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Short communication

# Molecular cloning and expression of chitin deacetylase 1 gene from the gills of *Penaeus monodon* (black tiger shrimp)

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ABSTRACT

Chitin deacetylases have been identified and studied in several fungi and insects but not in crustaceans. These glycoproteins function in catalyzing the conversion of chitin to chitosan by the hydrolysis of N-acetamido bonds of chitin. Here, for the first time, the full length cDNA of chitin deacetylase (CDA) gene from crustaceans was fully cloned using a partial fragment obtained from a transcriptome database of the gills of black tiger shrimp *Penaeus monodon* that survived White Spot Syndrome Virus (WSSV) infection employing Rapid Amplification of cDNA Ends (RACE) PCR. The shrimp CDA, named *Pm*CDA1, was further characterized by *in silico* analysis, and its constitutive expression determined in apparently healthy shrimp through reverse transcription PCR (RT-PCR). Results revealed that the *P. monodon* chitin deacetylase (*PmCDA*1) is 2176 bp-long gene with an open reading frame (ORF) of 1596 bp encoding for 532 amino acids. Phylogenetic analysis revealed that P*mCDA*1 belongs to Group I CDAs together with CDA1 and CDA2 proteins found in insects. Moreover, *PmCDA*1 is composed of a conserved chitin-binding peritrophin-A domain (CBD), a low-density lipoprotein receptor class A domain (LDL-A) and a catalytic domain that is part of CE4 superfamily, all found in group I CDAs, which are known to serve critical immune function against WSSV. Finally, high expression of *PmCDA*1 gene in the gills of apparently healthy *P. monodon* was observed suggesting important basal function of the gene in this tissue. Taken together, this is a first report of the full chitin deacetylase 1 (CDA1) gene in crustaceans particularly in shrimp that exhibits putative immune function against WSSV and is distinctly highly expressed in the gills of shrimp.

## 1. Introduction

The chitin exoskeleton of crustaceans is an important barrier against the external environment including bacteria and viruses, as well as for maintaining hemolymph and tissue integrity [1]. To form chitin, Chitin deacetylases (CDA) is needed as theyaid in the catalysis of the acetamido group in the *N*-acetyl-D-glucosamine units of chitin [2,3]. CDA is a member of the carbohydrate esterase 4 (CE4) superfamily, sharing the NodB homology domain or polysaccharide deacetylase domain, a conserved domain in the family's primary structure [4]. CDA has been associated with various biological roles. In marine bacteria, CDA function for: chitin catabolism supporting growth, adaptation to environmental nutrient gradients, tolerance to stress, and protection from predators; and cellular communication [5]. In fungi, CDA facilitate: cell wall synthesis and spore wall formation by increasing chitosan availability, which is a critical component that allows structural rigidity and resistance to various stresses of spores [6]. In insects, CDA are involved in tracheal development and tube elongation [7,8]. To date, however, CDA has never been fully cloned in crustaceans and has never been reported in shrimps.

One of the most lethal viruses that affect shrimp is the white spot syndrome virus (WSSV). It infects most shrimps by traversing into the nuclei of host cells causing disintegration and loss of cellular structure [9]. This virus can cause complete collective mortality within a week that makes a great burden in shrimp agriculture. Because of this, studies that focus on understanding WSSV and host interaction have been carried out including the generation of transcriptome databases that could shed light on

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genes that are involved in virus pathogenesis [9,10].

Expressed sequence tags (EST) libraries and transcriptome databases serve as a pool of genetic information that may contain valuable information in discovering significant novel genes. More so when there is differential gene expression analysis between healthy and infected organism. In this study, a partial fragment obtained from a transcriptome database extracted from the gills of black tiger shrimp (*Penaeus monodon*) that survived WSSV challenge [10] was fully cloned and identified to be CDA. This study represents the first report on the presence of CDA gene in crustaceans. In addition, it provides the structure and domains of the gene and its putative function, as well as its distinctly high basal expression in gills of shrimp.

## 2. Materials and methods

### 2.1. Sample collection

For the cloning part of this study, a live adult *P. monodon* was collected from a local market in Pasay City, Philippines. For constitutive expression analysis in different tissues, three (3) live, apparently healthy *P. monodon* were collected from shrimp farms in Vitali Island, Zamboanga City, Zamboanga Sibugay, and Barangay Sta. Monica, Hagonoy, Bulacan, Philippines. These 3 samples were tested negative for WSSV by Dr. Mary Beth Maningas of the Biochemistry Laboratory, University of Santo Tomas, Philippines using her Juan Amplifier WSSV Diagnostic Kit (JAmp Kit). Upon collection, shrimps were immediately dissected and tissues including: gills, hepatopancreas, intestine, lymphoid organ, heart and hemolymph were collected then preserved in microcentrifuge tube containing RNA later.

### 2.2. Sequencing of full length PmCDA1 cDNA gene

Partial sequence was obtained from transcriptome database of *P. monodon* that survived WSSV challenge [10] available at NCBI Sequence Read Archive with accession number SRR57708030 (http://www.ncbi.nlm.nih.gov/sra). Total RNA from gills of *P. monodon* was extracted using QIAzol® Lysis Reagent (QIAGEN) following the manufacturer's protocol. Subsequently, extracted RNA samples were subjected to RNA purification using RNEasy Mini Cleanup Kit (QIAGEN) following the manufacturer's protocol. RNA yield was determined using Implen™ nanophotometer.

In the first strand cDNA synthesis, RNA template had a concentration of 0.103 μg/μl. The 10 μl reaction mix had the total concentration as follows: 0.5X first strand buffer, 2 mM DTT, 0.1 mM dNTP mix, SMARTer Oligo IIA (for 5′ cDNA synthesis), 5 U RNAse inhibitor, 10 U SMARTScribe reverse transcriptase, and 1 mM of respective primers for 5′ and 3′ cDNA synthesis. The reaction mix was subjected to 42 °C for 90 min and heated to 70 °C for 10 min in a thermal cycler. Subsequently, the samples were diluted with 20 μl of tricine-EDTA buffer.

On the other hand, RACE was carried out with the in a 25 μl reaction mix with final concentration as follows: 1X PCR buffer, 1 mM dNTP mix, 1X polymerase mix, 1X universal primer, and gene specific primers. Specific primers designed for facilitating 5′ (*PmCDA*1 5RACE-1 and *PmCDA*1 5RACE-2) and 3′ RACE (*PmCDA*1 3RACE-1) extensions are shown in Table 1. Primer annealing sites are shown in Fig. S1. The reaction mixes were subjected to the following conditions: initial denaturation at 94 °C for 3 min, 38 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min, then final extension at 72 °C for 10 min. All PCR amplicons were visualized on a 1% gel stained with ethidium bromide and documented with SynGene G:BOX. Positive amplicons were sent to Macrogen Inc. (Korea) for sequencing.

**Table 1**
Primer sequences used to amplify and sequence chitin deacetylase 1 in *P. monodon* cDNA.

| Primer name | Sequence (5′ → 3′) |
|---|---|
| *PmCDA*1 5RACE-1 | TTCTTCCACTCTGCCTTCCTG |
| *PmCDA*1 5RACE-2 | TAGCAGTAGCAATCGGGGAGGCGGCAG |
| *PmCDA*1 3RACE-1 | GCGTCGTCGACGGCCCACCCTTC |
| GSP forward | GTCATGAACGAGATGGACCG |
| GSP reverse | CAGTACTTGCTCTACCTGGCC |
| EF1α forward | ATGGTTGTCAACTTTGCCCC |
| EF1α reverse | TTGACCTCCTTGATCACACC |

### 2.3. In silico analysis

The nucleotide sequence, translated amino acids, and average molecular weight were analyzed and determined using Geneious 6.1.8 (Biomatters). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) were utilized to predict signal peptide cleavage and *N*-glycosylation sites, respectively. Nucleotide and amino acid sequence identities were determined using BLASTn and BLASTp, respectively. Complete multiple alignments were conducted using ClustalW with default parameters. Conserved Domain Architecture Retrieval Tool (CDART) (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) was used to determine conserved domains. Sequences of the members of CE4 superfamily (GENBANK: cl15692) were retrieved from NCBI. Predicted 3-dimensional structure of the protein was generated through Protein Homology/analogY Recognition EngineV2.0 (Phyre$^2$) (www.sbg.bio.ic.ac.uk/phyre2/). Phylogenetic analysis was carried out in MEGA 6 (http://www.megasoftware.net/) for the generation of tree using Maximum Likelihood (ML) method based on the Whelan and Goldman (WAG) model [29] with 500 bootstrap tests and complete deletion of sites.

### 2.4. Expression analysis

Total RNA from different tissues of three (3) apparently healthy *P. monodon* samples were extracted using QIAzol® Lysis Reagent (QIAGEN) following the manufacturer's protocol. These are then used for cDNA synthesis. The reverse transcription (RT) reaction mix had the components with final concentration as follows: 1X Buffer RT, 0.5 mM dNTPs, 1 μM Oligo-dT primer, 10 units RNase inhibitor, 4 units Omniscript Reverse Transcriptase (QIAGEN), with 0.203 μg/μl of RNA template. RT – Polymerase Chain Reaction was used to determine expression of *PmCDA*1 from different tissues of healthy *P. monodon* following [12] with modifications. Primers used for the amplification of *PmCDA*1 (GSP forward and GSP reverse) and elongation factor 1 alpha (EF1α) (EF1α forward and EF1α reverse) are shown in Table 1. The following cycling parameters: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 10 min, were used for amplification of both *PmCDA*1 and EF1α. Negative control contained no template. All PCR amplicons were visualized on a 1% gel stained with ethidium bromide and documented with SynGene G:BOX. Relative expression based on light intensity was measured using ImageJ software (http://imagej.nih.gov/ij/) [11] and standard deviations were computed.

## 3. Results and discussion

This study serves as the first report on the presence of chitin deacetylase (CDA) gene in crustaceans particularly in shrimps.

Results revealed that the shrimp CDA (GENBANK: KU156741.1), has a length of 2176 bp with open reading frame (ORF) of 1596 bp encoding for 532 amino acids (AA), and a molecular weight of 61.271 kDa (GENBANK: ALO20448.1) (Fig. 1). BLAST analysis showed 63% identity of protein query with rice grasshopper *Oxya chinensis* chitin deacetylase 1 (*OcCDA*1) and red flour beetle *Tribolium castaneum* chitin deacetylase precursor [13,14] and 59% identity with cotton bollworm *Helicoverpa armigera* chitin deacetylase 1 (*HaCDA*1) [15]. The nucleotide sequence contains 59 bp upstream-untranslated region and 518 bp downstream-untranslated region, followed by poly-adenylation signal sequence (AATAAA) and A + T rich region. Further analysis revealed that it includes a potential signal peptide in its AA positions 23 and 24, and contains *N*-glycosylation sites at AA positions 236, 260 and 288. Chitin-binding peritrophin-A domain (CBD) (AA41 to AA95), low-density lipoprotein receptor class A domain (LDL-A) (AA 115 to AA 149) and catalytic NodB homology domain of the carbohydrate esterase 4 superfamily (CE4) (CDD accession: cl15692) (AA 191 to AA 460) that includes polysaccharide deacetylase (AA 192 to 301) were also found. CE4 domain was described to have several signature motifs (TFDD, H[S/T]xxHP, RxP[Y/F], DxxDW, GxxxFxx) that render different functions, and are variable that allows distinction in enzyme activity [16]. The predicted 3D structure of *PmCDA*1 is shown in Fig. S2. The model revealed alpha helices and beta strands in the protein structures as well as conserved sites based on Conserved Domain Database (CDD) including putative catalytic sites and NodB motif.

Chitin-binding peritrophin-A domain (CBD), the first conserved domain found, is known to be significant in enhancing enzyme activity to allow prolonged interaction with the substrate [17,18]. revealed that a group of CBD containing proteins is one of the four groups of proteins that are highly up-regulated after WSSV infection suggesting its immune-related function against the virus. CBD-containing proteins were included in the fifty most abundant genes and unique genes with increased differential abundance in WSSV-infected library [9]. This supports the transcriptome database of *P. monodon* that survived WSSV challenge generated by Ref. [10] wherein CBD-containing CDA was differentially expressed in

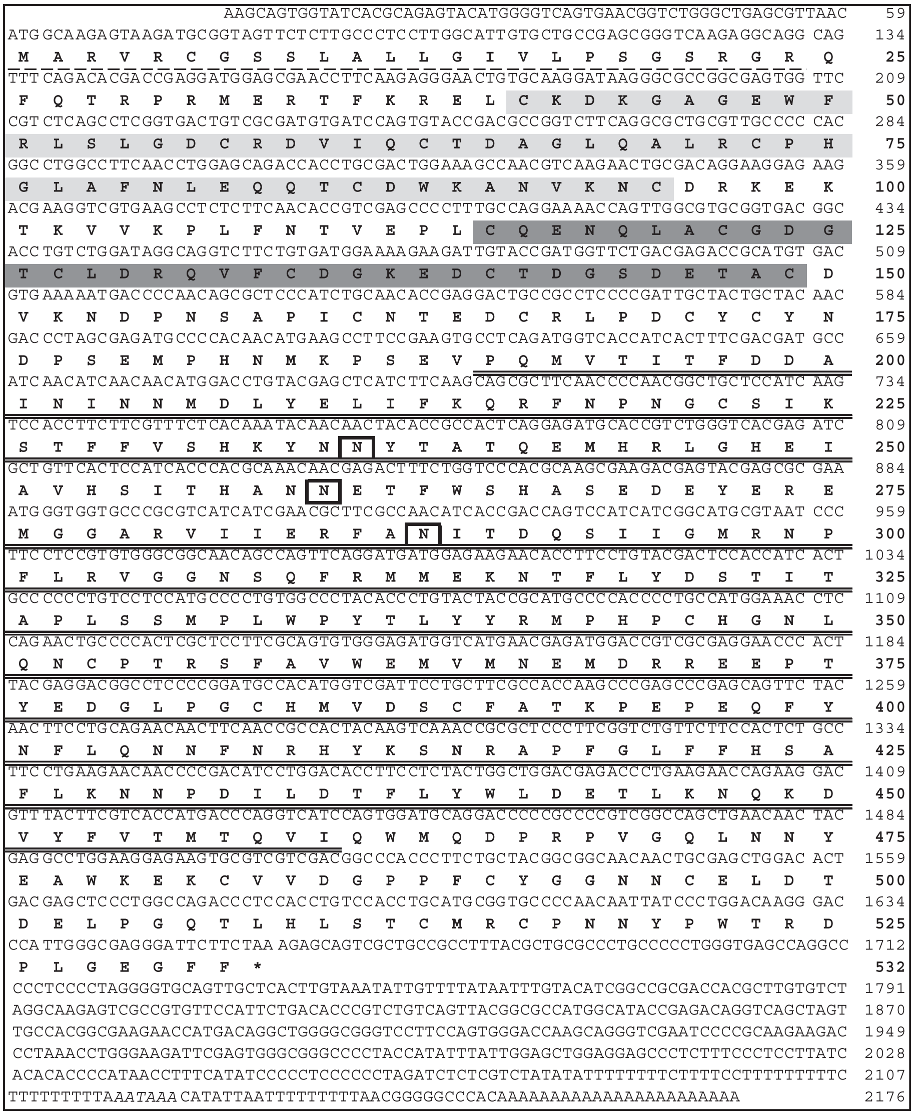

**Fig. 1.** The full-length cDNA sequence and deduced amino acid sequences of *PmCDA*1. The predicted signal peptide is underlined with a broken line. Chitin-binding peritrophin-A domain is highlighted in light gray, low-density lipoprotein receptor class A domain is highlighted in dark gray, and catalytic NodB homology domain of the carbohydrate esterase 4 superfamily is underlined with two lines and signature motifs are boxed with solid line. Predicted *N*-glycosylation sites are boxed with broken line and poly-adenylation signal is italicized.

survivor shrimp relative to apparently healthy shrimp. CBD is present in insects and animal chitinases as part of peritrophic matrix proteins, including peritrophins [19]. Peritrophin in fleshy prawn *Fenneropenaeus chinensis* was reported to have gram-negative bacteria binding activity and chitin binding activity, and was found to be highly induced in hemocytes, heart, stomach, gut, and gills of infected shrimp, predicting its role in immune defense and other physiological responses [20]. Previously reported *P. monodon* chitin-binding protein (PmCBP) and peritrophin-like protein from pacific white shrimp *Litopenaeus vannamei* (LvPT) revealed interaction between host shrimp and envelope protein of WSSV, which further strengthens the significance of the role of proteins with chitin-binding activity in shrimp-WSSV interaction [21,22]. Collectively, the presence of CBD in the shrimp CDA may also be involved in defense mechanism of *P. monodon* against WSSV.

Low-density lipoprotein receptor class A domain (LDL-A) was the second domain found in the shrimp CDA. In kuruma shrimp *Marsupenaeus japonicus*, C-type lectins (CTLs) which binds to WSSV causing decreased WSSV replication contain critical LDL-A to carry out pattern-recognition mechanisms during immune response [23]. In fact, disrupting CTLs caused increased WSSV replication and allows attachment and penetration of WSSV in shrimp [23]. Given this, LDL-A in shrimp CDA may also be predicted to have the same binding activity of pattern-recognition mechanisms in black tiger shrimp. LDL-A also occurs in insect and fungi CDAs, however no further function of the domain have been discussed in previous reports.

A putative catalytic domain of CDA proteins which serves as part of CE4 superfamily was also observed in shrimp CDA. The CE4 superfamily is comprised of different enzymes including chitin deacetylase, bacterial peptidoglycan *N*-acetylglucosamine deacetylases and acetylxylan esterases that aids in the catalysis of *N*- or *O*-deacetylation of their respective substrates [24]. Among the AA sequence of these enzymes, the NodB homology domain is always present. Previous reports about the domain mainly focuses on nitrogen-fixing bacteria that mediates synthesis ofhost-specific signal molecules [25]. However, the function of this domain has not been extensively studied in other organisms.

CDAs are suggested to occur from five to nine members, categorized into five groups (Group I – V), depending on the species [30]. These CDAs have variable assortment of domains that sometimes differ in organization. Group I includes CDA1 and CDA2 that have single and multiple isoforms, respectively. CDAs belonging to this group have a complete set of the three domains: CBD, LDL-A, and CE4. Group II includes CDA3 that has similar domain organization with CDAs from group I but are substantively different in the amino acid composition. Group III includes CDA4 that lacks LDL-A. Group IV includes CDA5 with similar organization with CDA4 only that CBD and CE4 are separated by a long glutamine-rich intervening region. Group V includes CDA5-9 that lacks CBD and LDL-A, then are only left with CE4. Since the shrimp CDA possess a complete set of CBD, LDL-A, and CE4 domains (Fig. S3), it can be grouped to Group 1.

Phylogenetic analysis using ML method involving eighteen (18) AA sequences with a total of 335 positions also revealed that the shrimp CDA belonged to group I CDAs, forming a clade together with reference sequences of CDA1 and CDA2 from various insects supported by a bootstrap value of 100% (Fig. 2). Previous studies revealed that CDA1 are found in all insect species whereas CDA2 are specific only to pupa, and that pre-mRNAs of CDA2 undergo RNA splicing and/or exon skipping to yield CDA isoforms [30,31]. These two CDAs were also distinctly differentiated based on their signature motifs in its CE4 domain, particularly TFDD motif 1 in CDA1 was substituted as TFNG in all isoforms of CDA2 that makes it inactive [30]. CDA from *P. monodon* was found to have TFDD motif 1 (Fig. 1) suggesting that it is CDA1 hence it is named here as *PmCDA1*. Differences in motifs from CDAs of different groups are further exemplified in multiple alignment of CDA sequences (Fig. S4).

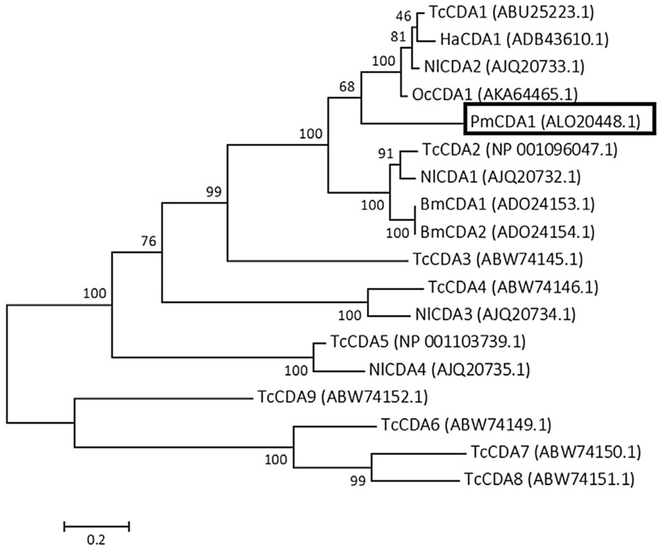

**Fig. 2.** Phylogenetic analysis of *PmCDA*1 and amino acid sequences of CE4 super family CDA members using ML method, and WAG model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Reference CDA sequences were obtained from the following organisms: red flour beetle *Tribolium castaneum* (Tc), cotton bollworm *Helicoverpa armigera* (Ha), brown planthopper *Nilaparvata lugens* (Nl), rice grasshopper *Oxya chinensis* (Oc), and silkworm *Bombyx mori* (Bm). All reference sequences obtained from GENBANK were labelled with database accession numbers.

The present study also observed that *PmCDA*1 was specifically highly expressed in the gills of black tiger shrimp (Fig. 3). Gills in shrimps serve as the predominant site for the formation of hemocyte nodules during injection of foreign particles and accumulation of viable bacteria during infection, suggesting its significant role in shrimp defense [32,33]. Gills mediate direct contact to the external environment, hence its cells play a vital role in response to biotic and abiotic factors [34]. In addition to this, *PmCDA*1 was also observed to be expressed in the muscle and lymphoid organ of shrimps. The lymphoid organ in penaeid prawns was predicted to be a major phagocytic organ that serves a major site for viral degradation [26,27]. Previous study revealed that muscle-related genes were upregulated after pathogen infection which serves as an effective way in clearing invading pathogens [28].

Expression of CDA from other species was reported to be high during early developmental stages of insects and pathogen infection in fungi. In rice grasshopper *O. chinensis*, highest expression of *OcCDA*1 was observed in the integument of early stage of its development [13]. In addition, expression of *NlCDA*1 peaked in the moulting stage of the brown planthopper *Nilaparvata lugens* suggesting its critical role in the turnover of the old insect cuticle [31]. Upregulation of CDA gene in the common mushroom *Agaricus bisporus* infected with mycoparasite *Verticillium fungicola* was previously observed [35]. In contrast, down-regulation of chitin deacetylase-like protein was observed during baculovirus infectivity in cotton bollworm *H. armigera*, which serves as a defense mechanism preventing entrance of baculoviruses in its host by decreasing peritrophic membrane permeability [15]. Given this, and the distinct high expression of *PmCDA*1 in gills of apparently healthy shrimp suggest an important functional role for the said gene at a possible entry site of infection. Such is worth pursuing in future studies to elucidate further “resistance” of WSSV “survivor”

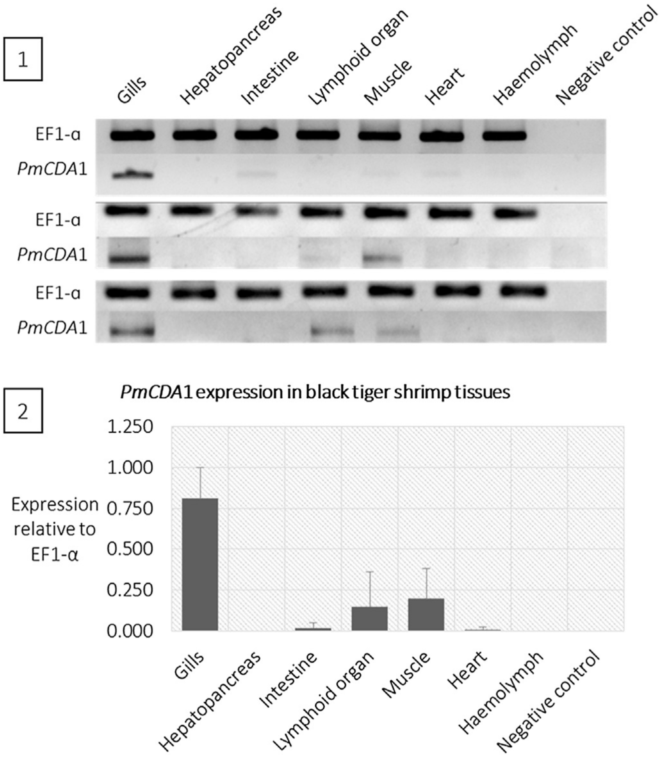

**Fig. 3.** 1. Gel electrophoresis of constitutive expression analysis of black tiger shrimp *PmCDA1* in various tissues relative to expression of EF1-$\alpha$ as determined by RT-PCR. 2. Constitutive expression of black tiger shrimp *PmCDA1* in various tissues relative to expression of EF1-$\alpha$ as determined by RT-PCR and ImageJ. The bars represent mean values of three shrimp samples plus Standard Deviation (SD).

shrimps.

In conclusion, the study fully cloned and identified the first CDA in crustaceans, particularly in black tiger shrimp *P. monodon*, named here as *Pm*CDA1. In addition, it provided the structure and domains of the gene and its possible role in the immune function of the gills of shrimp against WSSV. Such discovery is important in further understanding the effects of WSSV in the immune system of black tiger shrimp, an economically important culture species worldwide, and in providing long-term molecular-based solutions in combating the WSSV infection.

## Authors' contributions

KS carried out the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript. VP helped in drafting the manuscript. MS conceived the study, participated in its design and coordination, and structured the writing of the manuscript. All authors read and approved the final manuscript.

## Conflict of interests

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.06.025.

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