MOLECULAR ISOLATION AND CHARACTERIZATION OF AN IMMUNE DEFICIENCY (IMD) HOMOLOG GENE FROM *MACROBRACHIUM ROSENBERGII*

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การแยกและการศึกษาคุณสมบัติของยีน Immune deficiency (IMD) ของกุ้งก้ามกราม (*Macrobrachium rosenbergii*)

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BY

UTSANEE PINKAEW

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Author	UTSANEE PINKAEW
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Immune deficiency (IMD) is a death domain-containing protein, which plays a crucial role as a key adaptor protein in the IMD signaling pathway, one of the most important mechanisms in response to the viral and bacterial invasion of invertebrates. In this present study, an IMD gene was identified from *Macrobrachium rosenbergii* (*MrIMD*). The open reading frame (ORF) of *MrIMD* was comprised of 555 nucleotides and encoded a protein consisting of 184 amino acids residues, with a conserved death domain at the C-terminus. The *Mr*IMD protein demonstrated identity of 40-74% with the IMD homolog protein from other crustaceans, with the highest identity to *Mn*IMD from *M. nipponense*. In addition, *Mr*IMD protein also shared 12-20% identity with IMDs protein from insects. The phylogenetic analysis indicated that *Mr*IMD was clustered with the IMDs from other crustacean species. Gene expression analysis revealed that the tissue distribution of *MrIMD* mRNA levels was expressed in all examined tissues, with the high expression observed in the gills and hepatopancreas. In immune challenge studies, different patterns of the *MrIMD* mRNA expression were found between bacterial and viral infections. After *A. hydrophila* stimulation, *MrIMD* was significantly up-regulated in muscle, gills, and intestines whilst no significant difference observed in the hemocyte and hepatopancreas compared to that of control group. Conversely, the *MrIMD* was dramatically up-regulated after the *Macrobrachium rosenbergii* nodavirus challenge in the muscle and hepatopancreas, whereas the expression was down-regulated in the gills. These results suggested that the *Mr*IMD gene might have had a different role in response to Gram-negative bacteria and viral infection and plays a crucial role in innate immunity.

Keyword : Aeromonas hydrophila, Immune deficiency, Macrobrachium rosenbergii, Macrobrachium rosenbergii nodavirus

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CHAPTER 1
INTRODUCTION

Background

Macrobrachium rosenbergii, also known as giant freshwater prawn, is an economically important shrimp species because it is popularily consumed as food, making the giant freshwater prawn farming a high-income industry. The giant freshwater prawns farming has grown phenomenally since 1980 (New & Nair, 2012). The recent status from Food and Agriculture Organization of the United Nations shows that global production of giant freshwater prawns increases by more than 200,000 tonnes in 2018 (FAO, 2020). However, there are still several problems with the farming of the giant freshwater prawns. There are reports from many countries showing that the production rates of the giant freshwater prawns have declined mainly due to environmental degradation and disease problems, resulting in mass mortality and economic losses (New, 2007).

The causes of major diseases in freshwater prawns, are mostly viruses, bacteria and also fungi and protozoa. Infectious viruses such as *Macrobrachium rosenbergii nodavirus* (*Mr*NV) and extra small virus (XSV), which cause white tail disease (WTD) and lead to high mortality in hatcheries, and hepatopancreatic parvovirus (HPV) causing the inclusions in tubule epithelial cells of the hepatopancreas (Bonami & Sri Widada, 2011; Gangnonngiw et al., 2009). Bacteria such as *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp. are the cause of “black-spot” bacterial necrosis and gill obstruction (Sung, Hwang, & Tasi, 2000). Therefore, the immune system of shrimp is important for the elimination of pathogens.

Shrimps and invertebrates rely on the innate immune system. The innate immune system composes of cellular and humoral immune responses. Humoral response includes the prophenoloxidase (proPO) system, the blood clotting system, and the production of antimicrobial peptides (AMPs). The expression of AMPs is regulated by the Toll and the Immune deficiency (IMD) pathways (Li & Xiang, 2013b; Tassanakajon et al., 2018). The IMD pathway is an important signaling pathway in the

regulation of innate immunity to prevent pathogen invasion, such as Gram-negative bacteria and viruses. IMD is a key adaptor protein in the IMD pathway and similar to that of human receptor-interacting protein 1 (RIP1) in the tumor necrosis factor receptor (TNFR) pathway. IMD gene encodes a death domain-containing protein to initiate the signaling cascade to activate the NF-κB factor, Relish, which translocates to the nucleus and promotes the expression of many types of antimicrobial peptides (AMPs) (Georgel et al., 2001; Wang et al., 2009).

In shrimp, the *IMD* gene was firstly studied in *Litopenaeus vannamei* (*LvIMD*). It was found that *LvIMD* mRNA was expressed in hepatopancreas and hemocytes after immune challenge and could activate the expression of *Attacin A* and *PEN4* gene (Wang et al., 2009). Later, two *IMD* genes were identified from *Fenneropenaeus chinensis* (*FcIMD*) and *Procambarus clarkia* (*PcIMD*). Transcriptional expression of *FcIMD* was induced after stimulation by *Vibrio anguillarum*, *Micrococcus lysodeikticus* and white spot syndrome virus (WSSV), while *PcIMD* was upregulated after *V. anguillarum* infection. Furthermore, the results also showed that *FcIMD* and *PcIMD* could regulate the expression of three kinds of AMP genes including *crustins*, *anti-lipopolysaccharide factors* (*ALFs*) and *lysozymes* (Feng, Wang, Wen, & Li, 2014; Lan et al., 2013). In 2018, IMD was identified from *Macrobrachium nipponense*. After *Vibrio parahaemolyticus* challenge, *MnIMD* was continuously upregulated in gills. RNAi analysis showed that the silence of *MnIMD* could reduce the expression of *crustin4* and *crustin6*. Thus, *MnIMD* could regulate the expression of two AMP genes in the IMD pathway (Li et al., 2018). The recent study 2022, The role of IMD in *Cherax quadricarinatus* was investigated. The study's findings suggest that the *CqIMD* gene is involved in the immune response of crayfish to Vibrio parahaemolyticus infection, as the expression levels of this gene were upregulated early after exposure to the bacteria (Chen & Wang, 2022)

Based on the above studies of *IMD* genes from different shrimp species, it was shown that the expression of *IMD* genes as well as the synthesis of antimicrobial peptides could increase after infection by various pathogens leading to more resistant

against bacteria and viruses in shrimp. However, the role of the *IMD* gene in *M. rosenbergii* has not been reported. Therefore, in this study, an *IMD* homolog from *M. rosenbergii* was identified and characterized to understand the regulation mechanism in innate immunity of *M. rosenbergii*.

Objectives

1. To identify and characterize an IMD homolog from *M. rosenbergii* (*MrIMD*)

2. To analyze the expression pattern of *MrIMD* from various tissues in normal prawns by real-time RT-PCR

3. To analyze the expression of the *MrIMD* gene in *Aeromonas hydrophila* and *Macrobrachium rosenbergii* nodavirus challenged prawns using real-time RT-PCR

Research hypotheses

1. *Mr*IMD is highly conserved compared to various IMD proteins

2. The expression of *MrIMD* gene was increased after infection with *A. hydrophila*.

3. The expression of *MrIMD* gene was expected to be increased in response to infection of *Mr*NV

Scopes

1. Isolate *MrIMD* gene using rapid amplification of cDNA ends (RACE) and analyze the conserved domain of the *MrIMD*

2. Study phylogenetic relationships of *Mr*IMD and other crustaceans using data from amino acid sequences to construct a phylogenetic tree

3. Analyze tissue distribution and expression of *MrIMD* mRNA levels in healthy prawns using real-time RT-PCR

4. Investigate the transcription of *MrIMD* mRNA level in response to *A. hydrophila* and *Mr*NV infection using real-time RT-PCR

CHAPTER 2
LITERATURE REVIEW

1. *Macrobrachium rosenbergii*

Macrobrachium rosenbergii, also known as the giant freshwater prawn, is the one of the major important commercial aquaculture species. It is native throughout the Southern and Southeast Asian regions, as well as Northern Oceania and the Western Pacific Islands. The culture of *M. rosenbergii* is widespread in different regions of the world, and the major producing countries include Bangladesh, Brazil, China, Ecuador, India, Malaysia, Taiwan Province of China and Thailand (New, 2002). The global production between 2010 to 2018 was more than 200,000 tonnes/year and has gradually increased with the highest production in 2016 (238,400 tonnes) accounting for 2.5 percent of the total crustaceans (FAO, 2020).

1.1 Scientific classification of *M. rosenbergii*

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Suborder: Natantia

Superfamily: Palaeomonoidea

Family: Palaemonidae

Genus: *Macrobrachium*

Species: *Macrobrachium rosenbergii*

1.2 Morphology

M. rosenbergii is the largest species of the genus. The total body length of adult males can grow up to 33 centimeters, while for adult females can be up to 29 centimeters. Base on habitat, body is varied, usually greenish to brownish grey. The body of *M. rosenbergii* consists of the cephalothorax and abdomen (Figure 1) (New, 2002). The cephalothorax consists of the head and thorax which are fused together and covered by a dorsal and lateral shield, called the carapace. The rostrum has a very long and upwardly curved distal portion with 11-14 dorsal and 8-10 ventral teeth (Brown, New, & Ismael, 2009).

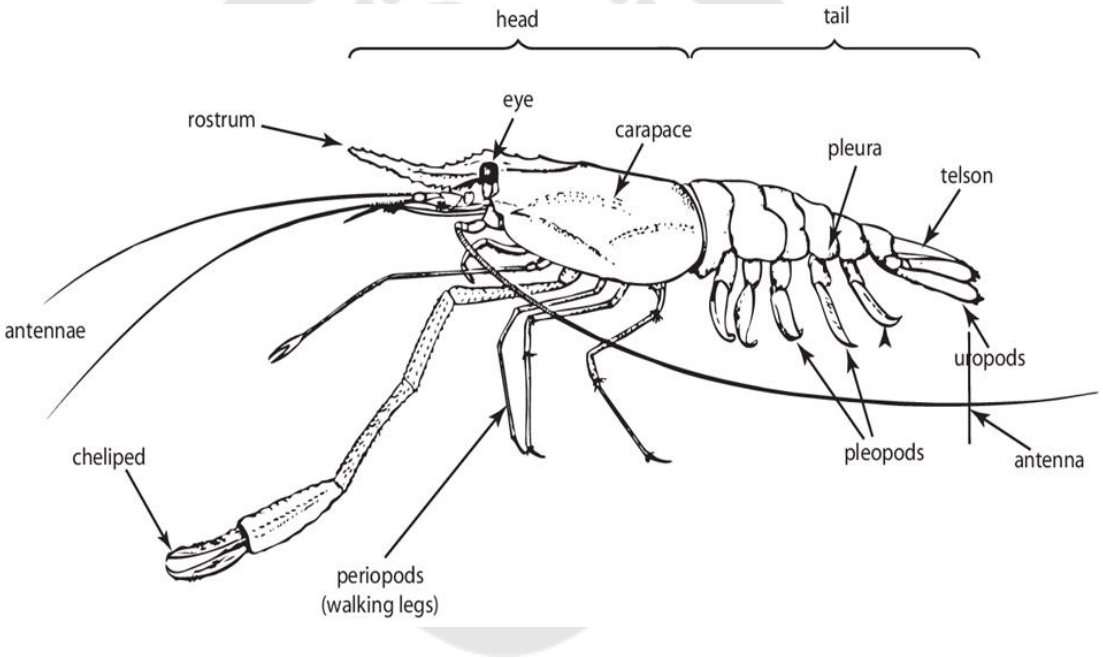


Figure 1 Anatomy of *Macrobrachium rosenbergii*

Source: Nandlal, S., & Pickering, T. (2004). Freshwater prawn *Macrobrachium rosenbergii* farming in Pacific Island countries - Volume One: Hatchery operation.

The front portion of the cephalothorax called the cephalon. The cephalon contains the eyes and five pairs of appendages (antennules, antennae, mandibles, maxillules, and maxillae). The rear portion of the cephalothorax celles the thorax. The thorax contains three pairs of maxillipeds (function as mouthparts) and five pairs of

pereiopods. The abdomen (tail) is divided into 6 segments, of which the first five pairs of pleopods or swimmerets use for swimming and a pair of uropods with the telson form the tail fan. The tail fan serves to move the prawn backwards to allow a rapid response to escape (New, 2002).

Adult male prawns are larger than female and exhibit larger cephalothorax and larger and thicker second chelipeds. Female prawns have a wider abdomen and longer pleura (the overlapping plates of the cuticle extending from the exoskeleton). The first, second and third tail segments of pleura form a chamber for incubating the eggs between laying and hatching. The genital pores are located between at the base of the fifth pair of walking legs in males and at the base of the third walking legs in females. (Ismael & New, 2000; New, 2002).

1.3 Habitat and life cycle

M. rosenbergii lives in a tropical freshwater environments adjacent to brackish water areas and in extremely turbid conditions. Generally, mating occurs throughout the year. Sexually mature females are not ready to mate until the completion of the pre-mating moult, while sexually mature males are capable to mate at any time (Ling, 1967). During copulation, the female receives a gelatinous mass or spermatophore from the male on the underside of the thoracic region between the female's walking legs (Chowdhury, Bhattacharjee, & Angell, 1993).

The incubation period of the eggs is averaged of 20 days (ranged from 18-23 days) (New, 2002). Gravid females migrate to estuaries areas for egg hatching as free-swimming larvae (Figure 2). The larval stages have developed through I-XI distinct stages before metamorphosis. From stage I to stage V, each molt leads to a new stage. From stage VI their size is variable. The larvae swim upside down by their thoracic appendages (Ling, 1967; New, 2002).

The larva stage requires brackish water until they metamorphose into postlarvae (PL), which resemble miniature adult prawns and are primarily crawling as adult prawns. Postlarvae begin to migrate upstream into freshwater conditions within one to two weeks after metamorphosis (Ismael & New, 2000; Michael B. New, 2002).

They move mainly crawling and generally swimming and can tolerate a wide range of salinities, which is a characteristic of freshwater prawns (Chowdhury et al., 1993; New, 2002).

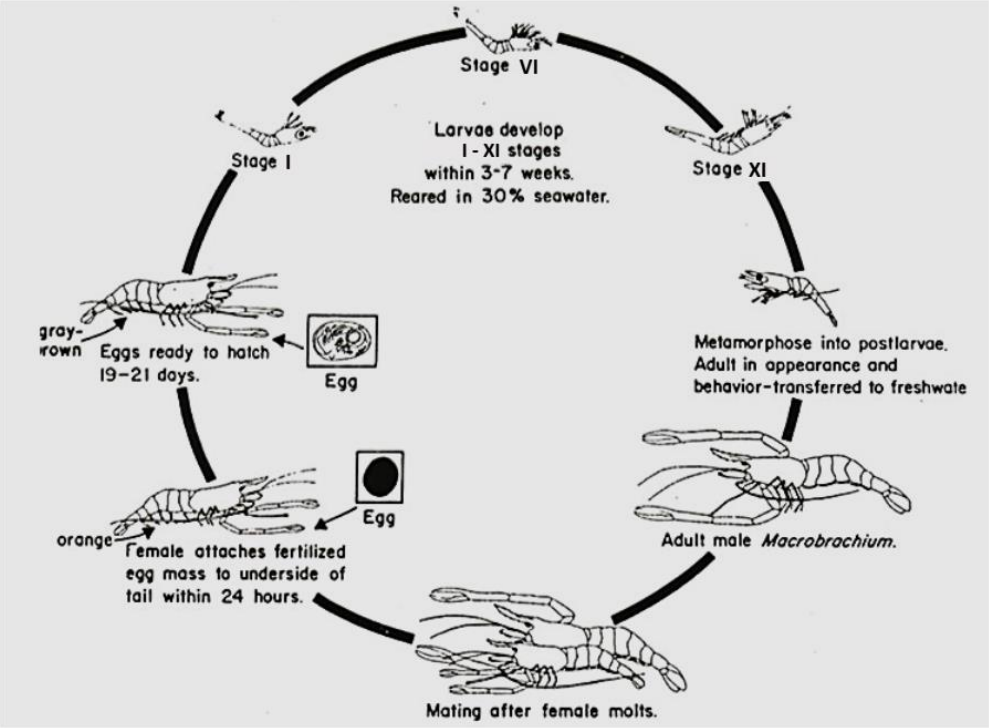


Figure 2 Life cycle of *M. rosenbergii* (After Sandifer and Smith, 1978)

Source: Lober, M. (2015). Investigation of the aquaculture potential of an Australian freshwater prawn, *Macrobrachium spinipes* (Schenkel, 1902), with emphasis on spawning induction, larval and nursery culture. Masters (Research) thesis, James Cook University.

Three main morphotypes are found in mature male *M. rosenbergii*, differing in morphology, physiology, and behavior (Karplus, Barki, & Goren, 1991). The most variable distinguishing feature is size, claw color and spines; blue-clawed males (BC), have extremely long deep blue color claws with longer and stronger spines. Orange-clawed males (OC), medium-sized, with orange claws that are generally shorter and have shorter, stronger spines than BC males. Small males (SM), smaller than BC and

OC males, delicate and translucent claws (Karplus et al., 1991; Kuris, Ra'anan, Sagi, & Cohen, 1987; New, 2002).

2. Immune system in shrimp

Shrimps lack an adaptive immune system and rely on the innate immune system to defend against pathogen invasion. The innate immune system recognizes invading pathogens through host pattern recognition proteins (PRPs) that bind to pathogen-associated molecular patterns (PAMPs) and transduce signals leading to the activation of cellular and humoral immune responses to eliminate foreign pathogens (Li, Wang, & He, 2019; Tassanakajon, Somboonwiwat, Supungul, & Tang, 2013). Molecular pattern PAMPs are usually the glycoproteins and polysaccharides on the surface of microbes, such as lipopolysaccharide (LPS) of Gram-negative bacteria, peptidoglycan (PGN) and lipoteichoic acid (LTA) of Gram-positive bacteria, and glucans of fungal cells. Furthermore, the patterns can be polynucleotides, such as bacterial and viral unmethylated CpG DNA, single-stranded and double-stranded RNA of viruses (Wang & Wang, 2013). The innate immune system consists of two distinct but coordinated systems, the cellular and humoral immune responses. Major cellular immune responses include phagocytosis, nodulation, and encapsulation leading to melanization (Kulkarni et al., 2020) while humoral immune responses include the prophenoloxidase (proPO) system, the blood clotting system, and the production of antimicrobial peptides (AMPs) (Tassanakajon et al., 2018).

2.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are distinct and diverse classes of molecules and have a wide range of activity against microorganisms, including antibacterial, anticancer, antiviral, antifungal, and immune modulation (Kumar, Kizhakkedathu, & Straus, 2018; Tassanakajon et al., 2013). The production of antimicrobial peptides is one of the key molecules of the innate immune system of crustaceans in humoral responses and act as the first line of defense to combat invading pathogens due to their rapid and effective antimicrobial activity against invading various pathogens (Maningas, Kondo, & Hirono, 2013; Tassanakajon et al., 2013). In general, AMPs are small proteins with less

than 150-200 amino acid residues and have amphipathic structures. Shrimp AMPs are produced as precursor proteins, stored in granule-containing hemocytes and can be released upon signal induction (Tassanakajon et al., 2018). There are many types of AMPs or effectors identified in shrimp, such as penaeidin (PEN), crustin (Cru), anti-LPS-factor (ALF), C-type lectin (CTL), lysozyme (Lyz), thioester-containing protein (TEP) and stylicins (Li et al., 2019; Tassanakajon et al., 2018). The expression of the specific AMPs is regulated by two distinct signaling pathways, the Toll and immune deficiency (IMD) signaling pathways (Li & Xiang, 2013a).

3. Immune signaling pathways

Signal transduction involves the binding between extracellular signaling molecules and ligands to cell surface receptors that trigger intracellular signaling within the cell and generate a response. The major signal transduction pathways in shrimp, including the Toll pathway, IMD pathway and JAK/STAT pathway, which play important roles in regulation of the immune response to various pathogens (Li & Xiang, 2013b).

3.1 Immune deficiency (IMD) signaling pathway

The IMD signaling pathway is one of the most important mechanisms in response to viral and bacterial invasion. The IMD pathway was firstly discovered in *Drosophila* by characterizing a mutation that causes severe immune deficiency, named immune deficiency (IMD). It was found while studying AMPs expression in mutant flies. In IMD mutants, the expression of several AMPs was impaired, in particular diptericin (Dpt), which is active against Gram-negative bacteria after infection with *Escherichia coli* and *Micrococcus luteus*, while the antifungal peptide drosomycin (Drs) remained inducible (Lemaitre et al., 1995; Li et al., 2019). Later in the year, the mutant Toll was shown to reduce the expression of the drosomycin gene following fungal infection. For this reason, the Toll signaling pathway responds to Gram-negative bacteria and fungi, while the IMD pathway responds to Gram-negative bacteria and some Gram-positive bacteria (Lemaitre, Nicolas, Michaut, Reichhart, & Hoffmann, 1996). After the discovery of the IMD gene, several components of the IMD pathway were identified and their functions have been well studied in *Drosophila* (Kleino & Silverman, 2014).

In shrimp, several members homologous to the components of the IMD pathway in *Drosophila* have been identified and the regulation of the IMD pathway in the shrimp has been further investigated and explained. However, the knowledge of the IMD pathway in shrimp is still very limited. In the shrimp IMD pathway, transmembrane receptors can recognize and bind to microbial meso-diaminopimelic acid (DAP)-type peptidoglycan (PGN), which induces dimerization or oligomerization of the receptors and leads to the activation of the adaptor protein IMD (Tassanakajon et al., 2018). The activated IMD induces the interaction of TAK1 binding to TAB2, leading to phosphorylation and activation of the IκB kinase (IKK) complex (Wang et al., 2016). The Relish transcription factor is activated by phosphorylation from the IKK complex (Li et al., 2019) and is cleaved away its C-terminal half, ankyrin repeats, and a death domain by a caspase enzyme. The activated Relish translocates to the nucleus and activates the expression of AMPs genes (Tassanakajon et al., 2018) (Figure 3).

3.2 Toll pathway

The Toll pathway plays a key role in response to Gram-positive bacteria, fungi, and some viruses to defend against pathogens in mammals, insects and shrimp (Li & Xiang, 2013b; Tassanakajon et al., 2018). Toll and Toll-like receptors (TLRs), are type I transmembrane proteins, a member of the pattern recognition receptors (PRRs) families, and are central to the innate immune system (Takeuchi & Akira, 2010; Vasselon & Detmers, 2002). Tolls were firstly discovered in *Drosophila melanogaster* as one of the essential genes for the development of dorsal-ventral patterning of the embryo (Belvin & Anderson, 1996) and later it was discovered that Tolls are also involved in innate immune responses. Mutant Tolls were found to have a dramatically reduced ability to stimulate the expression of antifungal peptides in adult *Drosophila* (Lemaitre et al., 1996). In 1997, the first mammalian Toll homolog was discovered and named Toll-like receptors (TLRs) (Medzhitov, Preston-Hurlburt, & Janeway, 1997). Subsequently, many Toll/TLR have been found in a variety of organisms, mammals and some invertebrates, including shrimp (Anthoney, Foldi, & Hidalgo, 2018; Li et al., 2019; Vasselon & Detmers, 2002).

Several genes involved in the shrimp immune system have been identified and the key molecules of Toll pathway are Toll-like receptor (TLR), MyD88, TRAF6, Tube/Pelle, Spätzle, Dorsal and Cactus (Deepika, Sreedharan, Paria, Makesh, & Rajendran, 2014). Activation of Toll leads to the initiation of signal transduction and results in the systemic production of specific antimicrobial peptides (AMPs) (Li et al., 2019).

Stimulation of the Toll pathway, when the pathogen infection occurs, the extracellular recognition factors can recognize and initiate proteolytic cascades that hydrolyze the proSpätzle into the active Spätzle that can bind to the Toll receptor (Ligoxygakis, Pelte, Hoffmann, & Reichhart, 2002; Sun et al., 2017a/2017b). Spätzle binds to the Toll receptor and Toll is activated. After Toll activation, the intracellular signaling occurs. The cytoplasmic TIR domains of the activated Toll receptor recruit a pre-existing heterotrimeric MyD88/Tube complex (MyD88-Tube Pelle complex) and form a signaling complex (MyD88-Tube Pelle complex) via their death domains downstream of the activated Toll receptor (Li et al., 2019; Li & Xiang, 2013b). The MyD88-Tube-Pelle complex interacts with Pellino and tumor necrosis factor receptor (TNFR)-associated factor 6 as a downstream target of Pelle, leading to phosphorylation and degradation of Cactus. Cactus is bound to the critical nuclear factor-κB (NF-κB) in the Toll pathway, namely Dorsal, and inhibits its activity and nuclear localization. Upon degradation of Cactus, Dorsal is released and translocated to the nucleus and activates transcription of immune-related genes (Li et al., 2014; Valanne, Wang, & Rämet, 2011; Wang et al., 2011) (Figure 3).

3.3 JAK/STAT pathway

The Janus kinase/signal transducers and activators of the transcription (JAK/STAT) signaling pathway is ubiquitous in vertebrates but is also found as an intact pathway in some invertebrates, including shrimp (Sun et al., 2017a/2017b) and is involved in both antibacterial and antiviral immunity (Sun, Shao, Zhang, Zhao, & Wang, 2011; Sun et al., 2017). However, the identification of other components and the

functioning of the JAK/STAT pathway in shrimp have been poorly studied. Generally, bacterial pathogens activate the JAK/STAT pathway through pattern recognition receptors (PRRs), e.g. C-type lectins (CTLs), as a cytokine-like ligands that can bind to polysaccharides of bacteria and bind to the Domeless receptor (Sun et al., 2017a/2017b), inducing combinations with associated JAKs and in turn recruiting STATs. STATs are phosphorylated and dimerized and translocated to the nucleus for expression of several AMPs (Yan et al., 2015) (Figure 3).

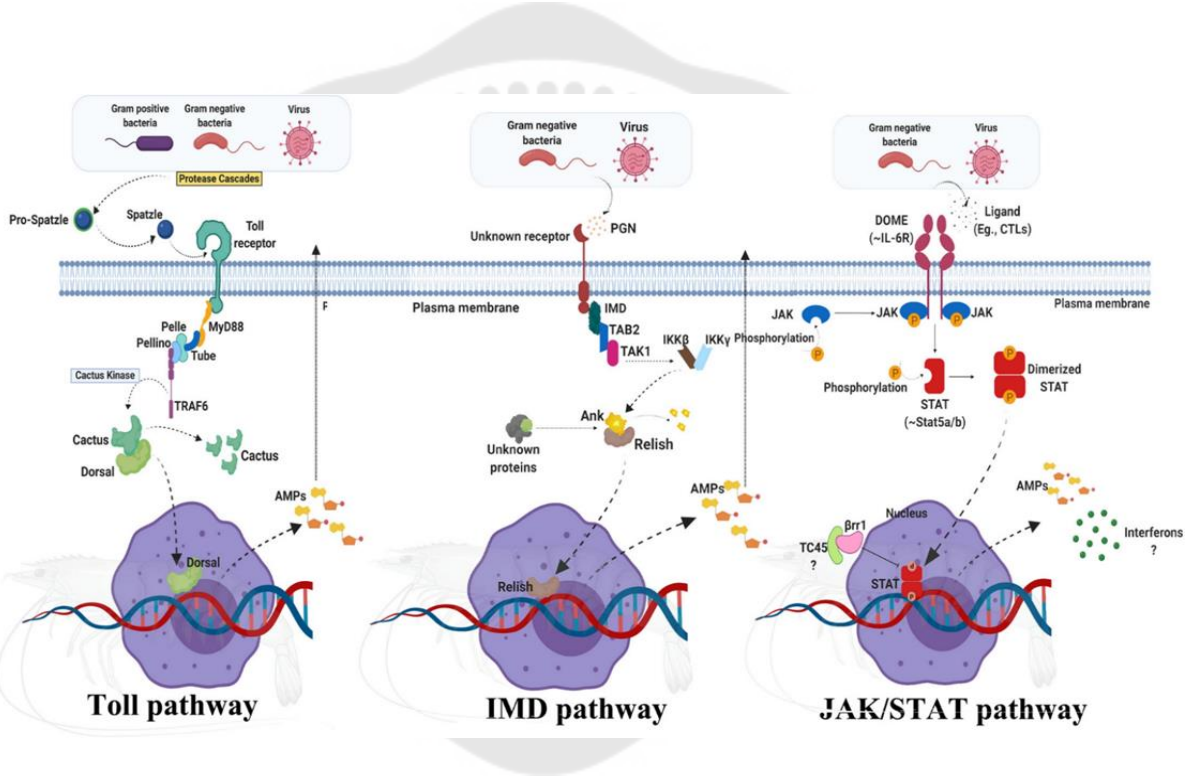


Figure 3 Schematic representation of innate immune pathways in shrimp

Source: Kulkarni, A., Krishnan, S., Anand, D., Kokkattunivarthil Uthaman, S., Otta, S. K., Karunasagar, I., & Kooloth Valappil, R. (2021). Immune responses and immunoprotection in crustaceans with special reference to shrimp. *Reviews in Aquaculture*, *13*(1), 431-459.

4. Immune deficiency (IMD)

The name of IMD refers to a mutation that causes severe immune deficiency. IMD is a death domain-containing protein (Georgel et al., 2001). The death domain of shrimp IMD is similar to that of *Drosophila* IMD and human receptor-interacting protein 1 (RIP1) of the tumor necrosis factor receptor (TNFR) pathway in mammal (Wang et al., 2009). In the TNFR1 signaling pathway, RIP is mandatory for NF-κB activation and apoptosis. RIP contains an N-terminal kinase domain and a C-terminal death domain but the kinase domain is not essential for NF-κB activation (Hsu, Huang, Shu, Baichwal, & Goeddel, 1996; Wang et al., 2009).

The first study of the IMD homolog in shrimp was in 2009, Wang and colleagues identified a *LvIMD* from *Litopenaeus vannamei*. *LvIMD* encoded a putative protein of 160 amino acids including a death domain at the C-terminus. The death domain of *LvIMD* was similar to that of *Drosophila* IMD and RIP1 of the TNFR pathway. *LvIMD* mRNA in hepatopancreas and hemocytes was induced by LPS and *V. alginolyticus* (Gram-negative bacteria), whereas it was not induced by *S. aureus* (Gram-positive bacteria), yeast (*S. cerevisiae*), or WSSV. *LvIMD* responded to Gram-negative bacteria, which is similar to *Drosophila* IMD; therefore, it is reasonable to assume that it has a similar function to *Drosophila* IMD. In addition, *LvIMD* can induce the expression of AMP genes, including *Drosophila Attacin A* and shrimp *penaeidin 4* (Wang et al., 2009).

In 2013, two IMDs gene were identified from the Chinese white shrimp, *Fenneropenaeus chinensis* (*FcIMD*) and the red swamp crayfish, *Procambarus clarkia* (*PcIMD*). Both *FcIMD* and *PcIMD* sequences consisted of 483 bp open reading frame that encoded a 160 amino acids protein and had a death domain at the C-terminus. *PcIMD* was very similar to *FcIMD* (99% similarity), which only two amino acid were substituted. The BLAST analysis showed that it also had similarities to vertebrate RIPs. *FcIMD* was upregulated after a WSSV challenge, but showed no change after challenging with *V. anguillarum* (Gram-negative bacteria). In contrast to *PcIMD*, it was upregulated after *V. anguillarum* challenge but showed no change after challenging with

WSSV. Immunocytochemical analysis showed that *FcIMD* was localized in the cytoplasm of hemocytes. Additionally, *FcIMD* and *PcIMD* also involved in regulating the expression of AMP genes, including *crustins*, *anti-lipopolysaccharide factors*, and *lysozymes* (Lan et al., 2013).

Later in 2014, Feng and colleagues isolated a *FcIMD* from *F. chinensis*. The full-length cDNA of *FcIMD* consisted of 767 bp with an open reading frame of 483 bp encoding 160 amino acids containing a conserved death domain at the C-terminus. Both Gram-positive and Gram-negative bacteria were able to induce the expression of *FcIMD*, differently from *Drosophila* IMD which only responded to Gram-negative bacterial infections, indicating the function of IMD in shrimp immunity to bacterial infection. In addition, *FcIMD* involved in regulation of the expression of Relish and AMP genes including *penaeidin*, *crustin* and *ALFs*, but not in the *Dorsal* or *JNK* regulated signaling pathway (Feng et al., 2014).

In 2018, Zhou and colleagues identified the IMD homolog and two NF-κB family members, Relish and Dorsal, in the mud crab *Scylla paramamosain*. The SpIMD protein contained a conserved death domain. The transcriptional expression levels of *SpIMD*, *SpRelish*, *SpDorsal*, and *SpIKKs* were significantly stimulated after infection with *V. alginolyticus*. When *SpIMD* was silenced, *SpIKKβ*, *SpRelish*, AMP genes including *crustin* and *ALFs* were downregulated except for *SpIKKε* and *SpDorsal* (Zhou et al., 2018).

Later in the same year, a new IMD gene was discovered in the oriental river prawn, *Macrobrachium nipponense* (*MnIMD*). The *MnIMD* encoded 182 amino acids with a conserved death domain at the C-terminus. Phylogenetic analysis showed that *MnIMD* has high similarity to the IMD of other crustaceans. *MnIMD* was induced within 24 hours after *V. parahaemolyticus* infection. After knockdown of *MnIMD*, the expression of *MnIMD* was decreased and the expression of both antimicrobial peptides *Cru4* and *Cru6* was downregulated. These results suggested that the crustin family was regulated by the IMD signal pathway (Li et al., 2018).

Recent study in shrimp studies in 2022, Chen and colleagues investigated the transcription of five genes, including Relish, Toll-like receptor (TLR), *tumor necrosis factor receptor-related factor 6* (*TRAF6*), *Akirin*, and *IMD*, in *Cherax quadricarinatus* after infection of *V. parahaemolyticus*. The results indicated a significant increase in the transcripts of all five genes in the hepatopancreas, while *TRAF6* and *IMD* remained down-regulation in muscle tissue. In gill tissue, *Relish*, *IMD*, and *Akirin* genes were significantly up-regulated during the early stage of infection (Chen & Wang, 2022).

5. Bacteria and virus

5.1 *Aeromonas hydrophila*

Aeromonas hydrophila is a type of Gram-negative bacterium found in freshwater that can cause significant harm to freshwater prawns. When infected with *A. hydrophila*, prawns may experience muscle damage, necrosis in various organs (such as the gills, hepatopancreas, and heart), and melanized lesions on the carapace and other parts of the body, which can lead to an increase in mortality rate. In studies on *M. rosenbergii*, it has been found that *A. hydrophila* can cause histopathological changes in various organs, such as the gills, hepatopancreas, and heart. These changes typically involve melanisation, tissue erosion and necrosis, infiltration and hyperplasia of gill lamellae, and both mild or severe haemocyte reactions in the infected organs (Abdolnabi, Ina-Salwany, Daud, Mariana, & Abdelhadi, 2015).

The genus *Aeromonas* includes several mesophilic aeromonads, including *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, *Aeromonas veronii*, and *Aeromonas schuberti*. These species have gained attention as emerging enteric pathogens that pose a public health concern. Mesophilic aeromonads can cause a variety of diseases in humans, including gastroenteritis, soft tissue and muscle infections, septicemia, and skin diseases (Fadel & Elamie, 2019).

5.2 *Macrobrachium rosenbergii* nodavirus (*Mr*NV)

The emergence of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and an associated satellite virus, extra small virus (XSV), has severely threatened the viability of

M. rosenbergii aquaculture by causing white tail disease (WTD) in infected *M. rosenbergii*.

*Mr*NV is a nonenveloped, icosahedral virus that is 26-27 nm in size and has a genome composed of two fragments of linear single-stranded RNA (ssRNA) of 2.9 and 1.3 kb, respectively. The first report of *Mr*NV was in hatchery-reared *M. rosenbergii* postlarvae. Clinical signs of WTD include whitish coloration of the abdominal and tail muscle, with the discolouration spreading from the tail towards the head as the infection progresses. Histopathological signs of WTD include hyaline necrosis of muscle fibers, moderate oedema, necrosis, haemocyte infiltration, and fibrosis in affected muscles. Pale to dark basophilic intracytoplasmic inclusions can also be observed in muscle cells and hepatopancreatic connective tissue cells (Fadel & Elamie, 2019).

6. Rapid Amplification of cDNA Ends (RACE)

Rapid cDNA amplification (RACE) is a technique for obtaining full-length cDNA sequences of the gene of interest from known partial sequence. RACE-PCR requires gene specific primers (GSPs) and nested primers, designed from the partial sequence and used to amplify both the 5' and 3' ends of the genes. The RACE-PCR method consists of reverse transcription of the mRNA and polymerase chain reaction (PCR) to amplify the 5' and 3' ends. The use of nested PCR is important to reduce non-specific amplification and amplify the cDNA product with high specificity (Oladapo & Michael, 2011). The sequence information obtained from the 5' and 3' cDNA ends was used to overlap with the partial sequence to generate full-length cDNAs (Frohman, 1994).

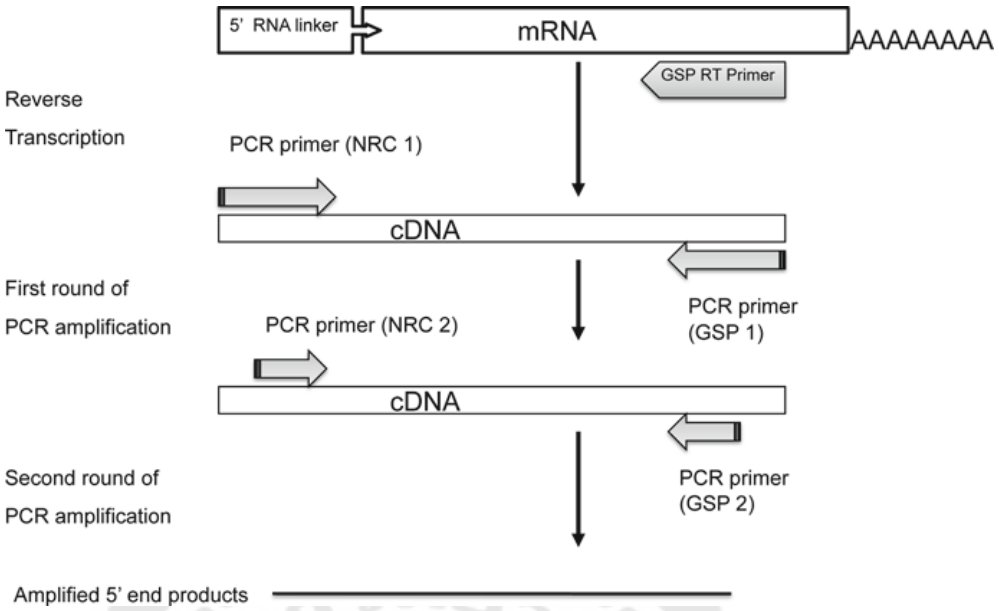


Figure 4 The general schematic of the 5' Rapid Amplification of cDNA Ends

Source: Oladapo, Y., & Michael, A. F. (2011). Rapid amplification of cDNA ends (RACE). *Methods in Molecular Biology*, *703*, 107-122.

To generate 5' ends, in the first step, reverse transcription with a gene-specific primer was used to generate the first-strand cDNA product. Subsequently, a homopolymeric tail is added to the 3' end of the cDNAs using terminal deoxynucleotidyl transferase (Tdt). In the amplification step of the first round PCR primer (GSP1) is used for the 5' end and combined with a universal primer that binds the homopolymeric tail. Finally, the second round of PCR is performed using nested primers (GSP2) in combination with a universal primer to increase the yield of the specific product (Figure 4) (Scotto–Lavino, Du, & Frohman, 2006b).

To generate the 3'-terminal sequences, an oligo-dT-adaptor primer (RT primer) is used. The oligo-dT primer binds to the poly(A)-tail at the 3' end of the mRNA to synthesize the first-strand cDNA by reverse transcriptase. Amplification is then performed using the PCR primer (GSP1) and combined with a universal primer. Nested

primer (GSP2) is used for the second amplification (Figure 5) (Scotto–Lavino, Du, & Frohman, 2006a).

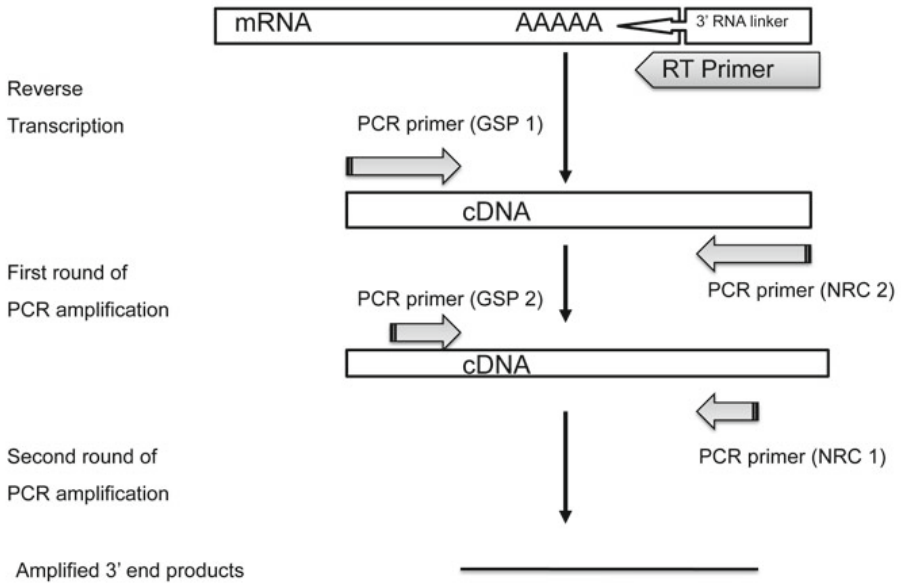


Figure 5 The general schematic of the 3' Rapid Amplification of cDNA Ends

Source: Oladapo, Y., & Michael, A. F. (2011). Rapid amplification of cDNA ends (RACE). *Methods in Molecular Biology, 703*, 107-122.

CHAPTER 3
MATERIALS & METHODS

1. Materials

1.1 Organisms

1.1.1 Juvenile *M. rosenbergii* (2 g)

1.1.2 Adult *M. rosenbergii* (10 g and 40 g)

1.2 Bacterial strains

1.2.1 *Aeromonas hydrophila* (VMARC1234)

1.2.2 *Macrobrachium rosenbergii* nodavirus (*Mr*NV)

1.2.3 *Escherichia coli* TOP10 strain (competent cell)

1.3 Experimental equipment

Table 1 Equipment used in the study

Equipment	Company
1. Analytical Balance TE313S	Sartorius (Germany)
2. Autoclave HA-36	Hirayama
3. Biological safety cabinet class II	NUAIRE
4. CFX Connect™ Real-Time system	Bio-Rad (USA)
5. Electrophoresis apparatus	Bio-Rad (USA)
6. Gel Doc™ XR + Gel Documentation System	Bio-Rad (USA)
7. Heating/Cooling Blocks	Bioer Technology (China)
8. Microcentrifuge D3024	Scilogex (USA)
9. Microfuge® 22R Centrifuge	Beckman Coulter (Germany)
10. NanoDrop™ Lite Spectrophotometer	Thermo Fisher Scientific (USA)
11. PCR thermal cycler	Bio-Rad (USA)
12. Refrigerated centrifuge 5804 R	Eppendorf (Germany)
13. Shaking incubator	Gibthai
14. Spectrophotometer S1200	Unico (china)

1.4 Chemicals and reagents

Table 2 Chemicals and kits used in the study

Chemicals	Company
1. Ampicillin, kanamycin antibiotics	Sigma (USA)
2. di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4)	CARLO ERBA
3. KOD One™ PCR master Mix	Toyobo (Japan)
4. NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel (Germany)
5. NucleoSpin® Plasmid Kit	Macherey-Nagel (Germany)
6. NucleoSpin® RNA XS total RNA isolation kit	Macherey-Nagel (Germany)
7. NucleoSpin® Tissue kit	Macherey-Nagel (Germany)
8. pCR™8/GW/TOPO® TA Cloning® Kit	Invitrogen (USA)
9. Platinum™ *Taq* DNA Polymerase	Invitrogen (USA)
10. Potassium chloride (KCl)	Merck (Germany)
11. Potassium dihydrogen phosphate (KH_2PO_4)	Ajax-Finechem
12. SensiFAST™ cDNA Synthesis Kit	Bioline (UK)
13. SensiFAST™ SYBR® No-ROX Kit	Bioline (UK)
14. Sodium chloride (NaCl)	Promega (USA)
15. SuperScript® III First-Strand Synthesis System for RT-PCR	Invitrogen (USA)
16. Zero Blunt® TOPO® PCR Cloning Kit	Invitrogen (USA)
17. 1 kb Plus DNA Ladder	New England Biolabs (USA)

2. Research methodology

This research consists of (1) isolation of the full-length *MrIMD* gene of *M. rosenbergii* ; (2) construction of the phylogenetic relationship of *Mr*IMD and IMDs from other species; (3) expression analysis of *MrIMD* in healthy *M. rosenbergii* by real-time RT-PCR (4) investigation the role of *MrIMD* after *A. hydrophila* and *Mr*NV challenged prawns using real-time RT-PCR (Figure 6).

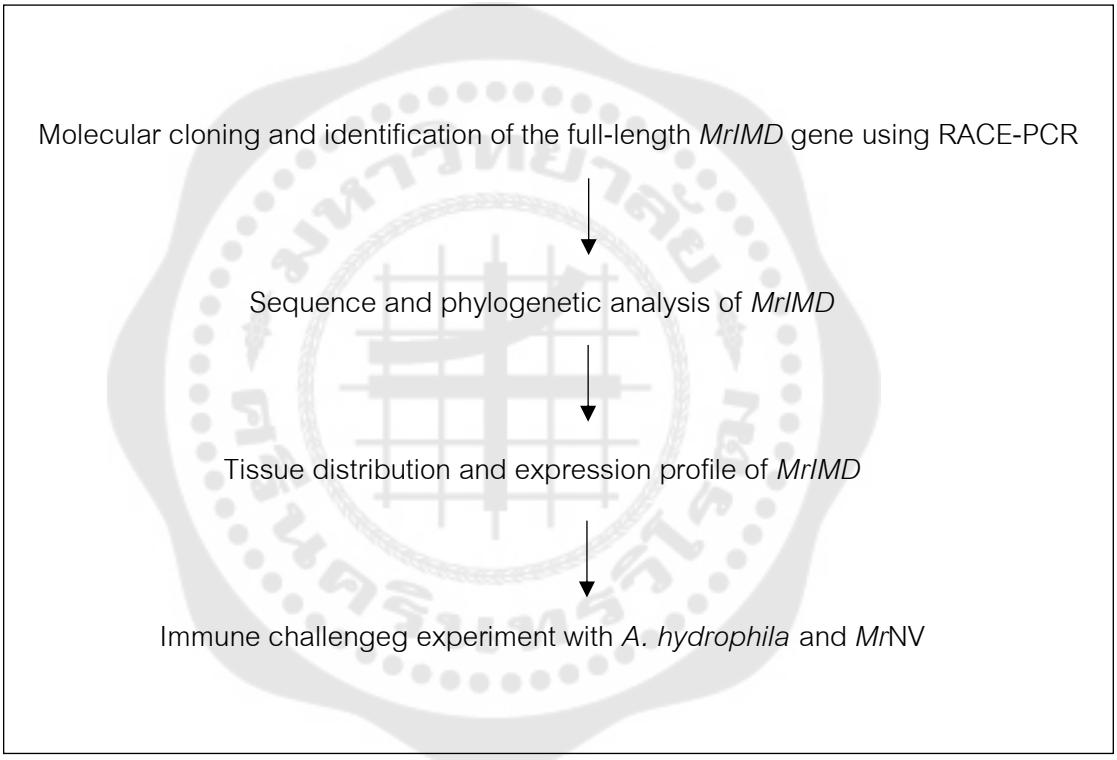


Figure 6 Overview of the research methodology

2.1 Molecular cloning and identification of full-length cDNA of *MrIMD* using Rapid Amplification of cDNA Ends (RACE)

In this study, the freshwater prawn weighing 40-50 g were purchased from local Market in Bangkok, Thailand. Hepatopancreas and stomach was collected for further RNA extraction.

2.1.1 RNA extraction

Total RNA from collected tissues was extracted using NucleoSpin® RNA XS total RNA isolation kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Firstly, hepatopancreas and stomach (5 mg) was homogenized in 200 µL RA1 buffer and 4 µL TCEP (tris-(2-carboxyethyl) phosphine hydrochloride). Then, 5 µL of carrier RNA working solution (Table 3) was added and mixed by vortexing for 5 seconds.

Table 3 Components of carrier RNA working solution

Component	Volume (µL)
RA1 Buffer	99
Carrier RNA stock solution	1
Total volume	100

The lysate was filtrated through NucleoSpin® Filter using centrifugation at 11,000 x *g* for 30 seconds. The filtrate was mixed with 200 µL of 70% ethanol by pipetting. The solution was transferred into NucleoSpin® RNA XS column and centrifuged at 11,000 x *g* for 30 seconds. 100 µL of Membrane Desalting Buffer (MDB) was added and centrifuged at 11,000 x *g* for 30 seconds. Then, 25 µL of rDNase reaction mixture (Table 4) was added into the center of silica membrane column and incubate at room temperature for 15 minutes.

Table 4 Components of rDNase reaction mixture

Component	Volume (µL)
Reaction Buffer for rDNase	27
rDNase	3
Total volume	30

In the silica membrane wash step, 100 µL of RA2 buffer was added, incubated at room temperature for 2 minutes and centrifuged at 11,000 x *g* for 30 seconds. The column was washed twice with 400 µL and 200 µL of RA3 buffer, consecutively, and then centrifuged at 11,000 x *g* for 30 seconds and 2 minutes for the second wash step to dry the silica membrane. The column was transferred to a new microcentrifuge tube. Finally, the total RNA was eluted from the column with 10 µL of RNase-free water and then centrifuged at 11,000 x *g* for 30 seconds. After purification, the total RNA concentration was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA) and stored at -70 °C until use.

2.1.2 Preparation of 3' and 5'-RACE-Ready cDNA

The 3' and 5' RACE-Ready cDNA was synthesized using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. Firstly, the RNA mixture of the 3' and 5'-RACE-Ready cDNA was prepared separately as shown in Tables 5 and 6, respectively, using extracted RNA as described in section 2.1.1.

Table 5 Components of RNA mixture for 3' RACE-Ready cDNA synthesis

Component	Volume (µL)
Total RNA from hepatopancreases (total 1 µg)	1
12 µM 3'-RACE CDS Primer A	1

Table 5 (Continued)

Component	Volume (µL)
Sterile H_2O	10
Total volume	12

3'-RACE CDS Primer A: 5'–AAGCAGTGGTATCAACGCAGAGTAC(T)30 V N–3'

Table 6 Components of RNA mixture for 5' RACE-Ready cDNA synthesis

Component	Volume (µL)
Total RNA from stomach (total 1 µg)	2
12 µM 5'-RACE CDS Primer A	1
Sterile H_2O	8
Total volume	11

5'-RACE CDS Primer A: 5'-(T)25V N-3' (N = A, C, G, or T; V = A, G, or C)

After mixing the contents and briefly centrifugation, both microcentrifuge tubes were incubated at 72°C for 3 minutes followed by 42°C for 2 minutes using the Thermal Cycler (Bio-Rad, USA). After cooling, the buffer mixture for the RACE-Ready cDNA synthesis reaction was prepared as shown in Table 7. In the case of 5' RACE-Ready cDNA synthesis, 1 µL of SMARTer II A oligonucleotide was added to the reaction.

Table 7 Components of buffer mix for the 3' and 5' RACE-Ready cDNA synthesis

Component	Volume (µL)
RNA mixture (Table 5 or 6)	12
5X First-Strand Buffer	4
20 mM DTT	0.5
10 mM dNTP Mix	1

Table 7 (Continued)

Component	Volume (µL)
RNase inhibitor (40 U/µL)	0.5
SMARTScribe Reverse Transcriptase (100 U)	2
Total volume	20

After mixing the reaction by gently pipetting and briefly centrifugation, the reaction mixture was incubated at 42 °C for 90 minutes followed by 70 °C for 10 minutes using the Thermal Cycler (Bio-Rad, USA). Finally, the RACE-Ready cDNA reaction was diluted with 90 µL of Tricine-EDTA buffer (10 mM Tricine KOH (pH 8.5), 1.0 mM EDTA) and stored at -20°C until use.

2.1.3 Rapid amplification of 3' and 5' cDNA ends (RACEs)

To isolate the 3' and 5' ends of the *MrIMD* cDNA sequence, four oligonucleotide primers were designed based on the partial sequence obtained from *M. resenbergii* transcriptome data (Pasookhush et al., 2019) (Table 8).

Table 8 Primer sequences used in the RACE-PCR reaction

Primer name	Sequence (5'-3')
IMD.GSP001	GCC CGA GGC TCC TCC ATG CCT GTC CCA GGT
IMD.NGSP001	CGG CGC CTA TGT GGA CTC CAC TGC TTC CA
IMD.GSP002	AGG CAT GGA GGA GCC TCG GGC GCG CCA TGA
IMD.NGSP002	CGG CGC CTA TGT GGA CTC CAC TGC TTC CA
UPM	
Long (0.4 µM)	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT
Short (2 µM)	CTA ATA CGA CTC ACT ATA GGG C

Table 8 (Continued)

Primer name	Sequence (5'-3')
Nested Universal Primer	AAG CAG TGG TAT CAA CGC AGA GT

The RACE-PCR reaction was prepared as shown in Table 9 and RACE-Ready cDNA from section 2.1.2 was used as DNA template.

Table 9 Components of RACE-PCR reaction

Component	Volume (µL)
2X SeqAmp Buffer	25
10X UPM	5
5'- or 3'-RACE-Ready cDNA from section 2.1.2	2.5
10 µM IMD.GSP001 or IMD.GSP002 primer	1
SeqAmp DNA Polymerase	1
Deionized H_2O	15.5
Total volume	50

The RACE-PCR amplification was performed in the different programs using the Thermal Cycler (Bio-Rad, USA). Touchdown PCR and conventional PCR was performed for the 3' and 5' RACE-PCR, respectively, according to the protocol of SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) as described below.

Touchdown PCR program for 3' RACE-PCR

Step 1	Denaturation	94°C	for 30 seconds
Step 2	Annealing and extension	72°C	for 3 minutes
Repeat steps (1) and (2) for 4 cycles			
Step 3	Denaturation	94°C	for 30 seconds
Step 4	Annealing	70°C	for 30 seconds

Step 5	Extension	72°C	for 3 minutes
Repeat steps (4), (5) and (6) for 4 cycles			
Step 6	Denaturation	94°C	for 30 seconds
Step 7	Annealing	68°C	for 30 seconds
Step 8	Extension	72°C	for 3 minutes
Repeat steps (8), (9) and (10) for 24 cycles			
Step 9	Holding	4°C	

PCR program for 5' RACE-PCR

Step 1	Denaturation	94°C	for 30 seconds
Step 2	Annealing	68°C	for 30 seconds
Step 3	Extension	72°C	for 3 minutes
Repeat steps (1), (2) and (3) for 29 cycles			

PCR products was analyzed on 1% agarose gel electrophoresis followed by SYBR™ safe staining (Invitrogen, USA) and visualized using Gel Doc™ XR + Gel Documentation System (Bio-Rad, USA). Then, the expected PCR product was diluted 50-fold with Tricine-EDTA buffer (10 mM Tricine-KOH (pH 8.5), 1.0 mM EDTA) and used as a template for nested RACE-PCR reaction as shown in Table 10.

Table 10 Components of nested RACE-PCR reaction

Component	Volume (µL)
2X SeqAmp Buffer	25
10 µM NUP	1
Diluted 3' or 5' RACE-PCR product	5
10 µM IMD.NGSP001 or IMD.NGSP002 primer	1
SeqAmp DNA Polymerase	1
Deionized H_2O	17
Total volume	50

The nested RACE-PCR was performed using the Thermal Cycler (Bio-Rad, USA), following the program as described below.

Step 1	Denaturation	94°C	for 30 seconds
Step 2	Annealing	68 °C	for 30 seconds
Step 3	Extension	72°C	for 3 minutes

Repeat steps (1), (2) and (3) for 29 cycles

The PCR products was analyzed on 1% agarose gel electrophoresis followed by SYBR™ safe staining (Invitrogen, USA) and visualized by Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA).

2.1.4 PCR purification

The PCR products with the expected size was excised from the agarose gel and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) according to manufacturer's protocol. Briefly, 200 µL of NTI buffer per 100 mg of gel was added to the PCR product, incubated at 50 °C for 5-10 minutes until the gel was completely dissolved. The mixture was loaded into NucleoSpin® Gel and PCR clean-up column and centrifuged at 11,000 x *g* for 30 seconds. Next, 700 µL of NT3 buffer was added to the column, centrifuged at 11,000 x *g* for 30 seconds to wash the silica membrane, and then centrifuged again at 11,000 x *g* for 1 minute to dry the column. After that, the column was transferred to a new microcentrifuge tube and the PCR product was eluted from the column with 30 µL of NE buffer, incubated at room temperature for 1 minute and then centrifuged at 11,000 x *g* for 1 minute. The purified PCR product was stored at -70 °C until use.

2.1.5 Ligation and transformation

The purified PCR product from section 2.1.4 was cloned into the pCR™-Blunt II-TOPO® vector (Figure 7) using Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, USA). The ligation mixture was prepared according to the manufacturer's protocol as shown in Table 11.

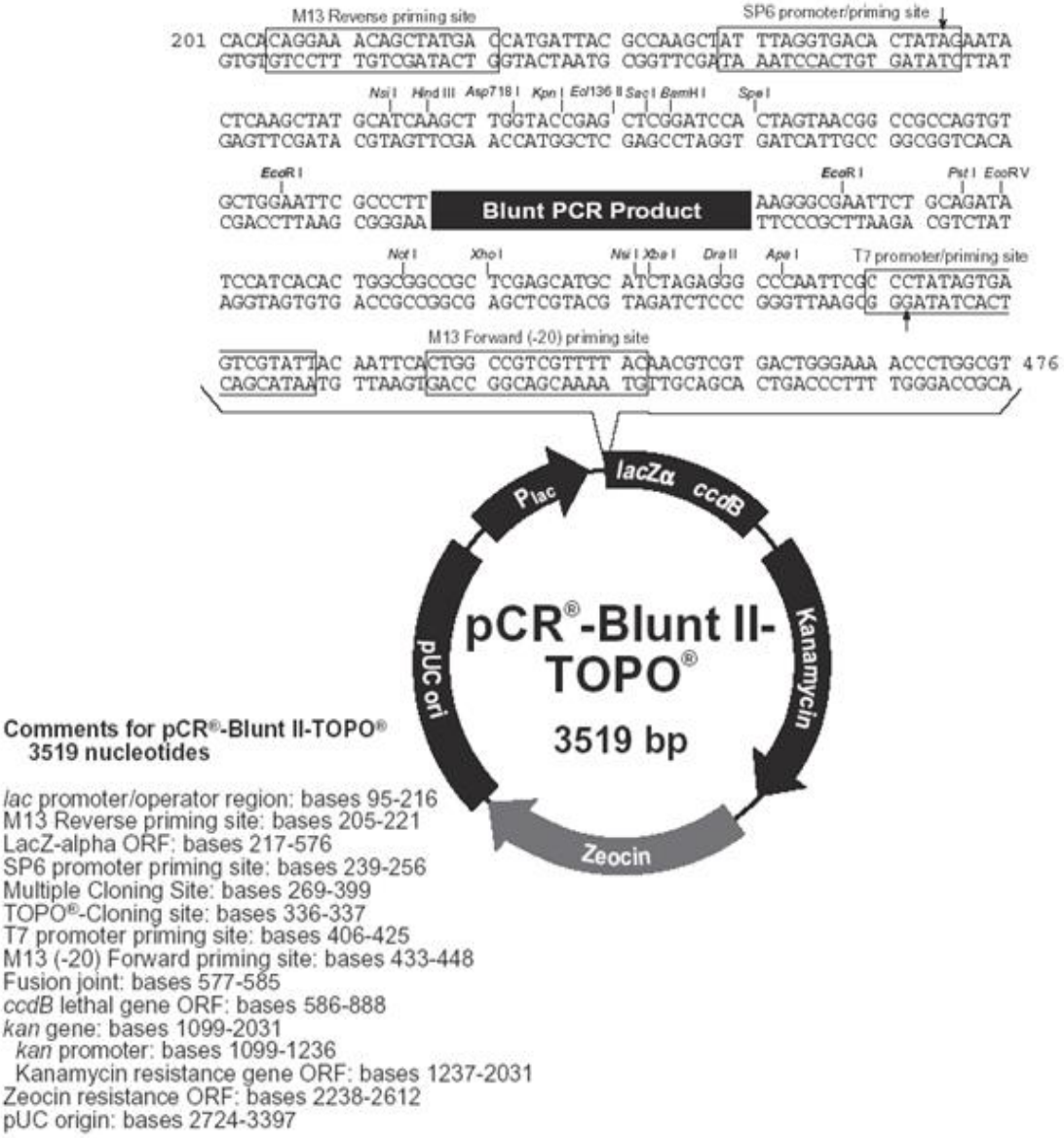
Table 11 Components of the pCR™II-Blunt-TOPO® ligation mixture

Component	Volume (µL)
Purified PCR products (Section 2.1.4)	4
Salt solution	1
pCR™II-Blunt-TOPO®	1
Total volume	6

The ligation mixture was incubated at 22°C for 30 minutes and transformed into *E. coli* TOP10 strain. For transformation, 200 µL of *E. coli* TOP10 strain was placed on ice for 5 minutes or until completely dissolved and then mixed with 6 µL of the ligation mixture and incubated on ice for 30 minutes. After incubation, the *E. coli* TOP10 was heat-shocked at 42°C for 2 minutes and immediately placed on ice for 20 minutes. Then, 600 µL of LB broth was added and incubated at 37°C with 225 rpm agitation for 2 hours. Finally, 100 µL of transformed *E. coli* was spread on LB agar plate supplemented with kanamycin at a final concentration of 100 µg/mL, 40 µg/mL X-gal and 50 µg/mL IPTG. The LB agar plate was incubated for overnight at 37°C for blue/white colony selection.

2.1.6 Colony selection

After incubation, the recombinant plasmid was selected by blue-white colony screening. The white colony contained recombinant plasmid whereas the blue colony contained a vector without the insert. The white colony was picked up to sub-cultured into 4 mL of LB broth supplemented with kanamycin at a final concentration of 100 µg/mL and incubated for overnight at 37°C with 225 rpm agitation.



Comments for pCR®-Blunt II-TOPO®
3519 nucleotides

lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-576
SP6 promoter priming site: bases 239-256
Multiple Cloning Site: bases 269-399
TOPO®-Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 (-20) Forward priming site: bases 433-448
Fusion joint: bases 577-585
ccdB lethal gene ORF: bases 586-888
kan gene: bases 1099-2031
kan promoter: bases 1099-1236
Kanamycin resistance gene ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2724-3397

Figure 7 Map and features of the pCR®-BluntII-TOPO® vector

Source: Zero Blunt® TOPO® PCR Cloning Kit. (Invitrogen, USA)

2.1.7 Plasmid extraction

The plasmid was extracted from the culture using NucleoSpin® Plasmid Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. To obtain the pellet, the culture was transferred into microcentrifuge tube and centrifuged at 11,000 x *g* for 30 seconds (repeat 2 times). The pellet was resuspended with 150 µL of A1 buffer and then 250 µL of A2 buffer added and mixing the tube by inverting 5 times and incubated at room temperature for 5 minutes to lyse the cell bacteria. Next, 350 µL of A3 buffer was added to the lysate and mixed by inverting the tube until the lysate has turned colorless, then centrifuged at 13,000 x *g* for 3 minutes to pellet the precipitate. The clear supernatant was transferred into a NucleoSpin® Plasmid EasyPure Column and centrifuged at 2,000 x *g* for 30 seconds. The column was washed with 450 µL of AQ buffer and then centrifuged at 13,000 x *g* for 1 minute. The column was placed into a new microcentrifuge tube. Finally, the plasmid was eluted from the column with 50 µL of AE buffer, incubated at room temperature for 1 minute, and centrifuged at 13,000 x *g* for 1 minute.

The extracted plasmid was further sequenced to identify the PCR products. The obtained sequencing data of 3' and 5' end was analyzed using BLASTX program (https://blast.ncbi.nlm.nih.gov/ blast/Blast.cgi). DNA sequences of 3' and 5' ends was assembled with the partial sequence of *MrIMD* generate the complete sequence of *MrIMD*.

3. Sequence confirmation of *MrIMD*

To confirm the full-length sequence of *MrIMD* cDNA; the first-strand cDNA was synthesized and subjected to the polymerase chain reaction for confirmation of *MrIMD*.

3.1 First-strand cDNA synthesis

To synthesized the first-strand cDNA, total RNA from hepatopancreases from section 2.1.1 as RNA template and reverse transcribed to cDNA using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to manufacturer's protocol. The RNA mixture was prepared as shown in Table 12.

Table 12 Components of the RNA mixture

Component	Volume (μL)
Total RNA from hepatopancreases	8
50 μM Oligo(dT)$_{12–18}$	1
10 mM dNTP	1
Total volume	10

After mixing the contents, the RNA mixture was incubated at 65°C for 5 minutes and placed on-ice at least 1 minute. Then, the 2X reaction mix was prepared as shown in Table 13.

Table 13 Components of 2X reaction mix for first-strand cDNA synthesis

Component	Volume (μL)
RNA mixture (Table 12)	10
25 mM $MgCl_2$	4
10X RT Buffer	2
0.1 M DTT	2
RNaseOUT™ (40 U/μL)	1
SuperScript® III RT (200 U/μL)	1
Total volume	20

After preparation, the reaction mixture was incubated at 42°C for 50 minutes followed by 85°C for 5 minutes and placed on-ice at least 1 minute. After that, 1 μL of *E. coli* RNase H (2 U/μL) was added and incubated at 37°C for 20 minutes.

3.2 Polymerase chain reaction for confirmation of *MrIMD*

A PCR amplification was performed using gene specific primers designed to cover of the coding sequences from start codons to stop codons (Table 14).

Table 14 Primers used for the confirmation of *MrIMD*

Primer name	Sequence (5'-3')	Product size (bp)
IMDcds-F	CCT TTC AGT CTG CAA AGC CA	711
IMDcds-R	CAC AGC TTA GCT ACA CTG GAA G	

The PCR reaction was prepared as shown in Table 15 and cDNA from section 3.1 was used as a DNA template.

Table 15 Components of the KOD One™ PCR Master Mix reaction

Component	Volume (µL)	Final concentration
KOD One™ PCR Master Mix	25	1x
10 µM IMDcds-F	1.5	0.3 µM
10 µM IMDcds-R	1.5	0.3 µM
cDNA from section 3.1	1	750 ng/µL
Deionized H_2O	21	-
Total volume	50	

After preparation, the PCR was carried out in a Thermal Cycler (Bio-Rad, USA) followed by the PCR program from KOD One™ PCR master Mix (Toyobo, Japan) with some modifications as described below.

Step 1	Denaturation	98°C	for 30 seconds
Step 2	Annealing	50 °C	for 30 seconds
Step 3	Extension	68°C	for 30 seconds

Repeat steps (1), (2) and (3) for 34 cycles

The PCR product was analyzed on 1% agarose gel electrophoresis followed by SYBR™ safe staining (Invitrogen, USA) and visualized by Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA). The PCR product with the expected size was purified and cloned into pCR™-Blunt II-TOPO® vector for sequencing as described in sections 2.1.4-2.1.7.

4. Bioinformatics analysis

4.1 Sequence analysis

The obtained *MrIMD* sequence was analyzed using the BLAST program to verify the correctness and similarity of the *MrIMD* gene (https://blast.ncbi.nlm.nih. gov/Blast.cgi). The ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was utilized to find the open reading frames (ORF) of the *MrIMD* cDNA. The deduced amino acid sequence was obtained using ExPASy Translate tool (http://www.expasy.org/). The *Mr*IMD protein was analyzed the molecular weight (Mw) and isoelectric point (pI) using Expasy Compute pI/Mw tool (https://web.expasy.org/compute_pi/).The putative domains were predicted by Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/).

4.2 Phylogenetic analysis

To study phylogenetic relationships of *Mr*IMD and IMDs from other crustaceans and insects. Multiple sequence alignment was performed using ClustalW. The phylogenetic tree was constructed using MEGA X software based on the deduced amino acid sequences of *Mr*IMD protein, IMDs protein from other species (crustaceans and insects) and receptor-interacting serine/threonine-protein kinase 1 (RIPK1) from *Danio rerio* (vertebrates) retrieved from GenBank database as listed in Table 16. The phylogenetic tree was constructed using Neighbor-Joining (NJ) with 1000 replicates of bootstrap analysis.

Table 16 IMD and RIPK1 proteins from various species used for construction of phylogenetic tree.

Species	Protein	Accession number
Macrobrachium rosenbergii	Immune deficiency	MT123546
Macrobrachium nipponense	Immune deficiency	-
Marsupenaeus japonicus	Immune deficiency	BAH86597.1
Homarus americanus	Immune deficiency-like	XP_042243584.1
Fenneropenaeus chinensis	Immune deficiency-like	AFU60551.1
Litopenaeus vannamei	Immune deficiency	ACL37048
Procambarus clarkii	Immune deficiency-like	XP_045613489.1
Penaeus monodon	Immune deficiency	AVD73295.1
Scylla paramamosain	Immune deficiency	AZK36044.1
Portunus trituberculatus	Immune deficiency	QYF10280.1
Eriocheir sinensis	Immune deficiency	QXO37021.1
Tribolium madens	Immune deficiency	XP_044256597.1
Tribolium castaneum	Immune deficiency	EFA11587.1
Harmonia axyridis	Immune deficiency	XP_045482153.1
Schistocerca gregaria	Immune deficiency-like	AFK75938.1
Bradysia coprophila	Immune deficiency	XP_037036042.1
Hermetia illucens	Immune deficiency	XP_037917390.1
Drosophila simulans	Immune deficiency	EDX07674.1
Sabethes cyaneus	Immune deficiency	XP_053687884.1

5. Tissue distribution and expression analysis of *MrIMD* gene

In this experiment, real-time RT-PCR was performed to analyze the tissue distribution of *MrIMD* mRNA level with elongation factor 1 alpha (*Ef1α*) as an internal control.

5.1 Tissue collection

Adult *M. rosenbergii* (approximately 40-50 g) was purchased from local Market in Bangkok, Thailand. The tissue from various organs including gills, hepatopancreas, heart, hemocyte, muscle, stomach, and intestine was collected for RNA extraction. For hemocyte collection, hemolymph was collected from the ventral sinus of prawn and mixed with 1 volume of modified Alsever's solution (0.055% citric acid, 0.8% sodium citrate, 2.05% D-glucose, and 0.42% sodium chloride (w/v)). The mixture was centrifuged at 2000 x *g* for 10 minutes to collect the hemocytes pellet. After centrifugation, the pellet was washed twice with 1 volume of Alsever's solution and centrifuged at 2000 x *g* for 5 minutes.

5.2 RNA extraction

Total RNA from the collected tissues was extracted using NucleoSpin® RNA XS total RNA isolation kit (Macherey-Nagel, Germany) as described in section 2.1.1. The extracted RNA concentration was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA) and stored at -70°C until use.

5.3 cDNA synthesis for real-time PCR

To synthesize cDNA for real-time PCR, the RNA concentration of each organ was adjusted to 1000 ng/µL and reverse transcribed into the cDNA using SensiFAST™ cDNA Synthesis Kit (Bioline, UK). The cDNA synthesis reaction was prepared as shown in Table 17.

Table 17 Components of cDNA synthesis reaction mixture

Component	Volume (µL)
Total RNA (total 1 µg)	1 to 15
5x TransAmp Buffer	4
Reverse Transcriptase	1
Sterile H_2O	0 to 14
Total volume	20

After preparation, the reaction mixture was carried out in a Thermal Cycler (Bio-Rad, USA), following the cDNA Synthesis program as described below.

Step 1	Primer annealing	25°C	for 10 minutes
Step 2	Reverse transcription	42°C	for 15 minutes
Step 3	Inactivation	85°C	for 5 minutes

The concentration and quality of cDNA was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA) and diluted to 500 ng/µL. The cDNA was used as a template for the qPCR reaction.

5.4 Real-time PCR analysis

To determine the expression pattern of the *MrIMD* in various tissues, Real-time PCR was performed using SensiFAST™ SYBR® No-ROX Kit (Bioline, UK). A total of 4 oligonucleotide primers was used in qPCR including two specific primers for *MrIMD*, designed by using Primer3Plus software (https://www.bioinformatics.nl/primer3 plus) and two specific primers for EF1α as an internal control (Table 18).

Table 18 Primers used for expression analysis of *MrIMD*

Primer name	Sequence (5'-3')
qMrIMD-F	GCT GCA GAG AAA TCG AGG AG
qMrIMD-R	GGA GCT CGA CAG TTT TGT CC
EF1α-F	TGC GCT GTG TTG ATT GTA GC
EF1α-R	ACA ATG AGC TGC TTG ACA CC

The real-time PCR reaction was prepared as shown in Table 19 and cDNA (500 ng/µL) from section 5.3 was used as a DNA template.

Table 19 Components of real-time PCR reaction mixture

Components	Volume (µL)	Final concentration
2x SensiFAST SYBR® No-ROX Mix	10	1x
10 µM qMrIMD-F or EF1α-F	0.8	0.4 µM
10 µM qMrIMD-R or EF1α-R	0.8	0.4 µM
cDNA from section 5.3	2	500 ng/µL
Deionized H_2O	6.4	-
Total volume	20	

The real-time PCR amplification was performed using the MiniOpticon Real-Time PCR system (Bio-Rad, USA), following the program as described below.

Step 1	Polymerase activation	95°C	for 2 minutes
Step 2	Denaturation	95°C	for 5 seconds
Step 3	Annealing	60 °C	for 10 seconds
Step 4	Extension	72°C	for 10 seconds
Repeat steps (2), (3) and (4) for 39 cycles			
Step 5	Melt curve analysis	65 °C to 95 °C	increment 0.5 °C

This experiment, all samples was analyzed in triplicate and the relative expression of *MrIMD* was calculated using the $2^{-\Delta\Delta CT}$ method.

6. Immune challenge experiment with *Aeromonas hydrophila*

To investigate the role of *MrIMD* against bacterial infection, the immune challenge experiment was conducted by injection of *A. hydrophila* into prawn and monitoring the *MrIMD* expression. The expression of *MrIMD* in infected prawn after *A. hydrophila* injection was analyzed by real-time PCR.

6.1 Confirmation of *A. hydrophila* (VMARC1234) isolate

To perform the immune challenge experiment, *A. hydrophila* was subjected to bacterial confirmation to ensure *A. hydrophila* (VMARC1234) strain before use in this experiment.

6.1.1 Purification of bacterial DNA

A. hydrophila (VMARC1234) isolate from stock (-70 °C) was dissolved and streaked onto the tryptone soy agar (TSA) plate and was incubated for overnight at 37°C. A single colony was selected and cultured in 4 mL of tryptone soy broth (TSB) and incubated for overnight at 37 °C with 225 rpm agitation.

The DNA from cultured bacteria was extracted using NucleoSpin® Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Briefly, 1 mL of bacterial culture was centrifuged at 8000 x *g* for 5 minutes and the pellet was resuspended in 180 µL of T1 buffer by pipetting. After that, 25 µL of proteinase K was added, vortex vigorously and incubated at 56 °C for 1-3 hours until the bacterial cells completely lysed. Next, 200 µL of B3 buffer was added to the lysate, vortexed vigorously and incubated at 70 °C for 10 minutes. Then, 210 µL of ethanol (96–100 %) was added and vortexed vigorously. Then, the solution was transfered to the NucleoSpin® Tissue Column and centrifuged at 11,000 x *g* for 1 minute to bind DNA. The column was washed twice with 500 µL of BW buffer and 600 µL of B5 buffer, respectively, and then centrifuged at 11,000 x *g* for 1 minute (2 times). The column was centrifuged at 11,000 x *g* for 1 minute to dry the silica membrane. The column was placed into a new microcentrifuge tube. Finally, the genomic DNA was eluted from the column using 100

µL of BE buffer, incubated at room temperature for 1 minute, and centrifuged at 11,000 x *g* for 1 minute.

6.1.2 Polymerase chain reaction for detection of *A. hydrophila*

To determine the bacterial isolate, a pair of specific primers (G. Wang et al., 2003) to detected hemolysin gene of *A. hydrophila* as shown in Table 20 was used for PCR amplification.

Table 20 Primers used in the PCR reaction

Primer name	Sequence (5'-3')	Product size (bp)
AHH1F	GCC GAG CGC CCA GAA GGT GAG TT	130
AHH1R	GAG CGG CTG GAT GCG GTT GT	

The PCR reaction was prepared as shown in Table 21 and the genomic DNA from section 6.1.1 was used as a template.

Table 21 Components of PCR reaction mixture for detection *A. hydrophila*

Component	Volume (µL)	Final concentration
10X PCR Buffer	5	1X
10 mM dNTP	1	0.2 mM
50 mM $MgCl_2$	1.5	1.5 mM
10 µM AHH1F	1	0.2 µM
10 µM AHH1R	1	0.2 µM
Template DNA	2	-
Platinum® *Taq* DNA Polymerase	0.2	1.0 unit
Distilled water	38.3	-
Total volume	50	

After preparation, The PCR was carried out in a Thermal Cycler (Bio-Rad, USA), following the PCR program from Platinum™ *Taq* DNA Polymerase (Invitrogen, USA) with some modifications as described below.

Step 1	Pre denaturation	94°C	for 5 minutes
Step 2	Denaturation	94°C	for 30 seconds
Step 3	Annealing	59 °C	for 30 seconds
Step 4	Extension	72°C	for 30 seconds
	Repeat steps (2), (3) and (4) for 29 cycles		
Step 5	Final extension	72°C	for 7 minutes

The PCR product was analyzed on 1% agarose gel electrophoresis followed by SYBR™ safe staining (Invitrogen, USA) and visualized by Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA). The PCR Product with expected size was purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) as described in section 2.1.4.

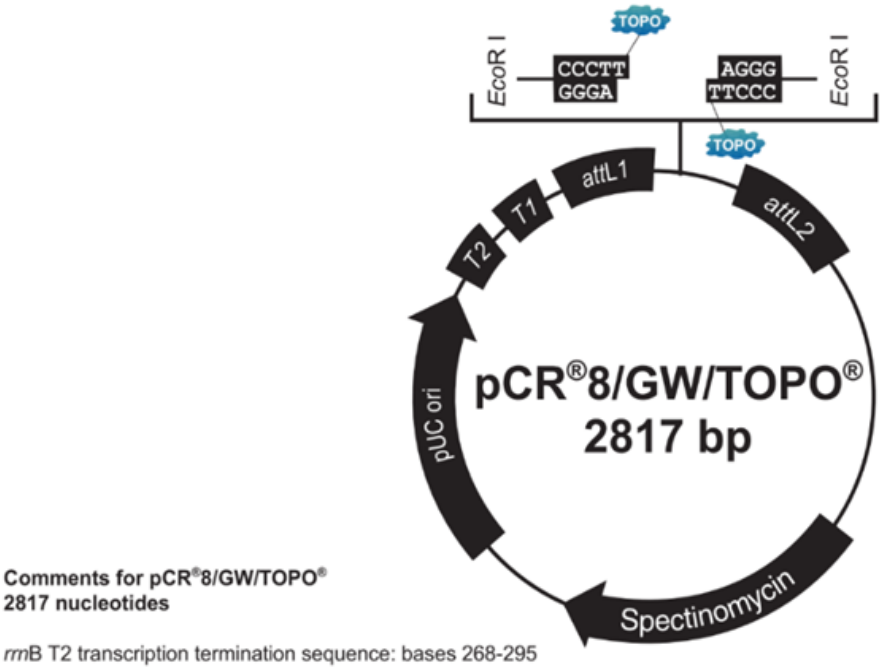
6.1.3 Cloning of *A. hydrophila* hemolysin gene

The PCR products from section 5.1.2 was cloned into pCR™8/GW/TOPO® vector (Figure 8) using pCR™8/GW/TOPO® TA Cloning® Kit (Invitrogen, USA) and transformed into *E. coli* TOP10 strain for sequencing. Briefly, the ligation mixture was prepared according to manufacturer's protocol as shown in Table 23.

Table 22 Components of pCR™8/GW/TOPO® cloning reaction

Component	Volume (µL)
Fresh PCR product (Section 6.1.2)	4
Salt solution	1
TOPO® Vector	1
Total volume	6

After that, the ligation mixture was incubated at 22 °C for 30 minutes and transformed into *E. coli* TOP10 strain as described in sections 2.1.5-2.1.7. The extracted plasmid was further sequenced to confirm the *A. hydrophila* isolate.



Comments for pCR®8/GW/TOPO®
2817 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*attL*1: bases 569-668
GW1 priming site: bases 607-631
TOPO® recognition site 1: bases 678-682
TOPO® recognition site 2: bases 683-687
*attL*2: bases 696-795
GW2 priming site: bases 733-757
T7 Promoter/priming site: 812-831 (c)
M13 reverse priming site: bases 836-852
Spectinomycin promoter: bases 930-1063
Spectinomycin resistance gene (Spn^R): 1064-2074
pUC origin: bases 2141-2814

(c) = complementary sequence

Figure 8 Map and features of pCR™8/GW/TOPO® vector

Source: pCR™8/GW/TOPO® TA Cloning® Kit. (Invitrogen, USA)

6.2 Preparation of *A. hydrophila* for immune challenge

In this experiment, *A. hydrophila* was prepared for injection into prawn, Briefly, *A. hydrophila* bacteria was cultured in 4 mL of TSB and then incubated for overnight at 37 °C with 225 rpm agitation. Then, 20 µL of bacterial culture was sub-cultured in 20 mL of TSB and then incubated at 37 °C with 225 rpm for about 3 hours or until an $O.D._{600}$ reached 0.5-0.7. The bacterial cell was harvested by centrifugation at 2,000 x *g* for 20 minutes. Resuspended the pellet with 20 mL of sterile 2X PBS (Phosphate Buffered Saline, 135 mM NaCl, 15 mM sodium phosphate, pH 7.2) and then centrifuged at 2,000 x *g* for 10 minutes. The cell suspension was adjusted with sterile 2X PBS until an $O.D._{600}$ reached 1 ($1x10^9$ CFU/µL) and then diluted concentration to $2x10^3$ CFU/µL.

6.3 Immune challenge with *A. hydrophila*

In immune challenge experiment, adult prawns (approximately 9-10 g) was acclimated to dechlorinate fresh water for 7 days before used in this study. The adult prawns was divided into two groups including *A. hydrophila* challenge group and 2X PBS group which serves as the control group. In *A. hydrophila* challenge group, prawns was intramuscularly injected with 100 µL ($2x10^4$ CFU/g) of *A. hydrophila* into the third abdominal segment using 1 mL insulin syringe. In the control group, prawns was injected intramuscularly with 100 µL of sterile 2X PBS. Prawns in each group was randomly collected the target organs at 0, 3, 6, 12, 24, 36, and 48 hours post-injection.

PCR was performed to confirm *A. hydrophila* infection in *A. hydrophila* challenge group. Hemolymph was collected, mixed with sterile 2X PBS, and serially diluted to 10^{-1}, 10^{-2} and 10^{-3} fold. Then, 100 µL of each hemolymph dilution was spread onto a TSA agar plate and incubated for overnight at 37 °C. The single colony which resembles that of *A. hydrophila* colony was selected and cultured in 4 mL of TSB and incubated for overnight at 37°C with 225 rpm agitation. After that, bacterial DNA was extracted as described in section 6.1.1. The PCR verification was performed as described in section 6.1.2.

6.4 Expression analysis of *MrIMD* after *A. hydrophila* challenge

Total RNA from the collected tissues was extracted using NucleoSpin® RNA XS total RNA isolation kit (Macherey-Nagel, Germany) as described in section 2.1.1. The extracted RNA was used as a template for cDNA synthesis for real-time PCR as described in section 5.3. Real-time PCR was performed as described in section 5.4. The experiment was performed in triplicate for each sample. Relative expression of *MrIMD* was calculated between *A. hydrophila* group compared to the control group. The data was analyzed using one-way analysis of variance (one-way ANOVA) and Student's t-test, followed by a post-hoc Tukey test. Significant differences were considered at $p < 0.05$

7. Immune challenge experiment with *Mr*NV

To investigate the immune response of *MrIMD* to *Mr*NV infection, all experiments were performed in the same manner as described in the previous report (Srisuk et al., 2022). Before this experiment, the *Mr*NV had to be confirmed to ensure the accuracy of the viral challenge experiment.

7.1 Verification of *Mr*NV

Viral nucleic acid was extracted from *Mr*NV-infected samples using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's protocol to ensure the accuracy of the *Mr*NV. Briefly, the sample was homogenized with 200 µL of lysis buffer and centrifuged at 3,500 rpm for 5 minutes. The supernatant was transferred into a microcentrifuge tube and mixed with 200 µL of the working solution (Table 23).

Table 23 Components of working solution mixture

Component	Volume (µL)
Binding Buffer	200
poly(A) carrier RNA	4
Total volume	200

Then, 50 µL of Proteinase K was added, mixed by vortexing, and incubated at 72°C for 10 minutes. Next, 100 µL of isopropanol was added and immediately mixed by inverting the tube. The supernatant was then transferred into a High Pure Filter Tube and centrifuged at 8,000 x *g* for 1 minute. After that, 500 µL of Inhibitor Removal Buffer was added and centrifuged at 8,000 x *g* for 1 minute. The column was washed twice with 450 µL of Wash Buffer and then centrifuged at 8,000 x *g* for 1 minute. Subsequently, the column was centrifuged at 14,000 rpm for 30 seconds to remove the Wash Buffer. Finally, the viral nucleic acids was eluted from the column with 50 µL of Elution Buffer, incubated at room temperature for 2 minutes, and centrifuged at 8,000 x *g* for 1 minute.

To carry out RT-PCR amplification, the SuperScript One-Step RT-PCR System (Invitrogen) was utilized with gene-specific primers for the RNA-dependent RNA polymerase (RdRp) gene (Mr-RdRP-F and Mr-RdRP-R) (Senapin et al., 2012). The size of the PCR product was 729 bp, and the primer sequences are listed in Table 24.

Table 24 Primers used in the RT-PCR amplification

Primer name	Sequence (5'-3')	Product size (bp)
Mr-RdRP-F	GCATTTGTGAAGAATGAACCG	729
Mr-RdRP-R	CATGTTCAACTTTCTCCACGT	

The RT-PCR reaction was prepared as shown in Table 25 and viral nucleic acid from section 7.1 was used as a template.

Table 25 Components of RT-PCR reaction mixture for detection *Mr*NV

Component	Volume (µL)	Final concentration
2X Reaction mix	12.5	1X
10 µM Mr-RdRP-F	0.5	0.2 µM
10 µM Mr-RdRP-R	0.5	0.2 µM
Template RNA	2.5	-
RT/Platinum™ *Taq* Mix Distilled	0.5	-
Distilled water	8.5	-
Total volume	25	

After preparation, The RT-PCR was carried out in a Thermal Cycler (Bio-Rad, USA), following the PCR program following as described below.

Step 1	cDNA synthesis	50°C	for 30 minutes
Step 2	Pre denaturation	94°C	for 2 minutes
Step 2	Denaturation	94°C	for 30 seconds
Step 3	Annealing	55°C	for 30 seconds
Step 4	Extension	68°C	for 1 minutes
Repeat steps (2), (3) and (4) for 29 cycles			
Step 5	Final extension	72°C	for 5 minutes

The PCR product was analyzed on 1% agarose gel electrophoresis followed by SYBR™ safe staining (Invitrogen, USA) and visualized by Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA). The PCR Product with expected size was purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) as described in section 2.1.4. The concentration of purified products was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA)

7.2 Preparation of *Mr*NV inoculum

The experimental methods and preparation of *Mr*NV inoculum were carried out following the steps of a previous study, using TN buffer (Tris-HCl and NaCl) as the control group (Srisuk et al., 2022).The copy number of *Mr*NV inoculum was 8.05×10^{11} copies/µg nucleic acid, which was detected by real-time PCR following the protocol (Srisuk et al., 2022)

7.3 Expression analysis of *MrIMD* after *Mr*NV challenge

In the viral challenge experiment, the juvenile prawns (approximately 1.5-2 g) were acclimated in dechlorinated freshwater for 7 days before used in this study. The juvenile prawns were divided into two groups, with each group containing 30 individual prawns, including *Mr*NV injection group, and TN injection group. In the *Mr*NV challenge group, 50 µL of *Mr*NV inoculum was injected into the abdominal segment of prawns, while 50 µL of TN buffer was injected in prawns in the control group. Target organs was randomly collected from the prawns in each group at 0,1, 2, 3, 4, 5, 6, and 7 days post-injection (3 prawns per time point). Total RNA from the collected tissues were extracted using NucleoSpin® RNA XS total RNA isolation kit (Macherey-Nagel, Germany) as described in section 2.1.1. The total RNA was used as a template for cDNA synthesis as described in section 5.3. Real-time PCR was performed as described in section 5.4 and the experiment was performed in triplicate for each sample. Relative expression of *MrIMD* was calculated between *Mr*NV group compared to the control group. The data was analyzed using one-way analysis of variance (one-way ANOVA) and Student's t-test, followed by a post-hoc Tukey test. Significant differences were considered at $p < 0.05$

CHAPTER 4
RESULTS

1. Molecular cloning of full-length cDNA of *MrIMD*

1.1 Primers design for isolation of the 3' and 5' ends

To isolate the complete sequence of the 5' and 3' ends of the *MrIMD* gene, 2 oligo nucleotide primers for 5' end and 2 oligo nucleotide primers for 3' end were designed from partial nucleotide sequences of *MrIMD* was analyzed with the nucleotide sequences of IMD genes of *M. japonicus* (BAH86597.1). The result showed that *MrIMD* shared high identity with *MjIMD*. Then, the sequences were aligned and the primers were designed as shown in Figure 9.

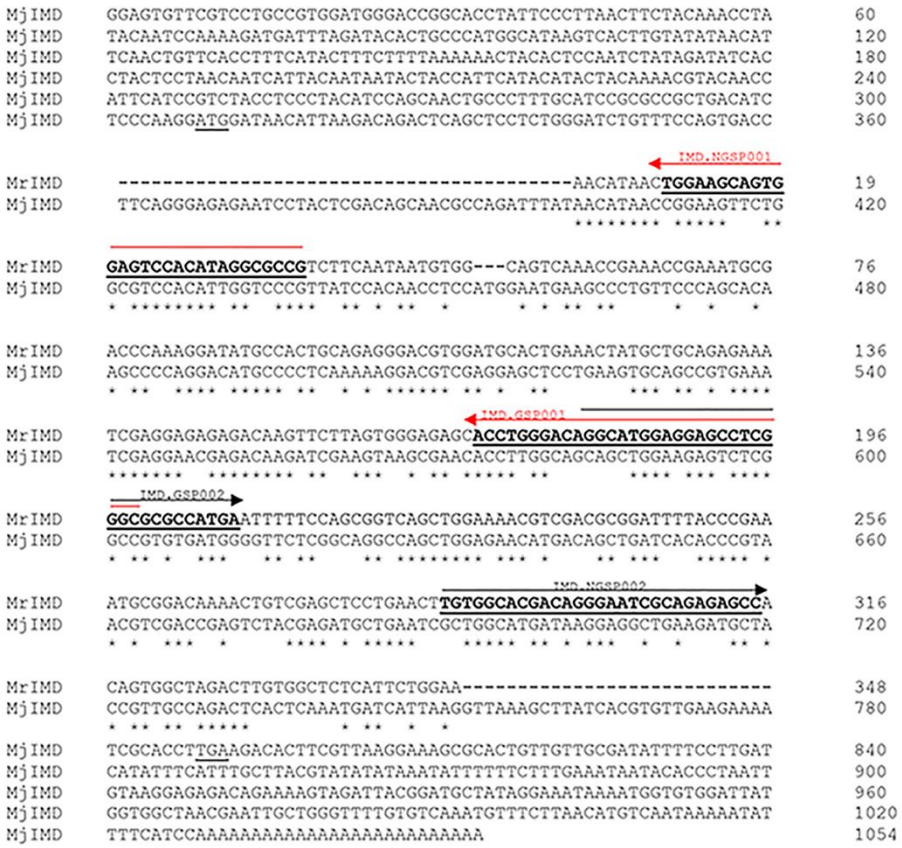


Figure 9 Primer design for isolate 3' and 5' end of *MrIMD* gene by sequence alignment of partial *MrIMD* and complete sequence of *MjIMD* gene

1.2 Identification of 3’ and 5’ cDNA ends of *MrIMD* using RACE-PCR

To amplified 3’ cDNA end, touchdown PCR and Nested RACE-PCR were performed using IMD.GSP002 primer and IMD.NGSP002 primer, respectively The gel electrophoresis results showed that the 3' touchdown PCR product was 500 bp whereas the 3' nested RACE-PCR products were 350 and 450 bp (Figure 10). For 5' cDNA end, the conventional PCR was performed using IMD.GSP001 primer and PCR product was 1,500 bp (figure 10). The PCR product with the expected size was purified and cloned into pCR™-Blunt II-TOPO® vector for sequencing

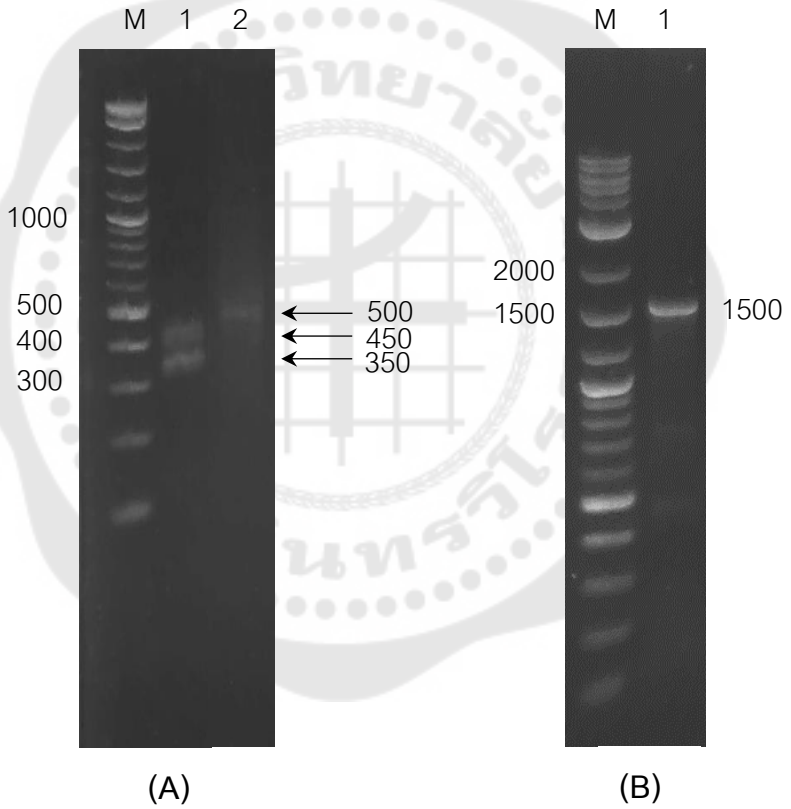


Figure 10 The PCR products of 3' and 5’ RACE-PCR of *MrIMD* gene

(A) Lane 1 is 3' nested RACE-PCR products with 450 and 350 bp
Lane 2 is 3' Touchdown PCR products with 500 bp
(B) Lane 1 is 5' conventional PCR products with 1500 bp
M is DNA Marker (1 kb DNA Ladder)

1.3 Sequence confirmation of the full-length sequence of *MrIMD*

To verify the full-length *MrIMD* sequence, a pair of primers was designed to cover the coding sequence region (from the start to stop codons) of the IMD gene. The PCR amplification was performed using KOD DNA polymerase, and the expected PCR product was 708 bp, as shown in Figure 11. The resulting PCR product was then purified and cloned into the pCR™-Blunt II-TOPO® vector. The recombinant plasmid, pCR™-Blunt II-TOPO® vector-*MrIMD* 700 bp, was verified for using *Eco*RI restriction enzyme digestion (Figure 11). Finally, the verified recombinant plasmid was sequenced to confirm the full-length of the *MrIMD* sequence.

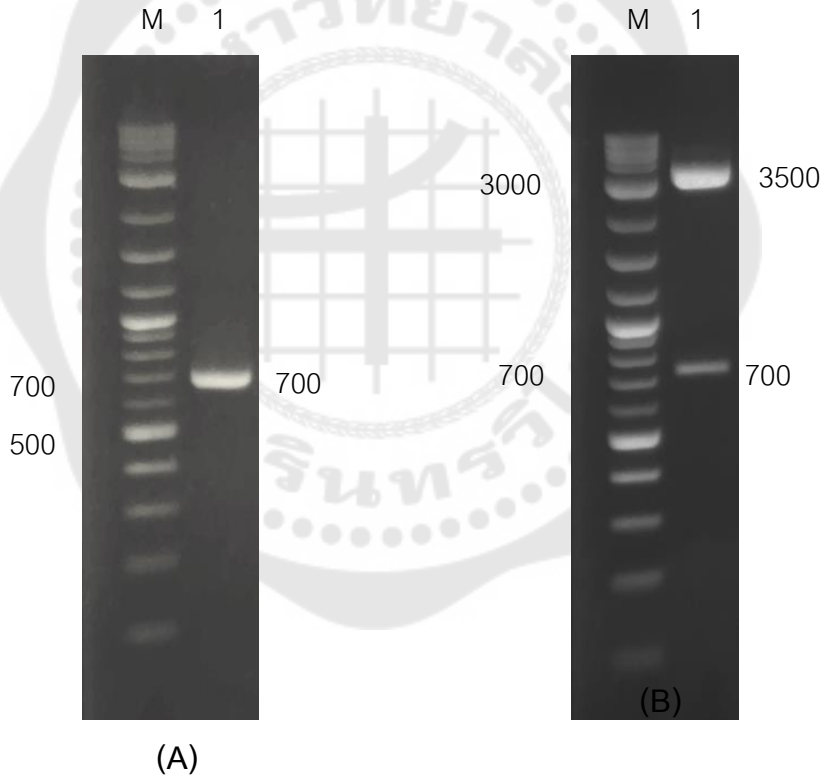


Figure 11 The PCR products of the open reading frame of *MrIMD* gene

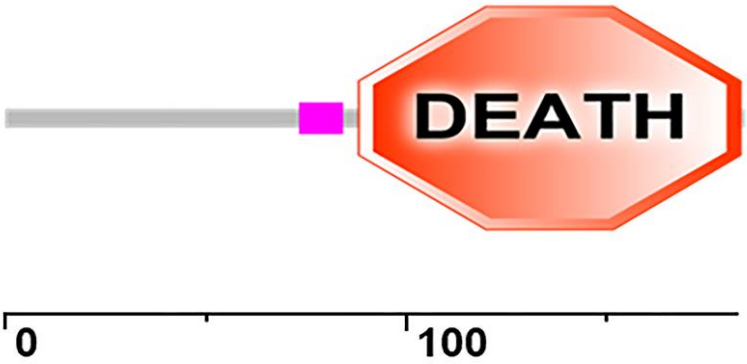
(A) Lane 1 is PCR product of cds-MrIMD with 700 bp

(B) Lane 1 is PCR Product of cds-MrIMD with 700 bp and pCR™II-Blunt-TOPO® vector with 3.5 kb

M is DNA Marker (1 kb DNA Ladder)

1.4 Characterization of *MrIMD* cDNA

The *MrIMD* cDNA was analyzed to investigate the open reading frame (ORF) region and deduce amino acid sequence. In addition, bioinformatics tools were used to determine the molecular weight, isoelectric point, and potential domains of the protein. The analysis revealed that the full-length of *MrIMD* cDNA consisted of 1,127 nucleotides, with 379 nucleotides in the 5'-untranslated region (5'-UTR) and 193 nucleotides in the 3'-UTR. The ORF consisted of 555 nucleotides and encoded a protein consisting of 184 amino acids (Figure 12). The nucleotide sequence of *MrIMD* was deposited in the GenBank database under accession number MT123546.



Confidently predicted domains, repeats, motifs and features:

Name	Start ▲	End	E-value
low complexity	73	84	N/A
DEATH	88	183	0.0000871

Figure 12 The putative domain analysis of *Mr*IMD protein using SMART program

The orange boxes represented a death domain at the C-terminus of protein and the pink boxes represented low complexity regions.

1.5 *Mr*IMD protein analysis

The *Mr*IMD protein of *M. rosenbergii* consisted of 184 amino acid residues with a conserved death domain at the carboxyl-terminus at amino acid positions 88 to 183 (Figure 13). The mature *Mr*IMD protein had a theoretical pI of 9.48 and molecular mass of 20.5 kDa.

```
-379   TAAATATTTTGCAATCCATCTATCATCCGTCACTCATCCTCCCTCATCCAAACTCCCATAACACATC
-312 CATCCATCAGAGCATTTTCACCCGTCCATTTCACTTAACGACAATTAAATAAATATTTTGCAATCCATC
-243 TATCATCCGTCACTCATCCTCTCTCATCCAAACGCCCCAACACACCTGTCAATCAAGGCAATTTCTCCC
-174 AACAGCAACTGGCTTCACCCTTCCTTGTCAACTGAACAATAAACAAATAAATATTCTACAATCCATCTA
-105 TCATCCGTCACTCATCCTCTCTCATCCGAACGCCCCATCACACCTGTCAACCAAGAAAGCTCCGCCCAA
 -35 CATCAACCTTTCAGTCTGCAAAGCCACCTGAAAAAGATGAATAACCTCAGGACGGATTCTTTAAGTTTG
   1                                    M  N  N  L  R  T  D  S  L  S  L
  34 GGCACAAGTTATCCCAAGGACCCACTCCGACCACATTCTCCTCCTCCCCCGCAGGACTCGAAGTCCTCC
  12  G  T  S  Y  P  K  D  P  L  R  P  H  S  P  P  P  P  Q  D  S  K  S  S
 103 TTCCAGAGGCCTGGCGTCCGTCAGGCACCTACTACGGGCCAGGGTCCTCGCGTCATCAACATAACTGGA
  35  F  Q  R  P  G  V  R  Q  A  P  T  T  G  Q  G  P  R  V  I  N  I  T  G
 172 AGCAGTGGAGTCCACATAGGCGCCGTCTTCAATAATGTGGCAGTCAAACCGAAACCGAAATGCGACCCA
  58  S  S  G  V  H  I  G  A  V  F  N  N  V  A  V  K  P  K  P  K  C  D  P
 241 AAGGATATGCCACTGCAGAGGGACGTGGATGCACTGAAACTATGCTGCAGAGAAATCGAGGAGAGAGAC
  81  K  D  M  P  L  Q  R  D  V  D  A  L  K  L  C  C  R  E  I  E  E  R  D
 310 AAGTTCTTAGTGGGAGAGCACCTGGGACAGGCATGGAGGAGCCTCGGGCGCGCCATGAATTTTTCCAGC
 104  K  F  L  V  G  E  H  L  G  Q  A  W  R  S  L  G  R  A  M  N  F  S  S
 379 GGTCAGCTGGAAAACGTCGACGCGGATTTTACCCGAAATGCGGACAAAACTGTCGAGCTCCTGAACTTG
 127  G  Q  L  E  N  V  D  A  D  F  T  R  N  A  D  K  T  V  E  L  L  N  L
 448 TGGCACGACAGGGAATCGCAGAGAGCCACAGTGGCTAGACTTGTGGCTCTCATTCTGGAAGTCAAGGCT
 150  W  H  D  R  E  S  Q  R  A  T  V  A  R  L  V  A  L  I  L  E  V  K  A
 517 TATCACGTGCTGAAGTATCTGAAGCCGAGATATTCATAGTCTTCTCAATCTTTCTTTTAATGAATGATG
 173  Y  H  V  L  K  Y  L  K  P  R  Y  S  *
 586 CCATTGTATCTTTCCTACTTGTGTCAAAGTTCATGTTTTAATAATTTTTTTTGTATATAAGAATCGTAT
 655 TTCTTCCAGTGTAGCTAAGCTGTGTAAACGAGTACTTAATCAGCATAAGTTATGATAGAAAAAAAAAAA
 724 AAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 13 The full-length cDNA and amino acid sequence of *MrIMD*

The numbers on the left indicated shown the positions of nucleotides and amino acids. The initiation codon (ATG) and stop codon (TAG) are in bold letters. The conserved death domain is enclosed in a blue frame.

Furthermore, Pairwise alignment of *Mr*IMD protein with IMD proteins from other species was analyze by Iden and Sim Webserver base on the full-length amino acid sequences of *Mr*IMD, *Macrobrachium rosenbergii* (MT123546); *Mn*IMD, *Macrobrachium nipponense* (accession no. -); *Mj*IMD, *Marsupenaeus japonicus* (BAH86597.1); *Ha*IMD, *Homarus americanus* (KAG7157047.1); *Fc*IMD, *Fenneropenaeus chinensis* (AFU60551.1); *Lv*IMD, *Litopenaeus vannamei* (ACL37048); *Pc*IMD, *Procambarus clarkii* (XP_045613489.1); *Pm*IMD, *Penaeus monodon* (AVD73295.1); *Sp*IMD, *Scylla paramamosain* (AZK36044.1); *Pt*IMD, *Portunus trituberculatus* (QYF10280.1); *Es*IMD, *Eriocheir sinensis* (QXO37021.1); *Tm*IMD, *Tribolium madens* (XP_044256597.1); *Tc*IMD, *Tribolium castaneum* (EFA11587.1); *Ha*IMD, *Harmonia axyridis* (XP_045482153.1); *Sg*IMD, *Schistocerca gregaria* (AFK75938.1); *Bc*IMD, *Bradysia coprophila* (XP_037036042.1); *Hi*IMD, *Hermetia illucens* (XP_037917390.1); *Ds*IMD, *Drosophila simulans* (EDX07674.1); and *Sc*IMD, *Sabethes cyaneus* (XP_053687884.1).

% similarity / % identity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *M. rosenbergii* (*Mr*IMD)		74.62	55.68	56.99	54.59	54.05	56.68	52.97	55.56	53.97	53.23	39.41	37.32	28.33	28.47	28.29	27.68	25.36	24.15
2. *M. nipponense* (*Mn*IMD)	74.11		56.83	63.39	55.19	54.64	58.29	53.55	55.32	55.61	57.53	37.02	35.98	25.51	27.01	27.24	26.87	24.54	24.05
3. *M. japonicus* (*Mj*IMD)	47.03	47.54		64.37	92.50	92.50	68.90	90.00	60.12	59.54	60.00	37.06	35.15	24.58	28.68	27.06	25.00	22.34	24.81
4. *H. americanus* (*Ha*IMD)	46.63	51.91	50.57		64.94	63.79	74.58	62.07	55.62	57.63	57.95	35.96	36.06	25.62	27.94	28.63	26.12	24.91	25.95
5. *F. chinensis* (*Fc*IMD)	45.95	46.45	88.13	51.15		98.75	68.90	96.25	60.12	58.38	58.82	37.56	36.14	26.27	28.68	26.27	25.37	23.08	25.57
6. *L. vannamei* (*Lv*IMD)	45.41	45.90	88.13	50.00	98.75		67.68	96.25	58.96	57.23	59.41	37.06	36.64	25.85	28.31	25.88	25.00	22.71	25.19
7. *P. clarkii* (*Pc*IMD)	44.39	46.52	54.88	64.41	54.88	53.66		65.24	54.91	57.80	57.65	37.37	36.45	27.85	27.84	25.39	24.91	24.45	26.24
8. *P. monodon* (*Pm*IMD)	43.78	44.26	84.38	47.70	95.00	95.00	51.22		57.80	55.49	55.88	36.55	35.15	24.58	28.31	26.27	25.37	23.08	25.57
9. *S. paramamosain* (*Sp*IMD)	42.33	43.09	43.35	47.19	45.09	44.51	46.24	42.20		88.30	82.35	41.12	40.10	28.69	25.00	25.10	27.99	23.44	22.14
10. *P. trituberculatus* (*Pt*IMD)	41.80	43.32	44.51	48.59	45.66	45.09	44.51	42.20	82.46		81.18	40.61	40.10	27.31	24.63	24.31	28.36	22.34	20.99
11. *E. sinensis* (*Es*IMD)	39.78	43.01	41.76	48.86	42.94	43.53	44.71	39.41	71.76	68.82		37.06	36.14	27.12	24.63	24.71	25.00	21.98	19.85
12. *T. madens* (*Tm*IMD)	20.20	18.75	17.26	20.69	15.23	15.23	20.20	13.20	22.34	22.84	21.32		86.43	47.48	30.71	33.20	33.96	31.50	29.7
13. *T. castaneum* (*Tc*IMD)	18.66	17.76	16.83	21.15	15.35	15.35	19.21	13.37	21.78	22.28	20.79	82.41		47.50	31.56	36.33	35.45	31.50	28.57
14. *H. axyridis* (*Ha*IMD)	16.67	14.40	12.71	15.29	12.29	12.29	16.46	11.02	16.03	14.29	15.25	35.29	33.75		36.65	32.73	31.32	29.68	36.84
15. *S. gregaria* (*Sg*IMD)	16.06	14.23	15.44	14.71	15.44	15.44	15.75	15.44	16.18	15.07	15.81	22.14	21.99	22.06		30.52	31.85	28.48	28.67
16. *B. coprophila* (*Bc*IMD)	15.50	14.40	13.73	16.86	12.94	12.55	16.41	12.55	15.69	14.90	14.90	22.66	23.44	20.36	19.81		43.01	40.34	37.86
17. *H. illucens* (*Hi*IMD)	14.42	13.43	12.31	15.30	12.31	11.94	14.87	11.19	16.04	15.67	16.04	21.64	22.01	19.57	19.43	27.24		46.83	39.79
18. *D. simulans* (*Ds*IMD)	13.41	13.19	13.19	17.58	13.92	13.55	15.69	13.19	13.92	13.55	13.55	21.61	21.61	20.14	17.72	27.59	34.51		39.08
19. *S. cyaneus* (*Sc*IMD)	12.45	11.83	12.6	14.59	11.83	11.45	14.89	11.45	10.69	9.92	10.31	17.29	16.54	21.80	14.00	22.14	22.18	22.54	

Figure 14 Percent identity and similarity between *Mr*IMD protein, IMDs protein from other crustaceans and invertebrates.

The result showed that the *Mr*IMD protein shared 40-74% identity with IMD proteins from crustaceans, with the highest identity to in *M. nipponense* (74% identity). The *Mr*IMD protein also shared 12-20% identity with IMDs from insects. (Figure 14). Moreover, the similarity percentage was higher than the identity percentage, analysis of the full-length amino acid sequence of *Mr*IMD showed a high level of similarity ranging from 53–74% to IMDs from crustaceans. While the level of similarity ranged from 24–38% with IMDs from insects, as shown in Figure 14.

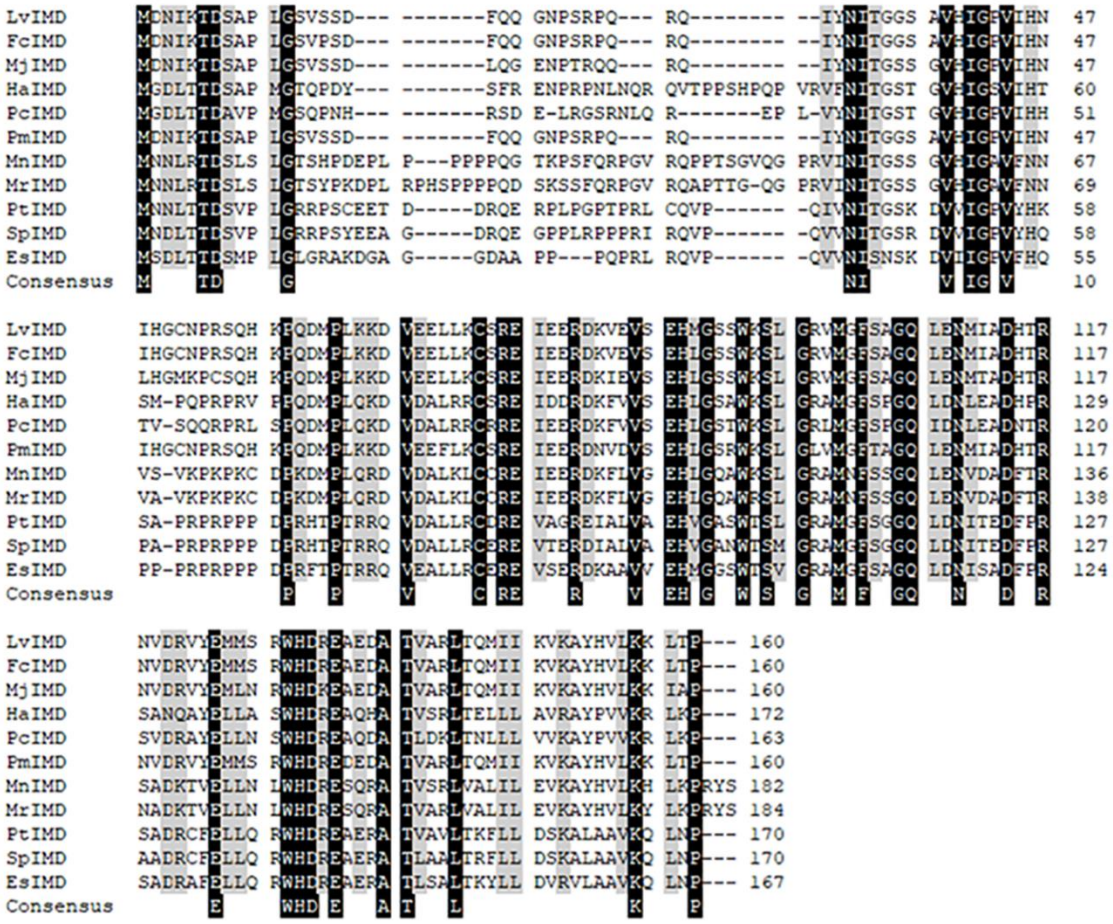


Figure 15 Multiple sequence alignment of IMD proteins from various shrimp species

Multiple sequence alignment was aligned based on the amino acid sequences of *Mr*IMD protein and IMDs protein from other shrimp species: *Lv*IMD, *Litopenaeus vannamei* (ACL37048.1); *Fc*IMD, *Fenneropenaeus chinensis* (AFU60551.1); *Mj*IMD , *Marsupenaeus japonicus* (BAH86597.1); *Pc*IMD, *Procambarus clarkii* (XP_045613489.1); *Pm*IMD, *Penaeus monodon* (AVD73295.1); *Ha*IMD: *Homarus americanus* (XP_042243584.1) and *Mn*IMD: *Macrobrachium nipponense*. The black highlighted regions indicate the consensus sequences, while the gray highlighted regions indicate similar sequences.

2. Phylogenetic analysis of *Mr*IMD protein

The results of the BLASTP analysis revealed that the *Mr*IMD protein shared identity with IMD proteins from crustaceans and insects. Additionally, the *Mr*IMD protein also shared identity to the receptor-interacting serine/threonine-protein kinase 1 (RIPK1). Therefore, a phylogenetic tree was constructed using amino acid sequences of *Mr*IMD, IMD proteins from other crustaceans and insects, and RIPK1 from *Danio rerio*, with 1000 replicates of bootstrap. The ML-tree tree could be divided into two groups: Group I consisted of IMD proteins from crustaceans, and Group II consisted of IMD proteins from insects. Group I could be further subdivided into two sub-clades: clade I include IMD proteins from other shrimp, and clade II included IMD proteins from crab species. The *Mr*IMD protein was clustered with IMD proteins from crabs and had a close relationship with the *Mn*IMD protein of *Macrobrachium nipponense* (Figure 15). The full-length amino acid sequences of *Mr*IMD protein, IMDs protein and RIPK1 protein from other species were included *Lv*IMD, *Litopenaeus vannamei* (ACL37048); *Fc*IMD, *Fenneropenaeus chinensis* (AFU60551.1); *Mj*IMD, *Marsupenaeus japonicus* (BAH86597.1); *Pc*IMD, *Procambarus clarkii* (XP_045613489.1); *Pm*IMD, *Penaeus monodon* (AVD73295.1); *Ha*IMD, *Homarus americanus* (KAG7157047.1); *Mn*IMD, *Macrobrachium nipponense* (accession no. -); *Mr*IMD, *Macrobrachium rosenbergii* (MT123546); *Pt*IMD, *Portunus trituberculatus* (QYF10280.1); *Sp*IMD, *Scylla paramamosain* (AZK36044.1); *Es*IMD, *Eriocheir sinensis* (QXO37021.1); *Tc*IMD, *Tribolium castaneum* (EFA11587.1); *Tm*IMD, *Tribolium madens* (XP_044256597.1); *Tm*IMD, *Tenebrio molitor* (QCS90310.1); *Hi*IMD, *Hermetia illucens* (XP_037917390.1); *Sc*IMD, *Sabethes cyaneus* (XP_053687884.1); *Sg*IMD, *Schistocerca gregaria* (AFK75938.1); *Ds*IMD, *Drosophila simulans* (EDX07674.1); *Nv*IMD, *Nasonia vitripennis* (NP_001135910.1); *Ha*IMD, *Harmonia axyridis* (XP_045482153.1); *Cg*IMD, *Colletes gigas* (XP_043255214.1); *Bc*IMD, *Bradysia coprophila* (XP_037036042.1); and outgroup *Dr*RIPK1, *Danio rerio* (CAP09375.1).

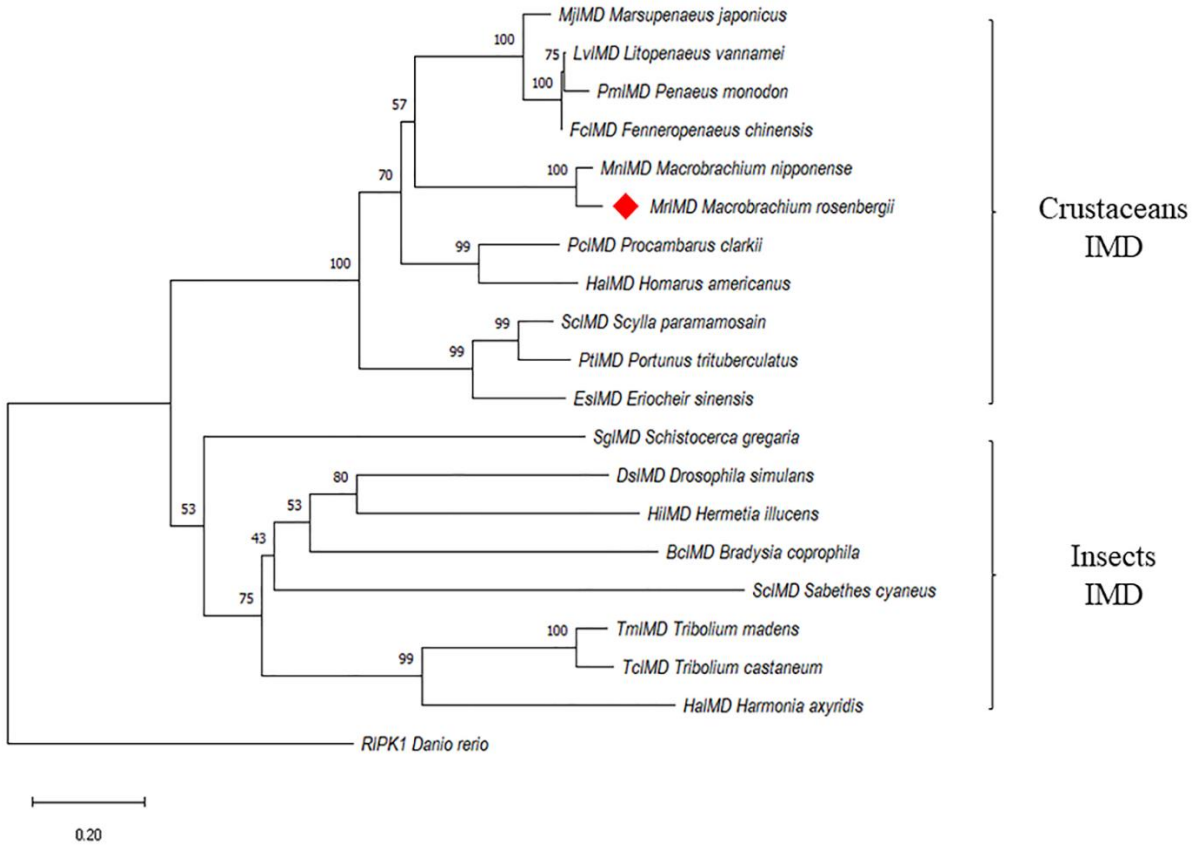


Figure 16 Phylogenetic relationship of IMD proteins from crustaceans and insects.

The phylogenetic tree was constructed using Neighbor-Joining (NJ) with 1000 replicates in MEGA X based on the full-length amino acid sequences of the *Mr*IMD protein and IMD proteins from crustaceans and insects, rooted by the RIPK1 protein from *Danio rerio* (outgroup).

3. Tissue distribution analysis of *MrIMD* using real-time RT-PCR

The expression pattern of *MrIMD* in healthy prawns was investigated by real-time RT-PCR using RNA samples extracted from various tissues, including gills, hepatopancreas, hemocytes, intestine, heart, stomach, and muscle. The expression levels of *MrIMD* mRNA in each tissue were normalized with Ef1α. The real-time RT-PCR results showed that *MrIMD* mRNA was expressed in all examined tissues, with the highest expression level observed in the gills, followed by the hepatopancreas. However, the lowest expression of *MrIMD* occurred in the stomach, intestine, heart, hemocytes, and muscle (Figure 16).

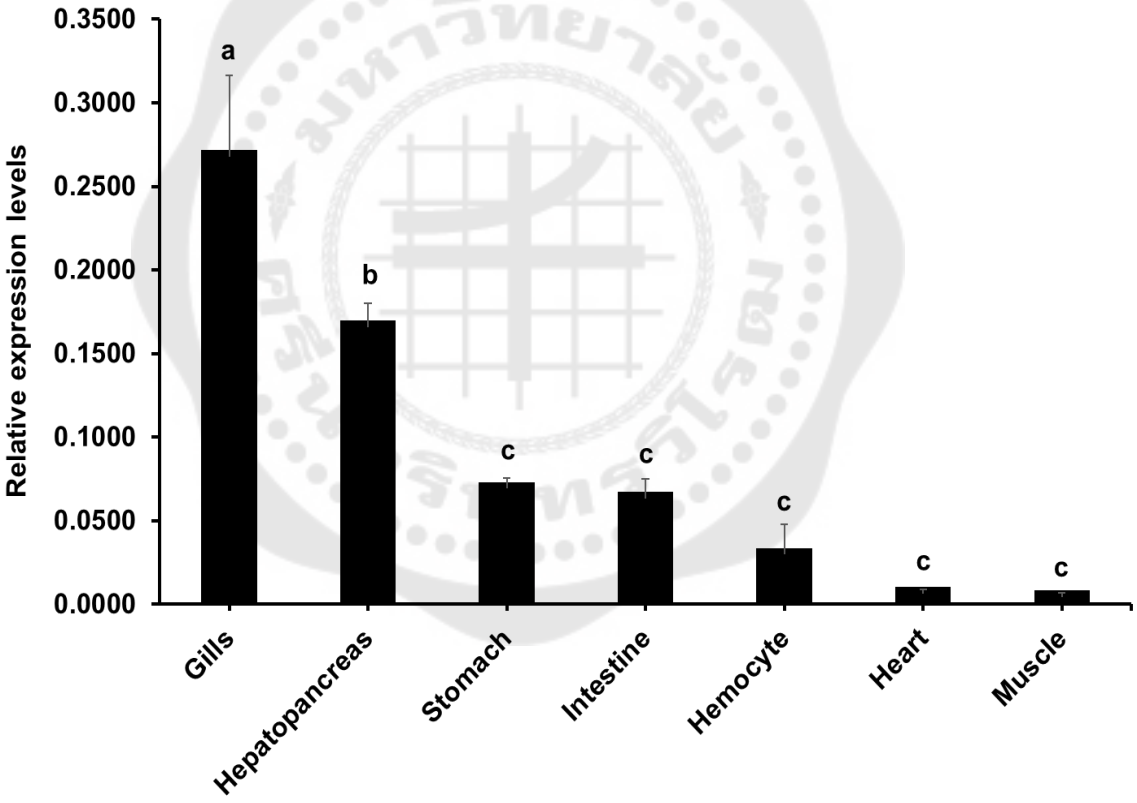


Figure 17 The expression pattern of *MrIMD* from various tissues in normal prawn

The relative expression of *MrIMD* was normalized to *Ef1α* gene using real-time RT-PCR. The data shows the mean ± SD (n=5) and the letters a, b, and c showed significant differences ($p < 0.05$)

4. Expression analysis of *MrIMD* in *A. hydrophila* challenged prawns

4.1 Confirmation of *A. hydrophila* strain

For the immune challenge experiment, bacterial DNA of *A. hydrophila* was extracted from cultured bacteria and used as a template for PCR amplification. The hemolysin gene PCR product, which was 130 bp in length (Figure 17A), was cloned into the pCR™8/GW/TOPO® vector (Figure 17B). The sequencing results revealed that the PCR product was identical to the hemolysin gene of *A. hydrophila* with 90% identity.

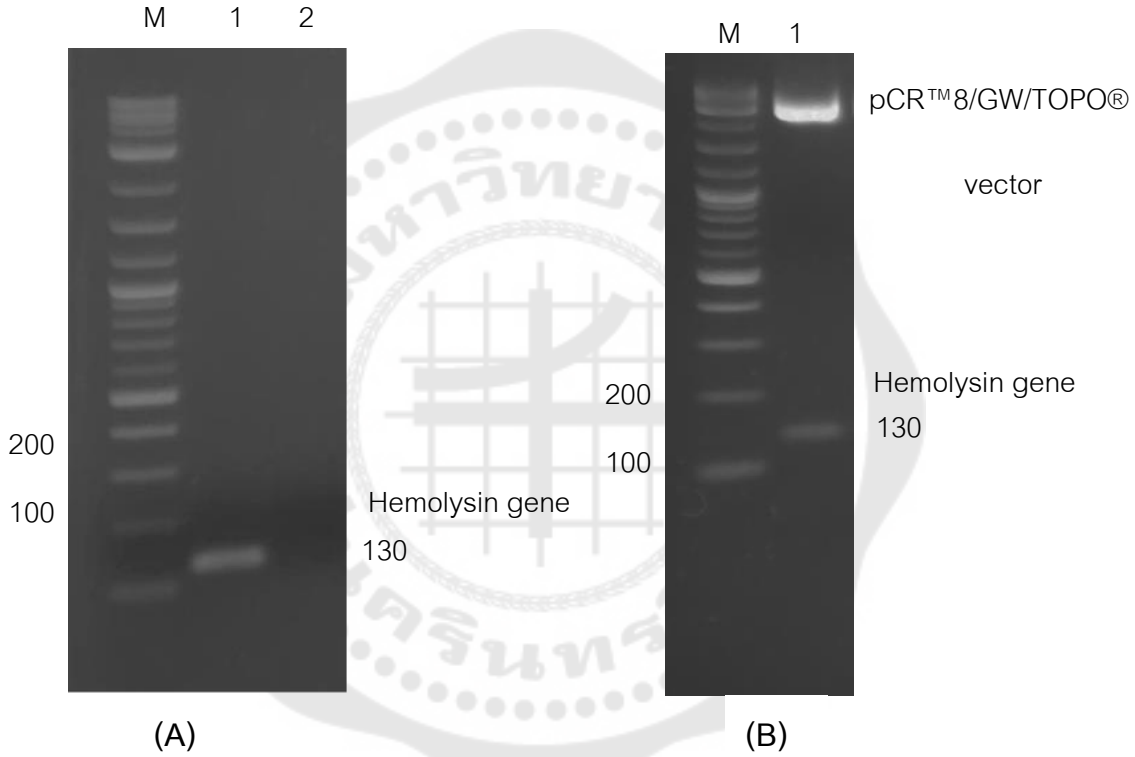


Figure 18 PCR products of hemolysin gene of *A. hydrophila*

(A) Lane 1 is PCR product of Hemolysin gene with 130 bp
Lane 2 is negative control

(B) Lane is PCR Product of hemolysin gene with 700 bp and pCR™8/GW/TOPO® vector with 2.8 kb

M is DNA Marker (1 kb DNA Ladder)

4.2 Expression analysis of *MrIMD* in response to *A. hydrophila* injection using real-time RT-PCR

The prawns were divided into two groups: an experimental group and a control group. In the experimental group, prawns were injected with 100 µL ($2x10^4$ CFU/g) of *A. hydrophila*, while Prawn in the control group were injected with 100 µL of sterile 2X PBS. The real-time RT-PCR results indicated that the relative expression of *MrIMD* in examined tissues had different patterns compared to the 2X PBS group. In muscle, the expression levels of *MrIMD* were significantly up-regulated at 6 hour-post injection (hpi), rapidly decreased at 12 to 36 hpi, and then returned to basal level at 48 hpi (Figure 19A). Contrary, in the gills, the expression level of *MrIMD* was significantly increased at 24 hpi (Figure 19B). However, in the intestine, the expression of *MrIMD* was significantly down-regulated at 3 to 24 hpi, and then significantly up-regulated at 36 hpi, but rapidly decreased at 48 hpi after *A. hydrophila* infection (Figure 19C).

Furthermore, similar expression patterns of *MrIMD* were found in hemocytes and hepatopancreas. In the hepatopancreas, the expression of the *MrIMD* increased at 36 hpi after *A. hydrophila* infection (Figure 19D). While there was no difference in the expression of the *MrIMD* in the hemocytes compared to the control group at all timepoint (Figure 19E).

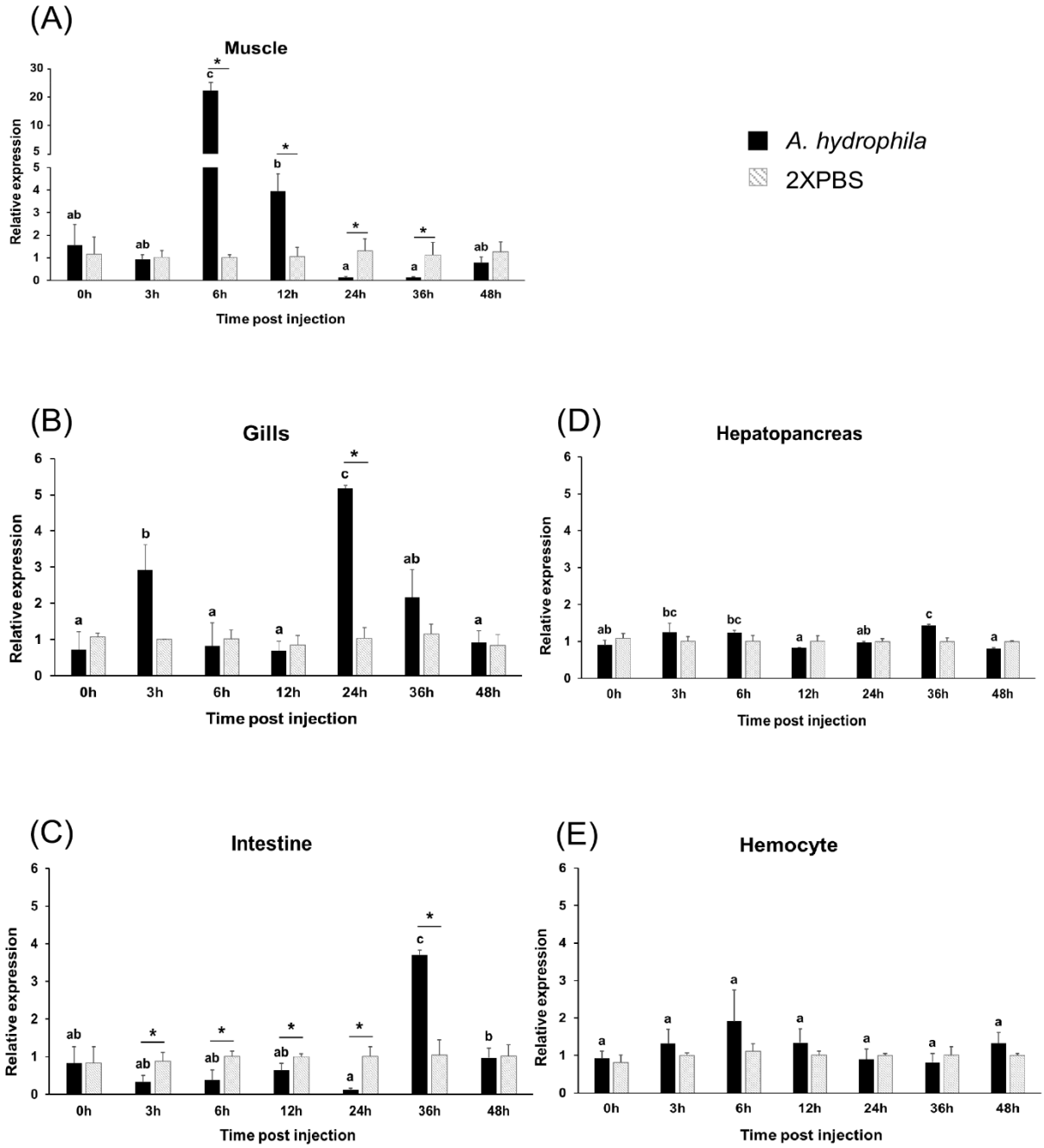


Figure 19 The expression pattern of *MrIMD* after *A. hydrophila* challenge.

The data shows the mean ± SD (n=5). Asterisks (*) indicated significant differences ($p < 0.05$) between *A. hydrophila* challenged group with the 2X PBS groups and the letters a, b, and c showed significant differences ($p < 0.05$).

Hour-post injection (hpi)	0	3	6	12	24	36	48
Average number of bacterial colony-forming units (CFU/mL)	0	$8.2x10^4$	$7.8x10^4$	$6x10^5$	$1.2x10^5$	$1.7x10^5$	$9.3x10^4$

Figure 20 Average number of bacterial colony-forming units (CFU/mL) at different time points in hemolymph after injection with *A. hydrophila*

The results of bacterial counting the number of bacterial colony-forming units in hemolymph (CFU/ml) at different time points (3, 6, 12, 24, 36 and 48 hpi) after injection with *A. hydrophila* showed an increase in CFU of *A. hydrophila* over time, which were $8.2x10^4$, $7.8x10^4$, $6x10^5$, $1.2x10^5$, $1.7x10^5$, and $9.3x10^4$ CFU/ml, respectively, with the highest count at 12 hpi ($6x10^5$ CFU/ml) (Figure 20). . The counts then decreased slightly at 24, 36, and 48 hours but remained relatively high. Colonies of *A. hydrophila* were randomly collected for bacterial confirmation using PCR analysis as described in section 6.1.2.

5. Expression pattern of *MrIMD* upon *Mr*NV challenge

This study dividing shrimp into two groups, the viral group and a control group. The viral group was subjected to viral injection with 50 µL of *Mr*NV inoculum, while the control group was injected with 50 µL of TN buffer. The real-time RT-PCR results demonstrated the relative expression of *MrIMD* response to the viral injection in various organs. Notably, the *MrIMD* was significantly up-regulated in the hepatopancreas of the experimental group at 6 and 7 day post-injection (Figure 21A). Conversely, a significant down-regulated expression of *MrIMD* was observed in the gills at 1 dpi (Figure 21B). In the muscle, the *MrIMD* was significantly up-regulated at 1, 2, and 7 dpi (Figure 21C).

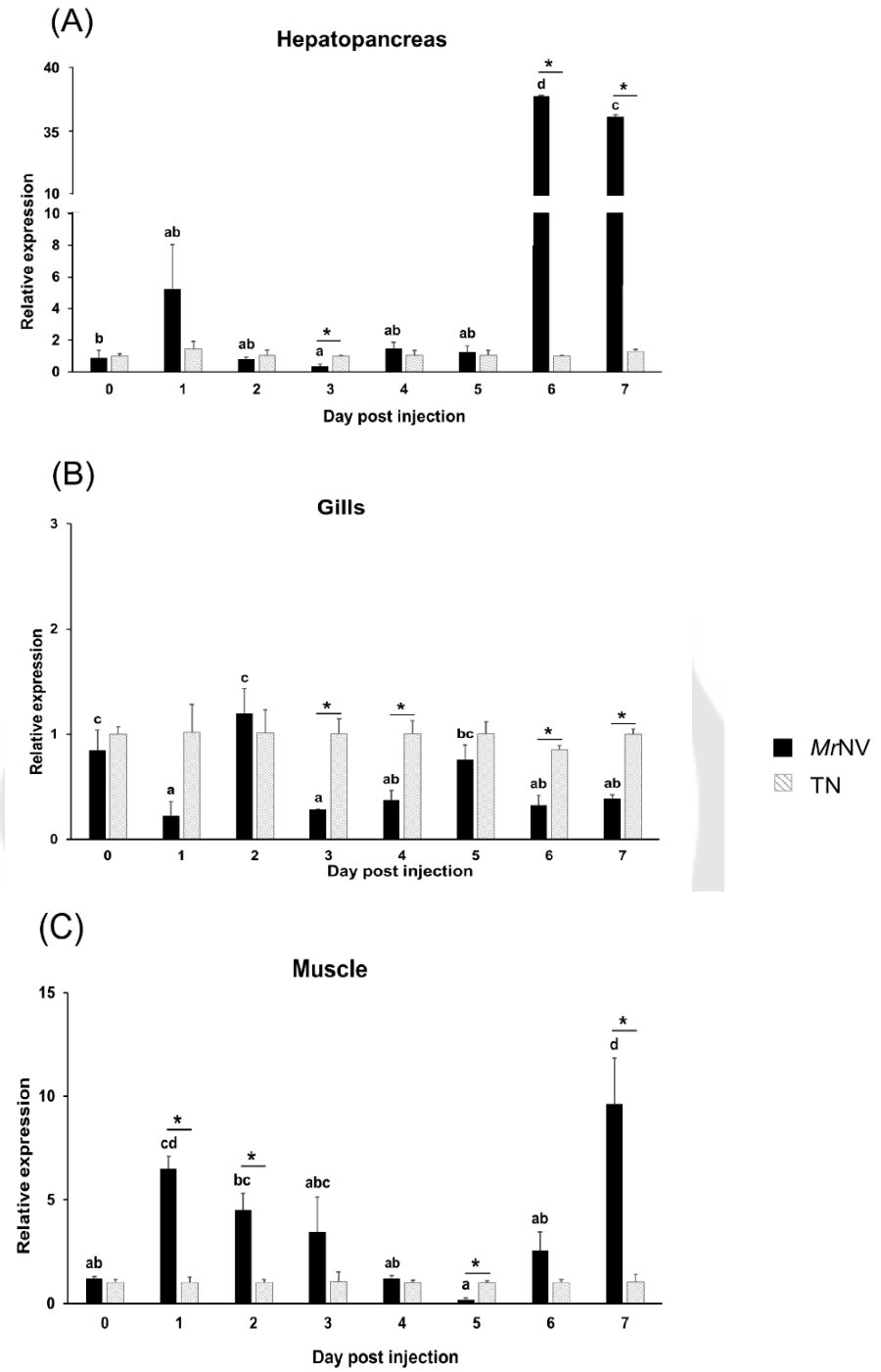


Figure 21 The expression of *MrIMD* after *Mr*NV challenge in different organs.

(A) hepatopancreas, (B) gills, and (C) muscle. The data was shown as mean ± SD (n = 3). Asterisks (*) and letters a, b, and c indicated significant differences ($p < 0.05$).

CHAPTER 5
DISCUSSIONS AND CONCLUSIONS

IMD protein is an adapter containing a death-domain protein and involved in intracellular signaling pathways. The death domain of IMD protein is a highly conserved region that mediates protein-protein interactions to activate the TAK1 protein, resulting in a signaling cascade that activates the transcription factors Relish. These results induces the expression of several antimicrobial peptides, which play a crucial role in the host defense against invading pathogens (Tassanakajon et al., 2018).

The IMD molecule was first observed in *Drosophila,* and component molecules of the IMD pathway in *Drosophila* have been fully identified. Currently, IMD proteins have been identified in several shrimp species including *MjIMD* from *Marsupenaeus japonicus* (BAH86597.1), *PmIMD* from *Penaeus monodon* (AVD73295.1) and *HaIMD* from *Homarus americanus* (XP_042243584.1) (unpublic journal), *LvIMD* from *L. vannamei* (Wang et al., 2009) , *FcIMD* from *F. chinensis* (Feng et al., 2014; Lan et al., 2013), *Pc*IMD from *P. clarkii* (Lan et al., 2013)*, MjIMD* from *M. japonicus* (BAH86597.1), *HaIMD* from *H. americanus* (XP_042243584.1), and *MnIMD* from *M. nipponense* (Li et al., 2018) and several crab species including as *SpIMD* from *Scylla paramamosain* (Zhou et al. 2018), *Pt-IMD* from *Portunus trituberculatus* (Zhou et al. 2022), *EsIMD* from *Eriocheir sinensis* (Bai et al. 2020), and the recent study, *cqIMD* from *Cherax quadricarinatus*, which have the ability to activate different types of antimicrobial peptides. However, the protein component and function of the IMD pathway in crustacean organisms have not been clearly identified.

In this study present, the IMD gene was isolated from *M. rosenbergii* for the first time, and named *MrIMD*. Sequence analysis results showed that the complete *MrIMD* gene nucleotide sequence was 1127 nucleotides long, consisting of a 379 nucleotides 5'-UTR and a 193 nucleotides 3'-UTR, with an open reading frame (ORF) of 555 nucleotides that translated into 184 amino acid residues. The results from the protein structure analysis revealed that the death domain (DD) region consisted of 96 amino acids (positions 88 -183) on the carboxylic end (C-terminus).

Pairwise alignment analysis revealed that the *Mr*IMD protein shared the highest identity and similarity with *Mn*IMD from *M. nipponense*, corresponding to the result of multiple sequence alignment which showed that *Mr*IMD proteins shared the highest consensus sequence with *M. nipponens* and the results also indicated that IMD proteins were highly conserved in various shrimp species. A phylogenetic analysis was conducted using the amino acid sequences of *Mr*IMD, IMDs from various crustaceans and insects, and RIPK1 from *Danio rerio*. The results showed that the phylogenetic relationships could be divided into two groups: IMD proteins from crustaceans and IMD proteins from insects. In IMD proteins from crustaceans group could be further subdivided into two sub-clusters: sub-cluster I containing IMD proteins from other shrimp, while sub-cluster II containing IMD proteins from crab species. The *Mr*IMD protein has been classified separately from the cluster of crab species and had a closely related to the *Mn*IMD protein from *M. nipponense* and clustered together with cluster of IMD proteins from several penaeid shrimps.

The real-time PCR results revealed that *MrIMD* gene was expressed all examined tissues, with the highest expression levels observed in gills and hepatopancreas, and low expression in stomach, intestine, heart, hemocyte, and muscle (Figure 16). This is consistent with previous studies such as in *L. vannamei* (Wang et al., 2009) and *F. chinensis* (Feng et al., 2014; Lan et al., 2013) which IMD genes were expressed in all organs, with the highest expression found in gills. In particular, the expression pattern of the *MnIMD* gene in *M. nipponense* was very similar to that of the *MrIMD* gene (Li et al., 2018), with high expression in the gills and hepatopancreas, but low expression in hemocytes and muscle. This may be due to the fact that *M. rosenbergii* and *M. nipponense* are in the same genus, and therefore their IMD genes may have similar functions.

To investigate the role and function of the *MrIMD* gene, immune response was studies by injection of *A. hydrophila* and *Mr*NV. The result showed that the *MrIMD* gene had different response patterns in organs studied. In the case of *A. hydrophila* infection, the expression of *MrIMD* gene was significantly increased in the muscle at 3 to 6hpi, in

the gills at 24 hpi and the expression levels decreased to basal levels at 48hpi. These findings corresponds to previous reports that the expression of IMD genes in other crustaceans, such as *F. chinensis*, *P. clarkii* (Lan et al., 2013), *C. quadricarinatus* (Chen & Wang, 2022) and *M. nipponense* (Li et al., 2018) were rapidly up-regulated within the first 6 hour after bacterial stimulations. In contrast, the expression of *Mr*IMD gene in the intestine was continuously down-regulated at 3 to 24 hpi and suddenly up-regulated at 36 hpi. While it has been reported in *C. quadricarinatus* that the expression of *Cq*IMD was decreased in muscles (Chen & Wang, 2022). The study of *M. rosenbergii* against *A. hydrophila* was found histopathological changes were observed in the affected organs including gills, hepatopancreas, and heart, as well as muscular damage, but there was no observed stimulation in the increase of hemocyte count (Sabili et al. 2015; Fadel and Elamie 2019). This is consistent with our report that no changes the expression of the *Mr*IMD gene in hemocytes after stimulation with *A. hydrophila* infection

Furthermore, when studying the response of the IMD gene to *Mr*NV infection, the results showed that the expression of the *Mr*IMD gene was predominantly induced in the hepatopancreas at 6 dpi and in the muscle at 7 dpi. Meanwhile, *Mr*IMD was decrease in expression was observed in the gills from 3 to 7 dpi. These findings are similar to previous reports in *F. chinensis* (Lan et al. 2013) and *L. vannamei* (Wang et al. 2009), which found that viral infection could repress the expression of the IMD gene after White Spot Syndrome Virus (WSSV) stimulation. This report suggested that IMD gene had the ability to respond to both bacterial and viral infections, with distinct patterns observed in different organs.

In summary, the *Mr*IMD gene was identified in *M. rosenbergii*. The *Mr*IMD consisted of a coding sequence of 555 bp that translated to a protein of 184 amino acids. The carboxylic end of the sequence contains a death domain. Our study showed that *Mr*IMD expression was significantly up-regulated and down-regulated during bacterial and viral infections, with distinct expression patterns observed in different organs. This study indicated the *Mr*IMD proteins as essential components of the IMD

signaling pathway, which plays a crucial role in the immune system's response to bacterial and viral infections.

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