

Molecular characterization and phylogenetic analysis of an anti-lipopolysaccharide factor from the crucifix crab, *Charybdis feriatatus*

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Abstract: Antimicrobial peptides are small cationic, gene-encoded, amphipathic, host defense peptides with a ubiquitous distribution in all living kingdoms. They are <10 kDa in size, with 15–100 amino acids having a net positive charge of +2 to +9. Anti-lipopolysaccharide factor (ALF) is a cationic antimicrobial peptide which constitutes one of the key effector molecules in the innate immune system of crustaceans, and is capable of binding and neutralizing lipopolysaccharides. In the present study, an ALF homolog (*Charybdis feriatatus* [Cf]-ALF1)-encoding cDNA sequence from the hemocytes of the crucifix crab, *C. feriatatus*, was cloned, identified, and characterized. The deduced peptide of Cf-ALF1 encoded for a 123 amino acid peptide with a 97 residue mature peptide (11.16 kDa) that had a net charge of +10. Two conserved cysteine residues and a putative lipopolysaccharide binding domain were observed in the Cf-ALF1 mature peptide. BLAST analysis of Cf-ALF1 nucleotides showed a 99% similarity to *Scylla serrata*. The spatial structure of Cf-ALF1 was composed of three α -helices packed against a four-strand β -sheet. Two of these helices were linked by a disulfide bond to form an amphipathic loop similar to the structure of anti-lipopolysaccharide factor isoform 3 from *Penaeus monodon* (ALF-Pm3). All these features suggest that Cf-ALF1 could play a significant role in the innate immune defense mechanism of *C. feriatatus*.

Keywords: antimicrobial peptides, crab, innate immunity, *Charybdis feriatatus*, anti-lipopolysaccharide factor

Introduction

Crustaceans represent one of the most abundant groups of animals, inhabiting both aquatic and terrestrial areas. By virtue of their diversity and abundance, they have been considered as a potential source of bioactive compounds. The lack of an adaptive immune system in crustaceans has led to the evolution of potent compounds, including antimicrobial peptides (AMPs) that are used as defense against pathogens. Anti-lipopolysaccharide factor (ALF) represents one of the evolutionarily-conserved cationic AMPs distributed among horseshoe crabs and crustaceans which can bind against lipopolysaccharides (LPS) by neutralizing the lipid-A moiety of LPS.¹

The first ALF was isolated from the amebocytes of two kinds of horseshoe crab, *Tachypleus tridentatus* and *Limulus polyphemus*.² Since then, numerous ALF homologs have been identified and characterized from decapod crustaceans, including penaeid shrimp,^{3–6} nonpenaeid shrimp,⁷ lobsters,^{8,9} crabs, and crayfish.^{10–13} ALFs belong to the group of single-domain AMPs with a signal peptide at the N-terminal region followed by a conserved LPS-binding domain.¹⁴ Different ALF isoforms and variants are either encoded by distinct genes or formed as a consequence of alternative mRNA splicing.¹⁵

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Functional characterization of ALFs has been conducted by chemically synthesizing the LPS domain region,^{16–22} and by recombinant expression of mature peptides.^{9,13,23–28} Biological activity reported so far for ALFs include antibacterial,^{6,9,20} antifungal,^{6,27} antiviral,^{29,30} immunomodulatory,³¹ antitumor,³² anti-inflammatory, and antiprotozoal activity.^{32,33} As per the previous studies, the structure of ALF was elucidated using nuclear magnetic resonance (NMR) spectroscopy, with the finding that it was composed of three α -helices packed against a four-stranded β -sheet.^{34,35}

Thus far, there are several studies concerning the molecular and phylogenetic characterization, gene expression analysis, and structural and functional analysis of ALFs from decapod crustaceans and chelicerates. However, there is little research of AMPs from the crucifix crab, *Charybdis feriatus*. *C. feriatus*, a member of the family Portunidae, is one of the important species of commercial crab sold in markets across East Asia, where it commands substantially higher premium prices than *Portunus* spp. (typically US\$8–\$15 per kg). Also it is a promising aquaculture species because of its meat quality, taste and size.

The present study focused on identifying an antimicrobial peptide from the crucifix crab, *C. feriatus*.

Materials and methods

Animals and sample collection

Live and healthy adult crucifix crabs, *C. feriatus*, were collected from Cochin estuary along Vypeen, Kerala, India. To ensure survival of the crabs during their transport to the laboratory, adequate ventilation was provided. The hemolymph was extracted from the base of the crabs' abdominal appendages using DEPC-treated, RNase-free capillary tubes that had been rinsed with precooled anticoagulant solution (RNase-free 10% sodium citrate, pH 7.0). The hemolymph was then homogenized in TRI reagent (Sigma-Aldrich Co, St Louis, MO, USA) and kept at -20°C until ready for total RNA isolation.

Total RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from hemocytes using TRI reagent, as per the manufacturer's instructions. Qualitative analysis of RNA was performed using agarose gel electrophoresis with a 0.8% agarose gel, as well as calculating the ratio of absorbance at 260 nm and 280 nm. RNA was quantified by spectrophotometry at 260 nm (Hitachi U-2900, Hitachi Ltd, Tokyo, Japan), and RNA samples with absorbance ratios (A₂₆₀:A₂₈₀) >1.8 were used for the present work. First-strand cDNA was generated in a 20 μL reaction volume containing 5 μg total RNA, 1 \times RT buffer, 2 mM dNTP,

2 mM oligo dT20, 20 U of RNase inhibitor, and 100 U of MMLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA). The reaction was conducted at 42°C for 1 hour, followed by an inactivation step at 85°C for 15 minutes. Gene-specific primers β -actin-F (5'-CTTGTTG-GTTGACAATGGCTCCG-3') and β -actin-R (5'-TGGT-GAAGGAGTAGCCACGCTC-3') were used to amplify the housekeeping gene *β -actin* (520 bp), which was used as an internal control to verify the reverse transcription polymerase chain reaction (RT-PCR).

PCR amplification and TA-Cloning

PCR amplification of 1 mL of cDNA was performed in a 25 μL reaction volume containing 1 \times standard Taq buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 3.5 mM MgCl₂, 200 mM dNTPs, 0.4 mM each primer, and 1 U Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA). Amplification was performed using the primers, ALF-F (5'-GGACAGAAGAAACATTGAGGACGACGCA-3') and ALF-R (5'-GGAAATCAAAAACATCCATTACAG-GTCA-3').¹¹ The thermal profile used was 95°C for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; final extension was at 72°C for 10 min. The PCR product was analyzed by electrophoresis in 1% agarose gels in TBE buffer, stained with ethidium bromide, and visualized using a gel documentation unit (Syngene, Synoptics Ltd, Cambridge, UK).

The purified PCR product was ligated into the pGEM[®]-T Easy Vector and transformed using DH5 α -competent *Escherichia coli* cells, as per the manufacturer's protocol (pGEM-T Easy TA Cloning Kit, Promega Corporation, Fitchburg, WI, USA). Transformed bacteria were cultured in Luria Bertani agar containing ampicillin, IPTG, and X-gal, at 37°C for 16 hours, and the recombinant clones with the inserts were selected by blue-white screening. Recombinant white colonies were selected and streaked onto fresh ampicillin plates, and screened by colony PCR using vector-specific primers (T7 F and Sp6 R) and the ALF sequence-specific primers. For vector-specific primers, the thermal profile used was 95°C for 3 minutes followed by 35 cycles of 94°C for 15 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; final extension was at 72°C for 10 minutes. Amplicons were examined on 1% agarose gels in TBE buffer, stained with ethidium bromide, and visualized using a gel documentation unit. The potentially positive recombinant clones were selected for plasmid isolation as per the manufacturer's protocol (GeneluteHp Plasmid Miniprep Kit Sigma-Aldrich Co, St Louis, MO, USA). Isolated plasmids were analyzed on a 0.8% agarose gel, and gene insertion was confirmed by PCR,

using vector-specific and insert-specific primers. Selected recombinant plasmids were sequenced using T7 F and Sp6 R primers with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 377 DNA Sequencer (Thermo Fisher Scientific, Waltham, MA, USA) at SciGenom Sequencing Facility, India.

Characterization of nucleotide and amino acid sequences

GeneTool software (Syngene, Synoptics Ltd, Cambridge, UK) was used for the nucleotide sequence analysis and assembly. The cDNA sequences acquired were translated using the Expert Protein Analysis System (ExPASy) (<http://web.expasy.org/tools/translate/>) to obtain their amino acid sequences. Homology searches of nucleotide and deduced amino acid sequences were accomplished using the BLASTn and BLASTp algorithms of the NCBI (<http://www.ncbi.nlm.nih.gov/blast>). Presumed signal peptide region was identified using the online program SignalP (<http://www.cbs.dtu.dk/services/SignalP>). The transmembrane region within the mature peptide was predicted using the TMPred tool of ExPASy (http://embnet.vital-it.ch/software/TMPRED_form.html). Physicochemical parameters of the protein sequence were predicted using the online protein analysis tool, ExPASy ProtParam (<http://web.expasy.org/protparam>).

Phylogenetic analysis of *C. feriatatus*-ALF I

Multiple sequence alignment of *C. feriatatus* (Cf)-ALF1's amino acid sequence was performed on ClustalW in BioEdit software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) for the comparison and alignment with other crustacean ALF-encoding sequences. The phylogenetic tree was constructed based on the neighbor-joining method, using Molecular Evolutionary Genetics Analysis (<http://www.megasoftware.net/>) version 6.

Structure analysis of Cf-ALF I

The spatial structure of *MnALF* was established with PyMOL (<https://www.pymol.org/>), using the SWISS-MODE (<http://swissmodel.expasy.org/>) prediction algorithm, which is based on homology modeling. The helical property of Cf-ALF1 was predicted using HeliQuest (<http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py>).³⁶ The cDNA sequence of Cf-ALF1 was converted to the corresponding RNA sequence (Attottron Biosensor Corporation, <http://www.attottron.com/>) and submitted to RNAstructure WebServer (<http://rna.urmc>

rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) to envisage the RNA structure with minimum free energy.

Prediction of Cf-ALF I antimicrobial peptide properties

The antimicrobial activity of Cf-ALF1 was predicted using the Antimicrobial Peptide Database's (APD2) calculation and prediction tool (<http://aps.unmc.edu/AP/main.php>).³⁷ The amphipathicity and antimicrobial activity of the LPS domain was interpreted using HeliQuest.

Results and discussion

In the present study, an AMP belonging to the ALF family (herein referred to as Cf-ALF1) was identified and characterized from the hemocytes of *C. feriatatus*. A 524 bp fragment of cDNA encoding an ORF of 123 amino acids was obtained from the hemocytes of *C. feriatatus* (Figure 1). The Cf-ALF1 nucleotide and deduced amino acid sequences were deposited in the GenBank sequence database (GenBank ID: KP688577). BLAST analysis of the 372 bp of cDNA sequence, as well as the homology search by BLASTn and BLASTp of the deduced amino acid sequence of Cf-ALF1, revealed that it belongs to the DUF3254 superfamily. This superfamily includes all the family of ALFs. BLASTn analysis of the Cf-ALF1 nucleotide sequence showed that it exhibited a 99% similarity to an ALF isoform from *Scylla serrata* (SsALF) (GenBank ID: ACH87655.1), a 97% similarity to an *S. paramamosain* ALF (GenBank ID: EF207786.1), a 96% similarity to a *P. pelagicus* ALF (GenBank ID: JQ899452.1), an 87% similarity to a *P. trituberculatus* ALF (GenBank ID: GQ165621.2), and a 72% similarity to an *Eriocheir sinensis* ALF (GenBank ID: HQ850572.1). SignalP analysis of the peptides determined the existence of a signal peptide composed of 26 amino acids and containing an exceedingly hydrophobic core region. The presence of a putative cleavage site after the 26th amino acid (CEA₂₆-Q₂₇Y) demarcated the highly cationic mature peptide of 97 amino acid residues.

Based on the ExPASy ProtParam analyses, Cf-ALF1 had an estimated molecular weight of 14.025 kDa, a net charge of +11, and a theoretical isoelectric point of 9.78. Comparatively, the mature peptide region measured 11.172 kDa, +10, and 10.01, respectively. Cationicity of the mature peptide was mainly attributed to the 18 positive amino acid residues (twelve lysine residues and six arginine residues), against eight negative residues (four aspartic acid residues and four glutamic acid residues). The estimated half-life of the mature peptide of Cf-ALF1 was found to be 0.8 hours (mammalian reticulocytes, in vitro),

ggacagaagaacattgaggacgacgcaaccaaaacttcocctcaagatcgccaccagggtg
M R T R V
atggccggcctgtgctgtggcgccggtggtgatgtgcctgtacatgcccagccgtgcgag
M A G L C V A P V V M C L Y M P Q P C E
gctcagtatgaagctctggtagcttccattcttggaaagctgtcgggactgtggcacagc
A Q Y E A L V A S I L G K L S G L W H S
gacacagtggacttcatgggacacacctgccacatccgccgcaagccgaagttcaggaaa
D T V D F M G H T C H I R R K P K F R K
ttaaagctgtaccacgagggcaagttttgggtgtcctggcctggacacatctcaggggcaat
F K L Y H E G K F W C P G W T H L E G N
tcgaggaccaagagcaggtcgggggtcaaccagggaagccaccaaggacttcgtgcacaaa
S R T K S R S G S T R E A T K D F V H K
gctttacaaaacaaactcatcacgaagaatagcgcgggacgcctggctgaaggggtgaggc
A L Q N K L I T K N S A D A W L K G *
aagtgatgccactctcatgtacgaggaggacgataggcatgagggtgaaaacaacaggg
aagtgactcgtgtctgacctgtaatggatgtttttgatttcc

Figure 1 Nucleotide and deduced amino acid sequence of the ALF isoform from the hemocyte mRNA transcripts of *Charybdis feriatus*-ALF1 (GenBank ID: KP688577).

Notes: Gray-colored highlighted portion specifies the 25 amino acid signal peptide. The bioactive mature peptide is highlighted in turquoise, and the underlined region within the mature peptide is the LPS binding domain.

Abbreviations: ALF, anti-lipopolysaccharide factor; LPS, lipopolysaccharides.

10 minutes (yeast, *in vivo*), and 10 hours (*E. coli*, *in vivo*). The predicted instability index was computed to be 34.37, and thus classified the protein as stable. The presence of a transmembrane helical region was determined in the mature peptide region, starting from L₄₃SGLWHSDTVDFMGHT₅₉, which may assist in the peptide and bacterial membrane interaction.

The multiple protein sequence alignment of Cf-ALF1 (with representatives of each crustacean's ALFs) revealed the existence of conserved sequence features (Figure 2), ie, cysteine residues (C₅₅-C₇₆) that established a disulfide bridge with a highly conserved sequence. This sequence contained a cluster of seven positively charged amino acid residues within the disulfide loop, forming the functional LPS domain

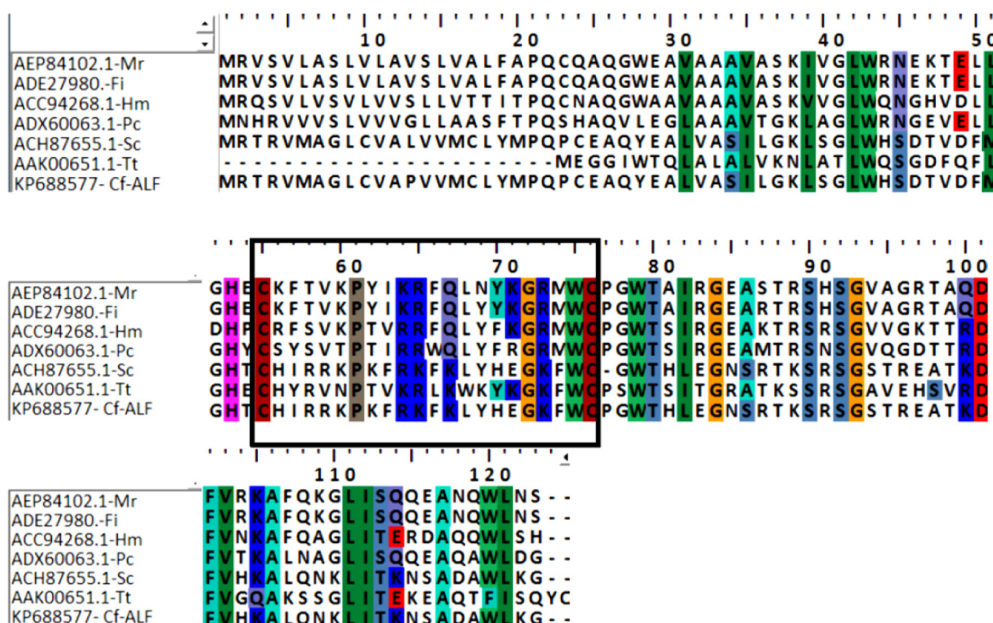


Figure 2 Multiple alignment of amino acid sequence of the *Carybdis feriatus* (Cf)-ALFI (GenBank ID: KP688577) with other crustacean and limulid ALFs obtained using BioEdit software: *Macrobrachium rosenbergii* ALF (Mr) (GenBank ID: AEP84102.1), *Fenneropenaeus indicus* ALF (Fi) (GenBank ID: ADE27980.1), *Homarus americanus* ALF (Hm) (GenBank ID: ACC94268.1), *Procambarus clarkii* (Pc) (GenBank ID: ADX60063.1), *Scylla serrata* ALF (Sc) (GenBank ID: ACH87655.1), and *Tachyplesus tridentatus* (Tt) (GenBank ID: AAK00651.1).

Notes: The LPS binding domains are enclosed within a square. The conserved residues are highlighted with uniform background colors.

Abbreviations: ALF, anti-lipopolysaccharide factor; LPS, lipopolysaccharides.

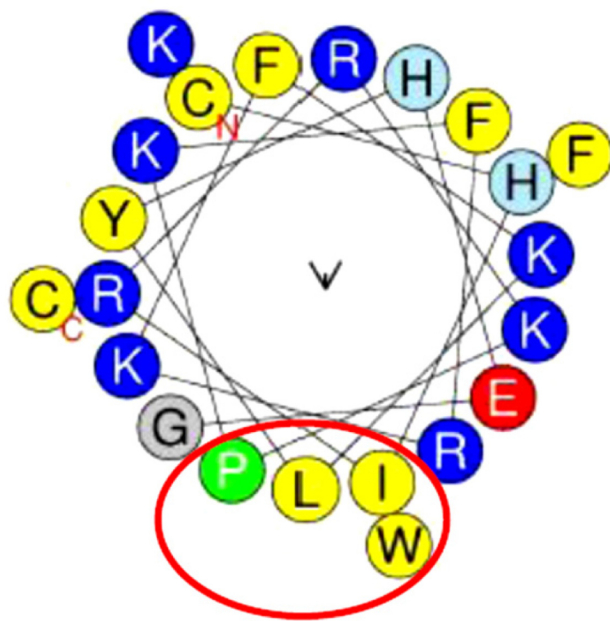


Figure 3 Helical wheel diagram of *Charybdis feriatus*-ALF1 (GenBank ID: KP688577) LPS domain predicted using the HeliQuest online tool.

Notes: The structure was built to identify the amphipathicity of the LPS binding domain. The Amino and Carboxy terminal ends are mentioned as N and C, respectively. The expected hydrophobic face IWLP is shown in the red circle.

Abbreviations: ALF, anti-lipopolysaccharide factor; LPS, lipopolysaccharides.

of ALF, which is mandatory for LPS binding and neutralization.³⁴ Sequence alignment also revealed that the amino acid sequence of the signal peptide was not conserved across different groups, but that of the mature peptide region showed a conserved pattern of the amino acid motif K₇₁GR/KM/FWC₇₉PGW₇₉ straddling the LPS domain; this domain interacts with microbial polysaccharides. The variation of sequences in the mature peptide leads to the functional divergence of ALF among different crustacean groups, and accounts for the differences in sequence characteristics such as theoretical isoelectric point, hydrophobicity, and net charge. In the case of Cf-ALF1,

it differs from SsALF by only one amino acid residue in the signal peptide, whereas the mature peptide region was found to be highly conserved.

The level of antimicrobial activity of Cf-ALF1 predicted by Antimicrobial Peptide Database 2 revealed Cf-ALF1 as a potent AMP with a protein-binding potential (Boman index) and Wimley–White whole-residue hydrophobicity of 2.1 kcal/mol and 15.8 kcal/mol, respectively. In silico analysis of the anticipated LPS binding domain of Cf-ALF1 (C₅₅HIRRKPKFRKFKLYHEGKFWC₇₆) based on amphipathicity, using HeliQuest, showed that Cf-ALF1 is composed of 22 amino acids. Polar residues comprised 45.45% of these amino acids, with the remaining amino acids being nonpolar residues, thus establishing a hydrophobic face with IWLP residues and a hydrophobicity of 0.342 H with a +7 net charge. Such properties could assist in the binding of the LPS domain with the highly negatively-charged lipid A moiety of LPS, and therefore confirm the antimicrobial activity (Figure 3).

Recombinant expression of SsALF has previously been carried out using a bacterial expression system, and it showed a stronger inhibition of Gram-negative bacterial growth than that of Gram-positive bacteria.²¹ Using homology modeling with the template recombinant-ALF-Pm3 (SMTL id: 2job.1) NMR structure (chosen for its shared similarity of 38.61%), prediction of the spatial 3D structure of Cf-ALF1 demonstrated the presence of three α -helices packed against four strands of β -sheets (Figure 4). Somboonwivat et al studied and elucidated the functional role of recombinant-ALF-Pm3 expressed in the *Pichia pastoris* system, and demonstrated a comprehensive spectrum of activity against the filamentous fungi, and both Gram-positive and Gram-negative bacteria.²⁵ In the current study, the predicted RNA sequence based on the Cf-ALF1 cDNA sequence, as well as its ideal secondary

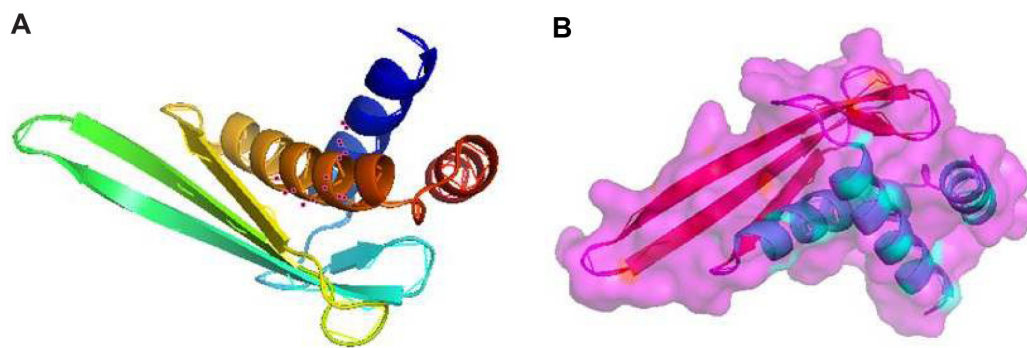


Figure 4 Spatial structure of *Charybdis feriatus*-ALF1 (GenBank ID: KP688577).

Notes: Structure created with the PyMOL software, using the pdb data generated by SWISS-MODEL server. The NMR structure of rALF-Pm3 (SMTL id: 2job.1) was used as template for the data generation. The diagrammatic representation (A) and the spatial structure (B) are presented.

Abbreviations: ALF, anti-lipopolysaccharide factor; NMR, nuclear magnetic resonance; rALF-Pm3, recombinant-ALF-Pm3.

AUGCGGACCAGGGUGAUGGCCGGCCUGUGCGUGGCGCCGUGGUGAUGUGCCUGUACAUGCCCCAG
 CCGUGCGAGGCUCAGUAUGAAGCUCUGGUAGCUUCCAUUCUUGGAAAGCUGUCGGGACUGUGCACAG
 CGACACAGUGGACUUAUGGGACACACUGGCCACAUCGCGCGCAAGCGAAGUUCAGGAAAUUUAAG
 CUGUACCACGAGGGCAAGUUUGUGUCCUGGCUGGACACAUCUCGAGGGCAAUUCGAGGACCAAGA
 GCAGUGCGGGGUCAACCAGGAAGCCACCAAGGACUUCGUGCACAAGCUUACAAAACAAACUCAUC
 ACGAAGAAUAGCGCGGACGCCUGGCUGAAGGGGUGA

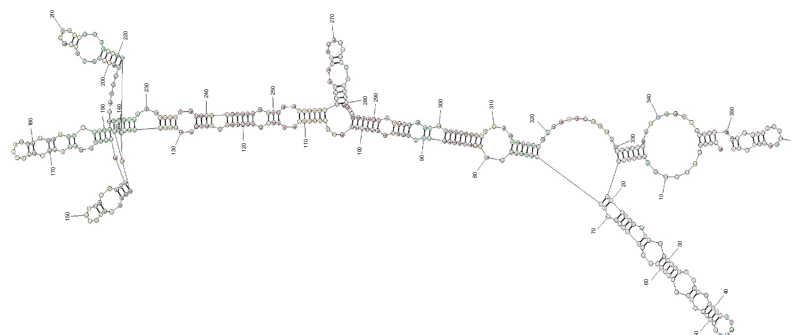


Figure 5 Predicted RNA sequence from cDNA sequence of *Charybdis feriatus*-ALF1 (GenBank ID: KP688577) and its optimal secondary structure of RNA with minimal free energy prediction.

Abbreviation: ALF, anti-lipopolysaccharide factor.

structure with minimum free energy, was found to consist of both double-stranded and looped regions (Figure 5).

Analysis of the phylogenetic tree constructed using the neighbor-joining method clearly showed that ALF sequences were clustered according to species (Figure 6).

The phylogenetic tree revealed that ALFs including Cf-ALF1 and SsALF shared a common ancestral origin and could have subsequently diverged in the course of evolution. The ALF family of AMPs were found to be evolutionarily conserved, with a diversity of ALF isoforms exhibiting the same

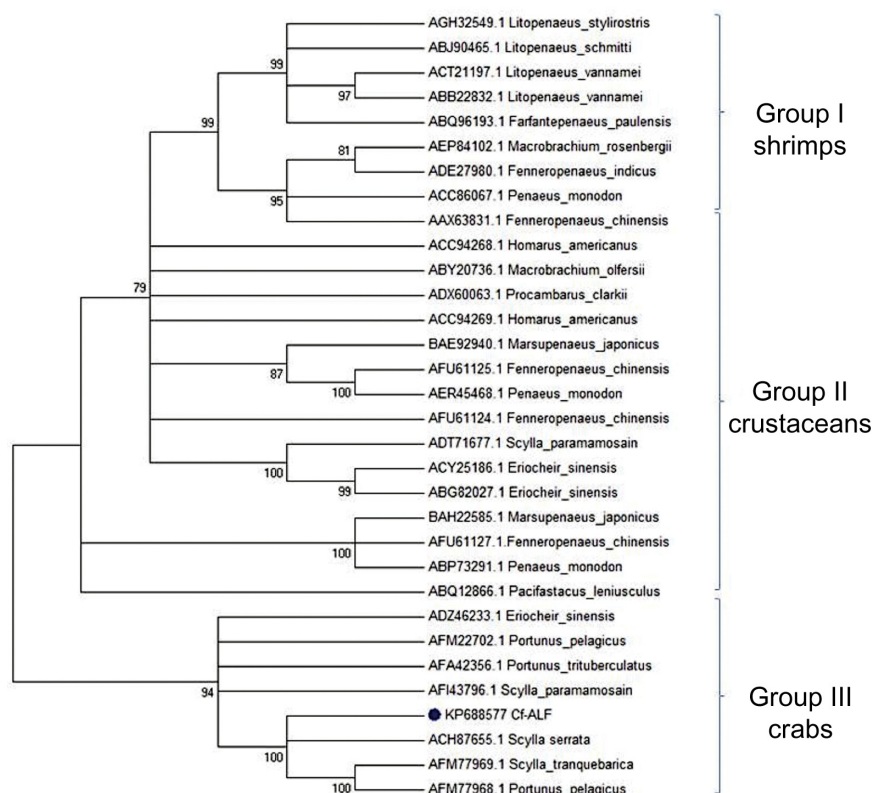


Figure 6 A bootstrapped neighbor-joining tree obtained using Molecular Evolutionary Genetics Analysis version 6, illustrating relationships between the deduced amino acid sequences of the *Charybdis feriatus*-ALF1 (GenBank ID: KP688577) with other ALFs of decapod crustaceans.

Notes: Values at the node indicate the percentage of times that the particular node occurred in 1,000 trees generated by bootstrapping the original deduced protein sequences. Branches corresponding to partitions reproduced in less than 75% bootstrap replicates are collapsed.

Abbreviation: ALF, anti-lipopolysaccharide factor.

physicochemical properties that formed one of the main AMPs involved in the removal of pathogenic microorganisms. The phylogenetic tree could be divided into three groups: Group I consisted of shrimp ALFs, including both penaeid and nonpenaeid shrimp ALFs; Group II comprised all crustacean ALFs; and Group III included crab ALFs. Cf-ALF1 was grouped along with SsALF and other portunid crab ALFs in Group III.

Conclusion

The present study reports the identification, cloning, molecular, and phylogenetic characterization of an isoform of ALF from the crucifix crab, *C. feriatius*, exhibiting an 100% identity to SsALF. The occurrence of sequence variation mainly in the mature peptide could be an important factor that has led to the functional divergence of ALF isoforms from limulids to decapod crustaceans. *C. feriatius* is now an emerging species in the aquaculture market, due to the lower quantities in wild catch and the requirement to meet the demands of the market. Also, *C. feriatius* has been suggested as one of the appropriate species for stock enhancement and aquaculture in the international forum on the culture of portunid crabs.³⁸

To date, there is little research regarding the physiological processes involved in the immune system or the host defense peptides from *C. feriatius*. As there are currently restrictions in antibiotic usage due to the emergence of multidrug resistant strains, the present study is of great significance, particularly in the aquaculture system because *C. feriatius* has shown its ability to combat pathogenic microorganisms. Since recombinant expression of SsALF has already been carried out using a bacterial expression system,²⁷ further work with Cf-ALF1 could include heterologous expression with post-translational modification, using a eukaryotic system such as Baculovirus-mediated insect cell line expression, and characterization of biological activity. These approaches would shed light on the possibilities of the Cf-ALF1 peptide as a therapeutic candidate in aquaculture and medicine. Bioprospecting for AMPs and other host defense molecules from potential aquaculture species would contribute to the understanding of crab immunology, and their potential role in prevention of microbial diseases in wild and domestic animals.

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Disclosure

The authors report no conflicts of interest in this work.

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