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Molecular identification and characterization of Type I crustin isoforms from the hemocytes of penaeid shrimps, *Penaeus monodon* and *Fenneropenaeus indicus*

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ABSTRACT

Aquaculture is considered one of the most sustainable animal protein sources and is the fastest-growing food sector. Production must increase to meet the rising global demand, but it should be sustainable for marine organisms. The emerging antibiotic resistance is a threat to sustainable aquaculture, and finding an alternative to this is getting prioritized by many scientists. Antimicrobial peptides (AMPs) are short, biologically active peptides seen in various organisms as an essential component of innate immunity. Crustins, a family of cationic AMPs of ca. 7–14 kDa, possess characteristic four-disulphide core containing a WAP domain. The present work attempts to characterize crustins, from *Penaeus monodon* (Pm-Crustin), commonly called Giant Tiger Shrimp, and *Fenneropenaeus indicus* (Fi-Crustin), the Indian White Shrimp. Briefly, total RNA was isolated from the haemolymph using TRI reagent according to the manufacturer's protocol. Gene-specific primers were used to amplify the cDNA, and the amplified bands were sequenced and then subjected to *in silico* analysis. The present study identified an isoform of crustin from both of the experimental organisms. A partial mRNA transcript of 67 bp belonging to the crustin family of AMPs encoding 22 amino acids could be amplified from the haemocytes by RT-PCR. BLASTp analysis reports the amino acid sequence as crustins and multiple alignments confirmed the BLAST analysis results. The bootstrap distance tree constructed based on both nucleotide sequence and amino acid sequence confirmed that both Pm-Crustin and Fi-Crustin possessed similarity to other crustins from *P. schmitti, L. vannamei* and *P. setiferus.* The phylogenetic analysis shows that they possess a close evolutionary relationship to other crustins, which might have subsequently diverged at some phases of evolution. Pm-Crustin and Fi-Crustin were closely related to crustins earlier discovered from shrimp rather than the other crustacean-derived crustin groups. The presence of varied isoforms of crustins in crustaceans indicates its importance in innate immunity. This work adds some useful knowledge for sustainable aquaculture practices dealing with microbial pathogen control in an eco-friendly way. Our results outlined two potent molecules, Pm-Crustin and Fi-Crustin, as a starting material to model novel and efficient antimicrobial drugs for aquaculture practices. Further study on this AMP would make it a way to design promising drugs for shrimp diseases.

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Antimicrobial peptides; Crustin; *Penaeus monodon; Fenneropenaeus indicus*

1. Introduction

The aquaculture field is at high risk due to emerging drug resistance and a shortage of new antibacterial drugs. The quest to find new alternatives has paved the way for exploring Antimicrobial Peptides (AMPs). AMPs are biologically active molecules seen in various organisms as essential to innate immunity against invading pathogens. Typically, they are short sequence peptides ranging from 10 to 100 amino acids, positively charged and amphipathic. Bacterial membranes are frequently targeted by AMPs, forming pores on them. The positively charged AMPs and the negatively charged components of the bacterial cell wall, such as lipopolysaccharides (LPS) and phospholipids (Yeaman et al., 2003), have strong interactions. Apart from their antimicrobial properties AMPs are known for their versatile nature in stress regulation, anti-cancer and immune modulatory activities (Chen et al., 2019; Sruthy et al., 2019; Xu et al., 2016). This ability to act on multiple fronts makes AMPs worth exploring (Hancock & Sahl, 2006; Haney et al., 2019), and the associated lower resistance risk factor has fascinated the researchers to unleash its unexplored and complex mechanisms of action for these classes of peptides (Raheem & Straus, 2019; Spohn et al., 2019).

Commercially, crustacean culturing has gained wide scope due to its high market value (Brauer et al., 2003). In 2015, shrimp catch only counted to 4.8 million tons and brought an annual economic value of up to ten billion US dollars (Guppy et al., 2018). However, the frequent hit of bacterial and viral diseases has tremendously reduced the world's economy (Leobert et al., 2015). Like other invertebrates, shrimps rely only on their innate immune system to protect them from pathogen invasion (Wang et al., 2014). Therefore, it is important to study the innate immune system and explore immune-related molecules for shrimp disease prevention and treatment.

Crustins are cationic AMPs possessing a single whey acidic protein (WAP) domain with a four disulfide core at the C-terminus. Crustins are classified into Type I-III based on their domain arrangement. Most of the shrimp crustins belong to the Type II crustin isoforms (Smith et al., 2008). Crustins have shown antibacterial activity both to the Gram-positive and Gram-negative bacteria (Supungul et al., 2008). In this paper, we have tried to identify and characterize an isoform of crustin from the haemocytes of two penaeid shrimps, *Penaeus monodon* and *Fenneropenaeus indicus,* adopting *in silico* techniques to better understand the potential of this particular isoform as a future drug.

2. Materials and Methods

2.1. Haemolymph Collection

Healthy live specimens of *Penaeus monodon* (Giant Tiger Shrimp) (approximate length of 19.5 cm; weight 47 g) and *Fenneropenaeus indicus* (Indian White Shrimp)

(approximate length of 14 cm; weight 9 g) were collected from a local shrimp farm located at Kuzhupilly, Edvanakad, Vypin, Kerala and were brought to the laboratory. Haemolymph was collected from the rostral sinuses using capillary tubes loaded with RNase-free, pre-cooled anticoagulant (10 % Sodium citrate, pH 7). Haemolymph was stored in TRI reagent till further processing.

2.2. RNA isolation and cDNA synthesis

Total RNA isolation from the haemocytes was done according to the manufacturer's protocol (Sigma, USA). Quantity of the RNA was measured by spectrophotometry at 260 and 280 nm and quality was assessed by 0.8% agarose gel electrophoresis. RNA with absorbance (A260:A280) ≥1.8 were proceeded into cDNA generation. cDNA generation was carried out in a 20 µl reaction mixture using 5 µg of total RNA, 2 mM dNTPs, 1× RT buffer, 2 mM oligo d(T20), 20 U of RNase inhibitor and 100 U of M-MLV Reverse transcriptase. The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min.

2.3. PCR amplification

Primarily to test the quality of mRNA, PCR amplification for the reference gene, β-actin, was carried out using β-actin specific primers. The PCR amplification of the crustin sequence of 1 µl cDNA was done using primers, crustin Type II a Forward (5′ CGAACCAGAGACACCTGTTG 3′) and crustin Type II a Reverse (5′ CAGCACACTTGTAGTCGTTG 3′) (Tharntada et al., 2008). The PCR condition used was as follows: 94 ºC for 2 min; 35 cycles of 94 ºC for 15 sec, 60 ºC for 30 sec, and 68 ºC for 30 sec; and a final extension at 68 ºC for 10 min. The PCR products were loaded in 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized using the Syngene G Box Gel documentation unit. Purified PCR products were sequenced using ABI Prism Sequencing Ready Reaction kit (BigDye Terminator Cycle) on an ABI Prism 377 DNA sequencer at SciGenom Sequencing Facility, Kochi, Kerala.

2.4. Sequence Analysis: an *in-silico* analysis

The sequences were coded, trimmed and edited using GeneTool and BioEdit software. The analysed nucleotide sequences were translated using Expert Protein Analysis System (http://au.expasy.org/) and GeneTool software. Homology searches of nucleotide sequences and the deduced amino acid sequences were performed using BLASTn and BLASTp algorithms of the National Centre for Biotechnological Information (http://www.ncbi.nlm.nih.gov/blast). Pre-deposited sequences of crustin AMPs were retrieved from NCBI and using ClustalW, MEGA 6.0 and GeneDoc computer programmes they were multi-aligned.

3. Results and Discussion

The present study reports the sequence of Crustin antimicrobial peptide from the haemocytes of the giant tiger shrimp, *P. monodon* and Indian White Shrimp, *F. indicus,* hereinafter designated as Pm-Crustin and Fi-Crustin. The sequences revealed the identity of the two crustins to be the same. Each with a 67 bp of PCR-amplified cDNA product and encodes 22 amino acids. The nucleotide and deduced amino acid sequence of the amplicon are shown in Fig. 1.

BLAST analysis of the nucleotide sequence revealed the relation of Pm-Crustin and Fi-Crustin to that of Crustin from other crustaceans (Table. 1 and Table. 2). According to the BLAST analysis, the maximum similarity was

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agtc cgt ccc aca tgc cca cgt ttc cat ggg ccc ccc acg acc tgt tcc aac gac tac aag
  V   R   P   T   C   P   R   F   H   G   P   P   T   T   C   S   N   D   Y   K
tgt gct
 C   A
```

Fig. 1. Nucleotide and amino acid sequence of Pm-Crustin and Fi-Crustin

Table 1. Result of BLASTn analysis of Pm-Crustin and Fi-Crustin

Genbank Accession Number	Description	Query coverage	e-value	% identity
EF182748.1	*Litopenaeus schmitti* crustin mRNA, complete cds	100 %	2e-24	100.00 %
AF430078.1	*Litopenaeus setiferus* clone PsB 164 putative antimicrobial peptide mRNA, complete cds	100 %	2e-24	100.00 %
AY486426.1	*Litopenaeus vannamei* crustin mRNA, partial cds	100 %	8e-22	97.01 %
EF182747.1	*Farfantepenaeus paulensis* crustin mRNA, complete cds	100 %	4e-20	95.52 %
EF450744.1	*Farfantepenaeus subtilis* crustin mRNA, complete cds	98 %	1e-19	95.45 %

Table 2. Result of BLASTp analysis of Pm-Crustin and Fi-Crustin

Genbank Accession Number	Description	Query coverage	e-value	% identity
AAS59735.1	crustin I [*Penaeus vannamei*]	100 %	6e-15	100.00 %
AAL36897.1	putative antimicrobial peptide [*Penaeus setiferus*]	100 %	6e-15	100.00 %
ABM63362.1	crustin [*Penaeus schmitti*]	100 %	6e-15	100.00 %
ACZ43781.1	crustin 1 [*Penaeus chinensis*]	100 %	1e-13	95.45 %
AAS57715.1	crustin [*Penaeus vannamei*]	100 %	3e-13	95.45 %

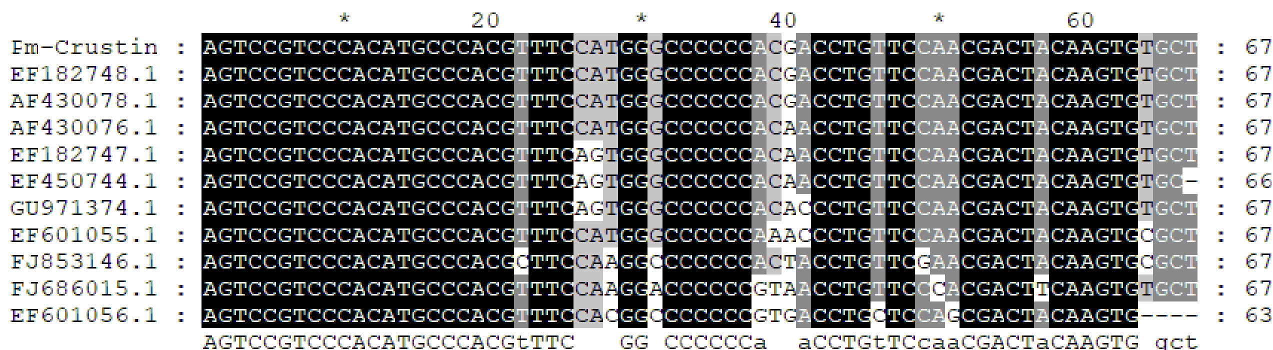

Fig. 2. Multiple alignment of the nucleotide sequence of Pm-Crustin and Fi-Crustin with previously reported crustins of various crustaceans showing sequence similarity obtained using GeneDoc program Version 2.7.0. Black and grey indicates conserved sequences

obtained for Crustin of *Litopenaeus schmitti* in case of BLASTn and to *P. vannamei* in case of BLASTp. The putative crustin genes have been explored extensively from the hemocyte and the gill-epipodite cDNA libraries of *P. monodon* (Supungul et al., 2004; Tassanakajon et al., 2008; Vatanavicharn et al., 2009). However, crustin genes from *F. indicus* are less explored.

BLAST analysis of the nucleotide sequence revealed that the sequence shared 100 % similarity to the crustin of *L. schmitti* for a query coverage of 100 % followed by similarity to crustin of *L. setiferus* (100 %). Great similarity was also found to crustin from *L. vannamei* (97.01 % for query coverage of 100 %) *Farfantepenaeus paulensis* (95.52 %), *Farfantepenaeus subtilis* (95.45 %). BLASTp analysis of the amino acid sequence also revealed the significant identity with crustin of *P. setiferus* (100 %) and *Penaeus schmitti* (100 %) for query coverage of 100 %. The deduced amino acid sequence also showed similarity with crustin of *P. chinensis,* having 95.45 % identity and 100 % query coverage and also with *P. vannamei* with 95.45 % identity and 100 % query coverage. The BLAST analysis of a 371 bp cDNA fragment coding 122 amino acid crustin gene from *F. indicus* has shown the greatest similarity to *F. chinensis* and *P. monodon* (Antony et al., 2010). Multiple alignments performed for the Pm-Crustin and FI-Crustin affirmed the BLAST analysis, by showing its similarity with other crustins from *L. schmitti, L. vannamei, F. paulensis* (Fig. 2 and 3).

A phylogenetic tree constructed to study the relationship between Pm-Crustin and Fi-Crustin with that of other crustins revealed that they are more closely related to *L. schmitti* and *L. setiferus,* than to the crustin of *P. monodon* and *M. japonicus,* in the case of nucleotide sequence. And about amino acid sequences of *P. vannamei* and *P. schmitti*. The tree could be broadly divided into 2 groups. Pm-Crustin and Fi-Crustin belonged to Group-1, which could be again divided into 8 subgroups. Group I include *L. schmitti* and *L. setiferus* other than Pm-Crustin and Fi-Crustin (Fig. 4).

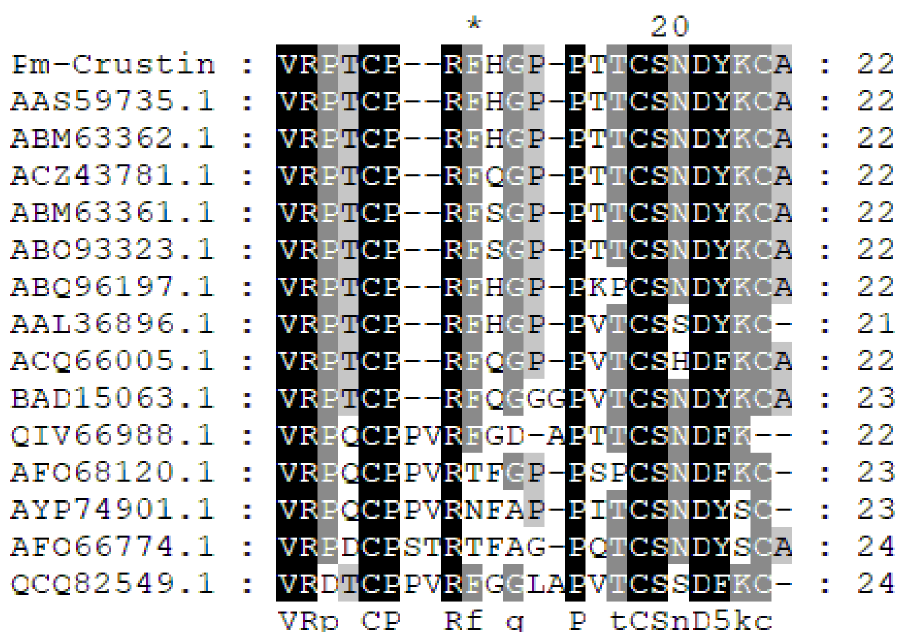

Fig. 2. HMultiple alignment of amino acid sequence of Pm-Crustin and Fi-Crustin with previously reported crustins of various crustaceans showing sequence similarity obtained using GeneDoc program Version 2.7.0. Black and grey indicates conserved sequences

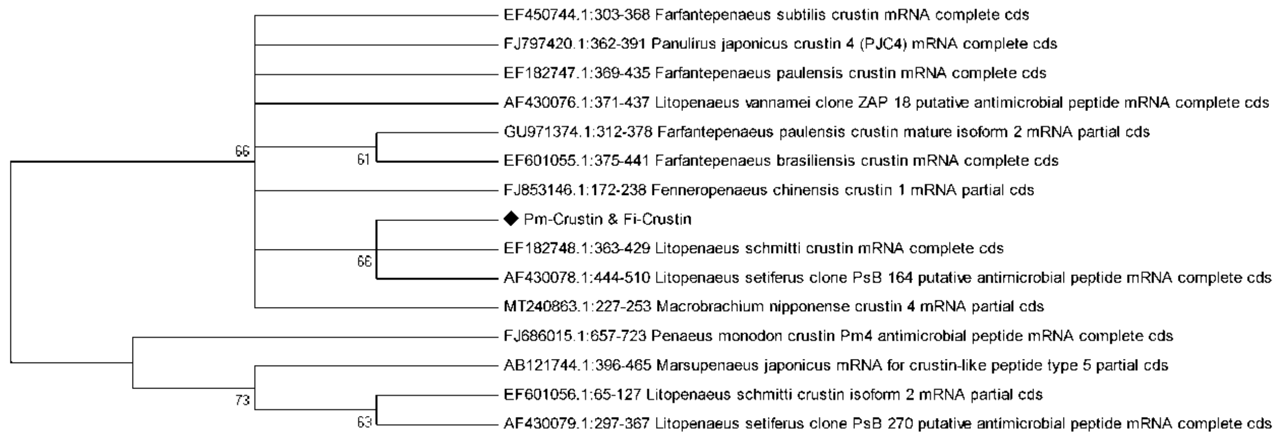

Fig. 4. MEGA version 6.0 illustrating bootstrapped neighbour-joining tree relationships between the amino acid sequence of Pm-Crustin and Fi-Crustin with other reported crustin in crustaceans. Node values indicate the percentage of times the particular node generated in 1000 trees by bootstrapping the original deduced amino acid sequences.

4. Conclusion

Exploring the wide range of elements of the antimicrobial peptide family from crustaceans might find a way to unleash any effective alternative to antibiotics. Further study on the functional characterization of the novel sequenced crustin gene would help us to design a better drug candidate for microbial infections in crustaceans.

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