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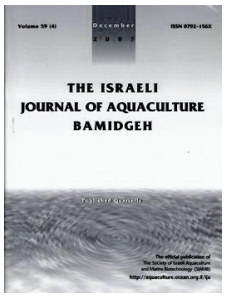


# Molecular Characterization of MyD88 in *Pinctada fucata* and its Response to Lipopolysaccharides and Polyinosinic-cytidylic Acid

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**Keywords:** *Pinctada fucata*, *MyD88*, LPS and poly (I: C), gene expression.

**Abstract**

Myeloid differentiation factor 88 (MyD88) is a key and essential adapter involved in the interleukin-1 receptor (IL-1R) and toll-like receptor (TLR)-mediated activation signaling pathway. To investigate molecular characterization of MyD88 and its gene expression profile in response to stimulation by lipopolysaccharide (LPS) and polyinosinic-cytidylic acid (poly (I: C)), we isolated the MyD88 cDNA sequence in *Pinctada fucata* and analyzed expression patterns using quantitative real-time PCR. Sequence analysis indicated that *Pf-MyD88* cDNA is 1463bp in length and contains a1050bp open reading frame that encodes a 349 α peptide. Pf-MyD88 has the highest similarity with homologues of *Crassostrea gigas* and highly conserved death and toll/IL-1R domains. Furthermore, during LPS and poly (I:C)-stimulated experiments in the gill, peak expression levels of *Pf-MyD88* were detected at 2h and 8h with a 1.58-fold and 3.58-fold increase, respectively. The results revealed the existence of a MyD88-dependent signaling pathway in *P. fucata* and contributed to understanding the potential role of *Pf-MyD88* in the TLR/IL-1R-mediated signaling pathway.

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## Introduction

The innate immune system is the first line of defense in an organism against invading pathogens and almost the only invertebrate defense mechanism to protect the host from microbial pathogens (Akira et al., 2006). The recognition of pathogen-associated molecular patterns (PAMPs) by various pattern recognition receptors (PRRs) can trigger signaling pathway-mediated immune responses to generate effectors and defend against these intruders (Medzhitov and Janeway, 2002). One well-characterized PRR is the family of toll-like receptors (TLRs), only present in microorganisms, that detect various kinds of PAMPs such as lipopolysaccharides (LPS), peptidoglycans (PGN), polyinosinic-cytidylic acid (poly(I:C)), β-glycan of fungi and lipoproteins of various pathogens (Akira et al., 2006; Mogensen, 2009).After PAMP recognition, the intracellular toll-IL-1R (TIR) domain of TLRs recruits the adaptor molecule myeloid differentiation factor 88 (MyD88) which can interact with the death domains (DDs)of interleukin-1 receptor (IL-1R)-associated kinase (IRAK) family members (including IRAK1, IRAK2, IRAK4, and IRAK-M) and tumor necrosis factor receptor-associated factor6 (TRAF6) (West et al., 2006; Kawai and Akira, 2011; Moresco et al., 2011; Ren et al., 2014).

Gene knockout studies in wild-type mice have indicated that the lack of MyD88 may lead to larger periapical lesions, with a severe inflammatory infiltrate and a significantly higher number of neutrophils (Bezerra da Silva et al., 2014). To date, MyD88 has been identified in mammals, birds, reptiles, amphibians, fishes and invertebrates (Deepika et al., 2014; Li et al., 2011; Prothmann et al., 2000; Wheaton et al., 2007). In invertebrates, *MyD88* cDNA has been characterized in *Apostichopus japonicus* (Lu et al., 2013), *Hyriopsis cumingii* (Ren et al., 2014), *Chlamys farreri* (Qiu et al., 2007), *Litopenaeus vannamei* (Zhang et al., 2012), *Ruditapes philippinarum* (Lee et al., 2011), and *Drosophila* (Horng et al., 2001). The MyD88 protein consists of three functional domains: the carboxyl terminal TIR domain, which is essential in the interactions between TLRs and MyD88; the intermediate domain; and the N-terminal DD, which is associated with the DD of IRAK family members and plays an important function in death signal transduction, regulation of apoptosis, and the inflammatory response (Kawai and Akira, 2007; West et al.,2006).

*Pinctada fucata* is an important commercial marine bivalve mollusk that is widely used to culture pearls. In both invertebrates and vertebrates, although MyD88 plays key roles as an adapter protein of toll in the toll signaling pathway, there is little information about innate immune systems in bivalve mussels, especially in *P. fucata*. Consequently, to characterize *Pf-MyD88* and further understand its role *in vivo* upon stimulation with LPS and poly(I:C), we analyzed *Pf-MyD88* sequence and the influence of these two immunostimulants on *Pf-MyD88* expression patterns in *P. fucata*. This research could provide useful information in improving understanding of the innate immune system in *P. fucata*.

## Materials and methods

*Animals and stimulation experiment. P. fucata* were obtained from Lingshui in Hainan Province, China. Before the initiation of the injection trial, adults (body weight 26.11 ± 2.52 g; shell length 5.30 ± 0.13 cm; shell width 5.01 ± 0.19 cm) were held (50 shell/tank) in 300 L tanks with circulating seawater (temperature 22 ± 0.5°C). After two weeks, *P. fucata* were randomly distributed into two groups with three replicates per group (n = 50). Shells were injected intramuscularly with LPS (0.1 mL, 1 mg/L) and poly (I:C) (0.1 mL, 1 mg/L, Sigma-Aldrich, St. Louis, MO, USA) or the same volume of PBS (the control).

In order to investigate the LPS and poly (I:C) effects on expression of *MyD88* in *P. fucata* gills, shellfish at 0, 2, 4, 8, 12, 24, 48 and 72 h post-injection were dissected and gills from all groups (n = 5) were immediately collected and snap-frozen in liquid nitrogen.

Total RNA isolation and reverse transcription. Total RNA samples were extracted from different tissues using a Trizol kit (Promega, Madison, WI, USA), and RNA quality and quantity (concentration) were measured by NanoDrop 2000 spectrophotometer (ThermoScientific, Waltham, USA). A PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) was used to synthesizec DNA. Two micrograms of RNA and 0.5 μg of Oligo $d(T)_{16}$ were reacted for 5 min at 70°C. After incubation for 2 min on ice, the mixture was reversely transcribed with 200 units of M-MLV reverse transcriptase,

5×buffer, 25 units RNasin and 0.8 mM dNTPs in a total volume of 25 μL for 1 h at 42°C.The cDNA was stored at −20°C until used.

*Molecular cloning and sequencing.* Based on the conserved sequences of *C. gigas* and *C. farreri* MyD88 (AFX68459.1, ABB76627.1), we designed gene-specific primers (Table 1) to clone the open reading frame (ORF) of *MyD88*. The PCR products were ligated into a pGEM®-T easy vector (Promega), respectively, and then sequenced on an ABI 3730XL Automated Sequencer using Sequencing Analysis 5.2.

**Table 1.** Primers used for cloning and expression.

| Gene | Primer sequences (5'–3') | Tm (°C) | Application |
|---|---|---|---|
| *MyD88-F* | CGGTGAACAATGGCTATG | 56 | ORF |
| *MyD88-R* | AATGTGGCGTTTCGTCTT | | |
| *MyD88-qRT -F* | CAGACAATAGTAGCATCAAGGACG | 58 | Real-time PCR |
| *MyD88-qRT -R* | AAGCCAGCACATTCAGCAAG | | |
| *18s RNA-F* | TCTCTGCCCTATCAACTTTC | 58 | Real-time PCR |
| *18s RNA -R* | TGTGGTAGCCGTTTCTCA | | |

*Bioinformatic analysis.* Nucleotide and amino acid sequence similarity searches were performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to predict the coding sequences of *MyD88*. The molecular weight (Mw), theoretical isoelectric point (pI) and features of the predicted proteins were obtained by ExPASy analysis (http://us.expasy.org/tools), and the SignaIP 4.1 Serverwas used for signal peptide prediction (http://www.cbs.dtu.dk/services/). Multiple sequence alignments were performed using Clustal X2 software, and phylogenetic trees were constructed with MEGA 5.1 program using the unweighted pair group method with arithmetic (UPGMA) method.

*Gene expression analysis.* Gene expression was analyzed by quantitative real-time PCR (qRT-PCR; Bustin et al., 2009). Specific primer pairs for *MyD88* and reference gene *Ef1a* (elongation factor 1, alpha) were obtained (Table 1). The qRT-PCR was performed in 20 μL total volume containing 10 μL SYBR Green qPCR Master Mix (Toyobo, Osaka, Japan), 50 ng cDNA, 0.3 μM of each primer and RNase-free $H_2O$. The qRT-PCR program consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of amplification 7 s at 95°C, 10 s at specific annealing temperatures (Table 1), 15 s at 72°C, and final extension for 10 min at 72°C in a Light Cycler® 480 II (Roche, Basel, Switzerland). Relative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

*Statistical analysis.* The data were presented as mean ± SE in triplicate for each sample. Statistical analysis was performed using one-way ANOVA, and Duncan's test was used for multiple comparisons. Differences were considered to be significant at $p < 0.05$.

## Results

*Cloning and sequence analysis of Pf-MyD88.* The ORF sequences of *Pf-MyD88* were amplified and identified. Bioinformatic analysis revealed that the *MyD88* ORF (GenBank accession no. KT894820) was 1463 bp in length and encoded a polypeptide of 349 amino acids. The predicted MyD88 protein had a molecular mass of 39 kDa with an isoelectric point of 5.58. No peptide signal was predicted in the amino acid sequence of *MyD88*. Signal P 4.1 analysis showed that a signal peptide was absent in MyD88.

*Multiple alignment and phylogenetic analysis of Pf-MyD88.* The MyD88 protein sequence contained two conserved domains, a typical DD and a conservative TIR domain, which are clearly identified in positions between13–109 and 168–305 (Fig. 1). The deduced amino acid sequence of *Pf-MyD88* shared 35–57% identity with other

species. Moreover, homologues of the DD and TIR domains were moderately conserved in all species tested (Table 2). These results suggest that the Pf-MyD88 protein probably has discriminative immune regulation functions as observed in other shellfish and vertebrates.

**Table 2.** Comparison of amino acid sequence, death and toll/IL-1R domain of *P. fucata* MyD88 with the orthologues of other species.

| *Species* | *Identity (%)* | *Full length amino acid* | *death domain* | *Toll/IL-1R domain* |
|---|---|---|---|---|
| *Crassostrea gigas* | (%) | 57 | 60 | 75 |
| *Mizuhopecten yessoensis* | (%) | 42 | 45 | 56 |
| *Chlamys farreri* | (%) | 41 | 41 | 57 |
| *Haliotis diversicolor* | (%) | 41 | 39 | 54 |
| *Lottia gigantea* | (%) | 39 | 35 | 51 |
| *Mytilus galloprovincialis* | (%) | 36 | 38 | 56 |
| *Apostichopus japonicus* | (%) | 36 | 33 | 46 |
| *Danio rerio* | (%) | 36 | 36 | 43 |
| *Gallus gallus* | (%) | 36 | 37 | 44 |
| *Mus musculus* | (%) | 36 | 40 | 42 |
| *Homo sapiens* | (%) | 35 | 36 | 42 |

A phylogenetic tree analysis of MyD88 in *P. fucata* and other metazoans was constructed (Fig. 2). It revealed that Pf-MyD88 was grouped together with other members of the Pterioida family, such as *C. gigas*. The homology of Pf-MyD88 from near to far was other mollusca, fishes, amphibians, birds, reptiles and mammals. These results correspond with conventional taxonomy.

Effect of LPS and poly (I:C) on MyD88 expression in *P. fucata* gills. As shown in Fig. 3, the transcript levels of *MyD88* indicated a strong response to LPS and poly (I:C) induction. *MyD88* expression predominantly increased to highest levels at 2h post-injection with LPS, approximately 1.57 times the level normally observed in *P. fucata*, then subsequently declined (Fig.3A). Moreover, the *MyD88* mRNA expression fluctuated during the whole experimental period, it reached a peak at 8 h post-injection with poly (I:C), then significantly reduced until 12 h post-injection, but was still lower than the control even at the end of the experiment (Fig. 3B).

**Fig. 1.**

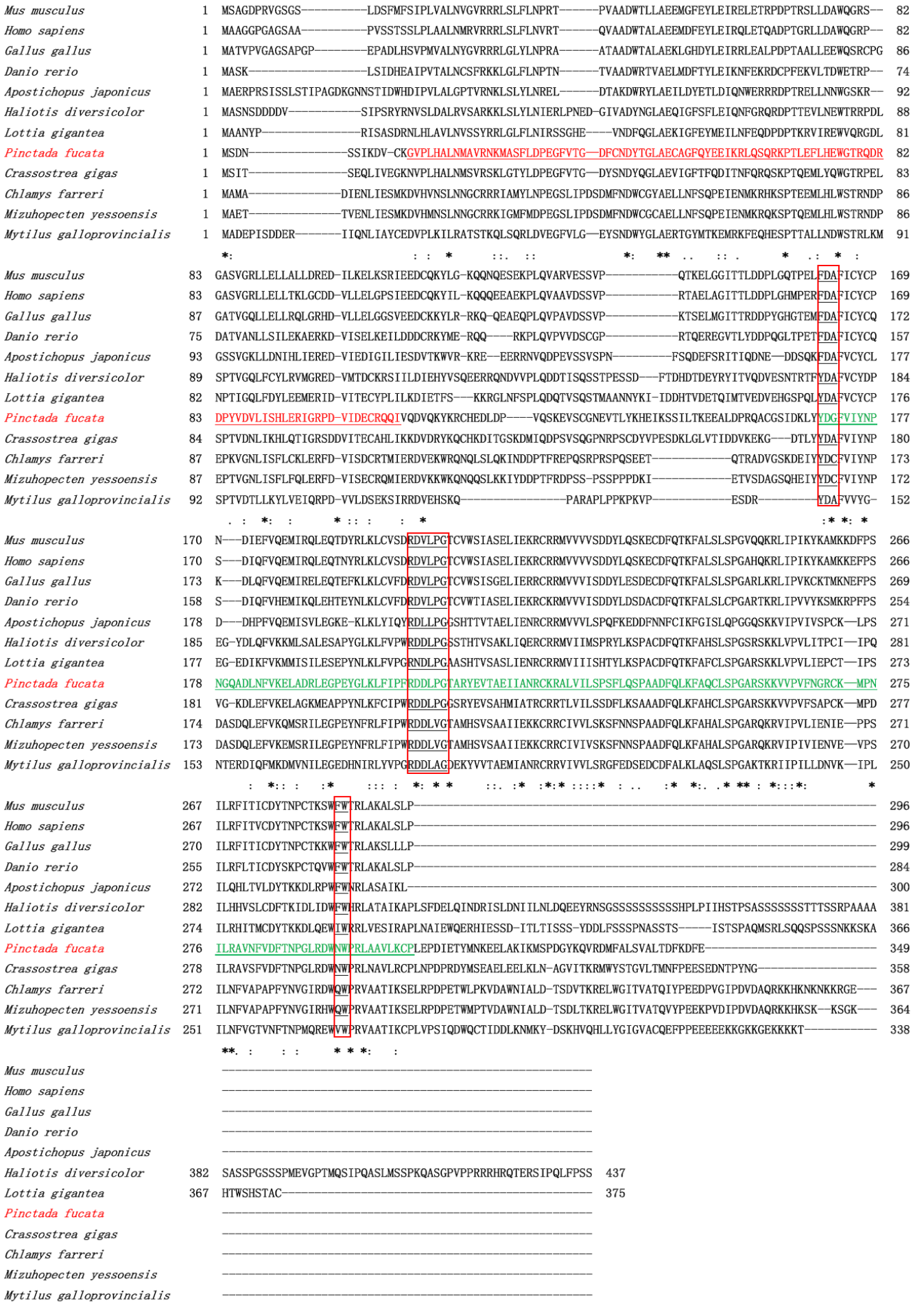

Mus musculus
Homo sapiens
Gallus gallus
Danio rerio
Apostichopus japonicus
Haliotis diversicolor
Lottia gigantea
Pinctada fucata
Crassostrea gigas
Chlamys farreri
Mizuhopecten yessoensis
Mytilus galloprovincialis

**Fig. 2.**

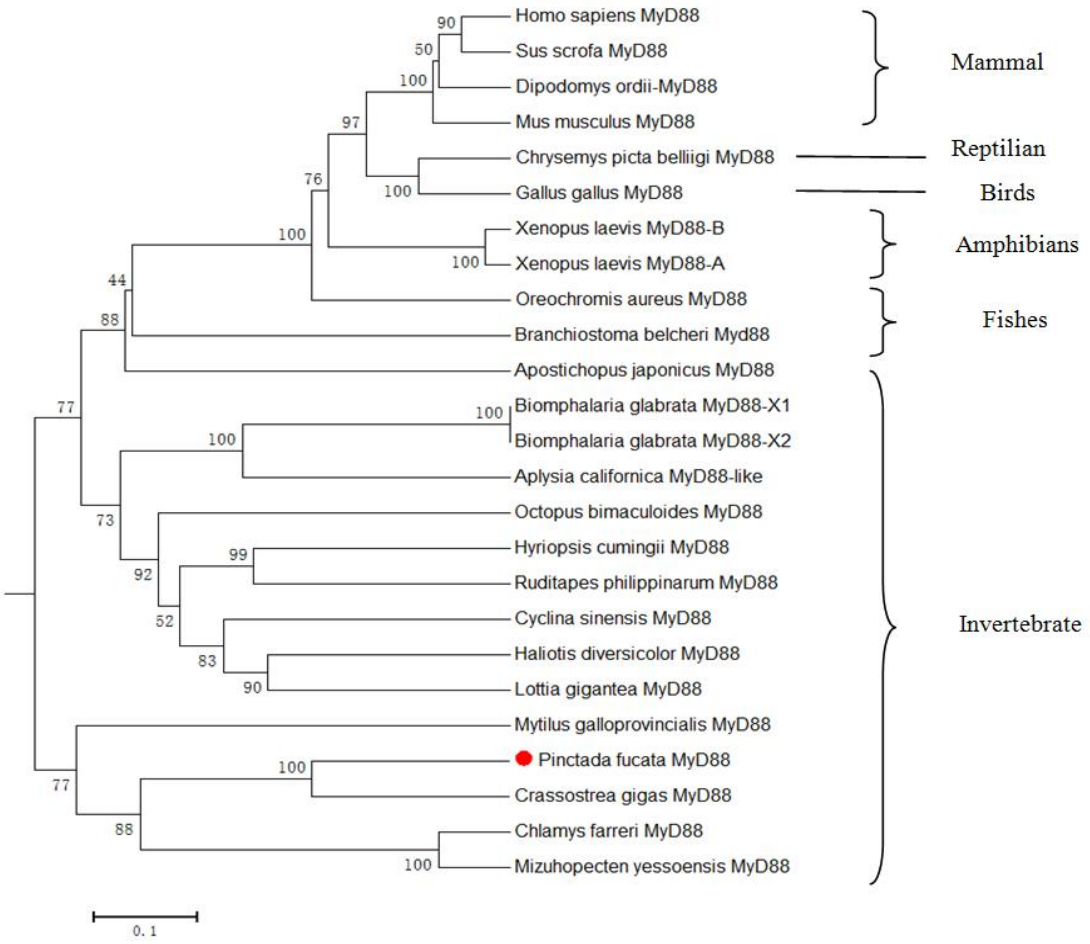
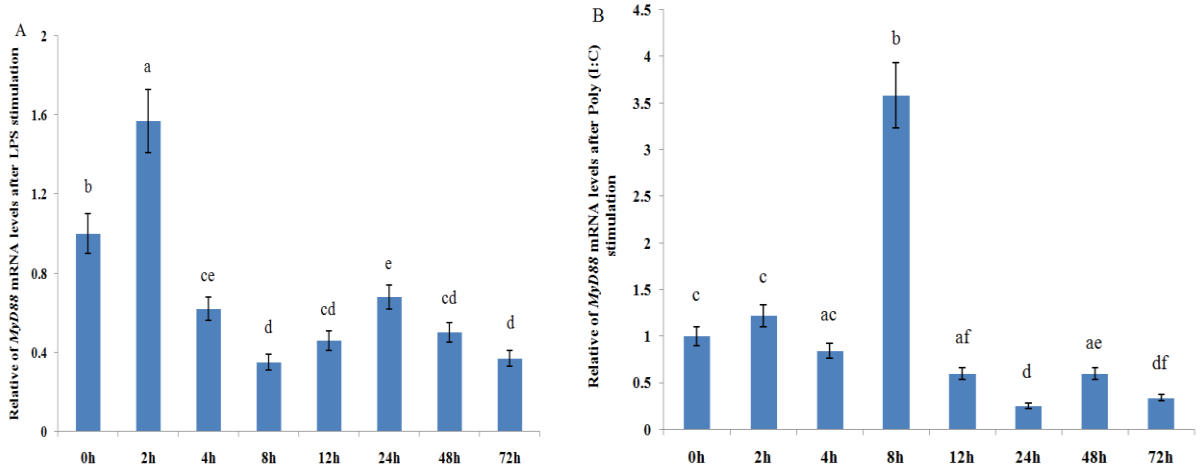

Homo sapiens MyD88
Sus scrofa MyD88
Dipodomys ordii-MyD88
Mus musculus MyD88
Chrysemys picta belliigi MyD88
Gallus gallus MyD88
Xenopus laevis MyD88-B
Xenopus laevis MyD88-A
Oreochromis aureus MyD88
Branchiostoma belcheri Myd88
Apostichopus japonicus MyD88
Biomphalaria glabrata MyD88-X1
Biomphalaria glabrata MyD88-X2
Aplysia californica MyD88-like
Octopus bimaculoides MyD88
Hyriopsis cumingii MyD88
Ruditapes philippinarum MyD88
Cyclina sinensis MyD88
Haliotis diversicolor MyD88
Lottia gigantea MyD88
Mytilus galloprovincialis MyD88
Pinctada fucata MyD88
Crassostrea gigas MyD88
Chlamys farreri MyD88
Mizuhopecten yessoensis MyD88
0.1
Mammal
Reptilian
Birds
Amphibians
Fishes
Invertebrate

**Fig. 3.**


A
Relative of MyD88 mRNA levels after LPS stimulation
0h
2h
4h
8h
12h
24h
48h
72h
B
Relative of MyD88 mRNA levels after Poly (I:C) stimulation

## Discussion

The ORF of *Pf-MyD88* was identified in this paper. Unfortunately, the full length of *Pf-MyD88* was not cloned. However, two typical conserved structural domains, DD and TIR, were determined in the Pf-MyD88 protein (Qin et al., 2015). This was consistent with MyD88 proteins in other species (Liu et al., 2007; Ren et al., 2014; Huang et al., 2014).

DD is typically located in the C-terminal sequence (Feinstein et al., 1995). However, the DD in MyD88 and IRAKs (Interleukin-1 receptor-associated kinases, IRAKs) of the TLR signaling pathway is located in the N-terminal region (Medzhitov et al., 1998). The Pf-MyD88 DD was found to be located at amino acid positions 13–109 of the N-terminal region in our study. In eukaryotic host organisms, the TIR domain is regarded nearly exclusively between TLRs and the association between TLRs and TIR domain-containing adaptors (Barton and Medzhitov, 2003). Three highly conserved regions (box1–3), which play a key role in TIR function and exist in most TIRs, were also present in Pf-MyD88. Nevertheless, *Artemia sinica* lacks box 3, and box1 and box2's positions are reversed (Qin et al., 2015). Two hydrophobic acids ($Leu^{210}$ and $Pro^{211}$), known to be essential for the interaction of TLRs with MyD88 in box2 of Pf-MyD88 (Xu et al., 2000), were consistent with MyD88 proteins from *C. gigas* (Du et al., 2013), *A. japonicus* (Qin et al., 2015), and *R. philippinarum* (Lee et al., 2011).While these are Ile and Pro in *H.cumingii* (Ren et al., 2014), Ile and Gly in shrimp (Zhang et al., 2012), and Leu and Val in *C. farreri* (Qiu et al., 2007). Overall, two amino acids (Leu and Pro) were identified unanimously in most vertebrates in box2, and were likely to be more diverse in invertebrates than in vertebrates (Zhang et al., 2012; Ren et al., 2014).

Protein alignments with other species indicated that the Pf-MyD88 shared the highest homology with *C. gigas*, consistent with the fact that both *P. fucata* and *C. gigas* were members of the Pterioida superfamily. However, there was a significant difference in protein sequences of MyD88 between invertebrates suggested that Pf-MyD88 demonstrated relatively low similarity to other species(Fig. 2).

In order to better evaluate the functional role of *Pf-MyD88*, particularly in relation to endotoxin exposure and virus analog induction, the expression of *Pf-MyD88* mRNA after immune stimulation was researched. The gram negative bacterial endotoxin LPS has been reported as a powerful stimulator of innate immunity and PAMP in various eukaryotic organisms (Qiu et al., 2007). Moreover, poly(I:C), a synthetic analog of double-stranded RNA, is another typical PAMP that mimics viral infection. It has been reported that *R. philippinarum* MyD88 was up-regulated in gills and hemocytes after immune challenge with both a *Vibrio tapetis* and LPS challenge (Lee et al., 2011). It was suggested there was an up-regulation of MyD88 transcript levels in response to LPS, CpG oligodeoxynucleotide (CpG-ODN) and turbot reddish body iridovirus (TRBIV) treatment in the *Scophthalmus maximus* head kidney, spleen, gills and muscle over a 7-day time course (Lin et al., 2015). Moreover, *MyD88* transcripts significantly increased in response to experimental exposure to LPS, PGN, and poly (I:C) in *Paralichthys olivaceus* peripheral blood leukocytes (Takano et al., 2006). In addition, during acute viral infection and periapical lesions in mice, MyD88 has been demonstrated to play an important role in regulating inflammatory responses (Bezerra da Silva et al., 2014; Butchi et al., 2015). In the present study, the up-regulation of MyD88 mRNA expression was stronger and arose earlier in the case of LPS treatment in gills during a 7-day time course, with 1.58-fold increases at 2 h post-injection, relative to poly (I:C) treatment which increased 3.58-fold at 8 h post-injection. Taken together, these experiments indicate that *Pf*-MyD88 may serve as an important innate immune response gene during the early stage of endotoxin and virus infections in *P. fucata*.

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## Figure Legends:

**Fig. 1.** Comparison of deduced amino acid sequences of *Pinctada fucata* MyD88 with published MyD88s in other species. The moderately conserved Death and TIR domains are underlined in red and green, respectively. Identical amino acid residues are represented by stars. Dashes represent gaps created to maximize the degree of similarity among all compared sequences. The red boxed areas are labeled to indicate the Box1, Box2 and Box3 positions in TIR domain. The accession numbers of the sequences used are listed in supplement table 1.

**Fig. 2.** Phylogenetic analysis of *P. fucata* MyD88 relative to the homologues of other vertebrates. Sequence alignment of MyD88 was analyzed using the MEGA 5.0 software with Neighbor-joining method. The numbers at each node indicate the percentage of bootstrapping after 1000 replications. The accession numbers of the sequences used in the phylogenetic analysis are listed in supplement table.

**Fig. 3.** Expression profiles of *MyD88* in gills of *P. fucata* after LPS (A) and Poly (I:C) (B) challenge. Significant differences at $P<0.01$ are labeled with different letters, mean ± SEM of each mRNA quantity is shown for each stage tested.

## Supplement table 1

The accession numbers of the sequences used in the phylogenetic analysis.

| *Species* | *GenBank No.* | *Species* | *GenBank No.* |
|---|---|---|---|
| *Danio rerio* | AAI64642.1 | *Chrysemys picta bellii* | XP_005297196.1 |
| *Apostichopus japonicus* | AHA83603.1 | *Gallus gallus* | NP_001026133.2 |
| *Xenopus laevis MyD88-B* | NP_001089255.1 | *Xenopus laevis MyD88-A* | NP_001081001.1 |
| *Mus musculus* | AAC53013.1 | *Homo sapiens* | AAC50954.1 |
| *Crassostrea gigas* | AFX68459.1 | *Azumapecten farreri* | ABB76627.1 |
| *Mizuhopecten yessoensis* | AKN04685.1 | *Haliotis diversicolor* | AHK60398.1 |
| *Cyclina sinensis* | AIZ97751.1 | *Mytilus galloprovincialis* | AFR54116.1 |
| *Biomphalaria glabrata-X1* | XP_013086371.1 | *Biomphalaria glabrata-X2* | XP_013086405.1 |
| *Lottia gigantea* | XP_009046476.1 | *Hyriopsis cumingii* | AHB62785.1 |
| *Ruditapes philippinarum* | AEF32114.1 | *Aplysia californica* | XP_005094456.1 |
| *Dipodomys ordii* | XP_012885778.1 | *Branchiostoma belcheri* | ABQ32299.1 |
| *Octopus bimaculoides* | KOF72031.1 | *Oreochromis aureus* | AEK87127.1 |
| *Sus scrofa* | ABW74617.1 | *Haliotis diversicolor* | AHK60398.1 |