Aquaculture 319 (2011) 298–301

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Molecular characterization and phylogenetic analysis of a penaeidin-like antimicrobial peptide, Fi-penaeidin from *Fenneropenaeus indicus*

Swapna P. Antony [a], I.S. Bright Singh [b], Rosamma Philip [a,*]

[a] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, CUSAT, Kochi-16, Kerala, India
[b] National Center for Aquatic Animal Health (NCAAH), CUSAT, Fine Arts Avenue, Kochi-16, Kerala, India

ARTICLE INFO

Article history:
Received 27 January 2011
Received in revised form 31 May 2011
Accepted 8 June 2011
Available online 23 June 2011

Keywords:
Antimicrobial peptide
Penaeidin
Shrimp
Fenneropenaeus indicus
Innate immunity

ABSTRACT

Antimicrobial peptides (AMPs) play a major role in innate immunity. Penaeidins are a family of AMPs that appear to be expressed in all penaeid shrimps. Penaeidins are composed of an N-terminal proline-rich domain, followed by a C-terminal domain containing six cysteine residues organized in two doublets. This study reports the first penaeidin AMP sequence, Fi-penaeidin (GenBank accession number HM243617) from the Indian white shrimp, *Fenneropenaeus indicus*. The full length cDNA consists of 186 base pairs encoding 61 amino acids with an ORF of 42 amino acids and contains a putative signal peptide of 19 amino acids. Comparison of *F. indicus* penaeidin (Fi-penaeidin) with other known penaeidins showed that it shared maximum similarity with penaeidins of *Farfantepenaeus paulensis* and *Farfantepenaeus subtilis* (96% each). Fi-penaeidin has a predicted molecular weight (MW) of 4.478 kDa and theoretical isoelectric point (*pI*) of 5.3.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Cationic antimicrobial peptides (AMPs) play important roles as effectors of the first line of defense in innate immunity of every animal, due to their broad-spectrum antimicrobial activity and rapid production. 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. In penaeid shrimps, three main families of AMPs have been currently described: anti-lipopolysaccharide factors (ALFs), crustins and penaeidins. ALFs possess a broader antimicrobial spectrum (Somboonwiwat et al., 2005), whereas, crustins are reported to have a more-restricted activity spectrum, affecting mainly marine Gram-positive bacteria (Relf et al., 1999). Penaeidins, first characterized from *Litopenaeus vannamei*, act mainly against Gram-positive bacteria, filamentous fungi (Destoumieux et al., 1997), viruses and protozoans (Bachere, 2003) and are also found to possess chitin-binding properties (Destoumieux et al., 2000). Recent studies have revealed the presence of penaeidin mRNAs in different penaeid shrimp species viz. *L. vannamei* (Destoumieux et al., 1997); *Litopenaeus setiferus* (Gross et al., 2001); *Penaeus monodon* (Supungul et al., 2002); *Penaeus japonicus* (Rojtinnakorn et al., 2002); *Litopenaeus schmitti* (Barracco et al., 2005); *Fenneropenaeus chinensis* (Kang et al., 2004); *Farfantepenaeus paulensis* (Barracco et al., 2005) and *Litopenaeus stylirostris* (Munoz et al., 2004). Gueguen et al. (2006) have developed a penaeidin database, named as 'PenBase' to provide comprehensive information about penaeidin properties, diversity and nomenclature.

The Indian white shrimp, *Fenneropenaeus indicus* is the most dominant shrimp species in the shallow water shrimp fishery along the west and east coast of India and an important shrimp species which is cultivated in Pokkali paddy fields (paddy-cum-prawn farming) practiced in the wetlands of Kerala. Though several research works have been carried out for unraveling the biochemical aspects of the defense mechanisms in *F. indicus*, few works have been carried out on AMPs in this species. So far no penaeidin sequences have been reported from *F. indicus*. Discovery of new penaeidin isoforms in *F. indicus* will certainly help us to unravel the host defense mechanism of this shrimp species. In the current study, a penaeidin cDNA has been characterized from the Indian white shrimp, *F. indicus*. This is the first report of this peptide molecule from *F. indicus*.

2. Materials and methods

2.1. Experimental animals and haemolymph collection

Healthy adult *F. indicus* (8–10 g body weight) were collected from a local shrimp farm in Vypeen, Kochi. They were transferred to aquarium tanks and maintained under laboratory conditions. Haemolymph was collected from the rostral sinus using specially designed capillary tubes (RNase-free) and rinsed using pre-cooled anticoagulant solution (RNase free 10% sodium citrate, pH 7.0). Haemolymph was suspended in TRI reagent (Sigma) for total RNA isolation.

* Corresponding author at: Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Kochi-682 016, Kerala, India. Tel.: +91 4842368120; fax: + 91 4842381120.
E-mail addresses: rosammap@gmail.com, rose@cusat.ac.in (R. Philip).

0044-8486/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2011.06.011

2.2. Total RNA isolation and reverse transcription

Total RNA was extracted from the haemocytes using TRI Reagent (Sigma) following 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 further experiments. 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, USA). 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

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), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer and 1 U Taq DNA polymerase (New England Biolabs, USA). Amplification was performed using the primers, Pen-F (5′-cctgaccctcacctgcagaggcc-3′) and Pen-R (5′-ttcgttgtcttctccatcaacc-3′). The thermal profile used was 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °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 Scigenom, Kochi, India.

2.4. Sequence analysis

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 proteins were performed with ExPASy (http://www.au.expasy.org/). The signal peptide was predicted by SignalP program (http://www.au.expasy.org/). Motif scan was performed using the software MotifScan of Expasy program. The multiple sequence alignment was done on amino acid sequences of known penaeidins using CLUSTALW and GENDOC. Amino acid sequences of penaeidins were retrieved from the NCBI GenBank and phylogenetic tree was constructed by the Neighbor-Joining (NJ) method using MEGA version 4.0 (Tamura et al., 2007). The nucleotide sequence and deduced amino acid sequence were submitted to GenBank.

3. Results and discussion

A 263 bp fragment of PCR amplified product was obtained from the mRNA of *F. indicus* haemocytes by RT-PCR (Fig. 1A). The penaeidin cDNA was found to consist of 186 bp nucleotides, encoding 61 amino acids and an ORF of 42 amino acids. The ORF encoded 42 amino acid residues with a predicted molecular weight (MW) of 4.478 kDa and theoretical isoelectric point (*pI*) of 5.3 as calculated by the PROTPARAM software. The analysis with the SignalP software revealed the presence of a signal peptide with 19 amino acids at the N-terminal region of the Fi-penaeidin (Fig. 1A). BLAST analysis of the amino acid sequence revealed the relation of Fi-penaeidin to that of penaeidins from *F. paulensis* and *F. subtilis* (Table 1). As per the nomenclature introduced by Gueguen et al. (2006), Fi-penaeidin could be named as *Fenind* PEN2-1 (HM243617).

The deduced amino acid sequence of Fi-penaeidin was found to be rich in amino acid residues Leucine (13.1%) and Serine (13.1%) followed by Cysteine (9.8%) and Proline (6.6%). Fi-penaeidin was characterized by 10 conserved amino acid sequence in the signal peptide; a threonine and two proline residues conserved in the N-terminal domain; and the conserved cysteine array of the C-terminal structured domain (Fig. 1A). This is in agreement with the penaeidin signature assigned by Gueguen et al. (2006). As per Destoumieux et al. (1997) and Gueguen et al. (2006) this overall structure of penaeidins is quite unique among the AMP families. Motif scan performed on the deduced amino acid sequence also confirmed that the sequence belonged to penaeidin AMPs (Fig. 1B).

A

```
atgcgtctcgtgatctgcctggtcttcttggcctccttcgccctggtctgccaaggccac
 M  R  L  V  I  C  L  V  F  L  A  S  F  A  L  V  C  Q  G  H

agggatacttatacttacccttttctctttatctagagctactggacctctacctctttca
 R  D  T  Y  T  Y  P  F  S  L  S  R  A  T  G  P  L  P  L  S

cccactgtctgctcttcctcttcccttattatcattaatgacactgatgcttgtgcttgt
 P  T  V  C  S  S  S  S  L  I  I  I  N  D  T  D  A  C  A  C

tgctga
 C  *
```

B

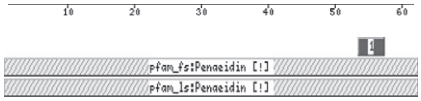
Matches map (Features from query are above the ruler, matches of the motif scan are below the ruler)	 **Legends: 1**, ASN_GLYCOSYLATION
List of matches	FT MYHIT 53 56 ASN_GLYCOSYLATION FT MYHIT 1 61 pfam_fs:Penaeidin [!] FT MYHIT 1 61 pfam_ls:Penaeidin [!]

Fig. 1. (A) Nucleotide and amino acid sequences of Fi-penaeidin from the haemocyte of the Indian white shrimp, *Fenneropenaeus indicus* (GenBank accession no. HM243617). The underlined amino acid residues indicate a putative signal sequence. Cysteine residues that form the C-terminal structure are bold printed. An asterisk is the stop codon. (B) Schematic representation of matches map and list of matches obtained from the Motif Scan search of Expasy program.

Multiple alignment performed for penaeidins confirmed the BLAST analysis which showed that Fi-penaeidin is similar to penaeidins of *L. setiferus*, *F. chinensis*, *F. paulensis*, *F. subtilis* and *L. schmitti* (Figs. 2A, 3A). Phylogenetic tree constructed to study the relationship of Fi-penaeidin with that of other penaeidins revealed that Fi-penaeidin is more closely related to *F. subtilis* and *F. paulensis* (Fig. 2B). The tree could be broadly divided into two groups. Group I included penaeidin-3 of *L. setiferus*, *L. schmitti* and *F. chinensis*. Whereas Group II included Fi-penaeidin and penaeidins of *F. subtilis* and *F. paulensis*. Group II consisted mainly of penaeidins belonging to subgroup 2, whereas the penaeidin subgroup of *F. subtilis* has not been identified. From the tree topology it is assumed that *F. subtilis* penaeidin also belongs to subgroup 2 (Fig. 3B). The phylogenetic tree revealed that Fi-penaeidin belong to Penaeidin-2 group.

4. Conclusion

The discovery of AMPs in crustaceans provides new clues for fundamental understanding of crustacean immunity. The studies on the shrimp penaeidins have largely contributed to this knowledge as they are the first AMPs fully characterized in crustaceans. This is the first report of penaeidin AMP from Indian white shrimp, *F. indicus*. The wide distribution of penaeidins in penaeid shrimps indicates the importance of these AMPs in the innate immunity and a detailed

Table 1
Result of BLASTp analysis of Fi-penaeidin (HM243617).

Accession no.	Description of the AMP	Query coverage	E value	Max identity
AAX58696	*Farfantepenaeus paulensis* (PEN2-2)	100%	8e-05	96%
AAX58695	*Farfantepenaeus paulensis* (PEN2-1)	100%	2e-04	96%
ABO93321	*Farfantepenaeus subtilis* (Penaeidin)	95%	3e-04	96%

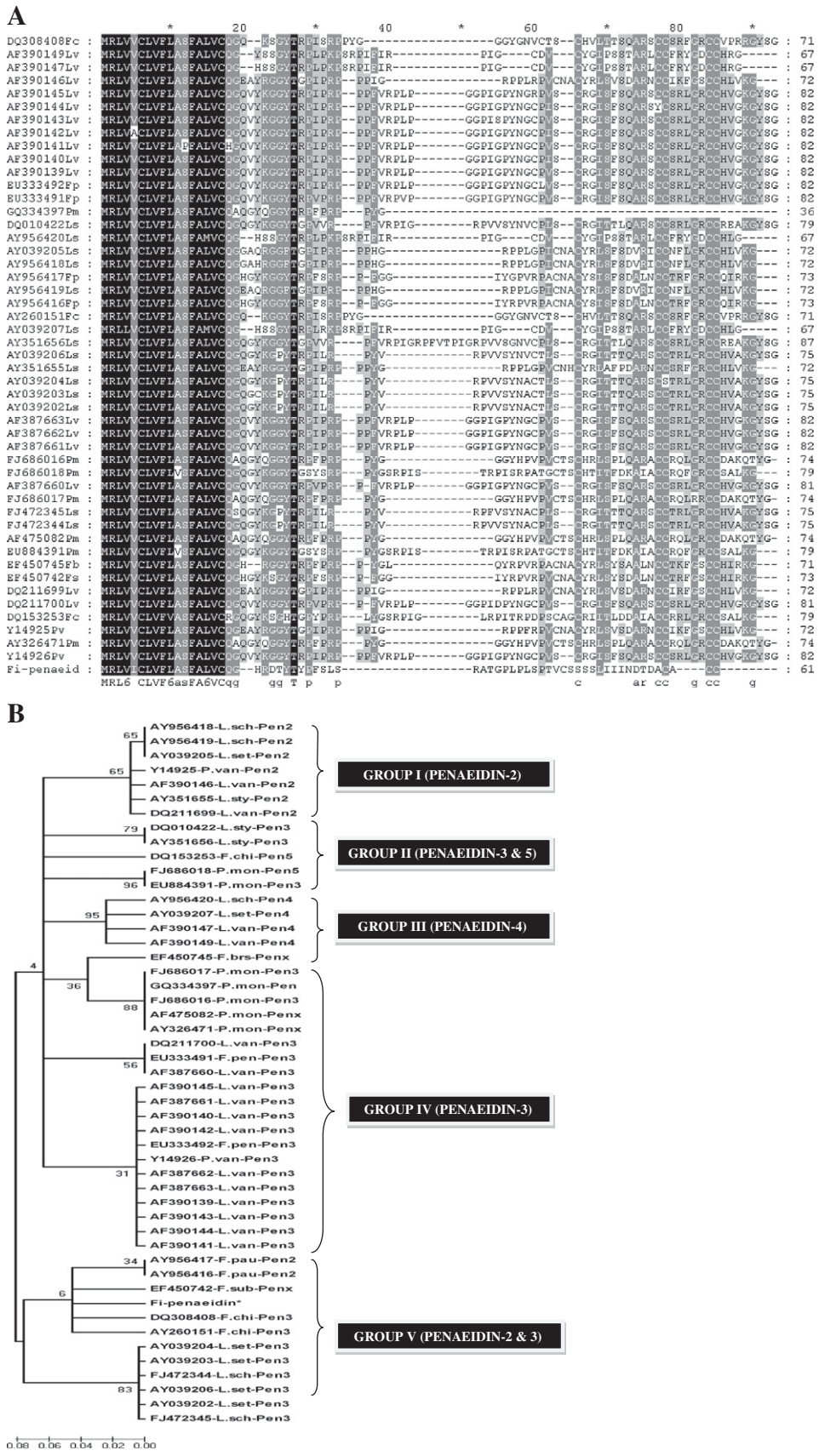


Fig. 2. (A) Multiple alignment of nucleotide sequence of the *Fenneropenaeus indicus* penaeidin-like antimicrobial peptide, Fi-penaeidin (HM243617) with all known penaeidins obtained using GenDoc programme Version 2.7.0. Black and gray indicate conserved sequences. (B) Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* penaeidin-like AMP, Fi-penaeidin (HM243617) with all known penaeidins.

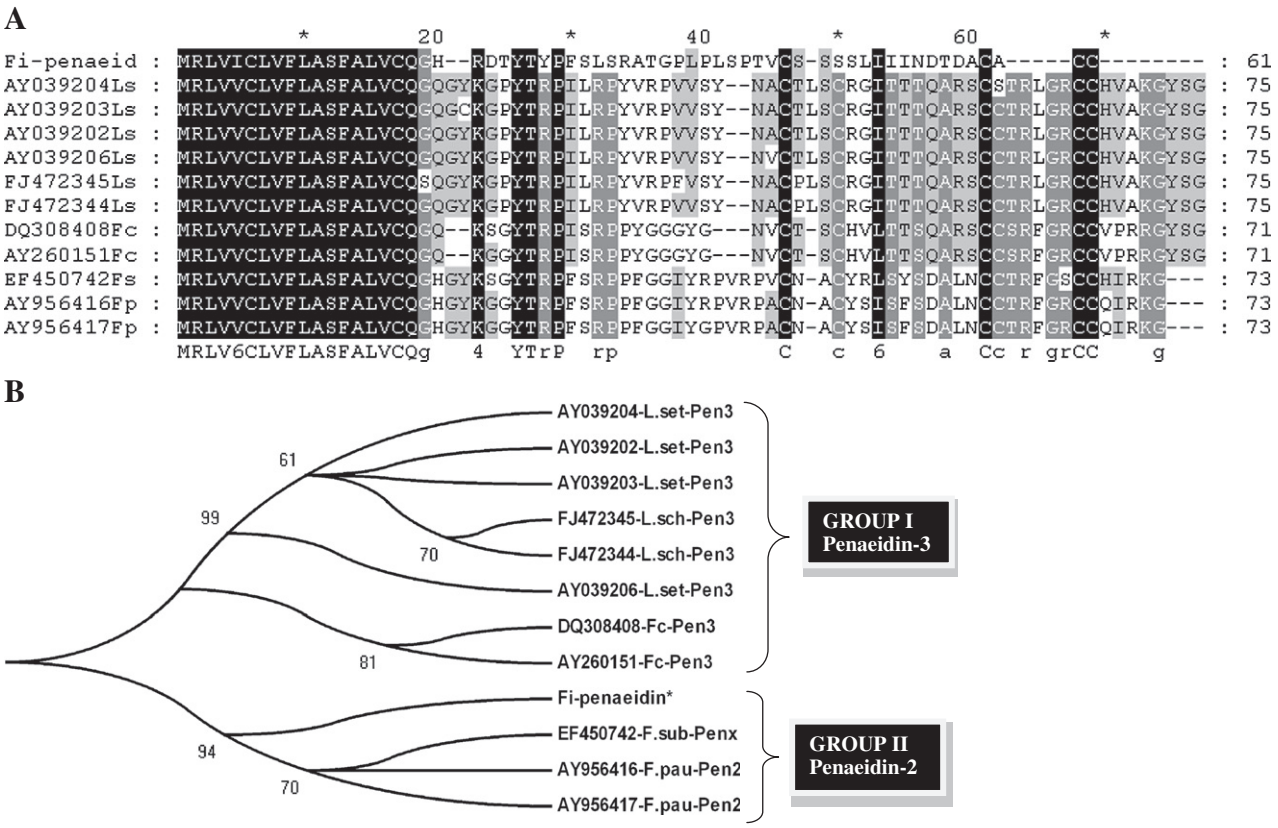


Fig. 3. (A) Multiple alignment of nucleotide sequence of the *Fenneropenaeus indicus* penaeidin-like antimicrobial peptide, Fi-penaeidin (HM243617) with other penaeidins that showed maximum similarity during BLAST analysis obtained using GenDoc programme Version 2.7.0. Black and gray indicate conserved sequences. (B) Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* penaeidin-like AMP, Fi-penaeidin (HM243617) with other penaeidins that showed maximum similarity during BLAST analysis.

research is required to reveal the inscrutable character of penaeidins in shrimp immunity. Discovery of novel penaeidins and its antimicrobial spectrum might pave way to unravel the obscurity of crustacean immunity. Further research on the expression profile of these molecules in response to various environmental conditions and microbial infection would reveal their role in the protection of the animals from the onslaught of diseases.

Acknowledgment

The authors are grateful to the Ministry of Earth Sciences (MoES), Govt. of India for the research grant (MoES/10-MLR/2/2007) with which the work was carried out. The first author gratefully acknowledges KSCSTE (Kerala State Council for Science, Technology and Environment) for the award of fellowship.

References

Bachere, E., 2003. Anti-infectious immune effectors in marine invertebrates, potential tools for disease control in larviculture. Aquaculture. 227, 427–438.

Barracco, M.A., de Lorgeril, J., Gueguen, Y., Bachere, E., 2005. Molecular characterization of penaeidins from two Atlantic brazilian shrimp species. Farfantepenaeus paulensis and Litopenaeus schmitti. FEMS Microbiology Letters. 250, 117–120.

Destoumieux, D., Bulet, P., Loew, D., Dorsselaer, A.V., Rodriguez, J., Bachere, E., 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. 272, 28398–28406.

Destoumieux, D., Munoz, M., Bulet, P., Bachere, E., 2000. Penaeidins, a family of antimicrobial peptides from penaeid shrimp Crustacea, Decapoda. Cell Mol. Life Sci. 57, 1260–1271.

Gross, P.S., Barlett, T.C., Browdy, C.L., Chapman, R.W., Warr, G.W., 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and Atlantic white shrimp, *Litopenaeus setiferus*. Dev. Comp. Immunol. 25, 565–577.

Gueguen, Y., Garnier, J., Robert, L., Lefranc, M.P., Mougenot, I., de Lorgeril, J., Janech, M., Gross, P.S., Warr, G.W., Cuthbertson, B., Barracco, M.A., Bulet, P., Aumelas, A., Yang, Y., Bo, D., Xiang, J., Tassanakajon, A., Piquemal, D., Bachere, E., 2006. PenBase, the shrimp antimicrobial peptide penaeidin database, Sequence-based classification and recommended nomenclature. Dev. Comp. Immunol. 30, 283–288.

Kang, C.J., Wang, J.X., Zhao, X.F., Yang, X.M., Shao, H.L., Xiang, J.H., 2004. Molecular cloning and expression analysis of Ch-penaeidin, an antimicrobial peptide from Chinese shrimp, *Fenneropenaeus chinensis*. Fish Shellfish Immunol. 6, 513–525.

Munoz, M., Vandenbulcke, F., Garnier, J., Gueguen, Y., Bulet, P., Saulnier, D., Bachère, E., 2004. Involvement of penaeidins in defense reactions of the shrimp *Litopenaeus stylirostris* to a pathogenic vibrio. CMLS, Cell Mol. Life Sci 61, 961–972.

Relf, J.M., Chisholm, J.R.S., Kemp, G.D., Smith, V.J., 1999. Purification and characterization of a cysteine-rich 11.5 kDa antibacterial peptide from the granular haemocytes of the shore crab, *Carcinus maenas*. Eur. J. Biochem. 264, 1–9.

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y., Aoki, T., 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. Fish Shellfish Immunol. 13, 69–83.

Somboonwiwat, K., Marcos, M., Tassanakajon, A., Klinbunga, S., Aumelas, A., Romestand, B., Gueguen, Y., Boze, H., Moulin, G., Bachere, E., 2005. Recombinant expression and antimicrobial activity of anti-lipopolysaccharide factor ALF from the black tiger shrimp *Penaeus monodon*. Dev. Comp. Immunol. 29, 841–851.

Supungul, P., Klingbunga, S., Pichyangkura, R., Jitrapakdee, S., Hirono, I., Aoki, T., Tassanakajon, A., 2002. Identification of immune related genes in haemocytes of black tiger shimp (*Penaeus monodon*). Mar. Biotechnol. 4, 487–494.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA 4, Molecular Evolutionary Genetics Analysis MEGA software version 4.0. Mol. Biol. Evol. 24, 1596–1599.