Fish and Shellfish Immunology 88 (2019) 102–110

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Evolution and function analysis of interleukin-17 gene from *Pinctada fucata martensii*

Yanfei Cao, Shuai Yang, Chuzhang Feng, Waner Zhan, Zhe Zheng, Qingheng Wang, Yuewen Deng, Yu Jiao*, Xiaodong Du**

Fishery College, Guangdong Ocean University, Zhanjiang, 524025, China

ARTICLE INFO

Keywords:
Interleukin-17
Immune
Pinctada fucata martensii
Mollusk
Genomic analysis

ABSTRACT

Interleukin-17 (IL-17) is a proinflammatory cytokine that plays an important role in immune responses. In this study, we identified 57 IL-17 genes from the genomes of six marine invertebrates, including *Pinctada fucata martensii*, *Crassostrea gigas*, *Lottia gigantea*, *Capitella teleta*, *Mizuhopecten yessoensis*, and *Mytilus galloprovincialis*. Phylogenetic analysis showed that all invertebrate IL-17 genes were clustered into one group, implying that invertebrate IL-17 evolved from one common ancestral gene. From the extron–intron analysis, we found many intronless IL-17 genes in mollusks, which may be caused by retroposition. Tissue and development transcriptomic analysis showed that the expression of PmIL-17 was tissue and developmental stage-specific. Moreover, we cloned the full length of the IL-17-2 gene from *P. f. martensii* (PmIL-17-2) and explored its function in the immune response. The full-length cDNA of PmIL-17-2 is 719 bp, containing an open reading frame of 564 bp, a 5′ -untranslated region (UTR) of 31 bp, and a 3′ -UTR of 124 bp with a 30 bp poly (A) tail. PmIL-17-2 had a strong response to lipopolysaccharide (LPS), indicating that the PmIL-17-2 participates in innate immune responses. In situ hybridization of hemocytes showed that PmIL-17-2 was mainly produced by granulosa cells, and the number of the stained granulosa increased after LPS stimulation. These results lay the foundation for the research of IL-17 family in marine invertebrates.

1. Introduction

Interleukin-17 (IL-17) belongs to an ancient cytokine family that is one of the major signaling molecule families involved in immune responses. IL-17 participates in the body's inflammatory response by regulating the NF-κB pathway [1]. In humans, six members of the IL-17 family (IL-17A-F) were identified and sequentially named in the order of discovery [2,3]. IL-17A-F in human bears greatest similarity within the C terminal and has four well-conserved cysteines [2]. To date, research on the function of IL-17 family is mainly focused on three members, namely, IL-17A, IL-17E, and IL-17F [2,4]. IL-17 family members have been cloned from mouse, zebrafish, chickens, and Japanese puffer fish, and they encode a cytokine associated with inflammation and immunity [4–8].

In invertebrates, the first IL-17 gene was found from the genome of sea squirt [9]. Thereafter, sequences similar to IL-17 and IL-17R have been found in other invertebrates, such as sea urchin and *Ciona intestinalis* [10,11]. In mollusks, IL-17 genes were cloned successfully from marine invertebrate species, including the pearl oyster *Pinctada fucata martensii* and Pacific oyster *Crassostrea gigas*; these genes can respond to lipopolysaccharide (LPS), heat-killed Vibrio alginolyticus (HKVA), and bacteria and are also involved in NF-κB signaling pathways [12–14]. Hence, IL-17 may play an important role in the inflammatory response of invertebrates.

Marine invertebrates such as pearl oyster, scallop, and Pacific oyster, are important marine economic species. To understand the innate immune response in the marine invertebrates, we present a comprehensive genomic annotation and transcriptomic analyses of a large subset of IL-17 in mollusks, including *P. f. martensii*, *C. gigas*, *Lottia gigantea*, *Capitella teleta*, *Mytilus galloprovincialis*, and *Mizuhopecten yessoensis*. Furthermore, we cloned and characterized IL-17-2 from *P. f. martensii* (PmIL-17-2), and analyzed the sequential expression of IL-17 in hemocytes after LPS stimulation to determine the role of the IL-17 genes in innate immunity. The results could contribute to our understanding of the evolution and function of the mollusca IL-17 in detail.

* Corresponding author.
** Corresponding author.
E-mail addresses: jiaoyu1981@hotmail.com (Y. Jiao), gdhddxd@Hotmail.com (X. Du).

https://doi.org/10.1016/j.fsi.2019.02.044
Received 13 November 2018; Received in revised form 20 February 2019; Accepted 21 February 2019
Available online 22 February 2019

2. Materials and methods

2.1. Identification of IL-17

Protein sequences of *Danio rerio, Homo sapiens*, *L. gigantea*, and *C. teleta* were downloaded from the National Center for Biotechnology Information (NCBI) database. For annotation, we queried all proteins against the functional databases Nr and KEGG [15] by using the basic local alignment search tool (BLAST) (E-value ≤ 1-e5) and accepted the results with best scores for each query protein. We also used InterProScan [16,17] to predict gene function on the basis of domain information. The annotation information of *P. f. martensii* and *C. gigas* was obtained from our previous research [17,18]. The IL-17 genes from *M. yessoensis* and *M. galloprovincialis* were extracted from the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/?term=gene) annotated as IL-17 genes. The protein domain of all of the identified IL-17 genes was re-analyzed and confirmed by the Simple Modular Architecture Research Tool (SMART) version 5.1 (http://smart.Embl-heidelberg.de/). Only the genes that were homologous with IL-17 (E-value ≤ 1-e5) and contain IL-17 domains were considered as IL-17.

2.2. Cloning the full-length cDNA of PmIL-17-2

5′/3′ -RACE was applied to obtain the full-length cDNA of PmIL-17-2 using the SMART RACE cDNA amplification kit (Clontech, Japan). Total RNA for the RACE reaction was extracted from the hemocytes of *P. f. martensii* by using Trizol reagent. Gene-specific primers were designed on the basis of the nucleotide sequences of the PmIL-17-2 cDNA fragment obtained from the transcriptome of *P. f. martensii* [19]. Table 1 shows the polymerase chain reaction (PCR) primers used in this study.

2.3. RNA extraction and cDNA synthesis

The gill (Gi) from *P. f. martensii* were collected, immediately stored in liquid nitrogen, and then saved in −80 °C before use. Total RNAs were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. RNA quantity was detected by measuring OD260/OD280 with NanoDrop ND 10000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and RNA integrity was analyzed through fractionation on 1.0% agarose gel. The total RNAs were transcribed into cDNA with oligo (dT)-adaptor primers (Sangon Biotech) and M-MLV reverse transcriptase (Clontech).

2.4. Immune challenge

In the challenged group, 100 μL of 10 μg/mL LPS (Sigma, USA) was injected into the adductor muscle of the pearl oyster *P. f. martensii*. In the phosphate-buffered saline (PBS) group, 100 μL of PBS was injected in the same way [20]. All the pearl oyster were placed back in the tanks with re-circulating filtered seawater after injection. Hemocytes were collected from six pearl oyster in 0, 6, 12, and 24 h after injection, immersed in Trizol (Invitrogen, USA), quick-frozen by liquid nitrogen, and then saved in −80 °C until use.

2.5. Localization of PmIL-17-2 in hemocytes

According to the full-length cDNA sequence of the acetylcholine receptor gene, primers with a length of 100 bp to 300 bp were designed, and a T7 promoter sequence (GCGTAATACGACTCACTATAGGG) was added before the forward primer. The target fragment obtained by PCR amplification were recovered and purified. The recovered PCR product was then in vitro transcribed using T7 RNA polymerase and DIG RNA Labeling Mix, and an RNA probe with digoxigenin label was finally obtained. The integrity of the RNA probes was detected by 1% agarose gel electrophoresis, and the quality of the probes was analyzed in conjunction with the RNA concentration and purity determined by the nucleic acid quantifier. Hemocytes after 0 h and 24 h treatment with PBS and LPS were subjected to in situ hybridization according to the kit instructions of Enhanced Sensitive ISH Detection kit I (POD) (BOSTER).

2.6. Quantitative real-time PCR (qRT-PCR) assay

The primer sequences used for qRT-PCR are listed in Table 1. The qRT-PCR assay was performed on a Roche LightCycler 480 (Roche, Switzerland) with Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific). β-actin [20] was selected as the reference gene to verify the expression of PmIL-17-2. The PCR program was conducted as follows: 5 min at 95 °C and 40 cycles (each cycle was for 30 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C).

2.7. Sequence analysis and statistical analysis

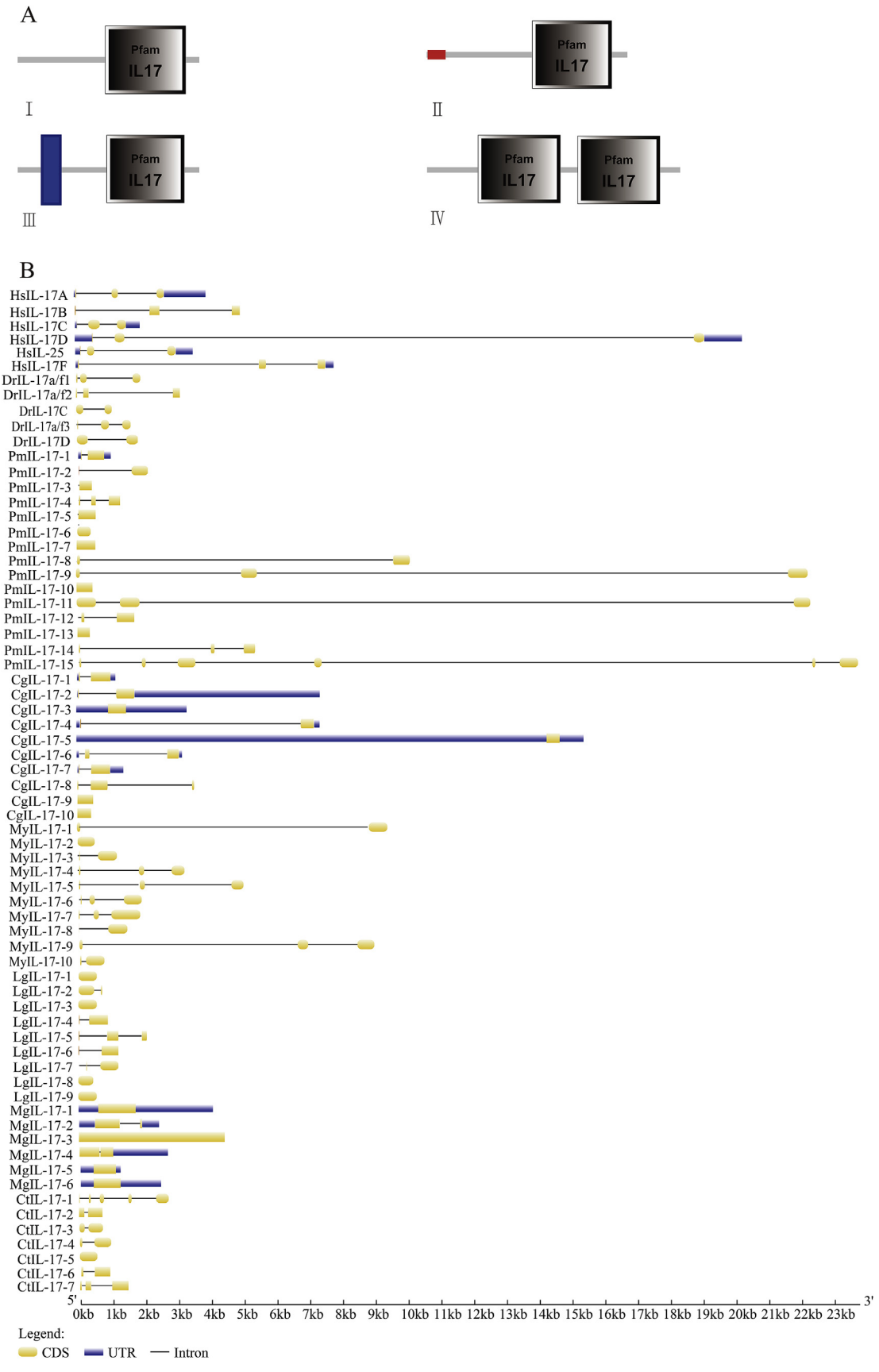
The resulting 5′ -end and 3′ -end sequences were sequenced using DNAMAN to obtain the full-length sequence of the gene. The full-length sequences of the obtained genes were analyzed using BLAST provided by NCBI (http://www.ncbi.nlm.nih.gov/). Open reading frame (ORF) was obtained using the ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). The protein domain was predicted by the SMART (http://smart.embl-heidelberg.de/smart/show_motifs.pl). SignalP 4.1 program was utilized to predict the presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight and the theoretical isoelectric point were analyzed by the ProtParam tool (https://web.expasy.org/cgi-bin/protparam/protparam). Based on amino acid sequences, comparison and phylogenetic analysis were performed with ClustalW multiple sequence alignment (http://www.genome.jp/tools-bin/clustalw). MEGA 6.0 software was used to construct the phylogenetic tree.

qRT-PCR data were analyzed using SPSS 22.0. $P < 0.05$ was considered statistically significant.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)
5′ Outer-PmIL-17-2	TACGGCATGTATTAGCTCGATTGTTAGCTGC
5′ inner-PmIL-17-2	AGTCTCTGACGGTTTTACTAAGGTTTGGTC
3′ outer-PmIL-17-2	GGAAACCAAAGGAATTAACAGAAGCCCAGTG
3′ inner-PmIL-17-2	ACACTCAGATTCTGGAACCTCTAGCAGCAGG
RT-PmIL-17-2-F	AAGAAAACTTTGAACATGCCGTAC
RT-PmIL-17-2-R	TAATCACATAATGCCAGGGACA
β-actin-F	CGGTACCACCATGTTCTCAG
β-actin-R	GACCGGATTCATCGTATTCC
ISH-PmIL-17-2-F	GCGTAATACGACTCACTATAGGGATAATCGAGCTAACAATCGACGTTGTTCAGTACGA
ISH-PmIL-17-2-R	GTGTACGTATAAATACCTTGATCGTCACAACCATT

Notes: The sequences underlined are the T7 promoter sequence.



(caption on next page)

Fig. 1. Structural characteristics of IL-17 in human, zebrafish and 6 marine invertebrates.
A: Three types of IL-17 genes. I: contains only one IL-17 domain. II: contains a C terminal signal peptide and IL-17 domain. III: contains a transmembrane domain and IL-17 domain. IV: Contains two IL-17 domains. Red represents signal peptide and blue represents the transmembrane domain. B: Gene structure of the IL-17 genes. Black lines represent the introns, and orange round-corner rectangle indicates the exons. The blue rectangle indicates the UTR regions. Hs: *Homo sapiens*; Dr: *Danio rerio*; Pm: *Pinctada fucata martensii*; Cg: *Crassostrea gigas*; My: *Mizuhopecten yessoensis*; Lg: *Lottia gigantean*; Mg: *Mytilus galloprovincialis*; Ct: *Capitella teleta*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

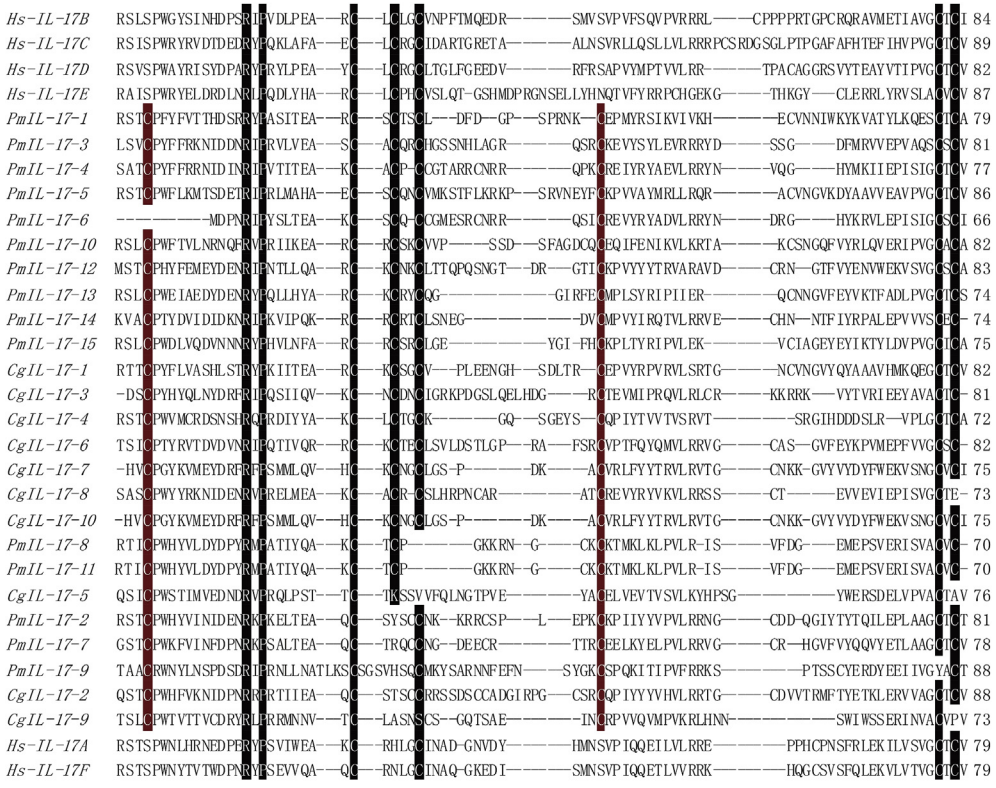


Fig. 2. Multiple sequence alignment of IL-17 domain of IL-17 gene from pearl oyster, Pacific oyster, and human.
Identical amino acids are highlighted in black. Red-labeled amino acids are cysteine residues that are only conserved in mollusks. Hs: *Homo sapiens*; Pm: *Pinctada fucata martensii*; Cg: *Crassostrea gigas*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Identification and gene structure analysis of IL-17 in marine invertebrates

Basing on the Nr and KEGG annotations combined with InterProScan analysis, we identified 57 IL-17 genes from six marine invertebrate genomes. Among which, 15 were found in *P. f. martensii* (including one previously reported homolog, designated as PmIL-17-1), 10 in *C. gigas*, 9 in *L. gigantean*, 7 in *C. teleta*, 10 in *M. yessoensis*, and 6 in *M. galloprovincialis*. Furthermore, 5 and 6 IL-17 genes were identified from *D. rerio* and *H. sapiens*, respectively. SMART results showed that all of the identified 68 genes had a typical IL-17 domain in the IL-17 family (Supple table 2). In the analyzed species, four types of domain combination were found on the basis of the domain analysis by SMART (Fig. 1A). We noted 38 genes with signal peptides and 24 genes without signal peptides, which may be caused by assembly and annotation errors. One IL-17, which was found in *P. f. martensii*, consisted of two IL-17 domains. Five IL-17 genes from pearl oyster, scallops, and Mediterranean mussel were predicted with a transmembrane domain in front of the IL-17 domain. This phenomenon was also found in the IL-17E isoform from human.

The genomic structure of the IL-17 family genes was analyzed, as shown in (Fig. 1B). Twenty intronless genes were found in the six marine invertebrates but none in humans and zebrafish. In pearl oyster, six intronless genes were found. Furthermore, four intronless IL-17 genes were identified in limpet, Pacific oyster, and Mediterranean mussel, respectively, whereas only one intronless gene was found in sea worms and scallops.

3.2. Multiple sequence alignment and phylogenic relationship

We performed multiple sequence alignment of IL-17 domains from pearl oyster, Pacific oyster, and human by ClustalW2 software. Most of the IL-17 members shared four highly conserved cysteine residues (indicated in black color) that participate in the formation of intrachain disulfide bonds (Fig. 2). In addition, the two other cysteine residues (indicated in red color) in the *P. f. martensii* and *C. gigas* IL-17 may be involved in the disulfide bond between chains to form homodimers.

Among the 15 IL-17 genes in *P. f. martensii*, 7 (47%) were linked in tandem arrays. In the phylogenetic tree, the five PmIL-17 tandem repeats (PmIL-17-2, PmIL-17-3, PmIL-17-4, PmIL-17-6, and PmIL-17-7) were clustered together and consisted of three types of domain combination. The PmIL-17 genes with transmembrane were clustered together with the IL-17 without transmembrane, indicating that these genes evolved from the same ancient gene (Fig. 3A). Furthermore, the unrooted phylogenetic tree based on the amino acid sequences of these analyzed mollusks and other vertebrates showed significant differences in the genes between invertebrates and vertebrates (Fig. 3B). The vertebrate IL-17 genes were clustered with their corresponding IL-17 family genes, including the IL-17A/F, IL-17B, IL-17C, IL-17D, and IL-17E groups, whereas all of the IL-17 genes from invertebrates were clustered together. The detail description of IL-17 proteins used in the phylogenetic analysis is shown in supple table 3.

3.3. Expression analysis of PmIL17 genes in pearl oyster

To characterize the expression of PmIL-17 in various tissues and developmental stages, we re-analyzed the published transcriptome data

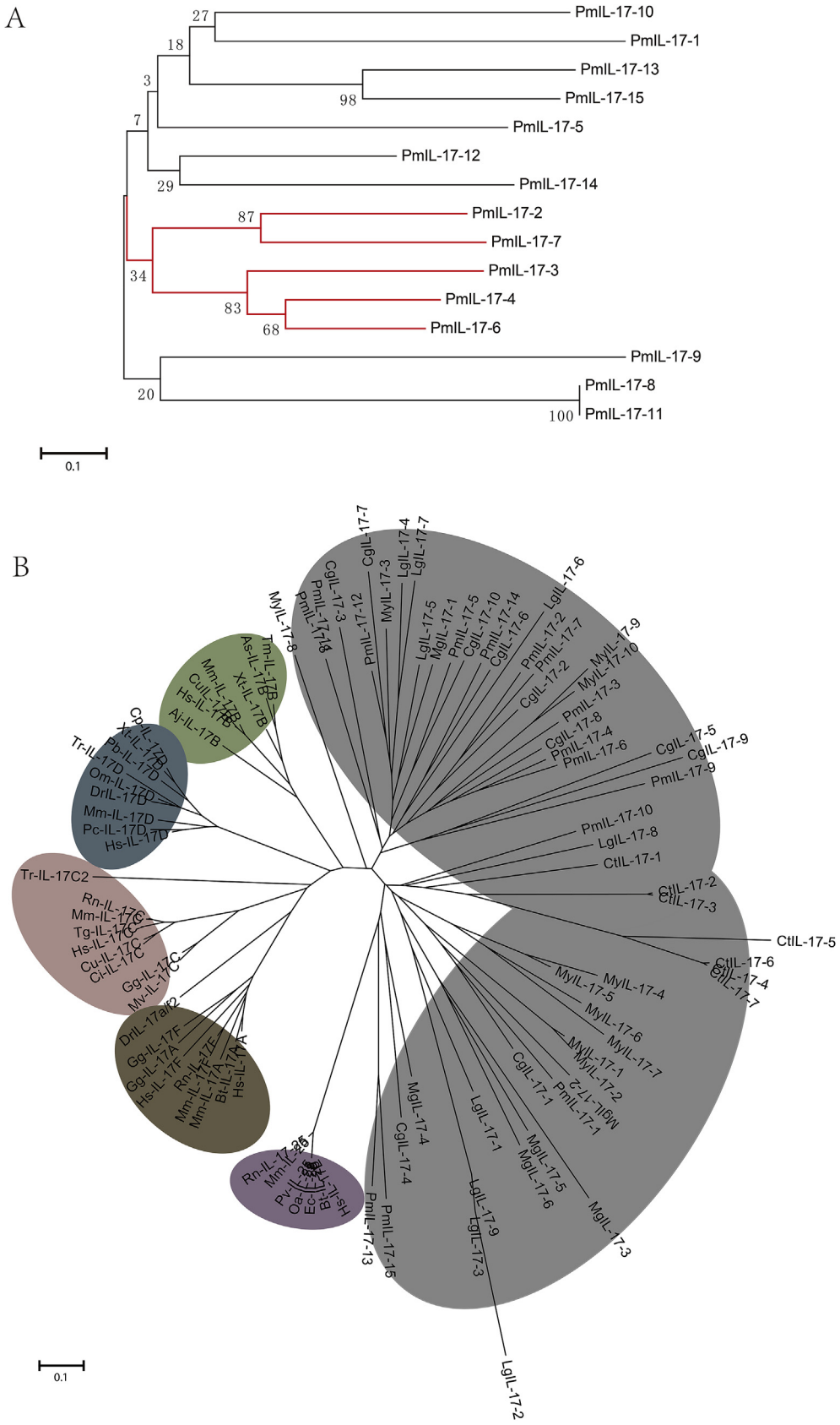


Fig. 3. Phylogenic analysis was performed using the maximum likelihood method in MEGA 6.0 based on the sequence alignment results.

A: Phylogenetic tree (constructed by adjacency) demonstrates the evolutionary relationship between IL-17 genes of different domain combination in *P. f. martensii*. Red lines indicate tandem repeat genes in *P. f. martensii*. B: Phylogenic tree showing the relationship between PmIL-17s and IL-17s from other species. The accession numbers of the IL-17 proteins are shown in supple table 3. The brown background represents the vertebrate IL-17A/F, the green background represents the vertebrate IL-17B branch, the pink background represents the vertebrate IL-17C, the blue background represents the vertebrate IL-17D branch, the purple background represents the vertebrate IL-17E branch and the gray background represents the invertebrate IL-17 family members. Hs: *Homo sapiens*; Mm: *Mus musculus*; Gg: *Gallus gallus*; Bt: *Bos Taurus*; Mm: *Mus musculus*; Aj: *Anguilla japonica*; Xt: *Xenopus tropicalis*; As: *Alligator sinensis*; Tm: *Terrapene mexicana triunguis*; Cu: *Callorhinus ursinus*; Tr: *Takifugu rubripes*; Cl: *Canis lupus familiaris*; Rn: *Rattus norvegicus*; Mv: *Manacus vitellinus*; Tg: *Theropithecus gelada*; Dr: *Danio rerio*; Om: *Oncorhynchus mykiss*; Pc: *Puma concolor*; Pb: *Python bivittatus*; Cp: *Chrysemys picta bellii*; Ec: *Equus caballus*; Oa: *Ovis aries*; Pv: *Pteropusvampyrus*

of *P. f. martensii*. Overall, the expression of PmIL-17 genes was tissue and developmental stage-specific (Fig. 4). PmIL-17-2 and PmIL-17-4 shared a similar expression pattern with high expression in eyed larvae and mantle central, whereas PmIL-17-7 was highly expressed in trochophore and gonad. PmIL-17-12 was highly expressed in the D-stage larvae, gill, and hepatopancreas. PmIL-17-10 was highly expressed in spat and gill; however, PmIL-17-13 was highly expressed in gastrula and mantle edge.

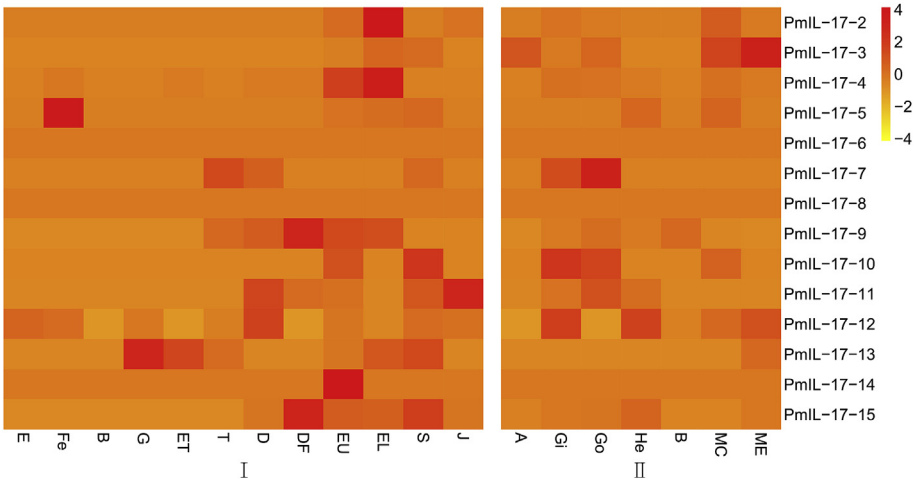


Fig. 4. Expression analysis of PmIL17s in pearl oyster.
I: Expression patterns of PmIL-17 genes mRNA in different tissues. Go: gonad, ME: mantle edge, He: hepatopancreas, F: foot, A: adductor muscle, Gi: gill, MC: mantle central and B: hemocytes. II: Analysis of expression patterns of PmIL-17 gene at different developmental stages. E: egg; Fe: fertilized egg at 30 min; B: blastula at 5 h 25 min; G: gastrula at 6 h 30 min; ET: early trochophore at 8 h 25 min; T: trochophore at 15 h 45 min; D: D-stage larvae at 19 h 5 min; DF: D-stage larvae before feeding at 4 d; EU: early umbo larvae at 14 d; EL: eyed larvae at 28 d; S: spat at 40 d; J: juveniles at 90 d.

```
1   acatgggggatcaaaatcagtttaagaaaagATGGAGAATTTCTTCATTTCCATTGTTTTAATTTCCTCAATATTTGTGAAATGTCCGTC
1                                   M  E  N  F  F  I  S  I  V  L  I  S  S  I  F  V  K  C  P  S
91  AGTTCTTGCGTCACGAATGTGCAGGGAACCTAATGATTTACCGAGCATCACGTCTGATTTGACGTCAGAGACTGCCAAAATGATTCCAAA
21  V  L  A  S  R  M  C  R  E  P  N  D  L  P  S  I  T  S  D  L  T  S  E  T  A  K  M  I  P  N
181 CCAGATTTTAGACACAGTAACTGGGGAATTAAGAACAATACCAAGAGGATATGTCAACATAACGAGAGAAAGAATTCAAGAAAACTTTGA
51  Q  I  L  D  T  V  T  G  E  L  R  T  I  P  R  G  Y  V  N  I  T  R  E  R  I  Q  E  N  F  E
271 ACATGCCGTACATAATCGAGCTAACAATCGACGTTGTTCAGTACGATCAACATGTCCCTGGCATTATGTGATTAATATAGATGAAAACAG
81  H  A  V  H  N  R  A  N  N  R  R  C  S  V  R  S  T  C  P  W  H  Y  V  I  N  I  D  E  N  R
361 GAAACCAAAGGAATTAACAGAAGCCCAGTGTTCTTACAGTTGTTGTAATAAGAAGAGAAGATGCTCGCCTCTCGAGCCGAAATGTAAACC
111 K  P  K  E  L  T  E  A  Q  C  S  Y  S  C  C  N  K  K  R  R  C  S  P  L  E  P  K  C  K  P
451 AATCATTTATTACGTACCAGTATTACGTAGAAATGGTTGTGACGATCAAGGTATTTATACGTACACTCAGATTCTGGAACCTCTAGCAGC
141 I  I  Y  Y  V  P  V  L  R  R  N  G  C  D  D  Q  G  I  Y  T  Y  T  Q  I  L  E  P  L  A  A
541 AGGGTGCACGTGTACTGAACAATCATTGATGACAGTTTCTATGCCTATGTATTAAttacagctcattcttacaaatatgcatttgtcaat
171 G  C  T  C  T  E  Q  S  L  M  T  V  S  M  P  M  Y  *
631 atgatcagtttcacattattcagctttatttgtgaatgaaaaataaacatatttcttacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Fig. 5. Nucleotide and amino acid sequence of PmIL-17-2.
The 5′ UTR and 3′ UTR are indicated with small letters. The 5′ UTR and 3′ UTR are indicated with small letters. The 23 amino acid signal peptide is indicated with underline and IL-17 domain with a gray background. Nucleotides with a frame represent the start and stop codons.

3.4. cDNA cloning and characterization of PmIL-17-2 gene

Our previous transcriptomic data analysis showed that only PmIL-17-2 was up-regulated after nucleus insertion during pearl production (data not shown). To further explore its function in the immune response, we cloned PmIL-17-2 gene and verified its function. The full-length cDNA of PmIL-17-2 is 719 bp, containing an ORF of 564 bp, a 5′-untranslated region (UTR) of 31 bp, and a 3′ -UTR of 124 bp with a 30 bp poly (A) tail (Fig. 5). The ORF of PmIL-17-2 cDNA encoded 187 amino acids with a theoretical isoelectric point of 8.44 and the predicted molecular weight of 21.4 KD. A signal peptide has 23 residues. The deduced amino acid sequence of PmIL-17-2 contained the characteristic IL-17 family motif as predicted by the SMART program.

3.5. Temporal expression pattern of PmIL-17-2 after LPS stimulation

To validate the function of PmIL-17-2 in the immune response, we analyzed the temporal expression of the PmIL-17-2 gene in hemocytes after LPS stimulation. In the LPS group, PmIL-17-2 expression was promptly and significantly up-regulated (approx. 20 fold) ($P < 0.05$), reached the maximum at 12 h, then fell and returned to the original level at 24 h (Fig. 6A). The results of in situ hybridization showed that PmIL-17-2 was mainly expressed in granulosa cells. In the LPS stimulation group, the number of positive hemocytes was approximately 60%, which was significantly higher than that in the PBS group at 30% (Fig. 6B).

4. Discussion

Innate immune system is the first barrier for marine invertebrates to resist pathogen infection [21]. IL-17 family is one of the major signaling molecules involved in innate immunity [4]. Basing on sequence analysis, we identified 57 IL-17 genes from six marine invertebrates, including *P. f. martensii*, *C. gigas*, *L. gigantea*, *C. teleta*, *M. yessoensis*, and *M. galloprovincialis*. Five and six IL-17 genes were found from sea worms and Mediterranean mussels, respectively. However, 9 to 15 IL-17 genes were found in mollusks, including Pacific oyster, pearl oyster, scallops, and limpets; this number exceeded the six IL-17 genes already reported in vertebrates [22,23]. We proposed that the expanded IL-17 genes in mollusks may help to effectively cope with the inflammation response.

Notably, five IL-17 genes with transmembrane domain were found in the analyzed species, and IL-17E with transmembrane was also found in human IL-17E isoform. From the phylogenetic tree, the IL-17E genes from vertebrates were closer with the IL-17 genes from invertebrate compared with the other IL-17 members in vertebrates. Similar results can also be seen from the phylogenetic tree constructed by Jun Li [13]. Hence, we proposed that IL-17E may be the ancient IL type, and the other IL members evolved from IL-17E. In addition, the IL-17 with the

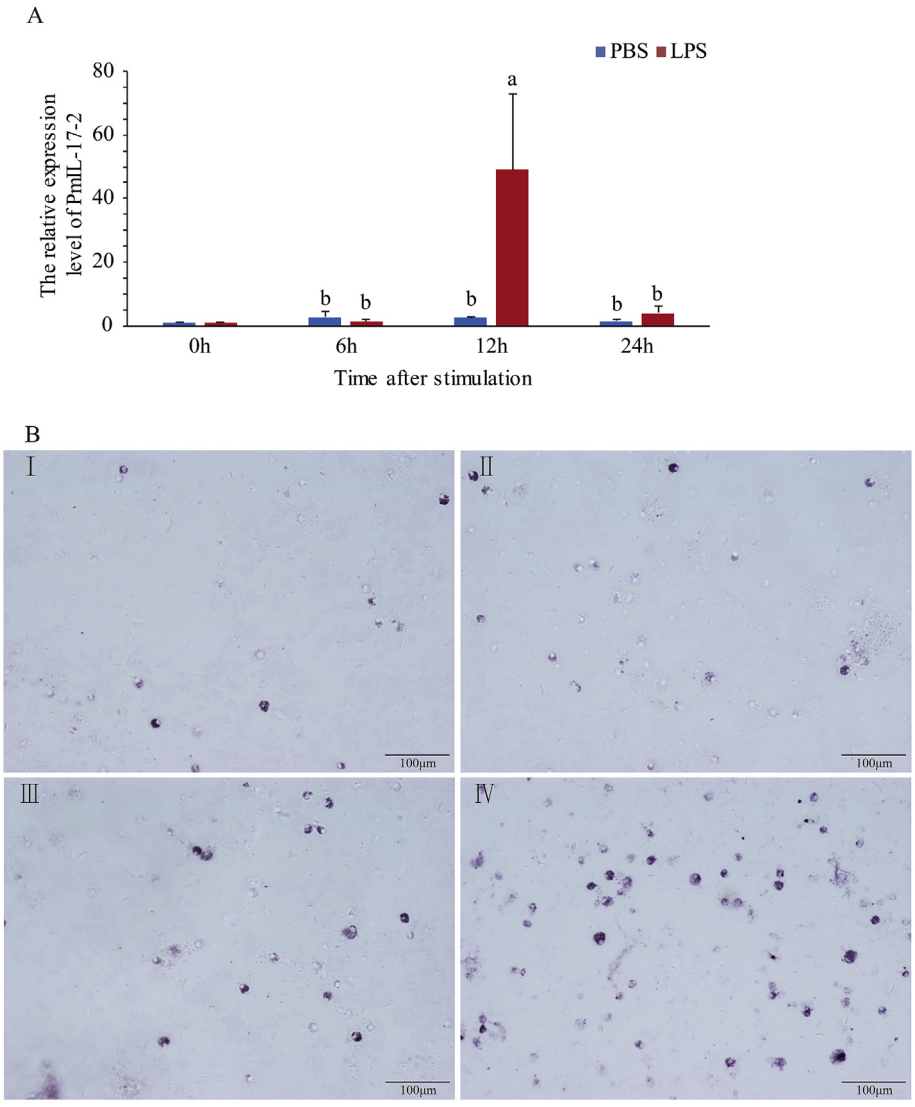


Fig. 6. Expression of PmIL-17-2 mRNA after LPS and PBS stimulation.
A: Expression patterns of PmIL-17-2 mRNA after LPS stimulation. Same letters above the bars represent no significant differences at the $P > 0.05$ level. Same letters above the bars represent no significant differences at the $P > 0.05$ level. Different letters above the bars represent significant differences at the $P < 0.05$ level. B: The expression PmIL-17-2 levels were determined by in situ hybridization. In the figure, Iand II indicate the expression of PmIL-17-2 at 0 h after stimulation with PBS and LPS, and in the figure, III and IV indicate the expression of PmIL-17-2 at 24 h after stimulation with PBS and LPS respectively.

transmembrane domain may be the ancient characteristic structure type of IL-17. During evolution, this gene may have lost its transmembrane domain and changed into one cytokine. The function of IL-17 with transmembrane may be different from that of the recent IL-17 and needs to be elucidated in detail in the future. From the extron–intron analysis, we found many intronless IL-17 genes in mollusks. This phenomenon may be caused by retroposition. Introns could delay the regulatory responses, and the genes that require rapid adjustment to survive in environmental challenges tend to have few introns [24]. The utilization of intronless opsins in *Chlamys farreri* was considered as an adaptive change for the improvement of transcription to support the scallop's unusual and advanced multi-eye visual system [25]. Thus, the intronless IL-17 genes in mollusk may be helpful for the immediate response to environmental stimulation.

Multiple sequence alignment revealed that most of the IL-17 genes in pearl oyster and Pacific oyster had four conserved cysteine residues. In humans, these cysteines are required for the formation of classical cysteine knots with two disulfide linkages [26], indicating that conserved amino acids may have conserved functions. In addition, the IL-17 gene in pearl oyster and Pacific oyster also has two other conserved cysteine residues that may participate in the linkage of the two sulfur bonds between the chains, thus forming two homologous polymers, which are common in members of the human IL-17 family [27,28]. The phylogenetic tree analysis showed that the IL-17 genes of vertebrates were clustered into five branches, namely, IL-17A/F, IL-17B, IL-17C, IL-17D, and IL-17E, suggesting that IL-17 family differentiated after the divergence of vertebrates and invertebrates. All of the IL-17 genes from marine invertebrate were clustered together. This finding strongly suggests that these marine invertebrate IL-17 genes may have evolved from the same ancestral gene.

Transcriptomic analysis showed that IL-17 expression in pearl oyster was tissue and developmental stage-specific. In general, PmIL-17 genes were mainly expressed in the gill, hepatopancreas, mantle, and gonad. Molluscan gill is the first line of defense against bacterial infection [29]. In addition, the hepatopancreas of mollusks is involved in digestive and defense functions [30]. Thus, the high expression of PmIL-17 in these tissues suggested that it may play a key role in the innate immune response of the pearl oyster. In mollusks, the mantle plays a vital role in the formation of the shell. The mantle edge also constitutes the first barrier between the environment and the mollusk and is involved in the immune homeostasis in oysters [31]. In vertebrates, IL-17 is involved in bone metabolism by reducing alkaline phosphatase, osteocalcin, and osterix [32]. The high expression of PmIL-17 may function as a regulator to inhibit the abnormal growth of the shell and maintain the homeostasis of shell formation. The high PmIL-17-3 expression in the gonad is consistent with the high IL-17D expression only in the ovaries of catfish, but the specific function of PmIL-17D in the gonad is still unclear [33]. In addition, PmIL-17 has

different expression levels at various developmental stages of pearl oyster. PmIL-17-5 and PmIL-17-13 were highly expressed in fertilized eggs and gastrula, respectively, whereas PmIL-17-6 and PmIL-17-8 were not expressed at any stage of development. Other PmIL-17 genes were mainly expressed after the trochophore stage. The bivalve immune system first appeared in the trochophore stage, whereas functional hemocytes and phagocytosis appeared in the early D-stage larvae and formed a favorable immune system in the umbo larva stage [34]. The specific expression of PmIL-17 genes after the trochophore stage suggests that they may help protect embryos and hatching larvae from pathogenic harsh aquatic environments. Moreover, D-type larvae are relatively rapid periods of embryonic development and organ formation [35]. In vertebrates, the IL-17 gene exhibits dynamic changes during development and may have certain functions during organ development [36–38]. The high PmIL-17 expression in D-type larvae also suggests that this gene may be involved in organ formation during the development of pearl oyster. In summary, IL-17 exhibited functional differentiation during the evolutionary process. This functional differentiation occurred in invertebrates and vertebrates after their differentiation possibly due to the biological environment changes when these organisms were exposed to different external stimuli and differentiation.

To analyze the role of PmIL-17 in the innate immune response of pearl oyster, we cloned the full-length sequence of PmIL-17-2 and verified its function in innate immunity of pearl oyster. Apart from IL-17 domain, the predicted IL-17 protein contains a predicted signal peptide with 23 amino acids, indicating that this gene secretes protein through Golgi/endoplasmic reactivity [39]. Hemocytes play a central role in the recognition of exogenous agents and defense against bacterial invasion in mollusks [40]. Hemocytes in mollusks are mainly divided into two types, granulocytes, and clear cells, in which granulocytes can phagocytize and clear pathogens [41,42]. We used in situ hybridization to localize PmIL-17-2 in hemocytes. Consequently, PmIL-17-2 was specifically expressed in granulosa cells. Thus, PmIL-17–2 may be synthesized in granulosa hemocytes and is involved in the innate immune response of pearl oyster. Moreover, we characterized the expression pattern of the PmIL-17-2 in response to the stimulation with LPS in hemocytes. PmIL-17-2 had immediately and drastically triggered LPS challenge. Similarly, the IL-17 expression increased under LPS stimulation in *C. gigas*, *P. f. martensii*, *D. rerio*, and *O. mykiss* [6,13,14,43]. LPS is the ligand of Toll-like receptor 4, which may trigger myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent pathways and consequently activate the NF-κB pathway [44]. The NF-κB signaling pathway is responsible for transcribing various proinflammatory cytokines [45]. These results indicate that the PmIL-17-2 gene is an inducible acute phase factor involved in the innate immune system against pathogens.

In conclusion, we identified 57 IL-17 genes from six marine invertebrate genomes, and genomic structural analysis found that genes are intronless in mollusks. Phylogenetic analysis revealed that all mollusk IL-17 genes share a common ancestral gene. The expression pattern of PmIL-17 is tissue and developmental stage-specific. Furthermore, we cloned the full length of the PmIL-17-2 gene. In situ hybridization of hemocytes showed that PmIL-17-2 is produced by the granulosa cells of hemocytes and participates in the immune response. Gene expression analysis indicated that PmIL-17-2 is involved in the innate immune response after LPS stimulation. To understand how PmIL-17-2 participates in innate immunity, we need to further research on signaling pathway.

Acknowledgments

The studies were financially supported by grants of the National Natural Science Foundation of China (grant numbers 31672626), the Guangdong Marine and Fishery Bureau (grant numbers Z2015004), Excellent Young Scientist Foundation of Guangdong Province (grant numbers 2015090), Graduate education innovation program of Guangdong Ocean University (grant numbers 201839), Project of Enhancing School with Innovation of Guangdong Ocean University (grant numbers GDOU2016050249).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.044.

References

[1] V. Valenzuela-Muñoz, C. Gallardo-Escárate, Molecular cloning and expression of IRAK-4, IL-17 and I-κB genes in Haliotis rufescens challenged with Vibrio anguillarum, Fish Shellfish Immunol. 36 (2014) 503–509, https://doi.org/10.1016/j.fsi.2013.12.015.
[2] M. Kawaguchi, M. Adachi, N. Oda, F. Kokubu, S.K. Huang, IL-17 cytokine family, J. Allergy Clin. Immunol. 114 (2004) 1265–1273, https://doi.org/10.1016/j.jaci.2004.10.019.
[3] N. Isailovic, K. Daigo, A. Mantovani, C. Selmi, Interleukin-17 and innate immunity in infections and chronic inflammation, J. Autoimmun. 60 (2015) 1–11, https://doi.org/10.1016/j.jaut.2015.04.006.
[4] S. Aggarwal, A.L. Gurney, IL-17: prototype member of an emerging cytokine family, J. Leukoc. Biol. 71 (2002) 1–8, https://doi.org/10.1189/jlb.71.1.1.
[5] J. Kennedy, D.L. Rossi, V.F. SM Zurawski Jr., R.A. Kastelein, J.L. Wagner, et al., Mouse IL-17: a cytokine preferentially expressed by alpha beta TCR + CD4-CD8-T cells, Journal of Interferon & Cytokine Research the Official Journal of the International Society for Interferon & Cytokine Research 16 (1996) 611–617, https://doi.org/10.1089/jir.1996.16.611.
[6] I. Gunimaladevi, R. Savan, M. Sakai, Identification, cloning and characterization of interleukin-17 and its family from zebrafish, Fish Shellfish Immunol. 21 (2006) 393–403, https://doi.org/10.1016/j.fsi.2006.01.004.
[7] W. Min, H.S. Lillehoj, Isolation and characterization of chicken interleukin-17 cDNA, Journal of Interferon & Cytokine Research the Official Journal of the International Society for Interferon & Cytokine Research 22 (2002) 1123–1128, https://doi.org/10.1089/10799900260442548.
[8] H. Korenaga, T. Kono, M. Sakai, Isolation of seven IL-17 family genes from the Japanese pufferfish Takifugu rubripes, Fish Shellfish Immunol. 28 (2010) 809–818, https://doi.org/10.1016/j.fsi.2010.01.016.
[9] V. Ayelet, N.F. Neff, S. Debashis, A.M. Newman, P. Dmitry, K. Winston, et al., The genome sequence of the colonial chordate, Botryllus schlosseri, Elife. 2 (2013) 1953–1965, https://doi.org/10.7554/eLife.00569.
[10] T. Hibino, M. Loza-Coll, C. Messier, A. Majeske, A. Cohen, D. Terwilliger, et al., The immune gene repertoire encoded in the purple sea urchin genome, Dev. Biol. 300 (2006) 349–365, https://doi.org/10.1016/j.ydbio.2006.08.065.
[11] A. Vizzini, F.F. Di, D. Parrinello, M.A. Sanfratello, C. Mazzarella, N. Parrinello, et al., Ciona intestinalis interleukin 17-like genes expression is upregulated by LPS challenge, Dev. Comp. Immunol. 48 (2015) 129–137, https://doi.org/10.1016/j.dci.2014.09.014.
[12] S. Roberts, Y. Gueguen, L.J. De, F. Goetz, Rapid accumulation of an interleukin 17 homolog transcript in Crassostrea gigas hemocytes following bacterial exposure, Dev. Comp. Immunol. 32 (2008) 1099–1104, https://doi.org/10.1016/j.dci.2008.02.006.
[13] J. Li, Y. Zhang, Y. Zhang, Z. Xiang, Y. Tong, F. Qu, et al., Genomic characterization and expression analysis of five novel IL-17 genes in the Pacific oyster, Crassostrea gigas, Fish Shellfish Immunol. 40 (2014) 455–465, https://doi.org/10.1016/j.fsi.2014.07.026.
[14] S.Z. Wu, X.D. Huang, Q. Li, M.X. He, Interleukin-17 in pearl oyster (Pinctada fucata): molecular cloning and functional characterization, Fish Shellfish Immunol. 34 (2013) 1050–1056, https://doi.org/10.1016/j.fsi.2013.01.005.
[15] S.A. Grando, R.M. Horton, E.F. Pereira, B.M. Diethelm-Okita, P.M. George, E.X. Albuquerque, et al., A nicotinic acetylcholine receptor regulating cell adhesion and motility is expressed in human keratinocytes, J. Invest. Dermatol. 105 (1995) 774–781, https://doi.org/10.1111/1523-1747.ep12325606.
[16] J. Levinton, Marine Biology: Function, Biodiversity, Ecology, fourth ed., (2013) Front Matter; 2013.
[17] G. Zhang, X. Fang, X. Guo, L. Li, R. Luo, F. Xu, et al., The oyster genome reveals stress adaptation and complexity of shell formation, Nature 490 (2012) 49–54, https://doi.org/10.1038/nature11413.
[18] X. Du, G. Fan, Y. Jiao, H. Zhang, X. Guo, R. Huang, et al., The pearl oyster Pinctada fucata martensii genome and multi-omic analyses provide insights into biomineralization, GigaScience 6 (2017) 1–12, https://doi.org/10.1093/gigascience/gix059.
[19] X. Zhao, Q. Wang, Y. Jiao, R. Huang, Y. Deng, H. Wang, et al., Identification of genes potentially related to biomineralization and immunity by transcriptome analysis of pearl sac in pearl oyster Pinctada martensii, Mar. Biotechnol. 14 (2012) 730–739, https://doi.org/10.1007/s10126-012-9438-3.
[20] Y. Jiao, Q.L. Tian, X.D. Du, Q.H. Wang, R.L. Huang, Y.W. Deng, et al., Molecular characterization of tumor necrosis factor receptor-associated factor 6 (TRAF6) in pearl oyster Pinctada martensii, Genetics & Molecular Research Gmr 13 (2014) 10545–10555, https://doi.org/10.4238/2014.December.12.17.
[21] S. Iwanaga, B.L. Lee, Recent advances in the innate immunity of invertebrate

animals, J. Biochem. Mol. Biol. 38 (2005) 128–150, https://doi.org/10.5483/BMBRep.2005.38.2.128.
[22] Q. Yang, Y. Sun, X. Su, T. Li, T. Xu, Characterization of six IL-17 family genes in miiuy croaker and evolution analysis of vertebrate IL-17 family, Fish Shellfish Immunol. 49 (2015) 243–251, https://doi.org/10.1016/j.fsi.2015.12.031.
[23] J.M. Reynolds, P. Angkasekwinai, C. Dong, IL-17 family member cytokines: regulation and function in innate immunity, Cytokine Growth Factor Rev. 21 (2010) 413–423, https://doi.org/10.1016/j.cytogfr.2010.10.002.
[24] D.C. Jeffares, C.J. Penkett, J. Bähler, Rapidly regulated genes are intron poor, Trends Genet. 24 (2008) 375–378, https://doi.org/10.1016/j.tig.2008.05.006.
[25] Y. Li, X. Sun, X. Hu, X. Xun, J. Zhang, X. Guo, et al., Scallop genome reveals molecular adaptations to semi-sessile life and neurotoxins, Nat. Commun. 8 (2017) 1721, https://doi.org/10.1038/s41467-017-01927-0.
[26] J. Witowski, K. Ksiazek, A. Jörres, Interleukin-17: a mediator of inflammatory responses, Cellular & Molecular Life Sciences Cmls 61 (2004) 567–579, https://doi.org/10.1007/s00018-003-3228-z.
[27] S.G. Hymowitz, E.H. Filvaroff, J.P. Yin, J. Lee, L. Cai, P. Risser, et al., IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding, EMBO J. 20 (2001) 5332–5341, https://doi.org/10.1093/emboj/20.19.5332.
[28] T. Starnes, Broxmeyer HE, M.J. Robertson, R. Hromas, Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis, J. Immunol. 169 (2002) 642–646, https://doi.org/10.4049/jimmunol.169.2.642.
[29] Y. Lee, I. Whang, M. Oh, N. Umasuthan, M.D. Zoysa, C. Oh, et al., Immune response-related gene expression profile of a novel molluscan IκB protein member from Manila clam (Ruditapes philippinarum), Mol. Biol. Rep. 40 (2013) 1519–1527, https://doi.org/10.1007/s11033-012-2196-5.
[30] T. Rőszer, The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism, Cell Tissue Res. 358 (2014) 685–695, https://doi.org/10.1007/s00441-014-1985-7.
[31] A. Herpin, C. Lelong, T. Becker, F.M. Rosa, P. Favrel, C. Cunningham, Structural and functional evidences for a type 1 TGF-beta sensu stricto receptor in the lophotrochozoan Crassostrea gigas suggest conserved molecular mechanisms controlling mesodermal patterning across bilateria, Mech. Dev. 122 (2005) 695–705, https://doi.org/10.1016/j.mod.2004.12.004.
[32] Y.G. Kim, J.W. Park, J.M. Lee, J.Y. Suh, J.K. Lee, B.S. Chang, et al., IL-17 inhibits osteoblast differentiation and bone regeneration in rat, Arch. Oral Biol. 59 (2014) 897–905, https://doi.org/10.1016/j.archoralbio.2014.05.009.
[33] X. Wang, C. Li, W. Thongda, Y. Luo, B. Beck, E. Peatman, Characterization and mucosal responses of interleukin 17 family ligand and receptor genes in channel catfish Ictalurus punctatus, Fish Shellfish Immunol. 38 (2014) 47–55, https://doi.org/10.1016/j.fsi.2014.02.020.
[34] X. Song, H. Wang, L. Xin, J. Xu, Z. Jia, L. Wang, et al., The immunological capacity in the larvae of Pacific oyster Crassostrea gigas, Fish Shellfish Immunol. 49 (2016) 461–469, https://doi.org/10.1016/j.fsi.2016.01.009.
[35] H. Zhang, Z. Ou, M. Xu, X. Huang, W. Liu, Y. Shi, et al., Molecular cloning and characterization of a putative mitogen-activated protein kinase (Erk1/2) gene: involvement in mantle immunity of Pinctada fucata, Fish Shellfish Immunol. 80 (2018) 63–70, https://doi.org/10.1016/j.fsi.2018.05.047.
[36] Q. Bie, C. Jin, B. Zhang, H. Dong, IL-17B: a new area of study in the IL-17 family, Mol. Immunol. 90 (2017) 50–56, https://doi.org/10.1016/j.molimm.2017.07.004.
[37] Z. You, G. Duraine, J.Y. Tien, C. Lee, T.A. Moseley, A.H. Reddi, Expression of interleukin-17B in mouse embryonic limb buds and regulation by BMP-7 and bFGF, Biochem. Biophys. Res. Commun. 326 (2005) 624–631, https://doi.org/10.1016/j.bbrc.2004.11.087.
[38] E.E. Moore, S. Presnell, U. Garrigues, A. Guilbot, E. Leguern, D. Smith, et al., Expression of IL-17B in neurons and evaluation of its possible role in the chromosome 5q-linked form of Charcot-Marie-Tooth disease, Neuromuscular Disorders Nmd 12 (2002) 141–150, https://doi.org/10.1016/S0960-8966(01)00250-4.
[39] SRP, J.E. Rothman, Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi, Annu. Rev. Biochem. 56 (1987) 829–852, https://doi.org/10.1146/annurev.biochem.56.1.829.
[40] B. Morga, I. Arzul, N. Faury, A. Segarra, B. Chollet, T. Renault, Molecular responses of Ostrea edulis hemocytes to an in vitro infection with Bonamia ostreae, Dev. Comp. Immunol. 35 (2011) 323–333, https://doi.org/10.1016/j.dci.2010.10.005.
[41] P.G. Tiscar, F. Mosca, Defense mechanisms in farmed marine molluscs, Vet. Res. Commun. 28 (2004) 57–62, https://doi.org/10.1023/B:VERC.0000045379.78547.23.
[42] J. Li, Y.H. Zhang, F. Mao, Y. Lin, S. Xiao, Z.M. Xiang, et al., The first morphologic and functional characterization of hemocytes in Hong Kong oyster, Crassostrea hongkongensis. Fish Shellfish Immunol. 81 (2018) 423–429, https://doi.org/10.1016/j.fsi.2018.05.062.
[43] T.H. Wang, S.A.M. Martin, C.J. Secombes, Two interleukin-17C-like genes exist in rainbow trout Oncorhynchus mykiss that are differentially expressed and modulated, Dev. Comp. Immunol. 34 (2010) 491–500, https://doi.org/10.1016/j.dci.2009.11.011.
[44] W.Y. Cui, M.D. Li, Nicotinic modulation of innate immune pathways via α7 nicotinic acetylcholine receptor, J. Neuroimmune Pharmacol. 5 (2010) 479–488, https://doi.org/10.1007/s11481-010-9210-2.
[45] T. Lawrence, The nuclear factor NF-κB pathway in inflammation, Cold Spring Harbor perspectives in biology 1 (2009), https://doi.org/10.1101/cshperspect.a001651 a001651-a001651.