including those encoding Wt1, Sry, Sox9 and Dmrt1 that promote testicular development [19], and Wnt4, RSpo1 and Dax1 that are conducive to ovarian development [20]. However, there are few reports on gender-related genes in *H. cumingii*, although the *fem-1c* gene is known to be involved in female gonad differentiation [21].

Several studies have found that *β-catenin* plays an important role in mammalian sex determination and differentiation. The activation of *β-catenin* in otherwise normal XY mice effectively impedes testicular development, including disruption of testosterone synthesis, and down-regulation of testicular marker genes, ultimately leading to male-to-female sexual reversal [22]. Although *β-catenin* is only necessary for ovarian differentiation and is dispensable for testis development, loss of *β-catenin* in ovaries can cause molecular and morphological defects, including the formation of testis-specific coelomic vessels, the appearance of androgen-producing adrenal-like cells, and loss of female germ cells [23]. *R-spondin* genes encode secreted proteins of the classical *β-catenin* pathway. *Sex-determining region Y* (*SRY*) genes in human and mouse interact with *β-catenin* in the nucleus to inhibit the transcriptional activation of the *Rspo1/Wnt* gene, ultimately promoting male development [24]. This indicates that *Rspo1* and *Wnt4* exert important effects on ovarian differentiation, hence *β-catenin* may also affect sex determination [25].

In order to further investigate the role of *Hc-β-catenin* in immune responses and sex formation processes in *H. cumingii*, we cloned and characterised the full-length *Hc-β-catenin* gene in the present work. Real-time quantitative PCR (qRT-PCR) was used to measure the relative expression levels in different tissues of male and female mussels, and during gonadal development. The results also revealed the dynamic expression of *Hc-β-catenin* following injection with the bacterial pathogen *Aeromonas hydrophila* (AH) or *lipopolysaccharide* (LPS). Additionally, the distribution of the *Hc-β-catenin* protein in male and female gland cells was observed by *in situ* hybridisation.

2. Materials and methods

2.1. Experimental animals

Larvae and 1-, 2- 3-year-old *H. cumingii* individuals were supplied by a breeding farm in Wuyi (Zhejiang Province, China). All mussels were transported to the laboratory and cultured in a cage at 26 ± 2 °C, regularly fed with *Chlorella vulgaris* with aerated water for 2 days before experimentation.

2.2. Tissue sampling and RNA isolation

Various tissues (gonad, gill, liver, spleen, mantle, foot and adductor) were collected from three untreated individuals for RNA extraction. Embryonic period samples were collected at 1 day (fertilised eggs), and 3, 5, 7 and 9 days, and stored in RNAstore Reagent (TIANGEN, Beijing, China). Fresh tissues were immersed in RNAstore at a ratio of 1:10 (tissue: RNAstore). The thickness of either side of the samples should not be > 0.5 cm. Tissues from juvenile mussels were collected from 3 month to 10 months (nine mussels each month), and all samples were immediately frozen in liquid nitrogen and stored at −80 °C.

The partial cDNA sequence of *Hc-β-catenin* was retrieved from our previous mantle transcriptome database [26]. Total RNA samples were extracted from various tissues of *H. cumingii* using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. RNA quality and quantity were determined by agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Scientific, USA). The cDNA was synthesised using a PrimerScript RT reagent Kit (TaKaRa, Japan).

Table 1
Primers used in this study.

Primer name	Primer sequence (5′ − 3′)	Purpose
β3′-1	ATGATGTTTGATGCCTGGGGTG	3′ RACE outer
β3′-2	ACAGGCTGGAGACAGAGAGGAC	3′ RACE inner
β5′-1	GATGGGCACAAAGCCAGGTTGCGGAT	5′ RACE outer
β5′-2	CGTGTGGTTTCGGCATCATTAGTATT	5′ RACE inner
HβF	CCAAGGTGGAGACCTGAACT	qPCR *Hc-β-catenin*
HβR	CCACTGGGTCATTCCCTGAT	qPCR *Hc-β-catenin*
EFl-αF	GGAACTTCCCAGGCAGACTGTGC	qPCR
EFl-αR	TCAAAACGGGCCGCAGAGAAT	qPCR
IβF	CCAAGGTGGAGACCTGAACT	ISH
IβR	TAATACGACTCACTATAGGGCCACT GGGTCATTCCCTGAT	ISH

2.3. Cloning of the full-length Hc-β-catenin cDNA

Purified RNA samples were diluted to 500 ng/μl for first-strand cDNA synthesis using a 5′/3′ SMARTer RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's instructions, with primers listed in Table 1. Thermal cycling included an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis, purified using a Gel Extraction Kit (TIANGEN), inserted into the PGEM-T vector (Promega, Madison, USA) at 16 °C for 16 h, and the resulting constructs were transformed into competent *Escherichia coli* DH5α cells. Selected clones were sequenced by Sangon Biotech (Shanghai, China).

2.4. Sequence analysis and phylogenetic tree construction

The sequence of *Hc-β-catenin* was confirmed using the BLAST algorithm and the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequence of *Hc-β-catenin* was determined using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). The physical parameters of the *Hc-β-catenin* protein were analysed using ProtParam (https://web.expasy.org/protparam/). Protein secondary structure was predicted using SMART (https://smart.embl-heidelberg.de/), and tertiary structure was predicted using SWISS-MODEL (https://swissmodel.expasy.org/). Multiple sequence alignments of amino acid and nucleotide sequences were performed using ClustalW2. The phylogenetic tree was constructed using the Neighbour-Joining (NJ) method in MEGA5.0 with 1000 bootstrap replicates were set to 1000.

2.5. Immune stimulation

For the bacterial challenge experiment, 2-year-old *H. cumingii* with a shell length of 7–8 cm were used. 72 healthy mussels were randomly divided into three groups of 24 mussels each. Challenge groups were injected with 100 μl of live *A. hydrophila* (AH) at a cell density of 10^9 colony-forming units (CFU) resuspended in phosphate-buffered saline (PBS), or with lipopolysaccharide (LPS) from *E. coli* 0111:B4 (1 mg/ml in PBS; Sigma-Aldrich) [27,28]. The control group was treated with PBS alone, injected into adductor muscle. Tissues (blood, gill) were collected at 0, 3, 6, 12, 24, 36, 48 and 96 h after injection (three mussels from each group at each time point). All samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA preparation.

2.6. Real-time quantitative PCR

For qRT-PCR, the house-keeping gene elongation factor 1 (*EF1*)-*α* served as an internal reference. Primers for *Hc-β-catenin* were designed using Primer 5.0 and are listed in Table 1. The cDNA templates for qRT-PCR were prepared using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. Relative gene expression levels were determined using TB Green *Premix Ex Taq* II (Tli RNaseH Plus; TaKaRa) with a CFX96 system (Bio-Rad). Reactions (20 μl) contained 10 μl of 2 × TB Green *Premix Ex Taq*, 6.8 μl of RNase-free water, 0.8 μl of forward and reverse primer, and 1.6 μl of cDNA. Thermal cycling included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Expression of *Hc-β-catenin* relative to the reference gene was calculated using the $2^{-\Delta\Delta CT}$ method.

2.7. In situ hybridisation (ISH)

Three-year-old mature male and female mussels were selected and gonads were removed. Tissues were fixed in 4% paraformaldehyde with diethylpyrocarbonate (DEPC) at 4 °C for 2 h, then in 20% sucrose for no longer than 1 week. Tissues were then frozen and sections were prepared using a microtome at a thickness of 8–10 μm. The probe was then prepared using a DIG RNA Labelling Kit (SP6/T7; Roche, Germany). The ISH experiment was carried out according to the manufacturer's instructions. The hybridisation signal was observed under a Leica DM 2500 microscope (Leica, Germany) and photographed.

2.8. Statistical analysis

Differences between tissues were analysed by one-way analysis of variance (ANOVA) with SPSS 18.0 software, and $p < 0.05$ was considered statistically significant. Bar charts were drawn using SigmaPlot 12.5.

3. Results

3.1. Sequence analyses of Hc-β-catenin

The complete cDNA sequence of *Hc-β-catenin* (MK078117) is 5544 bp long, including a 1355 bp 5′-untranslated region (UTR), a 1726 bp 3′-UTR, and an open reading frame (ORF) of 2463 bp encoding a putative protein of 820 amino acid residues. The amino acid sequence of *Hc-β-catenin* is predicted to have 12 armadillo (ARM) repeats, spanning amino acid residues 164–203, 204–246, 247–287, 288–329, 331–372, 373–413, 414–452, 453–496, 501–542, 543–607, 608–648 and 649–689 (Figs. 1 and 2). The predicted molecular weight of the protein is 89976.17 Da, the calculated isoelectric point (pI) is 5.71, and the protein does not appear to possess a signal peptide or transmembrane domains. The predicted secondary structure has an α-helical content of 48.29%, located in the centre of the sequence, a β-fold content of only 1.22%, and irregular random coil structure accounting for 50.49%, mainly distributed at both termini (Fig. 3). The prediction results of the tertiary structure are consistent with the secondary structure. Each of the three α-helical is arranged together to form one supercoil, which is the ARM domain, and a total of 12 armadillo (ARM) repeats are formed in the middle region.

3.2. Phylogenetic analysis

Comparison of the amino acid sequences of *Hc-β-catenin* and homologs from other species showed that *Hc-β-catenin* shares 86% identity with its counterpart in *Pinctada martensii*, 85% with homologs in *Patinopecten yessoensis* and *Mytilus coruscus*, and 73% and 72% with human and zebrafish sequences. Phylogenetic analysis revealed extremely high similarity with the *Hyriopsis schlegelii* gene/protein, with which the first cluster was formed, and *Hc-β-catenin* then clusters with other bivalve shellfish sequences. *Biomphalaria glabrata* and *Aplysia califomica* sequences formed a separate cluster., distant from human and mouse sequences. Thus, there is high sequence conservation between *Hc-β-catenin* from a wide range of species, indicating strong evolutionary and structural conservation, and hence functional similarity (Fig. 4).

3.3. Expression patterns of Hc-β-catenin in different tissues

Hc-β-catenin was expressed in both male and female tissues, but expression was generally higher in female tissues (Fig. 5). The *Hc-β-catenin* gene was strongly expressed in gill, liver, kidney and foot, and exhibited lower transcription levels in other tissues. In females, relative expression was highest in gill and lowest in mantle. In males, *Hc-β-catenin* was expressed highest in gonad and lowest in adductor. There were significant differences ($p < 0.01$) in expression between males and females, and although levels were slightly lower in the mantle of females, expression in all other tissues was higher in females than males (Fig. 5).

3.4. Transcriptional regulation of Hc-β-catenin in response to stimulation by A. hydrophila stimulation or LPS

In order to investigate *Hc-β-catenin* expression following immune stimulation, mussels were divided into three groups (two challenge groups and a blank control group). After injection with live *A. hydrophila* (AH), *Hc-β-catenin* expression in both gill and hemocytes was initially increased, then gradually down-regulated (Fig. 6). The highest expression level in the gill occurred at 6 h post-stimulation, and expression then decreased by 12 h. Expression reached a second peak at 24 h post-stimulation, but was low 96 h. In hemocytes, expression of *Hc-β-catenin* obviously increased at 24 h and 48 h (Fig. 6). Similarly, at 3 h after treatment with LPS, *Hc-β-catenin* transcript levels were markedly decreased, but were then gradually up-regulated and reached a peak at 24 h. Expression in gill decreased continuously and remained lower at 96 h than the PBS control group. *Hc-β-catenin* transcript levels in hemocytes were up-regulated at 36 h, then declined were lowest after 48 h (Fig. 6).

3.5. Expression of Hc-β-catenin at different developmental stages

To investigate the involvement of *Hc-β-catenin* in sex determination and differentiation, we selected embryos up to 8 months old and measured the relative expression of *Hc-β-catenin* in gonad tissue by qRT-PCR. At all stages, expression of *Hc-β-catenin* sharply declined after an initial rise (Fig. 7). The results revealed very high expression after 3 days, and lower expression thereafter. There was no obvious difference between months, and expression remained relatively stable level (Fig. 7). However, expression of *Hc-β-catenin* in 1-, 2- and 3-year-old animals was significantly different in females and males; *Hc-β-catenin* expression was markedly higher in females in all three age groups (Fig. 8).

3.6. In situ localisation of Hc-β-catenin in gonad tissue from 3-year-old male and female mussels

The results of *in situ* hybridisation showed revealed a significant hybridisation signal in female gonads, but no expression signal in male gonads (Fig. 9).

4. Discussion

The present results provide evidence for an immunoregulatory role of *Hc-β-catenin* in the triangle sail mussel *H. cumingii*. We cloned and characterised the full-length *Hc-β-catenin* gene, which includes a 2463

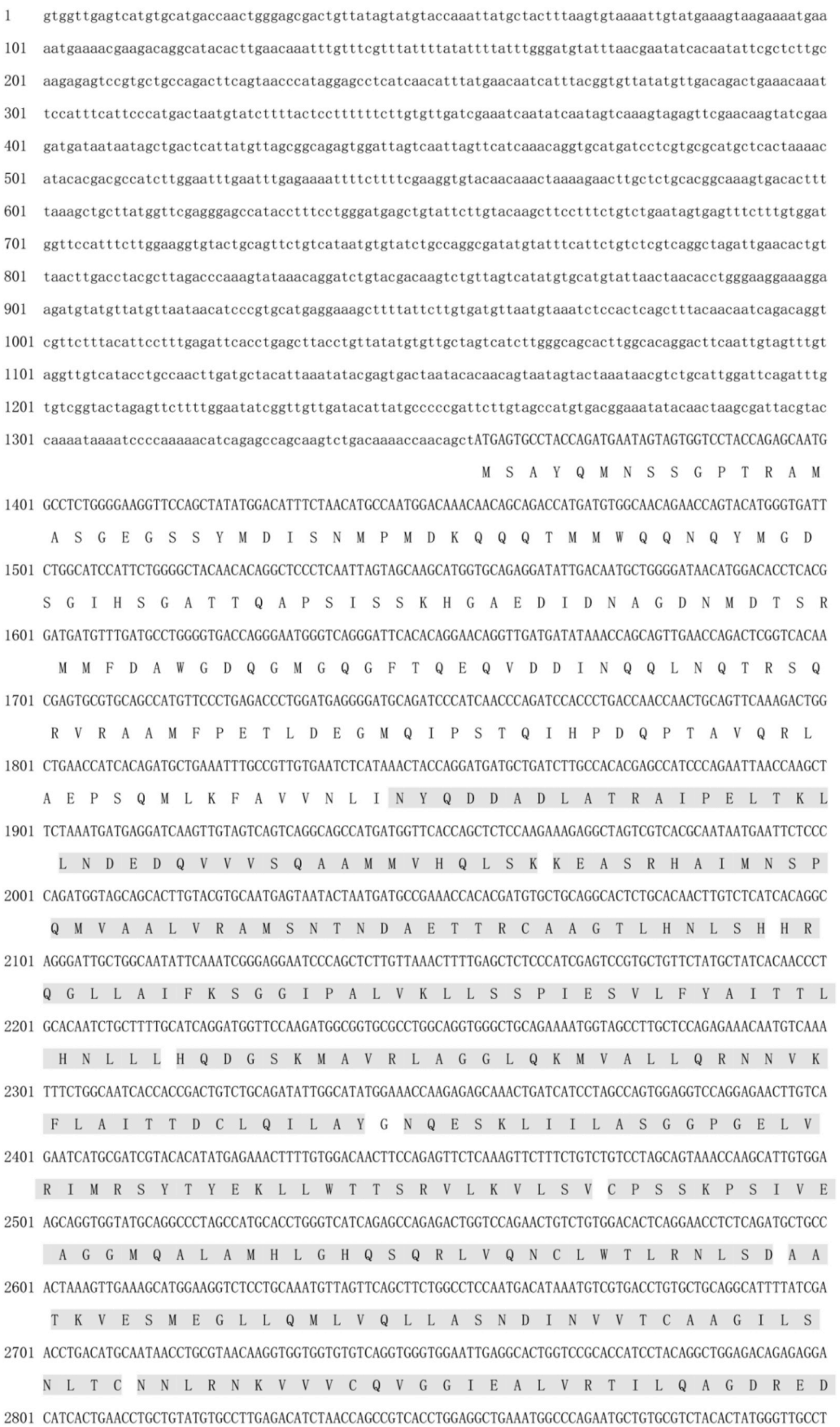

Fig. 1. Nucleotide and deduced amino acid sequences of *Hc-β-catenin* from *Hyriopsis cumingii*. Lowercase letters indicate 3′- and 5′-untranslated regions. Uppercase letters indicate the coding region (upper = nucleotides, lower = amino acids). * = stop codon. Putative polyadenylation signals (AATAA) are underlined. Shaded regions indicate armadillo (ARM) domains.

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      I  T  E  P  A  V  C  A  L  R  H  L  T  S  R  H  L  E  A  E  M  A  Q  N  A  V  R  L  H  Y  G  L  P
2901  GTTCTAGTCAAGCTTCTCCATCCCCCGAGCCGCTGGCCACTTATCAAGGCTGTCGTTGGACTGATCCGCAACCTGGCTTTGTGCCCATCTAATCATGCGC
      V  L  V  K  L  L  H  P  P  S  R  W  P  L  I  K  A  V  V  G  L  I  R  N  L  A  L  C  P  S  N  H  A
3001  CCCTGAGGGAACATGGAGCACTACCCCGAATTGTACAGCTCATGATCAGAGCACACCAGGATACACAGAGGAGAGCCTCAATCACCTCCAATGGACAGGG
      P  L  R  E  H  G  A  L  P  R  I  V  Q  L  M  I  R  A  H  Q  D  T  Q  R  R  A  S  I  T  S  N  G  Q  G
3101  CTCGGGCTATGTAGATGGTGTTAGAATGGAGGAGATTGTGGAGGGAACAGTAGGGGCACTCCACATTCTTGCTCGTGAGACCCACAACAGAGCCGTAATA
      S  G  Y  V  D  G  V  R  M  E  E  I  V  E  G  T  V  G  A  L  H  I  L  A  R  E  T  H  N  R  A  V  I
3201  CGTGACCTCAACTGCATACCACTCTTTGTCCAGTTGCTGTACTCTCCAATTGAGAACATTCAGCGTGTTGCAGCAGGAGTTTTGTGTGAACTGGCAGCTG
      R  D  L  N  C  I  P  L  F  V  Q  L  L  Y  S  P  I  E  N  I  Q  R  V  A  A  G  V  L  C  E  L  A  A
3301  ATAAGGAGGGGGCTGAGCGAATAGAACAGGAGGGGGCCACTGCTCCACTCACAGAACTTTTACACTCCAGAAACGAGGGAGTTGCTACATATGCTGCAGC
      D  K  E  G  A  E  R  I  E  Q  E  G  A  T  A  P  L  T  E  L  L  H  S  R  N  E  G  V  A  T  Y  A  A  A
3401  AGTGTTATTCCGCATGTCTGAAGATAAGCCCCAGGACTACAAGAAGAGATTGTCTGTGGAACTGACCAGCTCCCTCTTCAGAGGGGATCAAAACATGGCT
      V  L  F  R  M  S  E  D  K  P  Q  D  Y  K  K  R  L  S  V  E  L  T  S  S  L  F  R  G  D  Q  N  M  A
3501  TGGTCAGATCCTCCTGGATTTGACGATGTTGGTGGTTTTCCTGACGATGGATATCGTGACCAGATGTATCAGGCACATGACAGTCAGAGTCAGCATGACA
      W  S  D  P  P  G  F  D  D  V  G  G  F  P  D  D  G  Y  R  D  Q  M  Y  Q  A  H  D  S  Q  S  Q  H  D
3601  TGAGTCGCAGTGGATATGACCCTCAGGTTCCTGTCGACTCCATGCAAGGCCTTGACATTGGCAGTCACCATGGCAGCCACTACGGACCAATGGACAACAT
      M  S  R  S  G  Y  D  P  Q  V  P  V  D  S  M  Q  G  L  D  I  G  S  H  H  G  S  H  Y  G  P  M  D  N  M
3701  GCCTGATTTTGGACACCAAGGTGGAGACCTGAACTTTGAGAATCTGGAGTCCAGTCTCCCACATGGTCAGGAGACGGGTAATCAGGGAATGACCCAGTGG
        P  D  F  G  H  Q  G  G  D  L  N  F  E  N  L  E  S  S  L  P  H  G  Q  E  T  G  N  Q  G  M  T  Q  W
3801  TTTGATACAGATCTGTAAatactgcacacttgaatggatcactacaaactttcttggaatgagaagacttttgtatataacatttcagtttttataacat
      F  D  T  D  L  *
3901  gataatttgttttgcactgcctgggaactatttcttttccccccagaaacaggcatggaaaactagaatacagttacatgattgaatttcaggaagctaag
4001  ctgttaatatttttgtaattttaacttttagaatcacatcttttataaagaaaatacttttttttatttgttgacagtttgtatcagttgaagtcttgtgc
4101  tggtagcactgtcctgatgccatcattgaattggattgttgagtctttcagaaaatagggagcaagtttcatcataaagttttcatttcattacctaaac
4201  aatacttgtttgtttttatcttcagtaataaaatttttttgtatgtcttggaaagctataataataatcttgttgaatcacctgtttggtatggtcaagac
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4601  ctgcagttttctgaccaaataaatggtcttttgaacatattcccaattttgcatccaaaattagtcttggaagttaaatattaatgaacagatcttggca
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4901  cacttgtaaacagctgaatactgcatctctaattttctttcataaattaaaatttatcattgtctgtttaaatatacttaacactaaccaggtagacatg
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5401  tactcattatcttttaccatacagtaatagtgaaaatttgtatgtatttagccatggccacattggttttatgtaggaattatttctccattttcataca
5501  cgtttattagaataaagattacagtttttataaaaaaaaaaaaa
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Fig. 1. (*continued*)

bp ORF encoding a predicted protein of 820 amino acids. Like *β-catenin* in most other species, *Hc-β-catenin* has three conserved structural regions; N- and C-terminal regions, and a central region with 12 ARM repeat motifs [29]. The ARM motif is a tandem repeat of ~40 amino acid residues that was first discovered in *Drosophila melanogaster* when studying signalling *in vivo* [30]. Members of the ARM-containing repeat

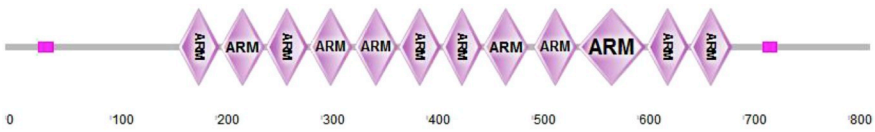

Fig. 2. SMART prediction of the conserved domains of *Hc-β-catenin*, including the central 12 ARM motifs.

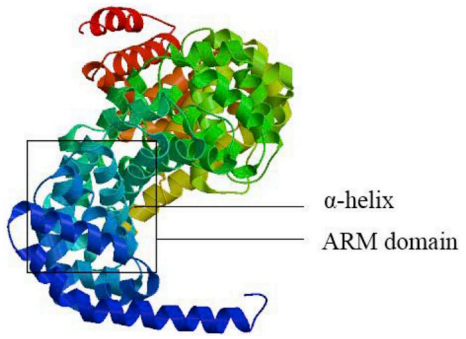

Fig. 3. *Hc-β-catenin* tertiary structure prediction.

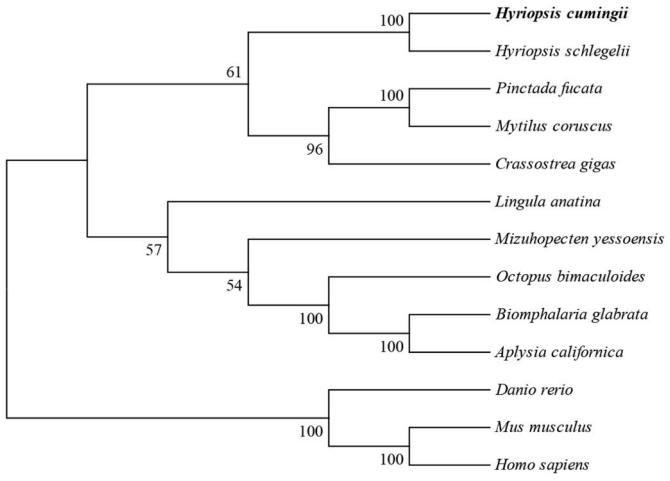

Fig. 4. Phylogenetic tree of *Hc-β-catenin* amino acid sequences from different species. The tree was constructed by MEGA5.0 using the neighbour-joining (NJ) method with 1000 bootstrap repeats.

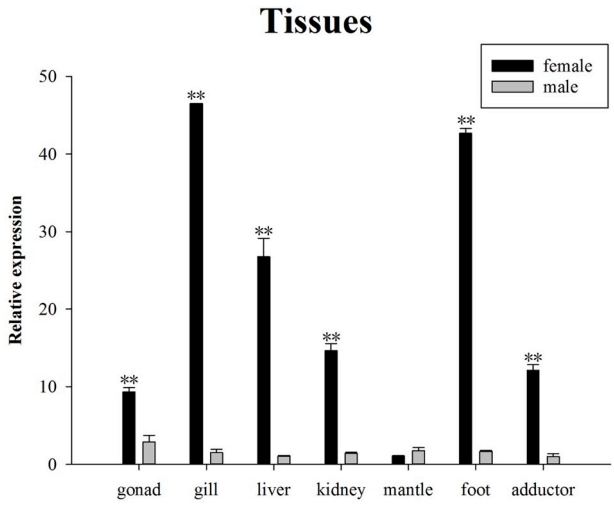

Fig. 5. Relative expression of *Hc-β-catenin* in different tissues of male and female *H. cumingii*. The results are based on three biological repeats and expressed as means ± standard error (SE). Significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

protein family are involved in cell proliferation, cell differentiation, cell adhesion, intercellular signal transduction, cytoskeletal regulation and tumorigenesis [31]. *β-catenin* is a key factor in the *Wnt/β-catenin* signalling pathway, and plays an important role in nervous system

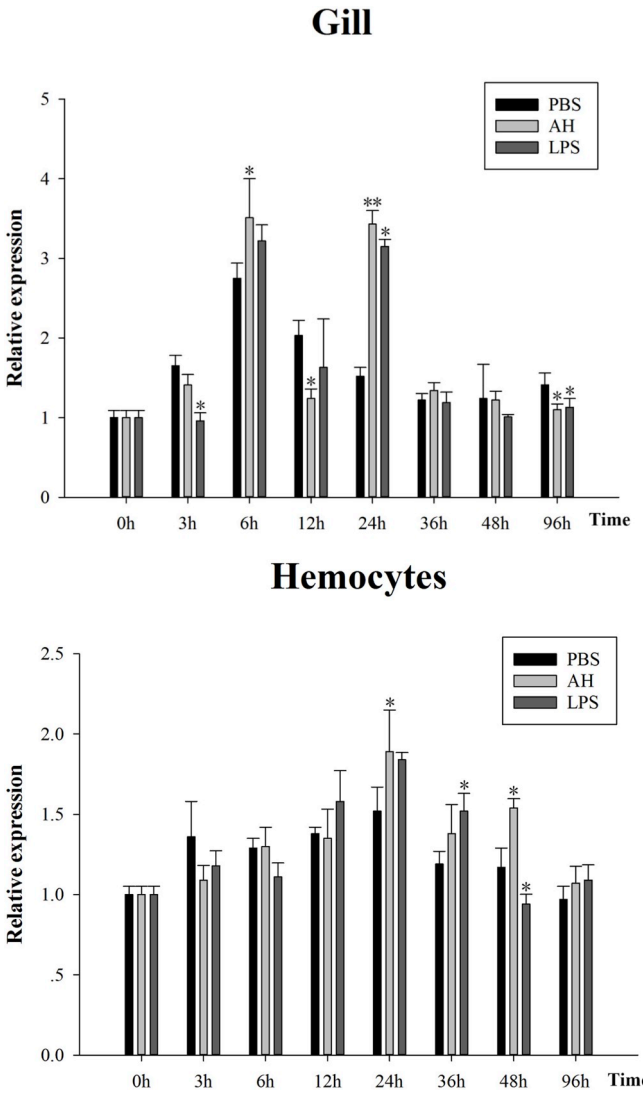

Fig. 6. Relative expression of *Hc-β-catenin* in hemocytes and gill following injection with *Aeromonas hydrophila* or lipopolysaccharide (LPS). Tissues were collected at 0, 3, 6, 12, 24, 36, 48 and 96 h after injection. Significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

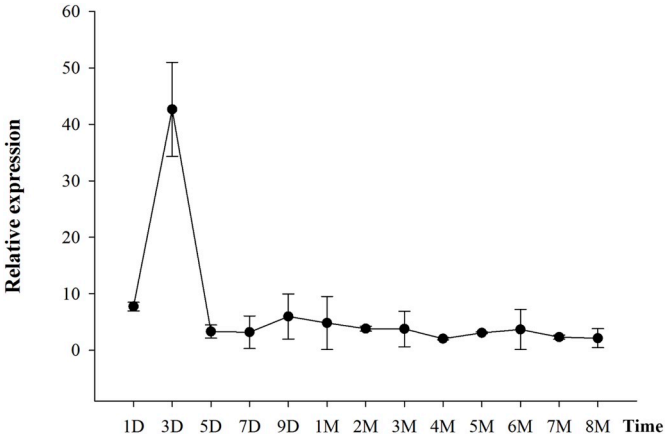

Fig. 7. Relative expression of *Hc-β-catenin* in male and female gonads of 1-, 2- and 3-year-old mussels. Vertical bars represent means ± SD (n = 3).

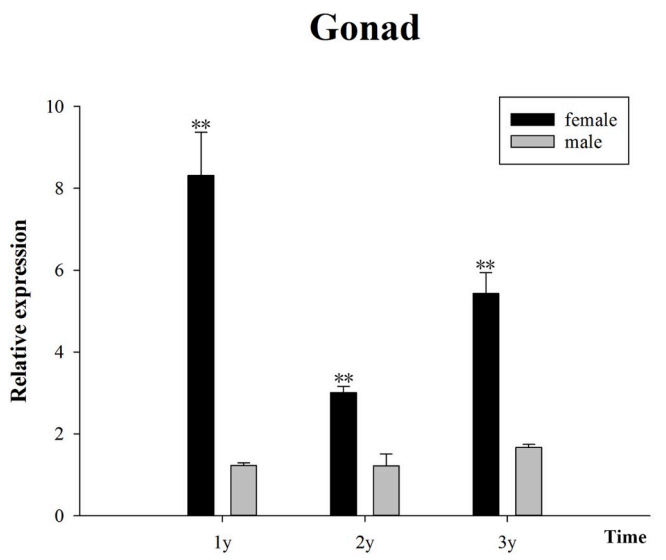

Fig. 8. Relative expression of *Hc-β-catenin* in gonads at different developmental stages.

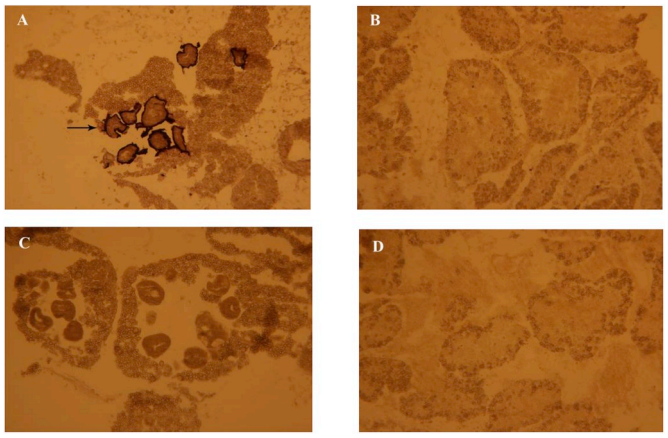

Fig. 9. *In situ* hybridisation of *Hc-β-catenin* in gonads. The upper images were obtained using antisense probes (10 × ; female on the left, male on the right). Lower images are from negative controls without probes (10 × ; female on the left, male on the right).

development, embryonic development, mammalian sex determination and differentiation [32].

Vertebrates not only have innate immunity, but also have acquired immunity, while invertebrates only have innate immunity and rely on innate immunity against invasion by pathogenic microorganisms [33]. As invertebrate, *H. cumingii* lacks a specific acquired immune system, hence the innate immune system acts as the first line of defence against pathogens [34]. Due to the open circulatory system of *H. Cumingii*, the gill is in direct contact with the environment, and hemolymph represents the extracellular fluid of the entire organism [34]. The gill is an important immune-related tissue because it provides a defence barrier that filters suspended matter, immune-related genes are usually highly expressed in this tissue [35]. Hemocytes in molluscs serve as the main protective tissue against external pathogens [36].

Immune-related research on *β-catenin* in shellfish is scarce. Herein, we found that *Hc-β-catenin* may play an important immunomodulatory role. After injection with live *A. hydrophila*, *Hc-β-catenin* expression was generally up-regulated in both gill and hemocytes, but was decreased at certain time points. After treatment with LPS, *Hc-β-catenin* transcript levels differed significantly from the control group, suggesting that *Hc-β-catenin* expression is affected by stress following challenge with LPS or bacterial pathogens. The results indicate that *Hc-β-catenin* participates in the immune responses of *H. cumingii*, and may play an important role in host resistance in shellfish. For example, infection with *Staphylococcus aureus*, *Vibrio anguillarum* or white-spot syndrome virus (WSSV) stimulated expression of *β-catenin* in the gill of the shrimp *Marsupenaeus japonicus* [37]. *β-catenin* is a positive regulator in the immunity of shrimp to bacteria and viruses. In *Litopenaeus vannamei*, *β-catenin* expression levels were increased by *V. parahaemolyticus* challenge [38], suggesting that *β-catenin* was involved in innate immune response of shrimp to bacterial and viral infection. Shrimp AMPs such as PENs and ALFs play an important role in anti-bacterial and anti-viral. Over-expression of *β-catenin* up-regulates promoter activity of AMP genes, suggesting that *β-catenin* could play a role in regulating the production of AMPs. However, surprisingly, WSSV infection reduced *β-catenin* levels in *Litopenaeus vannamei* [13]. By posttranslational modification, WSSV infection could inactive *β-catenin*. It may be the target of virus modulation. After WSSV infection, *β-catenin* transfered into the nucleus, indicating that *β-catenin* may play a negative role in the replicative cycle of WSSV. The immune system could be modulated by inhibiting the proliferation of the virus to protect the shrimp from WSSV infection. Similarly, in Chinese tongue sole, *β-catenin* and rspo2l expression in the gill was significantly down-regulated following *V. harvey*i challenge [14]. *β-catenin* is a downstream gene of R-spondin family. It is involved in the Wnt/*β-catenin* in the R-spondin family proteins (Rspos). These apparent contradictory immune responses may be related to differences between species or pathogens. Overall, the results indicated that *β-catenin* participates in innate immune responses to bacterial and viral infections.

During the experiment, we found that *Hc-β-catenin* was also related to gender determination. Expression of *β-catenin* in gonads has been studied in vertebrates, and it remains unclear whether it is linked to sex determination and differentiation, although it is believed to participate in female gonadal differentiation [25]. At present, some studies on the role of *β-catenin* in gonad determination and differentiation suggest that the developmental direction of mammalian undifferentiated gonads is determined by the competition between the female signalling pathway mediated by signalling molecules such as R-spondin1, Wnt4, *β-catenin* and the male signalling pathway mediated by signalling molecules such as Sry and Sox9. The gonads eventually develop towards the competitive dominant side [39]. In addition, two secreted ligands, Wnt4 and R-spondin1, can activate the *β-catenin* canonical signalling pathway [40]. In mice, during ovarian development, Rspo1 is essential for the activation of the canonical *β-catenin* signalling pathway [22]. In our present study, the qRT-PCR results showed that *Hc-β-catenin* was expressed in both male and female tissues, albeit differentially, with significantly higher levels in female gonads. Furthermore, in 1-, 2- and 3-year-old mature mussels, *Hc-β-catenin* expression was markedly higher in female gonads than in males, indicating that it may be involved in female gonad development. Expression was much elevated after 3 days, but then decreased and remained low, indicating an involvement in embryonic development. Thus, we speculate that *Hc-β-catenin* is a pre-ovarian anti-testis gene, consistent with some previous reports. In *C. farreri* gonadal development, *β-catenin* expression is high and sexually dimorphic, with maximum expression during the mature stage of the reproductive cycle, and higher levels in ovary is compared with testis [41]. In mice, *β-catenin* is a key anti-testis molecule. Activation of *β-catenin* in somatic cells of XY gonads disrupts the formation of the testis cord and down-regulates the expression of testis marker genes; it effectively blocks testis development and eventually leads to male-to-female sex reversal, indicating that *β-catenin* may be a key pro-ovarian and anti-testis signalling molecule [22]. Expression of the *β-catenin* gene increases during the development of gametes in mature female Pacific oysters, and peak levels are reached in mature females, whereas levels remain low in males [42]. It suggests that *β-catenin* is a potential regulator in the ovary. In *Marsupenaeus japonicus*, *β-catenin* is widely expressed in various tissues, with highest levels in testes and ovaries

[37]. In *Cynoglossus semilaevis*, *β-catenin* is transcribed in the gonads, but levels in ovaries are significantly higher than in testes. In addition, *CS-β-catenin1* mRNA levels are clearly up-regulated at 160 days, and continue to increase up to 2 years of age [43], indicating that *β-catenin* is involved in ovary growth. The differences in relative expression levels between male and female tissues observed in the present study are consistent with these previous findings. Our results showed that expression levels were higher in females in all tissues except for the mantle.

Our current *in situ* hybridisation results revealed a significant hybridisation signal in the cell membranes of female oocytes, while the male follicle wall displayed a weaker hybridisation signal, consistent with the qRT-PCR results, suggesting that the *Hc-β-catenin* was associated with female gonad differentiation and with oogenesis. In Chinese tongue sole, ISH experiments mainly detected *CS-β-catenin1* mRNA in oocytes [43], and in *Nile tilapia*, the ISH signal of *β-catenin* was greatest in the oogonia and oocytes of the ovary [44]. Together, these results suggest that *β-catenin* is a pro-ovarian, anti-testis gene. We therefore deduce that *Hc-β-catenin* influences gender determination and differentiation during the formation of female gonads.

5. Conclusion

Herein, we cloned the full-length cDNA *Hc-β-catenin* from *H. cumingii*. The predicted amino acid sequence contains 12 ARM repeats, similar to orthologs in other species. *Hc-β-catenin* was up-regulated in hemocytes and gills following pathogen/LPS exposure, the most strongest response occurred at 24 h (gills and hemocytes) after AH or LPS challenge, indicating an immunoregulatory role. qRT-PCR and *ISH* results linked *Hc-β-catenin* to female gonad determination and differentiation. Our results suggested that *Hc-β-catenin* play an important role in immune responses and sex determination.

Acknowledgements

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