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The crab Relish plays an important role in white spot syndrome virus and *Vibrio alginolyticus* infection

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ABSTRACT

Relish is a transcription factor and forms an important part of the immune deficiency signaling pathway. In the current study, a Relish homolog was cloned from the hemolymph of *Scylla paramamosain* using RT-PCR and RACE. The full length cDNA of *Relish* consists of 4263 base pairs (bp), including a 3552 bp open reading frame encoding a 1184 amino acid protein. The data showed that Relish was highly expressed in the gonad and digestive organs of *S. paramamosain*. Furthermore, the expression of Relish was up-regulated by infection with white spot syndrome virus (WSSV) or *Vibrio alginolyticus*. When Relish was knocked down, immune genes such as Janus Kinase, signal transducer and activator of transcription, crustin antimicrobial peptide, prophenoloxidase, C-type-lectin and myosin-II-essential-light-chain-like-protein were significantly down-regulated ($P < 0.01$), and Toll-like receptor was significantly up-regulated ($P < 0.01$) in hemocytes. The mortality of WSSV-infected or *V. alginolyticus*-infected crabs was enhanced following Relish knockdown. Thus, Relish is very important in the progression of WSSV and *V. alginolyticus* infection. It was found that Relish knockdown caused the highest level of apoptosis in the disease-free group, and higher levels of apoptosis in the WSSV group and *V. alginolyticus* group compared with that in the control group. Knockdown of Relish influenced the activity of phenoloxidase (PO) and superoxide dismutase (SOD), and total hemocyte count (THC) following WSSV or *V. alginolyticus* infection, indicating that Relish plays a regulatory role in the immune response to WSSV or *V. alginolyticus* infection in crabs. Thus, we conclude that Relish may anticipate host defense mechanisms against pathogen infection by affecting apoptosis, THC, PO activity and SOD activity.

1. Introduction

Innate immunity and acquired immunity are two components of the host immune system [1]. Due to the lack of complexity of the adaptive immune system, all crustaceans including *Scylla paramamosain* rely solely on innate immunity to maintain a highly efficient defense system against infections [2,3]. Immediate responses to pathogenic infection in animals involve innate immunity, which consists of humoral and cellular responses, such as the blood coagulation system [4], agglutination, nodule formation, phagocytosis, prophenoloxidase activating system [5] and various immune active proteins. Indeed, antimicrobial proteins are a group of immune active proteins in humoral response capable of inhibiting and eradicating pathogens [6,7].

The synthesis of antimicrobial proteins in innate immunity in response to infection is activated by two signaling pathways, the Toll and immune deficiency (IMD) pathways [8,9]. The κB-like transcription factor (NF-κB)/Rel transcription factors are central regulators of mammalian immunity and are also implicated in the induction of cecropins and other antibacterial peptides in insects [10]. Three Rel/NF-κB transcription factors, Relish, Dorsal [11] and Dif [12], are involved in the two aforementioned signal transduction pathways of innate immune reactions. Dorsal and Dif are activated in the successive signaling cascade of the Toll pathway for antifungal and antibacterial responses [13–15]. Relish is required for the IMD pathway to activate the gene expression of antimicrobial peptides (AMPs) [10]. Relish is a nuclear factor NF-κB whose N-terminal part enhances the synthesis of AMPs in *Drosophila* [16]. A Relish transcript is also detected in early embryos, suggesting that it acts in both immunity and embryogenesis. The presence of a compound Rel protein in *Drosophila* indicates that similar proteins were likely present in primordial immune systems and may serve unique signaling functions [17]. Relish is specifically required for induction of the humoral immune response, including both antibacterial and antifungal peptides [10].

As two of the most serious diseases in crabs, vibriosis and white spot

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syndrome virus (WSSV) infection have caused irreversible damage to the crab culture industry worldwide [18]. *Vibrio alginolyticus* is an important pathogen in oceans. Similar to other pathogens, many types of virulence factors that are based on the virulence gene mainly result in its pathogenicity [19]. The identification of effective treatments or preventive measures is essential for crustacean aquaculture.

In our previous study, we found that the expression level of Relish in *S. paramamosain* was up-regulated following infection with WSSV or *V. alginolyticus* [20]. In the present study, we aimed to investigate the role of Relish in the innate immune system of crabs.

2. Materials and methods

2.1. Crabs and tissue preparation

The normal adult *S. paramamosain* (approximately 100 g) were obtained from a aquatic product market of Hangzhou. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). The muscles, hepatopancreas, gills, digestive organs, gonad and hemolymph were collected from healthy or challenged crabs. The samples were used immediately for RNA extraction, aiming to prevent total RNA degradation. WSSV (AF 332093.3) was purified and used in challenge experiments, as described previously [21]. *V. alginolyticus* was cultured and used to challenge the crabs according to the previous report [22].

2.2. Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from hemocytes of *S. paramamosain* using PureLink™ RNA Mini Kit (Ambion, USA), following the protocol of the manufacturer. The concentration and quality of total RNA were determined by the Nanodrop Trace Spectrophotometer and 1% agarose gel electrophoresis detection, respectively. The RACE technique was utilized to clone the full-length cDNA sequence of the gene, based on the known middle fragment [23] using SMARTer® RACE 5′/3′ Kit, following the protocol of the manufacturer. The synthesized cDNA were kept at −20 °C, used for the 3′/5′ - RACE with 3′ gene-specific primer (3GSP1, 3NGSP1) or 5′ GSP (5GSP1, 5NGSP1), designed on the basis of middle known sequence (the primers sequences are shown in Table 1). The PCR products were purified using MiniBEST DNA Fragment Purification Kit Ver.3.0 (Takara, Japan), following the manufacturer's instruction. Amplified cDNA fragments were transferred into the pMD19-T vector (Takara, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR and sent to sequencing company (Sangon, China). Nucleotide sequences of the cloned cDNA were sequenced by double pass. All primers used in this experiment were designed using Primer Premier 5.0.

2.3. Nucleotide sequence and bioinformatics analyses

The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The 5′ and 3′ sequences from RACEs were assembled with the partial cDNA sequences corresponding to each fragmental sequence by DNAMAN 5.0. The protein prediction was performed using the open reading frame (ORF) finder tool. Multiple sequence alignment was created by using the DNAMAN 5.0. And the phylogenetic trees based on the amino acid sequences were performed using clustalx and Molecular Evolutionary Genetics Analysis, MEGA 7.1, with neighbor-joining method.

2.4. The quantitative real-time PCR

Relative MCM7 mRNA expression levels in various tissues were measured by qRT-PCR using a SYBR II® Premix Ex Taq (Tli Rnase Plus) (TaKaRa, Japan). Total RNA was isolated from various tissues of normal adult crabs and hemocytes of crabs challenged by intramuscular injection of 0.2 mL of viral or bacterial suspension including WSSV (10^5 copies/mL) or *V. alginolyticus* (10^5 colony-forming units [CFU]/mL) [24,25], respectively, for different times, using the EASY spin tissue/cell RNA extraction kit (Aidlab, China) according to the manufacturer's instructions. Experiments were performed in triplicate and at least three crabs were analyzed for each tissue type. cDNA synthesis was carried out using 200 μg of total RNA with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Code: FSQ-301; Toyobo, Japan). The synthetic cDNA was subpackaged and kept at −20 °C. qRT-PCR was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data were calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method by Office Excel [24], with GAPDH amplification as the internal control. The design and synthesis of the RT-qPCR primers were

Table 1
Primer sequences and purpose.

Name	Nucleotide Sequence (5′ to 3′)	purpose
3′ race GSP1	GTTGGTGGGGGAAAGCAAGAAAG	first primer for 3′ RACE
3′ race NGSP1	TGAAAGCCAAGAAAGTGCTGGGGAG	second primer for 3′ RACE
5′ race GSP2	CCACACCTCTCTGTCATTCTCATCC	first primer for 5′ RACE
5′ race NGSP2	GTCATACTGGAAAGCCTGGAACCGC	third primer for 5′ RACE
Relish-realtime-F	CAGGTACACCTTTGTGACCGT	for Relish expression
Relish-realtime-R	CCTTCTACTTAGGGCATTTCG	for Relish expression
GAPDH-realtime-F	ACCTCACCAACTCCAACAC	for GAPDH expression
GAPDH-realtime-R	CATTCACAGCCACAACCT	for GAPDH expression
Relish dsRNA F	AGCTTGCGTGACACAAGGGAGATAG	for Relish RNAi
Relish dsRNA R	TCGACTATCTCCCTTGTGTCACGCA	for Relish RNAi
JAK -F	ATTGCTGAGGGGATGGATT	for JAK expression
JAK -R	GCCCATCACATTCCCAAA	for JAK expression
STAT -F	GACTTCACTAACTTCAGCCTCG	for STAT expression
STAT -R	GAGCTGAGTCTGTCTTAATGTTATCC	for STAT expression
C-type-lectin-F	ACTGAGGGGAAAGTAGCC	for C-type-lectin expression
C-type-lectin-R	TGCCCGTGTTTATTCATC	for C-type-lectin expression
crustin-F	TCAGAGCACCCTGGTAAATGT	for crustin antimicrobial peptide expression
crustin-R	GGCAGAACTGCGAAAGAAAG	for crustin antimicrobial peptide expression
TLR-F	TGTTGCCAGAGCAGAAGGT	for toll-like receptor expression
TLR-R	TTCCGTGAATGAACGAAGG	for toll-like receptor expression
proPO-F	ATGAAAGAGGAGTGGAGATG	for prophenoloxidase expression
proPO-R	GTGATGGATGAGGAGGTG	for prophenoloxidase expression
myosin-F	GCCGAGATAAGTGTAGAGGAA	for myosin-II-essential-light-chain-like-protein expression
myosin-R	AGTGGGGTTCTGTCCAAG	for myosin-II-essential-light-chain-like-protein expression

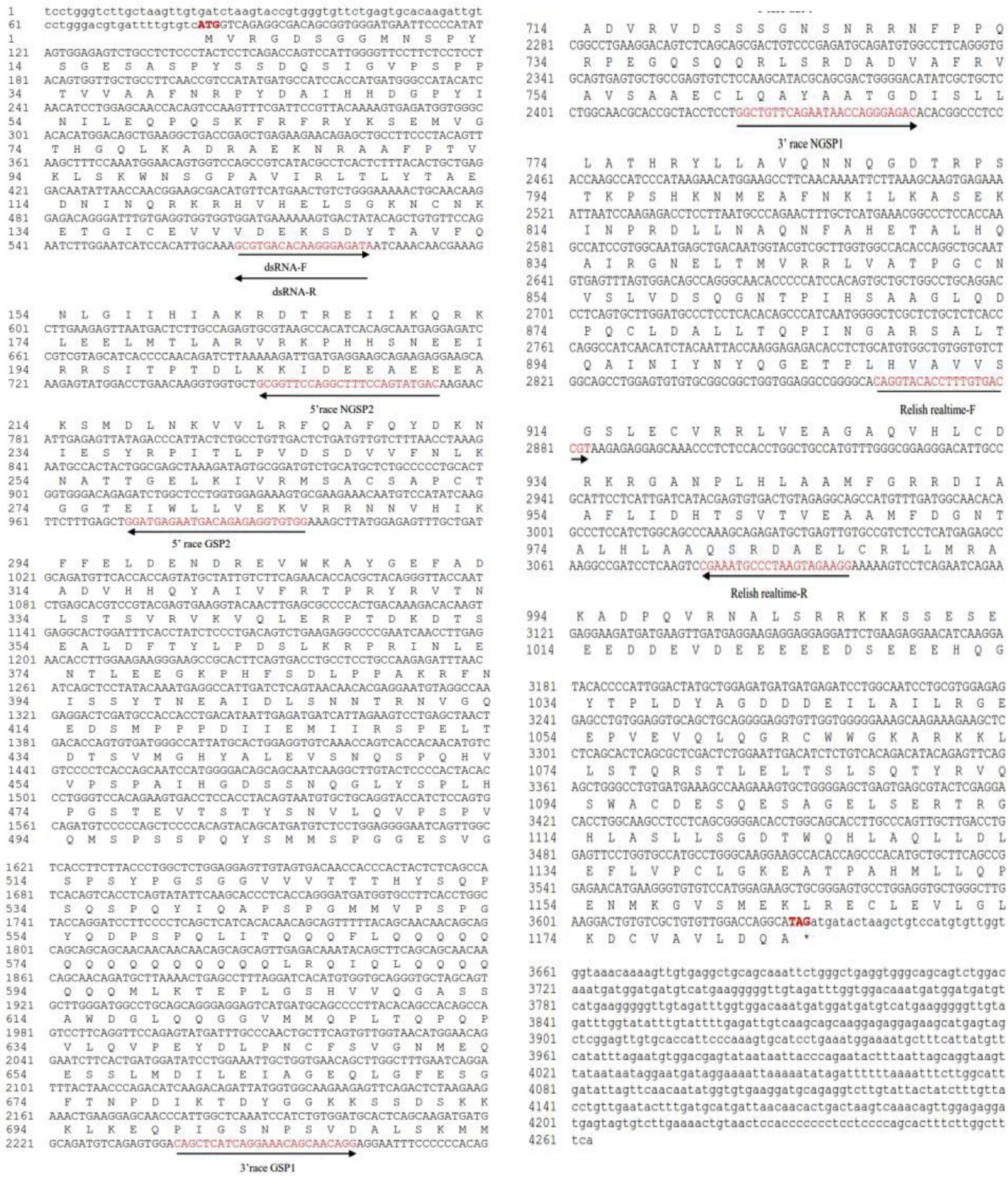


Fig. 1. Nucleotide and deduced amino acid sequences of Relish. The nucleotide sequence is displayed in the 5′–3′ direction and numbered at the left. The deduced amino acid sequence is shown in a single capital letter amino acid code. The 3′UTR and 5′UTR are shown with lowercase letters. Codons are numbered at the left with the methionine (ATG) initiation codon, and an asterisk denotes the termination codon (TAG). RACE and real-time qPCR primers are marked with arrows.

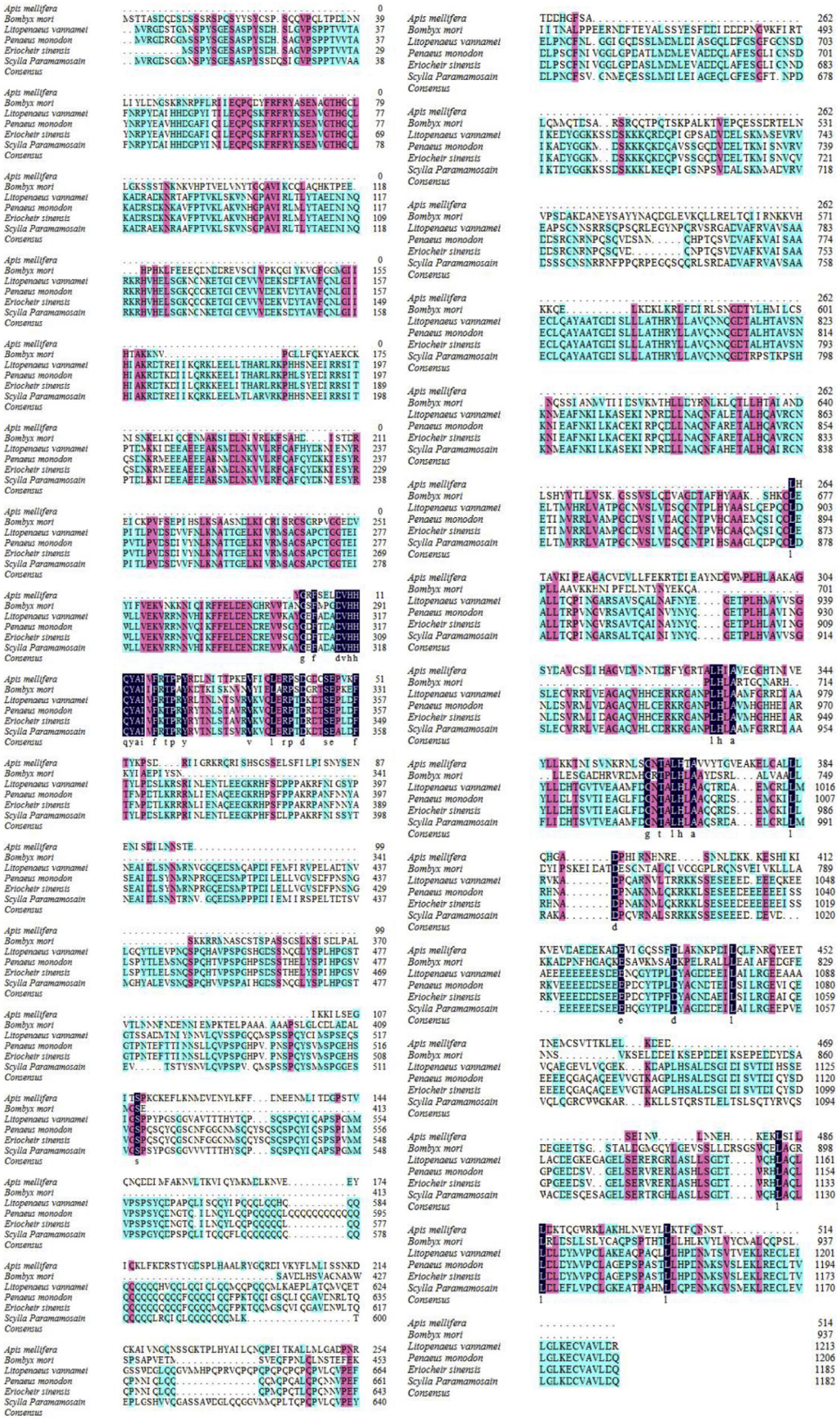


Fig. 2. Multiple alignments of the amino acid sequence of *S. paramamosain* Relish (in this study) with other Relish sequences of common animals including *Apis mellifera* (ACT66913.1), *Bombyx mori* (BAF74125.1), *Litopenaeus vannamei* (ABR14713.1), *Penaeus monodon* (AFH66691.1), and *Eriocheir sinensis* (ADM14334.1). Twelve conserved cysteines (C1 –C12) are shaded and boxed.

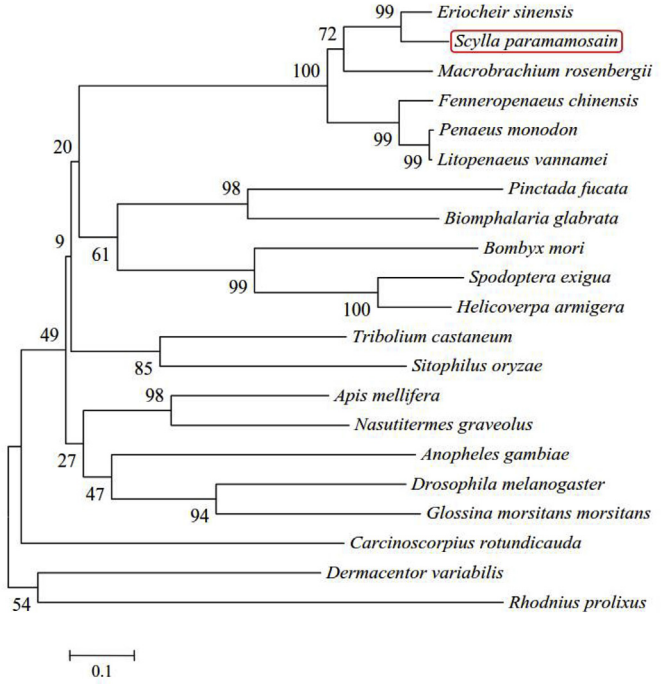


Fig. 3. The phylogenetic tree of Relish in different organisms based on amino acid sequence comparisons.

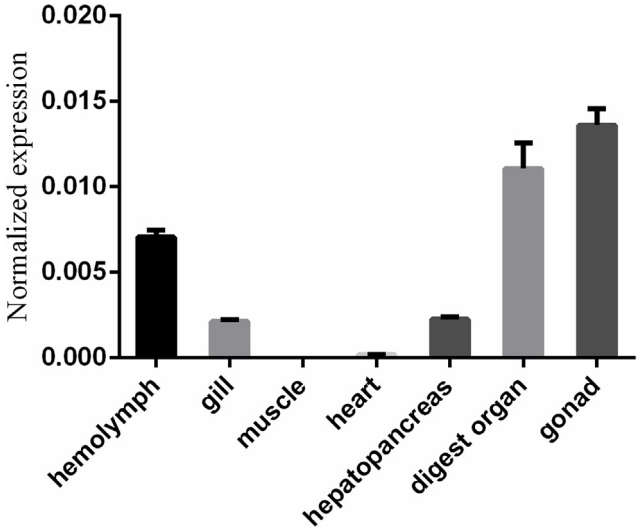


Fig. 4. Characterization of Relish expression in various tissues from normal *S. paramamosain* determined with quantitative real-time PCR. The amount of Relish mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD (standard deviation) of three separate individual tissues. Capital letters indicate expression of Relish in different adult tissues.

entrusted to Generay (Shanghai, China), based on the whole sequence. Table 1 lists all the primers that were designed and synthesized by Generay.

2.5. Prokaryotic expression, purification of Relish-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hin*d III in the forward primer and *Sal* I in the reverse primer) were designed from the cloned nucleotide sequence. PCR product digested with *Hin*d III/*Sal* I was subcloned into LIMTUS 38i Vector (NEB, MA, USA) digested with the same enzymes to gain plasmid L38-Relish. The constructed L38-Relish was verified by restriction enzyme digestion and DNA sequencing. The recombinant plasmid L38-Relish was transformed into HT115 (DE3) cells knocked out of RNase III. The following steps were performed as described previously [24].

2.6. Knock down of Relish by RNAi and challenge experiments

Total RNA was purified using an EASY spin tissue/cell RNA extraction kit (Aidlab, China), following the manufacturer's instructions. Relish-dsRNAs (75 μg/crab) [25] was immediately injected intramuscularly into the fourth pereopodcoxa of each crab, and Relish mRNA expression levels were detected by qRT-PCR following WSSV and *V. alginolyticus* challenge.

2.7. Kaplan–Meier survival analysis

For the pathogen challenge, normal crab were randomly distributed into six groups (n = 9 per group, three repeats). The control group received injections of PBS alone, the Relish-dsRNA group received injections of Relish-dsRNA alone, the WSSV group received injections of WSSV in PBS, and the Relish-dsRNA + WSSV group received injections of Relish-dsRNA and WSSV, the *V. alginolyticus* group received injections of *V. alginolyticus* in PBS, and the Relish-dsRNA + *V. alginolyticus* group received injections of Relish-dsRNA and *V. alginolyticus*. Each group of crabs was cultivated under the same condition. After every 12 h, the number of live and dead crab was counted. The survival data was arranged and analyzed in Microsoft GraphPad 5.0.

2.8. Apoptosis of crab hemocytes

The hemolymph was mixed with 20 mM EDTA at a ratio of 1:1, and the mixture was centrifuged at 2000 rpm at 4 °C for 10 min to collect hemolymph cells. The hemolymph cells were then suspended in highly alkaline PBS (12.585 g/L NaCl, 0.315 g/L KCl, 0.27 g/L KH_2PO_4, 0.948 g/L Na_2HPO_4, add ddH_2O to 1 L), counted and adjusted to a density of 3–5 × 10^6 cells/mL with PBS. The cells were stained using a BD Phrmingen™ FITC Annexin V Apoptosis Kit, and assessed by flow cytometry. And then, the cells were stained and assessed by FACScan at wavelengths of 530 nm and 575 nm. The cell numbers on quadrant 2 and 4, with high annexin V staining, were considered as apoptotic. The data, presented as means ± standard deviation (SD), were derived from at least three independent experiments.

2.9. Determination of immune parameters after RNAi

The immune parameters determined included total hemocytes numbers (THC), PO and SOD activities. THC was determined as described previously [25]. To determine PO and SOD activities, 500 μL of hemolymph was withdrawn into a 1 mL syringe containing 500 μL EDTA (20 mM) solution from each individual crab. PO activity was quantified in the hemolymph mixture based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [26]. SOD activity was quantified in hemocytes isolated from 300 μL of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [27].

2.10. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Data from three independent experiments were analyzed by one-way analysis of variance to calculate the means and standard deviations of the triplicate assays. Statistical differences were estimated using one-way ANOVA followed by least-significant differences (LSD) and Duncan's multiple range test. All statistics were measured using SPSS software version 19 (IBM, USA). A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$).

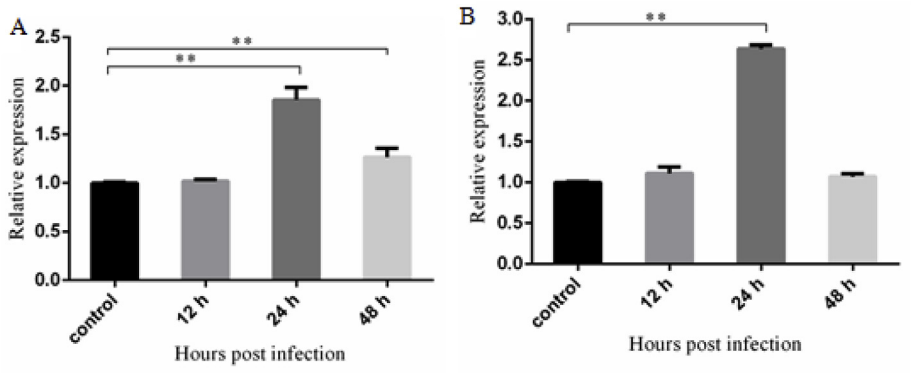


Fig. 5. Real-time RT-PCR analysis of Relish expression challenged with WSSV or *V. alginolyticus*. (A) Real-time RT-PCR analysis of Relish expression in the hemocytes of *S. paramamosain* challenged with WSSV. (B) Real-time RT-PCR analysis of Relish expression in the hemocytes of *S. paramamosain* challenged with *V. alginolyticus*. The amount of Relish mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD (standard deviation) of three separate individual tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

3. Results

3.1. Characterization of Relish cDNA

The full length Relish cDNA sequence was 4263 bp, including a 3552 bp open reading frame (ORF) which encoded a 1184 amino acid protein. The 5′ and 3′ untranslated regions (UTR) of Relish were 81 and 630 bp in length, respectively. The nucleotide and deduced amino acid sequences of the full-length cDNA are shown in Fig. 1.

3.2. Sequence homology and phylogenetic analysis

The putative intact amino acid sequence of Relish was then compared with the sequences of Relish in other species using DNAMAN version 6.0 (Lynnon Biosoft, USA). The results revealed a similarity of approximately 80% identity with that of *Eriocheir sinensis*, 67% with that of *Litopenaeus vannamei*, 66% with that of *Penaeus monodon*, and 22% with that of *Bombyx mori* (Fig. 2).

A condensed phylogenetic tree based on the deduced amino acid sequence was constructed by the neighbor-joining method using MEGA7.1 (Fig. 3). Phylogenetic analysis showed that *S. paramamosain Relish* showed the closest relationship to that of *E. sinensis*. The amino acid sequence showed that some highly conserved amino acid sites exist in it.

3.3. Tissue distribution of Relish mRNA

Expression profiling of the Relish gene in different tissues of *S. paramamosain* was examined with quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 4). Relish was significantly highly expressed in gonad compared with other tissues, and showed lowest expression in muscle tissue. Relish expression levels in gonad were 1.85-, 6.50-, 2600-, 5.90-, 65- and 1.18-fold greater than that in hemolymph, gills, muscle, hepatopancreas, heart and digestive organs, respectively. Thus, Relish expression in gonad was significantly higher ($P < 0.01$) than in any other tissues examined.

3.4. Time course of Relish expression after WSSV or V. alginolyticus challenge

We investigated the variation in Relish expression in crabs after immune challenge with WSSV or *V. alginolyticus*. Relish expression changed after immune challenge in a time-dependent manner. Relish expression was significantly up-regulated ($P < 0.01$) from 12 to 48 h post-challenge in crabs with WSSV infection, with the highest expression levels at 24 h (Fig. 5A). Relish expression was also significantly up-regulated ($P < 0.01$) at 12 and 24 h post-challenge with *V. alginolyticus*, but gradually returned to the control level at 48 h post-challenge (Fig. 5B). These results suggested that Relish may play an important role in crab innate immunity following infection with WSSV or *V. alginolyticus*.

3.5. Effects of Relish double-stranded RNA on the expression of immune genes

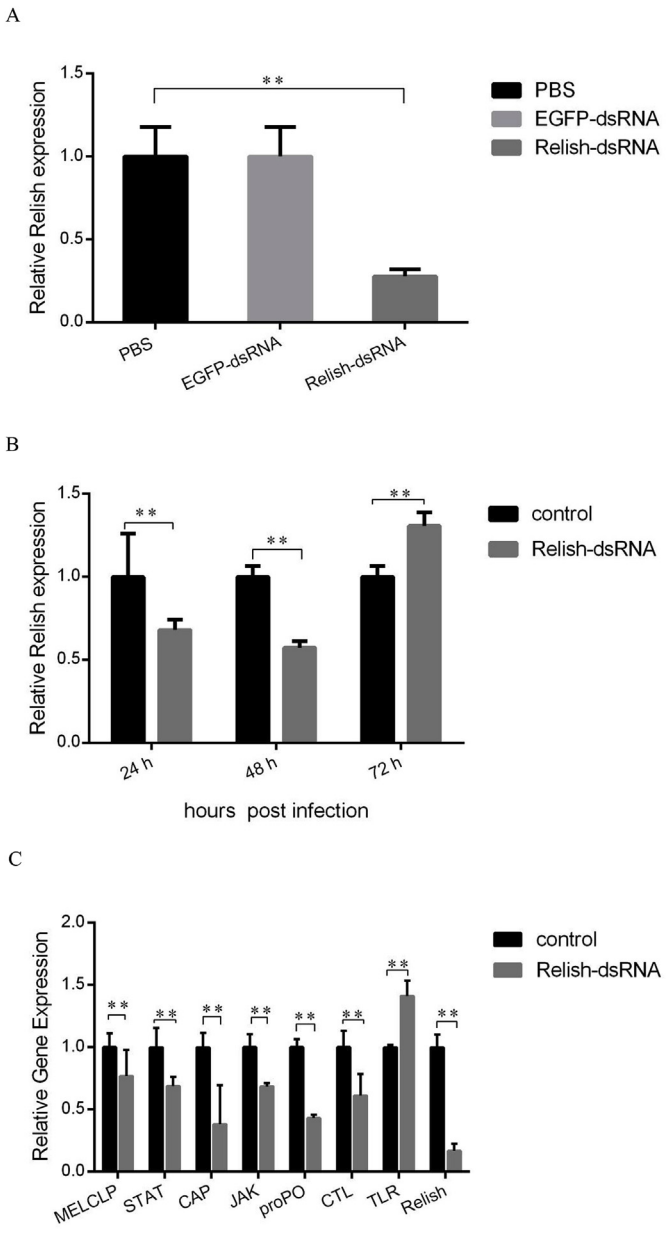
The effect of Relish-dsRNA on its mRNA expression was determined using RT-PCR. Relish expression in crab hemocytes was significantly knocked down by Relish-dsRNA ($P < 0.01$) (Fig. 6A). We also examined the effect of Relish-dsRNA on Relish gene expression in hemocytes of *S. paramamosain* at different times post-treatment. Relish-dsRNA inhibited the expression of Relish mRNA in hemocytes from 24 to 48 h post-treatment (Fig. 6B). We also examined the relationship between Relish and other immune-related genes by analyzing the expression of a variety of immune genes in hemocytes under Relish-dsRNA treatment. Among these genes, JAK, STAT, crustin antimicrobial peptide (CAP), prophenoloxidase (proPO), C-type-lectin (CTL) and myosin-II-essential-light-chain-like-protein (MELCLP) were significantly down-regulated ($P < 0.01$), while Toll-like receptor (TLR) was significantly up-regulated ($P < 0.01$) following Relish-dsRNA treatment (Fig. 6C). The expression levels of these genes in EGFP-dsRNA-treated crabs showed no obvious differences compared with those in the control.

3.6. Determination of immune parameters

The THC in the Relish-dsRNA group was significantly ($P < 0.01$) decreased at 48 h post-treatment compared with the PBS group (Fig. 7A). THC was increased in the WSSV group compared with the PBS group, but was significantly ($P < 0.01$) decreased in the WSSV + Relish-dsRNA group compared with the WSSV group (Fig. 7A). THC was increased in the PBS group at 24 h post-challenge. However, THC was increased in the *V. alginolyticus* + Relish-dsRNA group compared with the *V. alginolyticus* group at 48 h post-challenge (Fig. 7A). These results indicated that THC increased in crab after WSSV infection.

Phenoloxidase (PO) activity in the Relish-dsRNA group was significantly ($P < 0.01$) decreased at 24 h and 48 h post-treatment compared with the PBS group (Fig. 7B). PO activity was significantly ($P < 0.01$) higher in the WSSV + Relish-dsRNA group compared with the WSSV group at 24 h and 48 h post-challenge (Fig. 7B). These data suggested that Relish knockdown enhanced PO activity after WSSV challenge. However, PO activity was reduced at the 24 h and 48 h after *V. alginolyticus* challenge compared with that in the PBS group, but was significantly ($P < 0.01$) enhanced in the *V. alginolyticus* + Relish-dsRNA group compared with the *V. alginolyticus* group (Fig. 7B). These data suggested that Relish knockdown may increase PO activity in crabs infected with *V. alginolyticus*.

SOD activity in the Relish-dsRNA group was significantly ($P < 0.01$) decreased at 24 h and 48 h post-treatment compared with the PBS group (Fig. 7C). SOD activity was decreased in the WSSV group



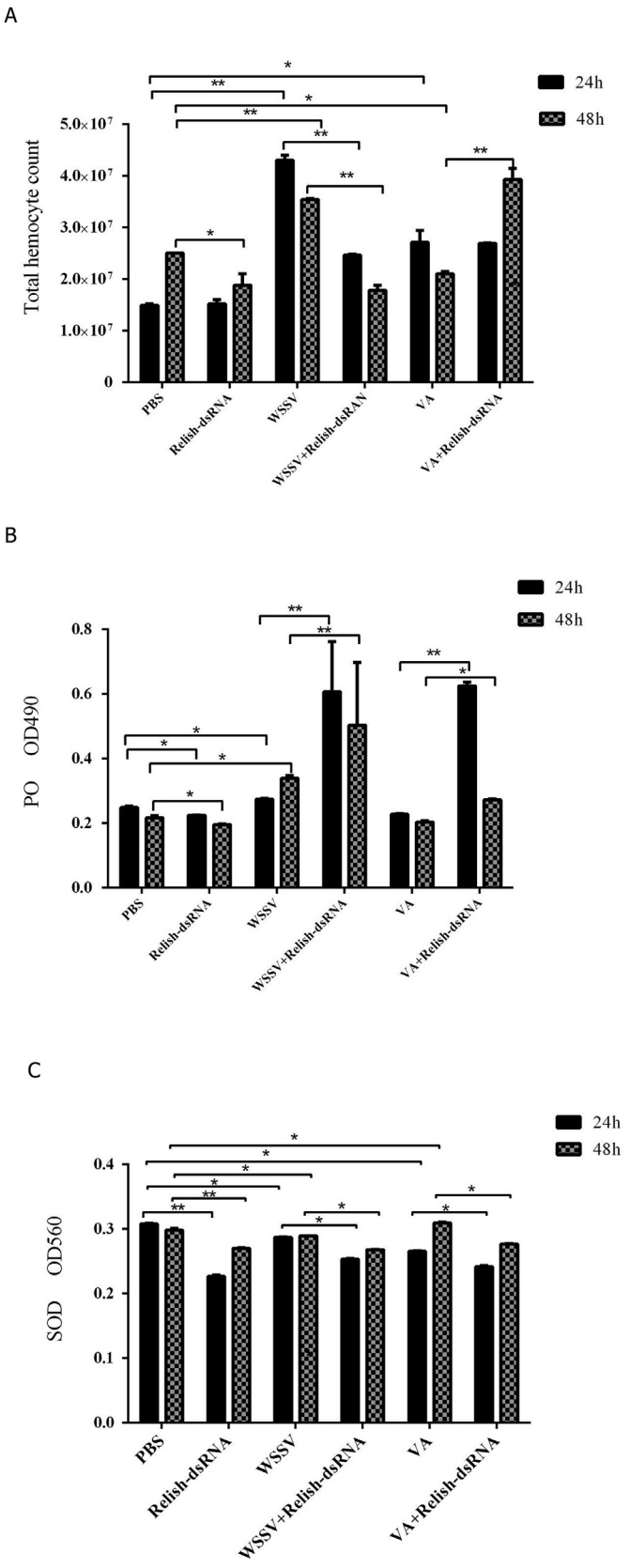


Fig. 6. Real-time RT-PCR analysis of the expression of Relish and immune genes. (A) Real-time PCR analysis of Relish expression in the hemocytes of *S. paramamosain* treated with Relish dsRNA (Relish-dsRNA) at 24 h post-treatment. The amount of Relish mRNA was normalized to the GAPDH transcript level. (B) Real-time RT-PCR analysis of Relish expression in hemocytes from *S. paramamosain* treated with Relish-dsRNA at different times post-treatment. (C) Real-time RT-PCR analysis of seven immune genes (JAK, STAT, crustin antimicrobial peptide [CAP], Toll-like receptor [TLR], prophenoloxidase [proPO], C-type-lectin [CTL] and myosin-II-essential-light-chain-like-protein [MELCLP]) expression in hemocytes from *S. paramamosain* treated with Relish-dsRNA. The amount of Relish mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD (standard deviation) of three separate individual tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

compared with the PBS group, and was significantly ($P < 0.01$) decreased in the WSSV + Relish-dsRNA group compared with the WSSV group (Fig. 7C). SOD activity was reduced at 24 h and induced at 48 h after *V. alginolyticus* challenge compared with that in the PBS group. However, SOD activity was significantly ($P < 0.01$) decreased in the *V. alginolyticus* + Relish-dsRNA group compared with the *V. alginolyticus* group (Fig. 7C). These results indicated that Relish knockdown may

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Fig. 7. Effects of Relish knockdown on crab immune parameters, including THC, PO activity, and SOD activity, as determined in normal, WSSV-treated or VA (*V. alginolyticus*)-treated crab. (A) THC after Relish-dsRNA, WSSV, WSSV + Relish-dsRNA, VA, VA + Relish-dsRNA treatment. (B) Hemocyte PO activity after Relish-dsRNA, WSSV, WSSV + Relish-dsRNA, VA, VA + Relish-dsRNA treatment. (C) Relative SOD activity after Relish-dsRNA, WSSV, WSSV + Relish-dsRNA, VA, VA + Relish-dsRNA treatment. All treatments, at each time point, included at least three crabs, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

block SOD activity in crab.

3.7. Effects of Relish knockdown on the survival of challenged crab

The WSSV + Relish-dsRNA group showed a significantly higher mortality rate ($P < 0.01$) than the WSSV group at 216 h post-challenge (Fig. 8A). The negative control showed a similar mortality rate to the Relish-dsRNA group post-treatment, indicating that Relish-dsRNA itself was non-toxic in crabs. However, Relish-dsRNA showed a similar effect on the cumulative mortality of *V. alginolyticus*-infected crabs. The cumulative mortality in the Relish-dsRNA + *V. alginolyticus* group was significantly higher ($P < 0.01$) than that in the *V. alginolyticus* group at 216 h post-challenge (Fig. 8B). Overall, these results indicated that Relish knockdown enhanced the cumulative mortality of crabs infected with WSSV or *V. alginolyticus*.

3.8. Effect of Relish knockdown on apoptosis

We investigated the role of Relish on apoptosis of crab hemocytes at 24 h post-treatment using the Annexin V-FITC Apoptosis Detection Kit I. The apoptosis rate was significantly enhanced ($P < 0.01$) in the Relish-dsRNA group compared with the PBS group as shown by flow cytometry (Fig. 9). In addition, the WSSV + Relish-dsRNA group showed a significantly higher apoptosis rate ($P < 0.01$) than the PBS group and WSSV group. The apoptosis rate was significantly decreased ($P < 0.01$) in the *V. alginolyticus* group compared with the PBS group, and the *V. alginolyticus* + Relish-dsRNA group showed a higher apoptosis rate than the *V. alginolyticus* group. These results suggested that Relish had an inhibitory effect on hemocyte apoptosis in crabs irrespective of whether they were infected with pathogens.

4. Discussion

Relish has the capacity to activate cecropin gene transcription, as does Dorsal and Dif, and the Relish gene is very strongly induced after infection, much more than either Dorsal or Dif [11,12,15]. Relish is a transcription factor and forms an important part of the IMD signaling pathway [13]. We examined the relationship between Relish and other immune-related genes by analyzing the effects of Relish-dsRNA on the expression levels of immune genes in crab hemocytes. The results strongly suggest that Relish is involved in affection of the immune response in *S. paramamosain*. To date, due to the absence of crab whole genome, the genes crucial for the immune system are being progressively characterized in crabs. In this experiment, *Relish*, possessing a 3552 bp ORF, was characterized from *S. paramamosain*. Relish was found to be highly expressed in the gonad, digestive gland and hepatopancreas of *S. paramamosain*. The digestive system is considered to be a complete and individual system, separate from the immune system, as well as the blood circulation system, and respiratory system. The mud crab intestine contains diverse microorganisms, and these microorganisms produce and secrete a variety of enzymes to maintain microbial metabolism and aid the conversion and absorption of food and medicine. In addition, it has been demonstrated that there are remarkable similarities and shared function in both nutrient acquisition and host defense. BLAST analysis of proteins showed that the amino acid sequence is highly conserved between crustaceans. The neighbor-joining tree method revealed a close evolutionary relationship between *S. paramamosain* Relish and *E. sinensis* Relish.

In crustaceans, it has been shown that Relish is involved in immune responses against bacteria, fungi and WSSV [23,27,28]. Previous research reported that the expression of Relish gene was positively stimulated by *Vibrio harveyi*, as well as WSSV or yellow head virus [29].

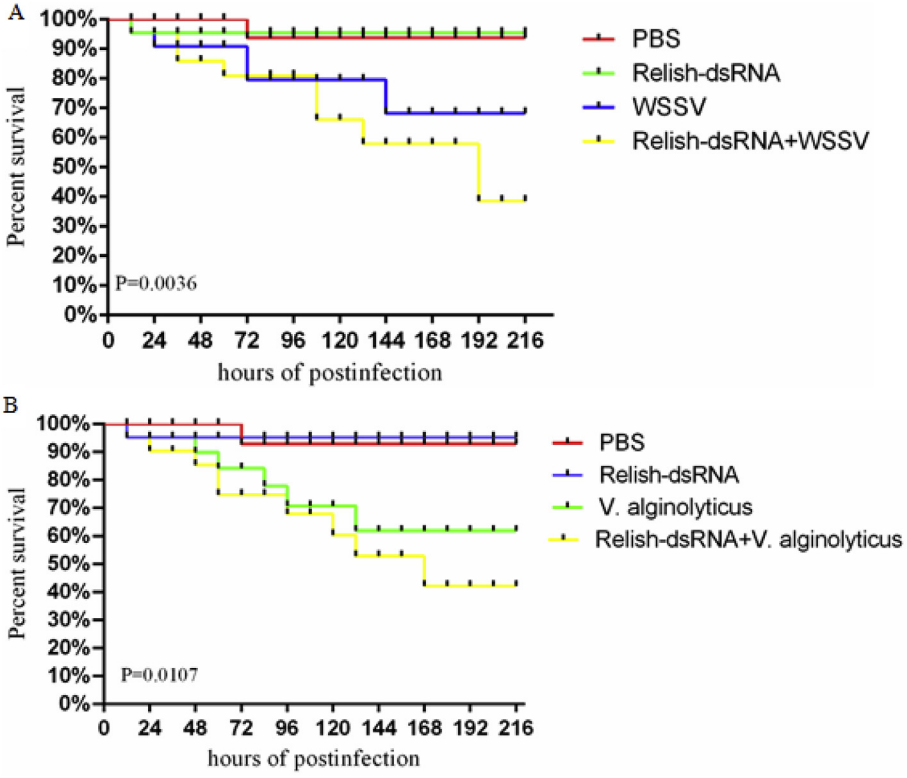

Fig. 8. The survival analysis of challenged crabs treated with Relish-dsRNA. (A) The Kaplan–Meier survival analysis of WSSV-challenged crabs treated with Relish dsRNA (Relish-dsRNA). (B) The Kaplan–Meier survival analysis of *V. alginolyticus*-challenged crabs treated with Relish-dsRNA. The solutions used for injection are shown on the left. Each group consisted of 9 individuals, respectively.

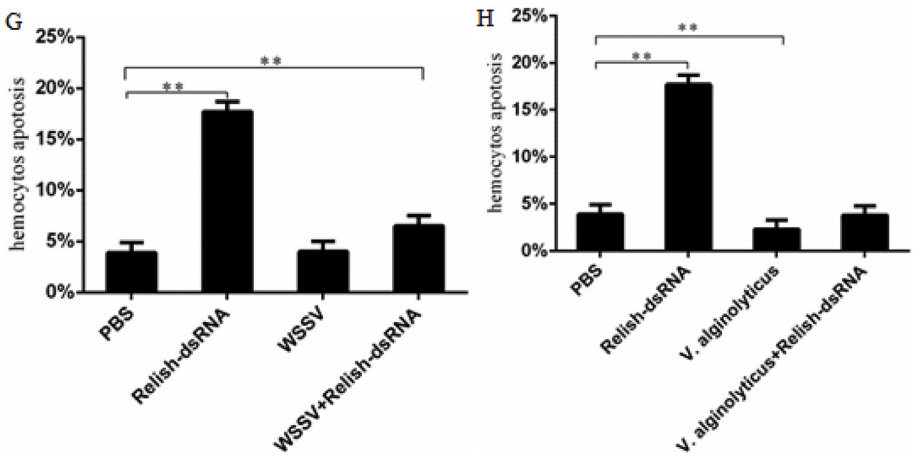

Fig. 9. Flow cytometry assay of apoptosis. (A) PBS; (B) Relish-dsRNA; (C) WSSV; (D) WSSV + Relish-dsRNA; (E) *V. alginolyticus*; (F) *V. alginolyticus* + Relish-dsRNA; (G) bar graph of apoptosis due to WSSV; (H) bar graph of apoptosis due to *V. alginolyticus*. Double asterisks indicate a significant difference ($P < 0.01$) between the sample and the challenge only.

The expression profile of the Dorsal homolog (FcDorsal), a type of Rel/NF-κB transcription factor similar to Relish, in *Fenneropenaeus chinensis* hemocytes was apparently modulated when shrimp were stimulated by bacteria or WSSV [30]. In the present study, similar results were observed and pathogen (WSSV or *V. alginolyticus*) stimulation led to up-regulation of Relish mRNA expression in the hemolymph of *S. paramamosain*. RNA interference has previously been used in the study of immunity in many invertebrate models in order to investigate the function of certain target proteins [23,31]. In the present study, we successfully inhibited the expression of Relish with specific double-strand RNA (Relish-dsRNA), which provided a practical way of examining the role of Relish in the innate immune system, and then normal and Relish-inhibited crabs were subsequently subjected to further experiments.

The Janus family tyrosine kinase and signal transducer and activator of transcription (JAK/STAT) signaling pathway has been proved to have a very important role in the antiviral process of vertebrates [32,33] and invertebrates [34–37]. In insects, such as mosquitoes and *Drosophila*, the JAK/STAT pathway also showed antiviral activity and in the Pacific white shrimp [38,39]. Toll-like receptors (TLRs) are key pattern recognition receptors (PRRs) of the innate immune system and can protect the host against pathogens in both arthropods and mammals [40–42]. The recognition of microbial pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) leads to activation of specific signaling pathways and a variety of cell-dependent responses, including pro-inflammatory cytokine release, phagocytosis and antigen presentation. Reports also show that the expression analysis of FcToll at the transcriptional level in shrimp was modulated after *Vibrio* or WSSV stimulation [43]. RNAi knock-down of shrimp *Litopenaeus vannamei* Toll gene would reduce the expression of Relish [44]. In this study, he significant change in Toll expression in Relish-knockdown crab suggested that Relish may affect the expression of Toll. Knockdown of Relish gene led to significant down-regulation of the innate immune factors JAK, STAT, CAP, proPO, CTL and MELCLP ($P < 0.01$), while TLRs were significantly up-regulated ($P < 0.01$) in the hemocytes of *S. paramamosain*. PO is a member of the tyrosinase group, and this enzyme is responsible for the activation of melanogenesis in invertebrates [45]. It has also been documented that PO is an important tool used against several pathogens [46]. Myosin is a key factor in the energy signal transduction pathway, participating in cell skeleton construction and motility processes [47]. In Kuruma shrimp (*Penaeus (Marsupenaeus) japonicus*), myosin and its light chain has been reported as a WSSV interacting protein involved in the antiviral defense mechanism of shrimp immune cells [48]. The role of myosin light chain and its partner (myosin) in the hemocyte defense mechanism has been demonstrated by its involvement in the regulation of phagocytosis in Kuruma shrimp [49].

Our study provides more evidence and a novel understanding of the function of Relish. Changes in the expression levels of the immune genes listed above indicated that Relish is likely to be associated with the innate immunity of crab. To determine whether Relish exerted any other effects on crab innate immunity, we investigated a variety of functional parameters to evaluate immune potential. We found that WSSV or *V. alginolyticus*-challenged crabs pretreated with Relish-dsRNA showed a higher mortality rate than that of the WSSV or *V. alginolyticus* group at 216 h post-challenge. In WSSV or *V. alginolyticus*-infected crabs, as Relish expression was inhibited, PO activity was enhanced, while SOD activity was reduced compared with the WSSV or *V. alginolyticus* group, THC activity was reduced compared with the WSSV-alone group in WSSV-infected crabs, and increased in *V. alginolyticus*-infected crabs. In this study, apoptosis was increased in Relish-dsRNA-treated crabs after WSSV or *V. alginolyticus* challenge. Apoptosis of crab hemocyte increases a lot with Relish-dsRNA treatment but not in conjunction with WSSV or *V. alginolyticus* infection. We suspect that WSSV or *V. alginolyticus* may affect the process of apoptosis to a great extent in crabs. This is an interesting phenomenon that we found, and it will be the next direction in our study. The results of the *V. alginolyticus* infection experiment revealed that Relish may play a positive role in the antibacterial process in crab. The findings of the present study suggest that Relish not only plays a positive role in bacterial infection, but also influences the immune response to WSSV in *S. paramamosain*.

In conclusion, our findings indicate that *S. paramamosain* Relish plays an important role in the innate immunity of crabs. This host protein could affect host defense mechanisms against pathogenic infections by regulating apoptosis, THC, PO activity and SOD activity.

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