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Short sequence report

Molecular characterization of a crustin-like, putative antimicrobial peptide, Fi-crustin, from the Indian white shrimp, *Fenneropenaeus indicus*

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Antimicrobial peptides are important innate immune defense, especially in those animals which lack adaptive immunity [1–8]. Due to their small size, amphipathic structure and cationic character they can rapidly diffuse to the point of infection [9], a mechanism that presumably makes it easier to circumvent microbial resistance against the peptides [10]. Besides providing an immediate and broad-spectrum microbicidal activity, AMPs can kill bacteria in micromolar range, are promptly synthesized at low metabolic cost, and are easily stored in large amounts and readily available shortly after an infection [11–13]. Many AMPs show a remarkable specificity for prokaryotes with low toxicity for eukaryotic cells; a phenomenon which has favored their investigation and exploitation as potential new antibiotics [14]. AMP gene expression and distribution are regulated through haemocyte reactions [15]. Transcripts of crustin-encoding genes have also been observed in gills, heart and intestine [16–18] but as these tissues are highly vascularised, it is assumed the transcripts from these organs are due primarily to the haemocytes.

In penaeid shrimps, four main families of AMPs have been currently described and characterized from the haemocytes: penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes. Penaeidins are mainly active against Gram-positive bacteria, filamentous fungi [19], viruses and protozoans [20] whereas ALFs have a broader antimicrobial spectrum including Gram-negative bacteria [21,22]. Conversely, crustins are reported to have a more-restricted activity spectrum, affecting mainly marine Gram-positive bacteria [17,23,24] Crustins, a widely distributed family of AMPs was first isolated from the shore crab, *Carcinus maenas* as an 11.5 kDa peptide [23]. Crustins are cationic, cysteine-rich antimicrobial AMPs having molecular weight of 7–14 kDa, with an isoelectric point in the range of 7.0–8.7, and contain one whey-acidic protein (WAP) domain at the carboxy terminus [25]. Crustins have been proved to be an important antimicrobial protein in the plasma and haemocyte granules of crustaceans and described as a component of the innate immune system [8]. These AMPs are dominantly synthesized and stored in haemocytes [4,8,16,18,23,24,26–30] and their release from haemocytes is induced by bacterial infection [15,27,31]. Crustin mechanisms of action and function are still largely unknown, although they contain a whey-acidic protein (WAP) domain common to proteinase inhibitory activities as well as antimicrobial activities [8].

Many full-length cDNA and several ESTs of crustins have been described in a wide range of penaeid prawns including *Litopenaeus vannamei* [8,24,30,32], *Litopenaeus setiferus* [24,32,33], *Penaeus monodon* [16,17,29,30,34–37], *Marsupenaeus japonicus* [17,38], *Litopenaeus schmitti* [33], *Fenneropenaeus chinensis* [17,29], *Farfantepenaeus brasiliensis* [33], *Farfantepenaeus paulensis* [33] and *Farfantepenaeus subtilis* [33]. However, no antimicrobial peptide sequences have been reported from *Fenneropenaeus indicus*. In the current study a crustin cDNA has been characterized from the Indian White Shrimp, *F. indicus*.

Healthy adult *F. indicus* (8–10 g body weight) were purchased from a local shrimp farm in Vypeen, Kochi. They were transferred to aquaria of 500 l capacity and acclimatized for one week under laboratory conditions. Prawns were fed with a standard feed (Higashimaru, India). Aeration was provided in all tanks during the experiment and bioreactor was set in all the aquaria for the removal of ammonia and nitrate. Only shrimps in the intermoult stage were sampled during the study.

Haemolymph was collected from the rostral sinus using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase-free, 10% sodium citrate, pH 7.0). Total RNA was extracted from the haemocytes using TRI

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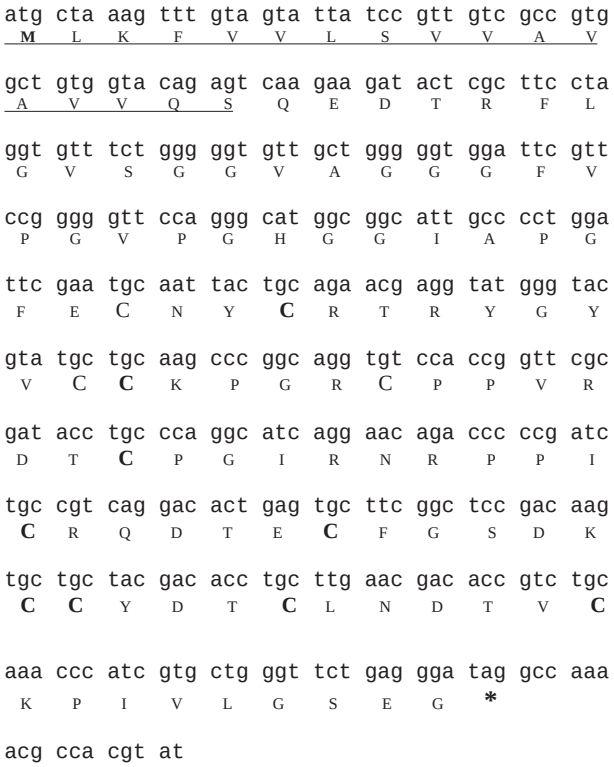

Fig. 1. Nucleotide and amino acid sequences of Fi-crustin from the haemocyte of the Indian white shrimp, *Fenneropenaeus indicus* (GenBank Accession No. GQ469987). The underlined amino acid residues indicate a putative signal sequence. Cysteine residues that participate in the formation of intramolecular disulphide bonds are bold printed. An asterisk is the stop codon.

Reagent (Sigma) following the manufacture's protocol. RNA was quantified by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratios (A_{260}:A_{280}) greater than 1.8 were used for the present work. First strand cDNA was generated in a 20 μl reaction volume containing 5 μg total RNA, 1x RT buffer, 2 mM dNTP, 2 μM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of M-MLV reverse transcriptase (New England Biolabs). The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min. PCR amplification of 1 μl of cDNA was performed in a 25 μl reaction volume containing 1x standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer and 1U Taq DNA polymerase (New England Biolabs). Amplification was performed using the primers, Crus F (5′- cgca cagccgagagaaacactatcaagat −3′) and Crus R (5′ggcctatccctcagaa cccagcacg −3′). The thermal profile used was 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. PCR product was analyzed by electrophoresis in 1.5% agarose gels in TBE buffer, stained with ethidium bromide and visualized under UV light. Purified PCR products were sequenced at Xcelris, India.

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Gene translation and prediction of deduced protein were performed with ExPASy (http://www.au.expasy.org/). The signal peptide was predicted by SignalP program (http://www.au.expasy.org/). The multiple sequence alignments were performed on amino acid sequences of known crustin or crustin-like peptides from shrimps using CLUSTALW and GENDOC computer programs. Amino acid sequences of shrimp crustins were retrieved from the NCBI GenBank and phylogenetic tree was constructed by the Neighbor-Joining (NJ) method and the Maximum Likelihood method (ML) based on amino acid sequences. Phylogenetic tree was drawn based on the sequences with WAP domain using MEGA version 4.0 [39]. The nucleotide sequence and deduced amino acid sequence was submitted to GenBank (GQ469987).

A 371 bp fragment cDNA encoding 122 amino acids and an ORF of 117 amino acids was obtained from the mRNA of *F. indicus* haemocyte by RT-PCR (Fig. 1). BLAST analysis of the nucleotide sequence revealed the relation of Fi-crustin to that of crustins from *F. chinensis* and *P. monodon* (Table 1). Multiple alignment and the bootstrap distance tree calculated for the resulting crustin sequences of BLAST analysis confirmed that Fi-crustin possessed more similarity to that of *F. chinensis* crustin than to the *P. monodon* crustins (Fig. 2). The ORF encoded 117 amino acid residues with a predicted molecular weight (MW) of 10.61 kDa and theoretical isoelectric point (pI) of 7.59 as predicted by the PROTPARAM software. The analysis with the Signal P software revealed the presence of a signal peptide with 17 amino acids at the N-terminal region of the Fi-crustin (Fig. 1).

The deduced amino acid sequence of Fi-crustin was found to be rich in amino acid residues Glycine (14.5%) and Valine (12.8%). At the N-terminal of the mature peptide, Fi-crustin contained a number of glycine-rich repeats between positions 25 and 49. Following the repeat region is a cys-rich region just like that of the crustin-like peptide from *M. japonicus* and *F. chinensis*; however they have no proline-rich domain compared with those of *M. japonicus* and hence is more similar to crustin of *F. chinensis* [17,38]. The C-terminal segment included a high proportion of Cysteine-rich region (10.3%), which contained 12 Cysteine residues that participate in the formation of disulphide bonds. The 12 cysteines in Fi-crustin are considered to be important for maintaining the tertiary structure of the peptide just as that reported in *L. setiferus*, *L. vannamei* and *F. chinensis* crustins [32,17].

As predicted by the ScanProsite program, a whey-acidic protein (WAP) domain signature exists in the C-terminal. According to the previous reports on the crustin-like proteins, the four-disulfide core domain has proved to play important roles in the biological function of crustins [17]. The position of the conserved cysteines for the 'four-disulfide core' domain is Cys^{68}-Cys^{98}, Cys^{75}-Cys^{102}, Cys^{85}-Cys^{97}, and Cys^{91}-Cys^{108}. In addition, searching the Prosite database, analysis of Fi-crustin revealed the existence of WAP-type 'four-disulfide core' domain signature, C1-(Xn)-C2 (Xn)-C3-(X5)-C4-(X5)-C5-C6-(X3-5)-C7-(X3-4)-C8 [24] and Fi-crustin followed the same pattern with 4 residues between C_7 and C_8 (C_7 X_4 C_8). Several other consensus sequences also appear in the 4-DSC domain: (1) the consensus KXGXCP containing C1; (2) a conserved aspartate (D) residue between C3 and C4; (3) KCC with C5 and C6; (4) CXXP with C8. [24] Fi-crustin follows this pattern as 'CXP with C_8' instead of 'CXXP with C_8'.

The amino acid sequence of Fi-crustin was also compared with crustins of decapod crustaceans and it revealed maximum identity to that of *F. chinensis,* and *P. monodon* (Fig. 3). Multiple alignments of

Table 1
Result of BLAST analysis of Fi-crustin (GQ469987).

Accession No.	Description of the AMP	Query coverage	*E* value	Max identity
DQ097703	*Fenneropenaeus chinensis* crustin-like protein fc-1 mRNA, complete cds	94%	1e-78	91%
GQ334395	*Penaeus monodon* crustin-like antimicrobial peptide type 2 mRNA, complete cds	70%	2e-80	97%
EF654659	*P. monodon* crustin-like antimicrobial peptide gene, complete cds	70%	2e-75	97%
DQ097704	*F. chinensis* crustin-like protein fc-2 mRNA, complete cds	58%	2e-27	96%

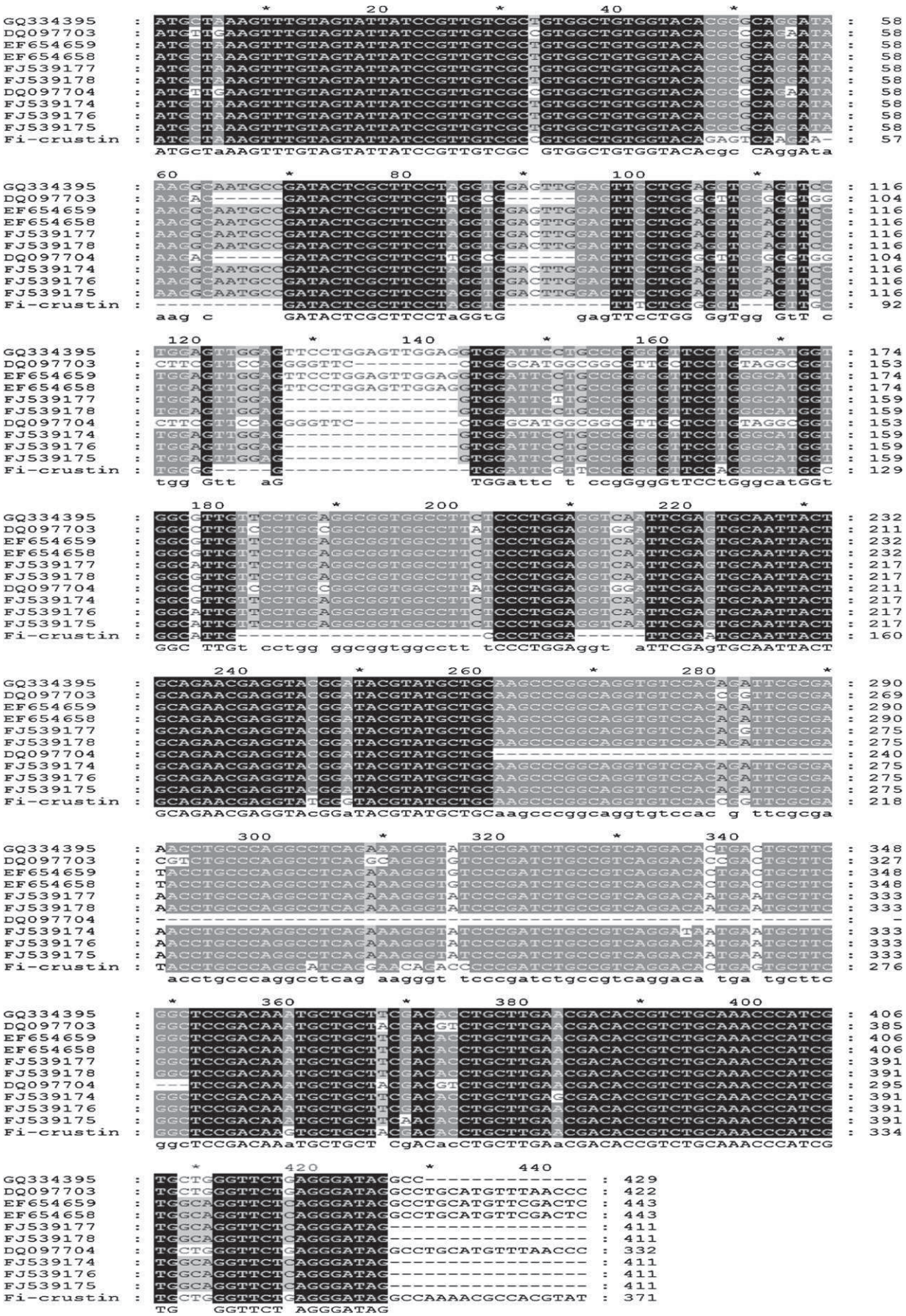

Fig. 2. Multiple alignment of nucleotide sequence of the *Fenneropenaeus indicus* crustin-like antimicrobial peptide, Fi-crustin (GQ469987) with other shrimp crustins (*Penaeus monodon* GQ334395, *Fenneropenaeus chinensis* DQ097703, *P. monodon* EF654659, *P. monodon* EF654658, *P. monodon* FJ539177, *P. monodon* FJ539178, *F. chinensis* DQ097704, *P. monodon* FJ539174, *P. monodon* FJ539175, *P. monodon* FJ539176) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences.

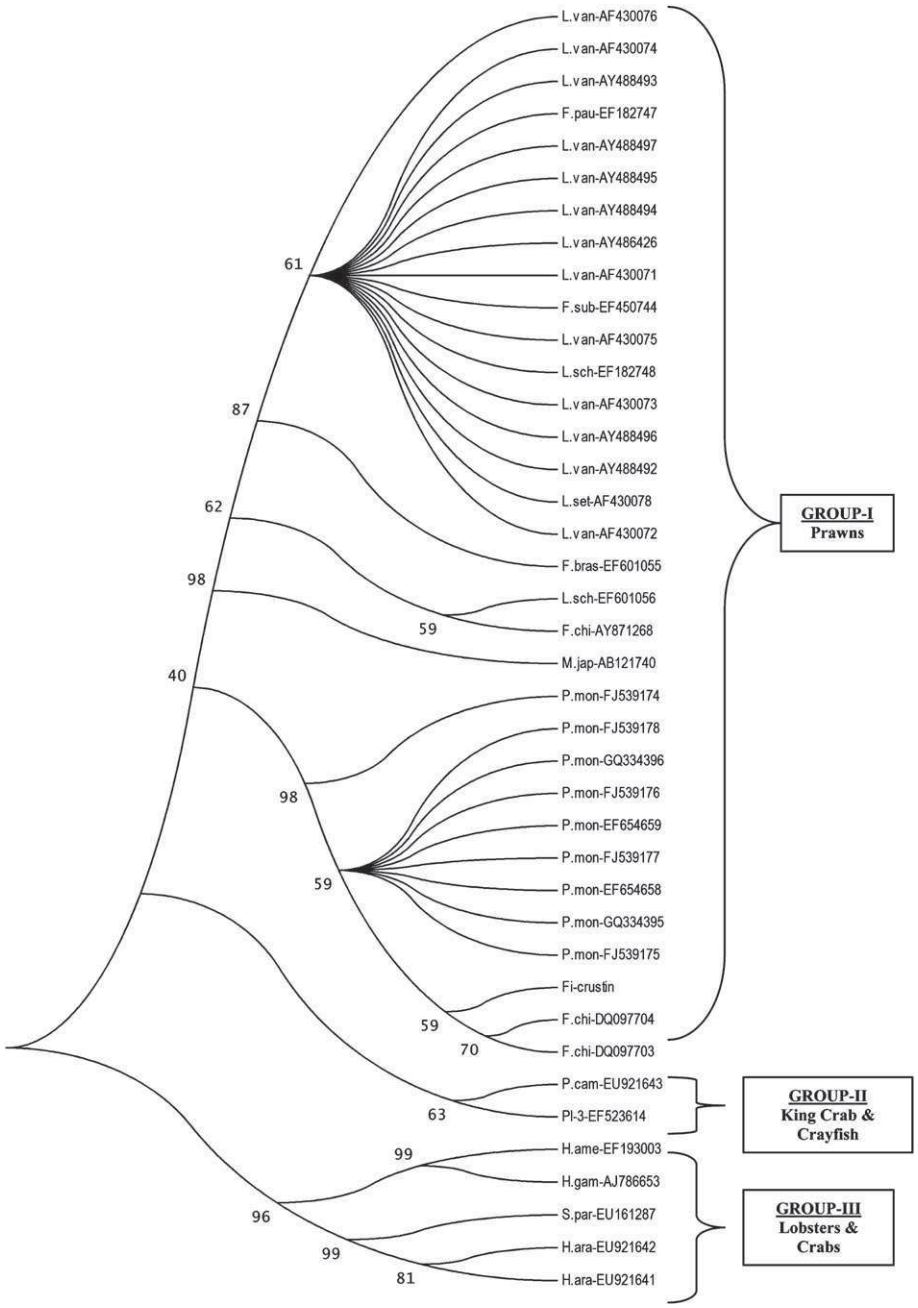

Fig. 3. A bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Fenneropenaeus indicus* crustin-like AMP, Fi-crustin (GQ469987) with other crustins of decapod crustaceans (*Litopenaeus vannamei* AF430071, *L. vannamei* AF430074, *L. vannamei* AY488497, *L. vannamei* AY488492, *Litopenaeus setiferus* AF430078, *Farfantepenaeus paulensis* EF182747, *L. vannamei* AY488493, *L. vannamei* AF430073, *L. vannamei* AY486426, *L. vannamei* AF430072, *Farfantepenaeus subtilis* EF450744, *Litopenaeus schmitti* EF182748, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AF430075, *L. vannamei* AF430076, *L. vannamei* AY488494, *Farfantepenaeus brasiliensis* EF601055, *L. schmitti* EF601056, *Fenneropenaeus chinensis* AY871268, *Marsupenaeus japonicus* AB121740, *Penaeus monodon* FJ539174, *P. monodon* EF654659, *P. monodon* FJ539178, *P. monodon* EF654658, *P. monodon* GQ334395, *P. monodon* GQ334396, *P. monodon* FJ539175, *P. monodon* FJ539177, *F. chinensis* DQ097704, *F. chinensis* DQ097703, *Paralithodes camtschaticus* EU921643, *Pacifastacus leniusculus* 1 EF523614, *Homarus americanus* EF193003, *Homarus gammarus* AJ786653, *Scylla paramamosain* EU161287, *Hyas araneus* EU921642, *Hyas araneus* EU921641). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

amino acid sequences of crustins also showed that the deduced amino acids of Fi-crustin shared relatively high identities with those of *F. chinensis* and *P. monodon*. Multiple alignment of the nucleotide sequences of Fi-crustin and other shrimp crustins showed high similarity in the signal peptide region, major gaps could be observed in the ensuing region. The 58–70th nucleotide sequences were found to be absent in the Fi-crustin when compared to *P. monodon* crustin sequence. A similar gap could be observed for the *F. chinensis* crustins between the 64–70th position. Similarly, another missing sequence region could be observed for Fi-crustin between the 88–98th position that matches with a similar gap for the *F. chinensis* crustins at the 88–93rd position, when compared to *P. monodon*. Great variation between the sequences of *P. monodon* and *Fenneropenaeus* sp. could be observed at the 122nd–144th position. Other major missing sequences of the Fi-crustins were found at 182–202nd and also between 211th and 216th position. *F. chinensis* showed a major gap for the nucleotide sequences at 262–352nd position whereas *P. monodon* and Fi-crustin did not.

The phylogenetic relationships between Fi-crustin and other crustins with WAP domain are shown in Fig. 3. The tree topologies revealed the relationships of Fi-crustin with other invertebrate crustin-like peptides. The molecular phylogenetic tree based on amino acid sequences suggests that all the crustin members possess the same ancestral origin, which has subsequently diverged at different phases of evolution. Out of all the species, crustins of prawns are found to be evolutionarily distantly related to crustins of other decapod species. The tree could be broadly classified into three major groups, one major group (Group I) which included the crustins of prawns; another (Group II) with that of king crab/crayfish crustins and Group III containing the lobster/crab crustins. The bootstrap distance tree calculated for the crustin sequences clearly indicate that the Fi-crustin possessed great similarity to crustins isolated from *F. chinensis* and *P. monodon*. Great variability could also be noticed in the crustin sequences of various decapods.

This is the first report of an antimicrobial peptide from Indian white prawn, *F. indicus* .The reported AMP belonged to the class of crustins with its characteristic WAP domain and showed 91% similarity to *F. chinensis* crustins. The phylogenetic tree analysis showed that the crustins diverged from an ancestral sequence to three major groups i.e. Group 1 with prawns, Group II with Cray fishes/King crabs and Group III with Lobsters/Crabs. Under prawn – crustins, three sub groups were noticed 1) *L. vannamei* 2) *P. monodon* and 3) *Fenneropenaeus* sp. The wide distribution of crustins in crustaceans indicates the importance of these antimicrobial peptides in the innate immune system.

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