Developmental and Comparative Immunology 48 (2015) 324–341

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Sequence diversity and evolution of antimicrobial peptides in invertebrates

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ARTICLE INFO

Article history:
Available online 17 June 2014

Keywords:
Antimicrobial peptides
Sequence diversity
Defensins
Cecropins
Crustins
Antilipopolysaccharide factors

ABSTRACT

Antimicrobial peptides (AMPs) are evolutionarily ancient molecules that act as the key components in the invertebrate innate immunity against invading pathogens. Several AMPs have been identified and characterized in invertebrates, and found to display considerable diversity in their amino acid sequence, structure and biological activity. AMP genes appear to have rapidly evolved, which might have arisen from the co-evolutionary arms race between host and pathogens, and enabled organisms to survive in different microbial environments. Here, the sequence diversity of invertebrate AMPs (defensins, cecropins, crustins and anti-lipopolysaccharide factors) are presented to provide a better understanding of the evolution pattern of these peptides that play a major role in host defense mechanisms.

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1. Introduction

Antimicrobial peptides (AMPs), which are found in all living organisms ranging from bacteria to humans, are an evolutionarily conserved component of the innate immune system (Hancock and Diamond, 2000; Zasloff, 2002). AMPs exhibit a broad spectrum of activity against bacteria, fungi, yeast, protozoa and viruses. Besides their antimicrobial activity, some AMPs are also recognized for their immunomodulatory properties (Bowdish et al., 2005). AMPs are typically less than 100 amino acids in length, display hydrophobic and cationic properties, and adopt an amphipathic structure (Hancock and Sahl, 2006; Zasloff, 2002). Some AMPs have been discovered to originate as the processed form of other larger proteins, such as buforin II from histone 2A (Kim et al., 2000) and astacidin1 from hemocyanin (Lee et al., 2003). AMPs are grouped into four major classes based on their secondary structure: β-sheet, α-helical, loop and extended peptides (Giuliani et al., 2007). Most AMPs disrupt the target cell membrane and exhibit selectivity for prokaryotic cells over eukaryotic cells (Brogden, 2005; Yeaman and Yount, 2003). The mechanism of membrane permeation may vary for different AMPs depending on the amino acid sequence of the peptides as well as the membrane lipid composition of the microorganism.

As of February, 2014, more than 2000 AMPs have been reported in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php), and they exhibit tremendous sequence diversity. It has been found that AMPs have retained their antimicrobial activity during evolution since they have been used for hundreds of millions of years and yet they have remained effective against microbial targets. Thus, the sequence diversity of AMPs likely indicates the adaptation of organisms to survive in different microbial (pathogen) containing environments (Peschel and Sahl, 2006; Zasloff, 2002).

Invertebrates, which lack an adaptive immune system, rely on an effective innate immunity to protect themselves against microbial infections. Insects, in particular, possess various types of AMPs, including defensins and cecropins, which appear to be major defense molecules. Insect AMPs are predominantly produced by the fat body and are released into the hemolymph (Bulet and Stöcklin, 2005). Upon microbial infection, a single insect might produce up to 10–15 AMPs that can effectively kill the microbial invaders. In the fruit fly *Drosophila*, distinct inducible AMPs have been found to exhibit activity against Gram-positive (defensins) and Gram-negative (cecropins, diptericin, drosocin, attacins and mature prodomain of attacin (MPAC)) bacteria, as well as against filamentous fungi (drosomycin and metchnikowin) (Imler and Bulet, 2005). The simultaneous release of multiple AMPs during an immune response might be a key factor that constrains the evolution of the pathogens (Dobson et al., 2013). AMPs are among the insect immune proteins that evolve more rapidly than non-immune proteins and even more so in the social insects, such as ants and honey bees (Bulmer et al., 2010; Patil et al., 2004; Viljakainen et al., 2009), since they are attributed to a sustained

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http://dx.doi.org/10.1016/j.dci.2014.05.020
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arms race between the host and pathogens and to rapid environmental changes that expose the insects to new pathogens.

In crustaceans, especially shrimps, different AMP families have been reported and characterized (Rosa and Barracco, 2010; Tassanakajon et al., 2010). Shrimp AMPs are primarily produced by circulating hemocytes and are released upon pathogen infection. To overcome the fairly diverse variety of harmful microbes that they are exposed to, shrimps have evolved and use a diverse array of AMPs as an important part of their host defense system. Shrimp AMPs, such as penaeidins, crustins, anti-lipopolysaccharide (LPS) factors (ALFs), lysozymes and stylicins, are comprised of multiple classes or isoforms (Rolland et al., 2010; Tassanakajon et al., 2010).

In this review, we restrict our focus on the evolution of the major AMPs in insects (defensins and cecropins) and crustaceans (crustins and ALFs). Analysis of the sequence diversity found in these invertebrate AMPs should provide an improved understanding of the evolutionary dynamics of the peptides and how the host immune system uses them to counteract different pathogens.

2. Invertebrate defensins

Defensins are an evolutionarily ancient family of cationic AMPs that are commonly characterized by the presence of six or eight cysteine residues that form three or four intramolecular disulfide bonds, respectively, in a complex folded arrangement of two or three antiparallel β-sheets with or without an α-helix structure (Bulet et al., 2004; Froy, 2005). Defensin and defensin-like genes have been discovered in diverse species of fungi, plants, vertebrates and invertebrates (Bulet et al., 2004; Froy, 2005; Hughes, 1999; Mygind et al., 2005; Semple and Dorin, 2012; Zhu, 2008). Generally, the primary structure of defensins can differ considerably between different isoforms according to the spacing patterns between the conserved cysteine residues (Bulet et al., 2004; Froy, 2005; Hazlett and Wu, 2011). In vertebrates, it is well known that defensins are key components of the host innate immune system. Vertebrate defensins can be classified into the three sub-families of α-defensins (three disulfide bonds are formed by the linkage of C1–C6, C2–C4 and C3–C5), β-defensins (C1–C5, C2–C4 and C3–C6), and θ-defensins (a cyclic peptide containing three disulfide bonds), based on the spacing pattern of the six cysteine residues. The α- and β-defensins of vertebrates adopt a three-stranded antiparallel β-sheet structure. The diversification between these three families of vertebrate defensins indicated a likely common evolutionary origin and suggested that the origin of the vertebrate defensin family comes from β-defensins (Dimarcq et al., 1998; Hughes, 1999; Hazlett and Wu, 2011).

Although the evolutionary relationship between invertebrate and vertebrate defensins remains obscure, phylogenetic and three-dimensional structure analyses revealed that there is a closer relationship between invertebrate defensins and vertebrate β-defensins than between vertebrate α- and β-defensins. This then suggests that defensins are ancient molecules that are conserved across the eukaryotic kingdom (Zhu and Gao, 2013). In fungi, defensin-like peptides with a high degree of sequence and structural similarity to invertebrate defensins have been discovered, supporting that invertebrate defensins and fungal defensin-like peptides share a common evolutionary and genetic origin (Mygind et al., 2005; Zhu, 2008). Recently, the likely ancestor of defensins in invertebrates and fungi was traced to a bacterial defensin-like peptide of myxobacterium (Gao et al., 2009), suggesting that myxobacterial defensins are the potential origin of eukaryotic defensins (Zhu, 2007).

In invertebrates, defensins have also been identified in a different phylogenetic group composed of arthropods, mollusks and nematodes, in which they act as key effectors of the innate immune system (Bulet et al., 2004; Dimarcq et al., 1998; Froy, 2005). The evolutionary history of invertebrate defensins has already been reviewed elsewhere (Froy, 2005; Froy and Gurevitz, 2003, 2004; Zhu and Gao, 2013). Invertebrate defensins are secreted AMPs that exhibit similarity in their amino acid sequences, mode of action and three-dimensional structure to each other and are expressed ubiquitously. The defensins are synthesized as prepropeptides, which are then processed through various events to different extents before being released as the active peptides. Several insect defensins have been isolated from the hemolymph of infected animals and also found in the granular hemocytes of non-infected mollusks, termites and scorpions (Bulet et al., 2004; Dimarcq et al., 1998; Froy, 2005). Several defensins have been reported to be active against the membrane of invading microbes. Mammalian α- and β-defensins disrupt the microbial membrane integrity (Froy and Gurevitz, 2003; Risso, 2000), whereas invertebrate defensins (defensin A and sapecin) devastate the bacterial membrane by interacting with the membrane phospholipids and forming complexes that are not miscible in the lipid phase (Bulet et al., 2004; Froy and Gurevitz, 2003; Maget-Dana and Ptak, 1997; Matsuyama and Natori, 1990). Additionally, defensins from lepidopteran insects not only function as membrane-disrupting agents, but also interact with the fungal glucosylceramides (Thevissen et al., 2004). Moreover, mammalian, invertebrates (non-lepidopteran insects and mollusks like oysters), and fungal defensins can act as specific inhibitors of the bacterial peptidoglycan biosynthesis pathway (de Leeuw et al., 2010; Sass et al., 2010; Schneider et al., 2010).

Two major types of invertebrate defensins have been classified. The first type is the largest group and is comprised of peptides that contain six cysteine residues (C1–C4, C2–C5 and C3–C6), and includes the arthropod, insect and mollusk defensins (oyster and abalone). Members of the second type are characterized by having eight cysteine residues (C1–C5, C2–C6, C3–C7 and C4–C8) and contain the mollusk (mussel and oyster) and nematode (worms) defensins (Bulet et al., 2004; Froy, 2005).

In this review, based on the sequence analysis of the conserved cysteine spacing pattern within the defensin motif, invertebrate defensins can be further classified into the five major groups (Fig. 1) of: (I) arthropod and mollusk-type 6-cysteine defensins (known as arthropod defensins), (II) mollusk-type 8-cysteine defensins, (III) nematode-type 8-cysteine defensins, (IV) invertebrate big defensins and (V) invertebrate (putative) β-defensin-like peptides. Alignment of all known invertebrate defensins revealed the consensus defensin motif of each group (Fig. 2) as follows. (I) Arthropod and mollusk-type 6-cysteine defensins: C–X_{5-16}–C–X_3–C–X_{9-10}–C–X_{4-7}–C–X–C; (II) mollusk-type 8-cysteine defensins: C–X_{5-6}–C–X_3–C–X_{4-6}–C–X_{3-4}–C–X_{7-8}–C–X–C–X_2–C; (III) nematode-type 8-cysteine defensins: C–X_{6-15}–C–X_3–C–X_4–C–X_4–C–X_{4-8}–C–X–C–X_2–C; (IV) invertebrate big-defensins: C–X_6–C–X_3–C–X_{13-14}–C–X_4–C–C; and (V) invertebrate β-defensin-like peptides: C–X_6–C–X_4–C–X_{7-9}–C–X_5–C–C, which is similar to the β defensins: C–X_{4-8}–C–X_{3-5}–C–X_{9-13}–C–X_{4-7}–C–C, Bulet et al., 2004).

2.1. Arthropod and mollusk-type 6-cysteine defensins

This defensin family is the major group of invertebrate defensins and contains insect and mollusk defensins (Bulet et al., 2004; Dimarcq et al., 1998; Froy, 2005; Froy and Gurevitz, 2003). Along with plant and fungal defensins, they contain six cysteine residues that form the three disulfide linkages of C1–C4, C2–C5 and C3–C6 and are composed of an α-helix linked to an antiparallel two-stranded β-sheet by these disulfide bridges, referred to as the cysteine-stabilized α-helix/β-sheet (CSαβ) motif (Bulet et al., 2004). This leads to a tertiary structure that is completely different

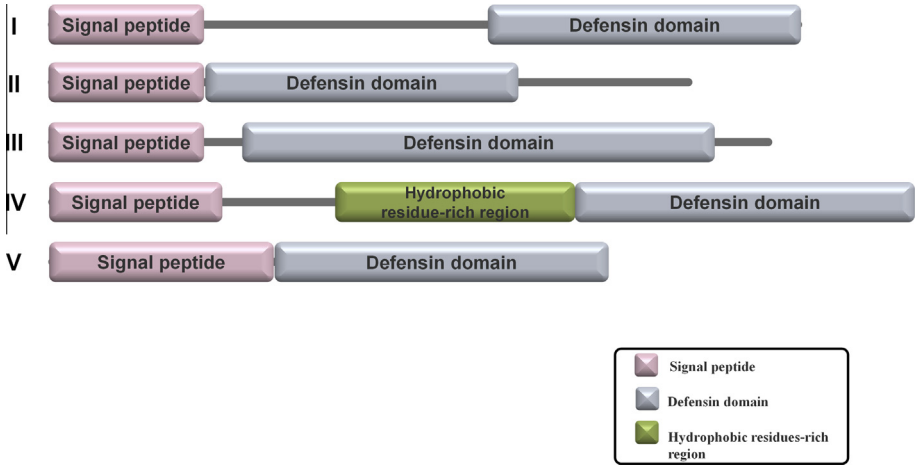

Fig. 1. Schematic illustration of the primary structure of the invertebrate defensins. (I) Arthropod and mollusk-type 6-cysteine defensin (*Aedes aegypti* defensin A; P91793), (II) Mollusk-type 8-cysteine defensin (*Mytilus galloprovincialis* MGD2; AAD45118), (III) Nematode-type 8-cysteine defensin (*Ascaris suum* ASABF; BAA89497), (IV) Invertebrate big defensins (*Tachypleus tridentatus* big defensin; P80957) and (V) β-defensin-like peptide (*Panulirus japonicus* β-defensin-like peptide PjDef1: ACM62357).

to that of vertebrate defensins because the α-helix is located at the N-terminus of defensins (Sawai et al., 2001).

In insects, in which the evolutionary selective trends have been analyzed, defensin can further be classified into the two main lineages of; (I) mainly lepidopteran insects and (II) hemipteran, coleopteran, dipteran and hymenopteran insects. These defensins are diverse, suggesting a potential large diversity in invertebrate immune mechanisms. Overall, the evolutionary analysis indicated that the CSαβ motif of insect defensins has evolved by duplication followed by divergence, to produce a diverse cluster of paralogous genes (Dassanayake et al., 2007).

2.2. Mollusk-type 8-cysteine defensins

In mollusks, several defensins have been discovered, including from mussels, scallop, oyster and abalone (Adhya et al., 2012; De Zoysa et al., 2010; Gonzalez et al., 2007; Gueguen et al., 2006; Schmitt et al., 2010a; Yang et al., 2000). However, based on their sequence comparison, mollusk defensins can be classified into the two major sub-groups of (a) six-cysteine defensins, which belong to a group of arthropod and mollusk-type 6-cysteine defensins, and eight-cysteine defensins, which belong to a group of mollusk- and nematode-type 8-cysteine defensins.

In the mussel *Mytilus galloprovincialis*, an eight-cysteine defensin (named MGD-1) has been isolated from the hemocytes. The structure of MGD-1 consists of an α-helix and two antiparallel β-strands stabilized by four disulfide bridges (C1–C5, C2–C6, C3–C7 and C4–C8), instead of by the three disulfide bonds commonly found in arthropod and mollusk-type 6-cysteine defensins (C1–C4, C2–C5 and C3–C6). MGD-1 possesses antimicrobial activity against Gram-positive bacteria (Bulet et al., 2004; Roch et al., 2008; Yang et al., 2000).

Although the two types of defensin (arthropod and mollusk-type 6-cysteine defensins and mollusk-type 8-cysteine defensins) have different primary structures, they are similar in the CSαβ structural motif and exhibit similar antimicrobial activity, which suggests that the fourth disulfide bond of the mollusk-type 8-cysteine defensin is not essential for the biological activity, but contributes to their stability (Bulet et al., 2004; Schmitt et al., 2010a; Yang et al., 2000).

In the oyster *Crassostrea gigas*, the primary structures of the three defensins (*Cg*-Defm, *Cg*-Defh1 and *Cg*-Defh2) share the same motif with MGD-1. The transcripts of the three different oyster defensins could be detected in one individual oyster in different tissues and at different expression levels. They were all found to be active against Gram-positive bacteria only and were inhibitors of a bacterial biosynthesis pathway, but bound differentially to lipid II. Amino acid sequence analyses suggested that the residues that were involved in lipid II binding had been conserved through evolution and that residues that conferred an improved activity by modifying their charge distribution (improved electrostatic interactions with the bacterial membranes) were under diversifying selection (Gonzalez et al., 2007; Gueguen et al., 2006; Schmitt et al., 2010a,b; Yang et al., 2000).

2.3. Nematode-type 8-cysteine defensins

A well-characterized AMP of nematode is defensin. *Ascaris suum* antibacterial factors (ASABFs), the first nematode defensins, and their homologs in *Caenorhabditis elegans* (CeABFs) contain eight conserved cysteines that form four disulfide bonds and are clearly similar to the mollusk-type 8-cysteine defensins (Bulet et al., 2004; Froy, 2005; Kato and Komatsu, 1996) with the exception of ASABF-6Cys-α that is a six-cysteine defensin (Minaba et al., 2009). The extra disulfide bond (the fourth bond) in nematode defensins (ASABF-α) is similar to that in the mollusk-type 8-cysteine defensin (MGD-1) that contributed to its stability in a high-osmolarity environment (Bulet et al., 2004; Schmitt et al., 2010a; Yang et al., 2000), suggesting a potential similar role of increased stability of ASABF-α defensins. Nematode defensins (ASABF-α and CeABF-2) have the greatest activity against Gram-positive bacteria, with less activity against Gram-negative bacteria and yeasts. The antimicrobial activity of these defensins is probably due to their ability to form pores in microbial membranes (Bulet et al., 2004; Froy, 2005). Recently, a defensin (similar to ASABF) was identified from the demosponge *Suberites domuncula*. The sponge defensin shares a high amino acid sequence similarity to the nematode defensins and showed the characteristic consensus of the nematode CSαβ motif. The recombinant sponge defensin peptide was found to exhibit microbiocidal activity, anti-fungal activity and hemolytic activity (Wiens et al., 2011).

Based on their gene organization, the arthropod and mollusk defensin families (6- and 8-cysteine defensins) exhibit a diverse number and location of introns, suggesting the existence of potential diversity in their immune mechanisms. Their introns are phase-I and they lack introns between the exons of the CSαβ motif, which indicates that arthropod and mollusk defensins are genetically related and have most likely evolved via exon shuffling of autonomous modules (Bulet et al., 2004; Froy, 2005; Froy and Gurevitz, 2003). In nematode defensins, the defensin gene organization

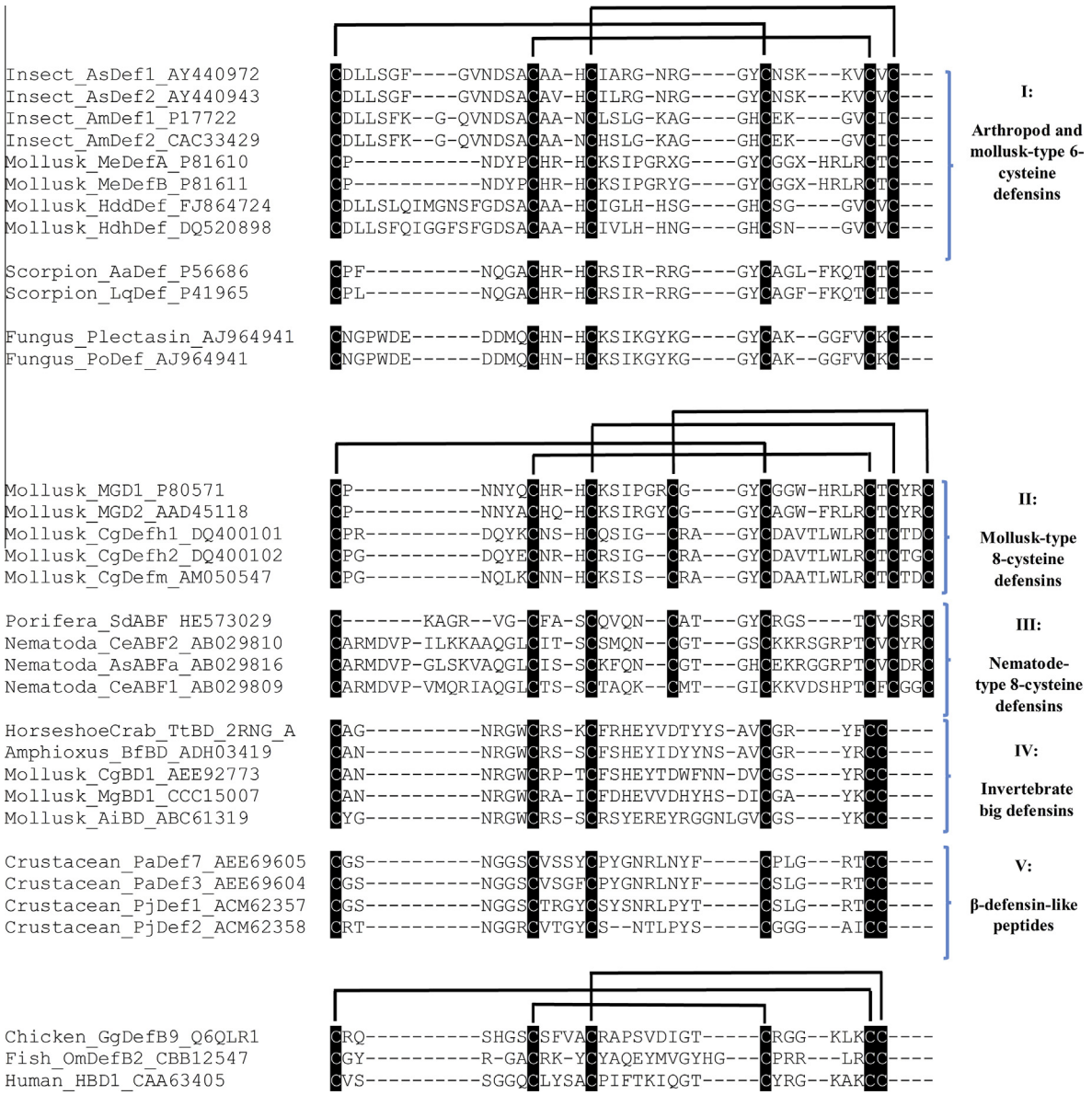

Fig. 2. Multiple alignment of the deduced amino acid sequences of defensin domains in invertebrate defensins. The five major groups are shown as: (I) Arthropod and mollusk-type 6-cysteine defensins, (II) Mollusk-type 8-cysteine defensins, (III) Nematode-type 8-cysteine defensins, (IV) Invertebrate big defensins and (V) β-defensin-like peptides. Black highlights indicate complete conservation of the six or eight conserved cysteine residues of defensins. The disulfide linkages are shown in black lines. GenBank accession numbers are shown in Figure.

contains a phase-0 intron at a conserved location. The intron phase and location are congruent with the postulated intron gain rules, suggesting a gain of an intron before duplication and divergence of the ancestral gene. Thus, based on previous comparisons of the primary structure, antimicrobial activity and genomic organization, arthropod and mollusk defensins and most likely do not share a common ancestor with nematode defensins, but rather have developed via convergent evolution to exhibit similar antimicrobial properties (Bulet et al., 2004; Froy, 2005).

2.4. Invertebrate big defensins

Big defensin is an AMP initially characterized from the hemocytes of the horseshoe crab *Tachypleus tridentatus* that has antimicrobial activities against both Gram-positive and Gram-negative bacteria and fungi (Kawabata, 2010; Saito et al., 1995), and the later from several species of mollusk (mussels, clams, scallop and oysters) and the amphioxus (Gerdol et al., 2012; Rosa et al., 2011; Saito et al., 1995; Teng et al., 2012; Zhao et al., 2007).

The mature peptide of big defensin is divided into the two distinct structural domains of an N-terminal hydrophobic residue-rich region, which was more effective against Gram-positive bacteria, and a cationic C-terminal region containing six-cysteine residues (or defensin-like domain), which was more effective against Gram-negative bacteria. Additionally, the native mature big defensin displays LPS-binding properties, whereas the two separated regions do not (Kouno et al., 2009; Saito et al., 1995; Zhu and Gao, 2013).

Based on the predicted tertiary structure of the six-cysteine big defensin and the pattern of the defensin-like domain of the horseshoe crab big defensin, this domain is likely to form a β-sheet structure folded by three disulfide bonds, which is similar to the β-defensin of human (HBD-2). Moreover, the defensin-like domain structure of the big defensin is different from that of the

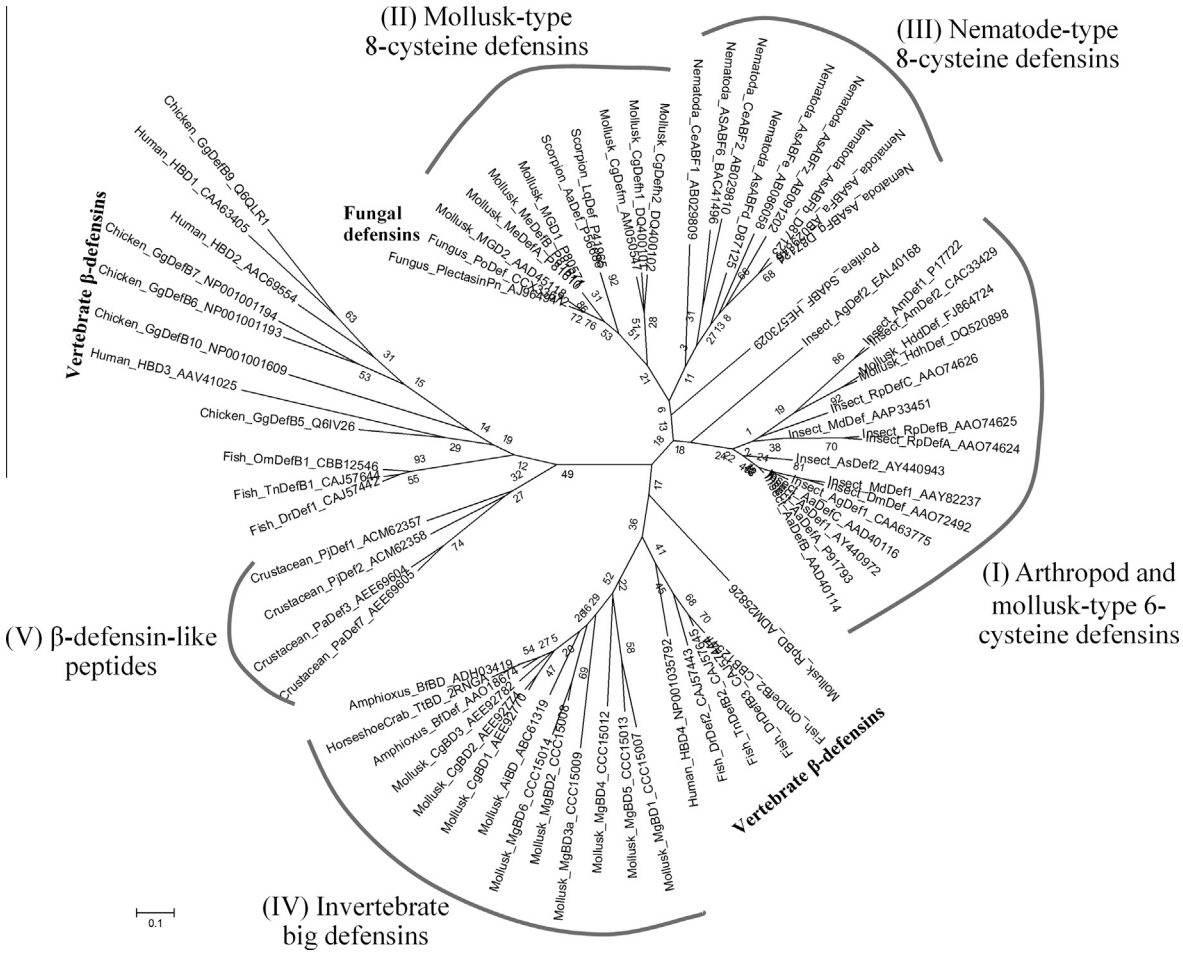

Fig. 3. Phylogenetic analysis of defensins among invertebrates and vertebrates. Neighbor-Joining distance-based phylogenetic tree (unrooted) of the invertebrate defensin genes, including the (I) arthropod and mollusk-type 6-cysteine defensins, (II) mollusk-type 8-cysteine defensins, (III) nematode-type 8-cysteine defensins, (IV) invertebrate big defensins and (V) invertebrate β-defensin-like peptides and the vertebrate β-defensin genes. Bootstrap analysis (1000 replications) values are shown at each node. Sequences of defensin genes were obtained from the NCBI GenBank database (Accession codes in parentheses after the species name). Scale bar represents.

invertebrate CSαβ-containing defensins. These data suggest a possible evolutionary relationship between vertebrate β-defensin and the defensin-like domain of invertebrate big defensins (Zhu and Gao, 2013).

According to the genomics data, the vertebrate β-defensin genes have a conserved exon–intron structure with one phase-1 intron located at the end of the signal peptide. In mollusks and amphioxus big defensins, a single conserved phase-1 intron was found to be situated in the loop interlink of the N- and C- domains, but not in the defensin-like domain. A similar case was also observed in invertebrate CSαβ-containing defensins. Thus, the genomic organization data supports the origin of vertebrate β-defensins from an ancestral invertebrate big defensin via intronization of exonic sequences or exon shuffling (Froy and Gurevitz, 2003; Zhu and Gao, 2013).

2.5. Invertebrate β-defensin-like peptides

To date, β-defensin-like peptides have been reported only in the crustacean lobster *Panulirus* genus, including the Japanese spiny lobster *P. japonicus* (Pisuttharachai et al., 2009a) and the spiny lobster *P. argus* (Montero-Alejo et al., 2012). Due to the limited information available on crustacean genomes, no significant homology of these sequences was found in genome database of other crustaceans and invertebrates.

Analysis of the primary structure (Fig. 2) and phylogenetic tree (Fig. 3) indicated crustacean β-defensin-like peptides do not possess any homology to those of other invertebrate defensins, but rather are somewhat similar to vertebrate β-defensins. The crustacean β-defensin-like peptides also showed the presence of the six-cysteine pattern (C–X_6–C–X_4–C–X_{7-9}–C–X_5–C–C) that is characteristic of vertebrate β-defensins (Bulet et al., 2004). The transcripts of the β-defensin-like peptide of the lobster *P. japonicus* were detected in several of the examined tissues, including hemocytes. Although the phylogenetic origin of crustacean β-defensin-like peptides is still unclear, one possible hypothesis is their convergent evolution from invertebrate defensins. Moreover, to date, crustacean β-defensin-like peptides are still considered as putative peptides because their biological activity has not yet been demonstrated. Further studies are required to demonstrate the antimicrobial activity of these peptides.

3. Cecropins

Cecropins are one family of AMPs that were first isolated from the hemolymph of the giant silk moth, *Hyalophora cecropia* (Hultmark et al., 1980). Many cecropin family members, cecropins and cecropin-like peptides, have been identified and characterized in various lepidopteran, coleopteran and dipteran insects, such as *Sarcophaga peregrina* (Okada and Natori, 1985), *Bombyx mori*

(Morishima et al., 1990), *Drosophila melanogaster* (Kylsten et al., 1990), *Musca domestica* (Liang et al., 2006), *Acalolepta luxuriosa* (Saito et al., 2005), *Artogeia rapae* (Yoe et al., 2006), *Helicoverpa armigera* (Wang et al., 2007), *Papilio xuthus* (Kim et al., 2010), *Spodoptera exigua* (Pascual et al., 2012) and *Ostrinia furnacalis* (Shen et al., 2014). Asides in insects, cecropins have been also identified in the bacterium *Helicobacter pylori* (Pütsep et al., 1999), tunicate (Zhao et al., 1997), ascarid nematodes (Andersson et al., 2003; Tarr, 2012) and mammals (Lee et al., 1989). As observed in other gene families, the cecropin multigene family consists of both functional and pseudo-genes. The cecropin family is classified into five sub-types (cecropin A–E) and the members of the cecropin multigene family varied among the species. Cecropin from *B. mori* was comprised of 13 genes divided into members of four cecropin sub-types (A, B, D and E but not C) (Ponnuvel et al., 2010). Recently, transcriptomic analysis of pathogen-related genes in *S. exigua* identified 10 different genes coding for cecropin that can be divided into six subfamilies (A–F) (Pascual et al., 2012). In *Drosophila*, it was evident that different species contain a different number of cecropin genes. *D. melanogaster* has three functional genes (*CecA1*, *CecA2* and *CecB*), and two pseudogenes (*Cecψ1* and *Cecψ2*), but *CecA1* and *CecA2* both encode for the same peptide, cecropin A (Kylsten et al., 1990). *Drosophila psuedoobscura* has five *Cec* coding sequences *CecI–V*, the products of which are all functional, but *CecIII*, *CecIV* and *CecV* encode the same peptide. *Drosophila subobscura* has seven functional genes (*CecIA*, *CecII1*, *CecIII1*, *CecIV1*, *CecIIV2*, *CecIII2* and *CecII2*) as well as one psuedogene (Cecψ*1*) (Quesada et al., 2005).

Generally, cecropins are synthesized as precursors that consist of 58–64 amino acid pre-propeptides, and the mature peptides are then generated via post-translational processing cleaving the 22–23 amino acid signal peptide (Cheng et al., 2006). The primary translation product contains a well-conserved *Cec* structure (Fig. 4A). Within this subfamily the amino acid sequences of the signal peptide are highly conserved. The mature cecropins contain no cysteine residue and form two amphipathic α-helices (an N-terminal amphipathic α-helix and a C-terminal hydrophobic α-helix) joined by a hinge region. The mature cecropins are basic peptides of approximately 4 kDa in size. A typical feature of most insect cecropins is the presence of a Trp residue at position 1 or 2, and an amidated C-terminus (for review: Bulet and Stöcklin, 2005). The proposed cecropin signature was [KRDEN]-[KRED]-[LIVMR]-[ED]-[RKGHN]-X(0,1)-[IVMALT]-[GVIK]-[QRKHA]-[NHQRK]-[IVTA]-[RKFAS]-[DNQKE]-[GASV]-[LIVSATG]-[LIVEAQKG]-[RKQSGIL]-[ATGVSFIY]-[GALIVQN] (Tarr, 2012). Cecropins can be linear or ring-shaped. These polypeptides are small molecules with a high thermal stability and solubility.

Each cecropin gene has a conserved genome structure with two exons separated by one intron. Note that, among the cecropin subtypes and in different species, the intron lengths are different. Like in other gene families, cecropins are generally arranged in a compact cluster. In *Drosophila*, the *Cec* gene members are arranged in tandem fashion. In *D. pseudoobscura*, *D. melanogaster* and *D. virilis*, the *Cec* genes constitute a single cluster whereas in *D. subobscura*, eight members of the family are split into two clusters located in different regions of the same chromosome. At the Cec83B region, the six *Cec* genes are split into two clusters of three copies each. In each cluster, the three copies are in the same orientation whereas they are in the opposite direction between the two clashes (Quesada et al., 2005). Single genome sequence analysis revealed that the evolution of cecropin originated via gene duplication. The analyses of cecropin families have been reported in model organisms, such as *Drosophila* and *B. mori* (Ponnuvel et al., 2010; Quesada et al., 2005). The presence of transposable elements (TE) in the 5′ and 3′ flanking regions of each paralogous gene confirmed the gene duplication occurrence.

Phylogenetic analysis of cecropin genes from *S. exigua* and *B. mori* also supported that cecropins have evolved independently between these insect taxa by a gene duplication event (Pascual et al., 2012). Considering the insect cecropins from lepidopteran and dipteran species, phylogenetic analysis of the mature peptide sequences revealed that the lepidopteran cecropins cluster apart from the dipteran sequences. In Diptera, the cecropins from each genus (*Drosophila* and *Anopheles*) were grouped together forming a unique branch but separate from the two genera (Fig. 4B).

Although positive selection plays an important role in the evolution of *Drosophila* antibacterial peptides, analysis of cecropin family members suggested that the cecropin multigene family evolved differently from the other *Drosophila* antibacterial peptides. Rather the evolution of the cecropin family in the genus *Drosophila* can be explained by Nei's birth-and-death model due to the presence of the following characteristics: repeated gene duplication within species, nonfunctionalization and loss of some *Cec* copies, and the presence of highly divergent and highly similar copies within species. In this case, the loss of duplicated loci has no deleterious effects if there are enough functional cecropin genes to build up an effective antibacterial response in *Drosophila* (Quesada et al., 2005).

Due to the variation in the cecropin amino acid sequences among the cecropin family members, the antimicrobial activity of each cecropin is different. Thus, in *H. cecropia* the cecropins (A, B, and D) are effective against Gram-negative and Gram-positive bacteria but are more active against Gram-negative bacteria (Hu et al., 2013), with cecropin B being the most active of them (Hultmark et al., 1982). Cecropin B also exhibited activity against bacterial pathogens of fish (Sarmasik et al., 2002), plants (Jan et al., 2010) and porcine (Hu et al., 2013). In *B. mori*, BmcecB6 and BmcecD had a wide antimicrobial spectrum and a strong biological activity. Indeed, BmcecB6 had the highest antimicrobial activity compared to the four other paralogs (Yang et al., 2011). BmcecA1 and BmcecE had a similar antimicrobial spectrum but a narrower range than that for BmcecB6 and BmcecD. In contrast, BmcecC was not able to inhibit the growth of any of all the tested bacterial strains.

Cecropin A from *H. cecropia* has 37 amino acid residues in which seven Lys, one Arg, one Glu, and one Asp are responsible for a net charge of +7 at a neutral pH. The cecropins form random structures in water, and adapt an amphipathic α-helices structure upon binding to a membrane or are present in other hydrophobic environments (Christensen et al., 1988; Silvestro and Axelsen, 2000; Steiner, 1982). The organized amphipathic structure induced changes in the bacterial membrane structure and reduces its integrity (Chen et al., 2003), and the mechanism of action of cecropin A has been established to be by forming ion-permeable channels on synthetic lipid vesicles (Silvestro et al., 1997).

H. cecropia cecropin A and the cecropin-like peptide papiliocin have strong permeabilizing activities against bacterial and fungal cell membranes. Their hydrophobic side-chains, including the aromatic rings of Trp2 and Phe5, make a close contact with the acyl chains of the hydrophobic lipids and this interaction with the bacterial and fungal cell membranes leads to a disruption of the phospholipid structure of the membrane (Lee et al., 2013). Sarcotoxin IA, a cecropin-type antibacterial peptide from the flesh fly *Sarcophaga peregrine*, has 100% amino acid identity to the *D. melanogaster* cecropin A and was shown to exhibit antibacterial activity against Gram-negative bacteria (Okada and Natori, 1985). The molecular mechanism of sarcotoxin IA on binding to lipid A, the component of LPS, was revealed by NMR spectroscopy. Upon interaction with aqueous micelles, the strongly cationic N-terminal segment (Leu3 to Arg18) of sarcotoxin IA forms an amphiphilic α-helix and the Lys4 and Lys5 residues are key residues that take part in this lipid A interaction (Yagi-Utsumi et al., 2013).

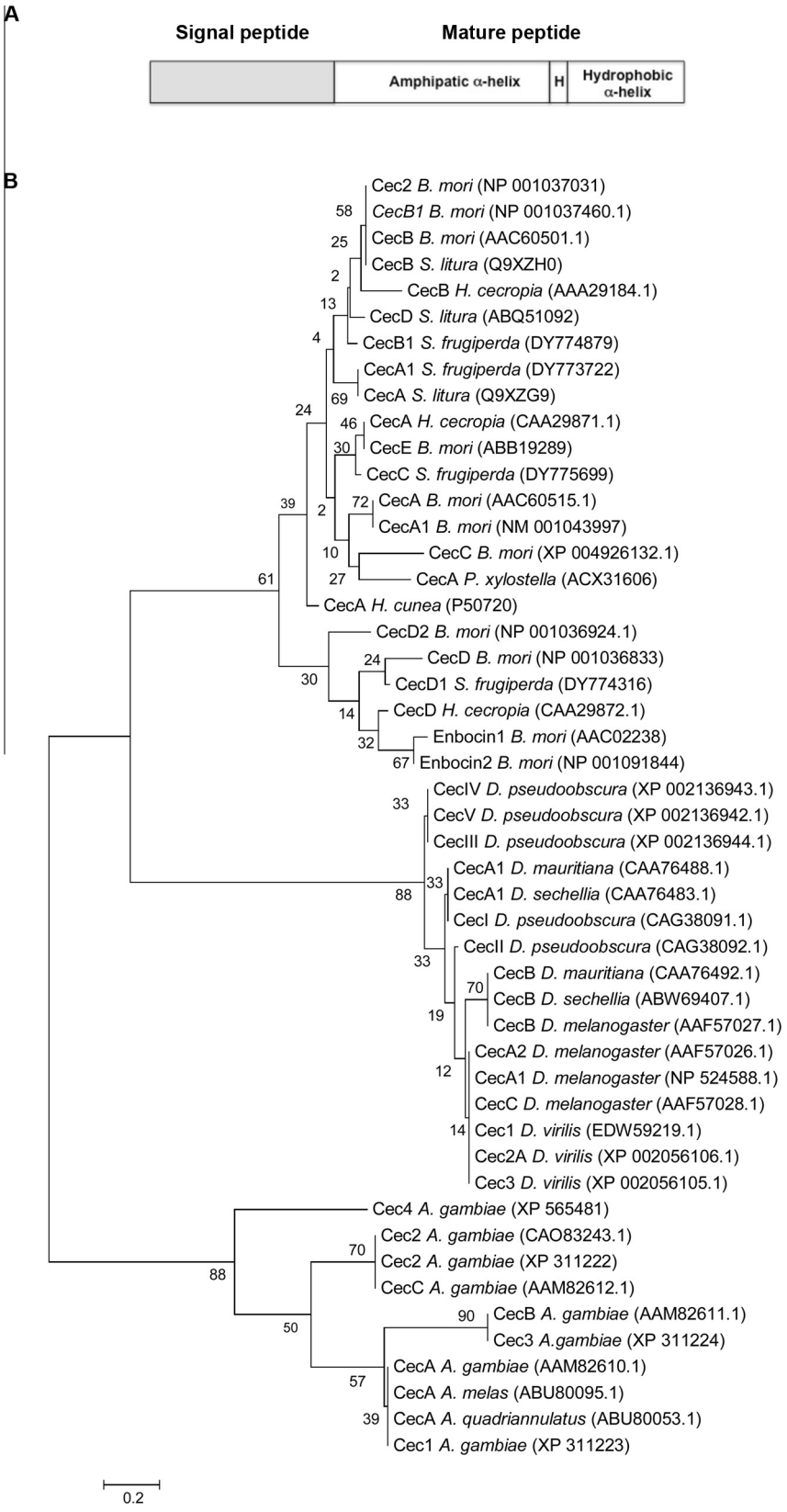

Fig. 4. The simple primary structure of insect cecropin and their phylogenetic analysis. (A) The cecropin structure, based on that of cecropin A of *H. cecropia*, is shown with the N-terminal signal peptide (~22 amino acids) and the mature peptide (~42 amino acids). The N-terminus part of the mature peptide is an amphipathic α-helix that is connected to the hydrophobic C-terminus α-helix by a Gly-Pro hinge region (H). (B) Neighbor-Joining distance-based phylogenetic tree (rooted) showing the relationships between the insect cecropins. The mature cecropin peptides from Lepidoptera; *Bombyx mori*, *Spodoptera litura*, *Hyalophora cecropia*, *Spodoptera frugiperdai*, *Plutella xylostella* and *Hyphantria cunea*, and from Diptera; *Drosophila pseudoobscura, D. mauritiana*, *D. sechellia, D. pseudoobscura, D. mauritiana, D. virilis*, *Anopheles gambiae*, *Anopheles melas* and *A. quadriannulatus*, were selected for the tree construction. Their sequences and accession numbers were retrieved from the GenBank database and are shown in the tree. Bootstrap values, calculated from 1000 replications, are presented.

Cecropin A was identified in the hemolymph of immunized *D. melanogaster* flies, and was found to be able to kill some Gram-negative and Gram-positive bacteria at a concentration of 25–50 μM, except for *Serratia marcescens*, a *Drosophila* pathogen (Samakovlis et al., 1990). Comparative evaluation of the biological activity revealed that mosquito cecropins showed a broader range of activity than *Drosophila* cecropin A, with activities against Gram-negative bacteria, Gram-positive bacteria and yeast. Although, a Trp residue at the beginning of the N-terminal α-helix was thought to be important to cecropin activity, mosquito cecropins are devoid of any Trp residue at this position suggesting that the presence of Trp may inhibit the activities of cecropins against yeast and some Gram-positive bacteria (Vizioli et al., 2000).

Being devoid of adaptive immunity, insects employ their innate immunity to fight against invading pathogens, where cecropins, like other AMPs, are induced and released into the blood stream following synthesis by immune cells upon infection. *H. cecropia* cecropin genes were shown to be controlled at the transcriptional level and induced by bacterial infection. Although *CecA*, *CecB* and *CecD* are encoded for in the same locus, the expression of *CecA* and *CecB* are in parallel but that of *CecD* is different, being delayed (relative to *CecA* and *CecB*) upon bacterial infection (Gudmundsson et al., 1991).

In *Drosophila*, the fat body is the major site of expression of various cecropin genes. No specialized cell types of the fat body lobes are uniquely responsible for the production of *Cec* in *Drosophila*. Rather, not only the fat body but also hemocytes contribute to cecropin production (Samakovlis et al., 1990). *CecA1*, *CecA2* and *CecB* are strongly induced upon bacterial infection in adult, pupae and third instar larvae, but *CecB* is mainly expressed in the pupae whilst *CecA1* is induced in the fat body of hatched larvae by the inducing signal transmitted from the bacterial-infected late stage embryos (Tingvall et al., 2001).

In *B. mori*, different cecropin gene paralogs are expressed in response to microbial stimulation to different degrees. Yang et al. (2011) reported that upon systemic infection (by injection) with the Gram-negative bacterium *Escherichia coli*, Gram-positive bacterium *Staphylococcus aureus* and fungus *Pichia pastoris*, of the pupae, the *BmcecA1*, *BmcecB6*, *BmcecD* and *BmcecE* genes were induced at 6 h post injection with an obviously higher induction level following the fungus infection than after either bacterial infection. The induced expression levels of cecropin were positively correlated with their antimicrobial activities, where *BmcecB6* and *BmcecD*, which have a stronger and broader range of antimicrobial activity than other family members, were induced at higher levels than other paralogs, whilst *BmcecE*, which had a low induction level, had a limited antimicrobial spectrum and weak antimicrobial activity. The correlation between antimicrobial activity and induced expression of AMPs suggested that whilst the major genes likely have their main functions in the antimicrobial defenses, the other members of each family might function as facilitators of antimicrobial defense. Indeed, the possibility of synergistic interactions between cecropin paralogs remains to be evaluated.

Signal transduction pathways are the key processes that cells use to transmit signals from the different stimuli recognized by pattern recognition proteins to then activate the appropriate expression of specific genes encoding AMPs. Two signaling pathways, the Toll and Immune-deficiency (Imd) pathways, are involved in regulating the expression of cecropins in insects. In *D. melanogaster*, the cecropin A genes are selectively induced by the Gram-positive bacteria, *Micrococcus luteus* and *S. aureus*. Induction of *CecA1* requires the Imd/Relish pathway whereas the induction of *CecA2* requires the Toll/Dif pathway (Hedengren-Olcott et al., 2004).

Regulation at the post-transcriptional modification stage also contributes to the control of cecropin gene expression upon an immune response. The cis-acting AU-rich element (ARE) at the 3′ UTR of *Drosophila CecA1* mRNA specifically interacts with the RNA destabilizing protein transactor Tis11 resulting in the control of transient expression of the highly expressed *CecA1* upon LPS induction (Wei et al., 2009).

4. Crustins

Crustin AMPs were originally identified in the crab *Carcinus maenas* as an 11.5 kDa antibacterial peptide named carcinin with anti-Gram-positive bacterial activities (Relf et al., 1999). Subsequently, based on genomics research, several cDNAs of crustin and crustin-like peptides have been identified from several tissues of crustaceans (Afsal et al., 2011, 2013; Antony et al., 2010, 2011; Arockiaraj et al., 2013; Battison et al., 2008; Brockton and Smith, 2008; Cui et al., 2012; Donpudsa et al., 2010; Hauton et al., 2006; Imjongjirak et al., 2009; Jiravanichpaisal et al., 2007; Kim et al., 2012; Liu et al., 2011; Mu et al., 2010, 2011; Pisuttharachai et al., 2009b; Smith, 2011; Smith et al., 2008; Soonthornchai et al., 2010; Sperstad et al., 2009, 2010; Sun et al., 2010; Tassanakajon et al., 2010; Yue et al., 2010) and different species from the insect order Hymenoptera (Zhang and Zhu, 2012).

Crustins are cationic cysteine-rich AMPs that contain a multi-domain region (glycine-rich, proline-rich or cysteine-rich) at the N-terminus and a four-disulfide core (4-DSC) with a whey acidic protein (WAP) domain at the C-terminus (Smith, 2011; Smith et al., 2008; Tassanakajon et al., 2010). Crustins are currently classified into three major groups (types I–III) (Smith et al., 2008), but recently double WAP domain (DWD)-containing proteins have been discovered in crustaceans and found to exhibit antimicrobial activities (Chen et al., 2008; Du et al., 2009; Jiménez-Vega and Vargas-Albores, 2007; Li et al., 2012, 2013; Smith, 2011; Suthianthong et al., 2011), whilst based on comparative genomic analysis, a novel type of insect crustin has been identified in an ant (Zhang and Zhu, 2012). In this review, we include DWD-containing proteins (types I–III) and also the novel ant crustins as type IV and type V crustins, respectively. To date, all crustin types contain a signal peptide sequence, which implies that they are secretory peptides, but they have high sequence diversity within each crustin family. At the N-terminus of the mature protein, crustins have different domain organizations and vary greatly in length of amino acid sequences. Nevertheless, the structural conservation of the WAP domain suggests that its function might be similar to that in vertebrates.

The basic structures of the crustins (types I–V) are shown schematically in Fig. 5. Type I crustins are characterized by the presence of a cysteine-rich domain with two disulfide bridges at the N-terminus and a single WAP domain at the C-terminus. The type II crustins contain a long glycine-rich hydrophobic region (20–80 amino acids) at the N-terminus and a cysteine-rich domain and C-terminal single WAP domain. Unlike type I and II crustins, type III crustins (single WAP domain (SWD) containing proteins) are characterized by the presence/absence of a short proline and arginine-rich region at the N-terminus and a single WAP domain near the C-terminal (Smith, 2011; Smith et al., 2008; Tassanakajon et al., 2010). Nevertheless, the (putative) type IV crustins (DWD crustins) differ from the other types of crustins in that they have two WAP domains (Smith, 2011). Finally, the (putative) type V crustins, known from within the insect order Hymenoptera, resemble type I crustins but with an aromatic amino acid-rich domain between the cysteine-rich domain and WAP domain (Zhang and Zhu, 2012).

Based on the NJ phylogenetic analysis of the amino acid sequences of the WAP domains, the WAP proteins are divided into two main branches: Group I contains the type I, II and V crustins and group II contains the type III and IV crustins and the other

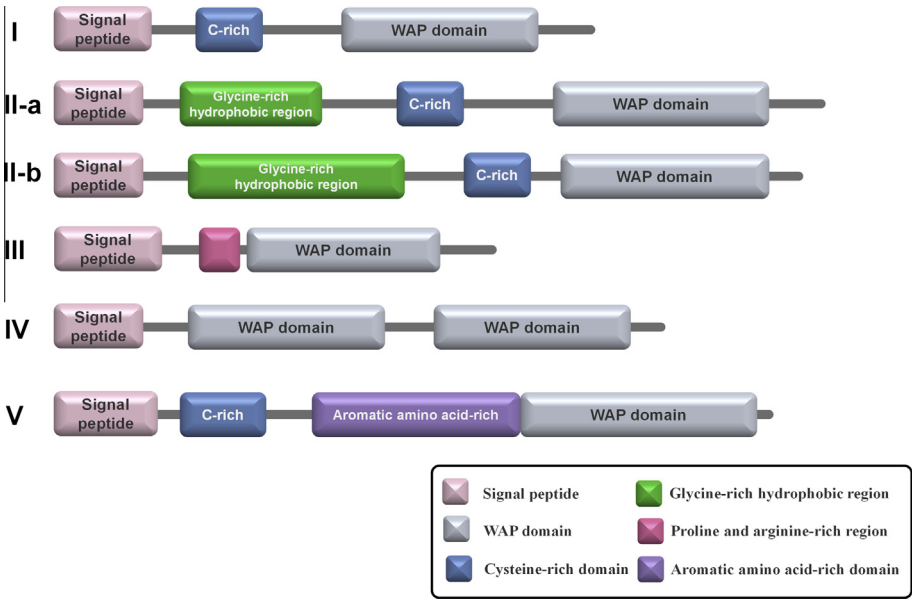

Fig. 5. Schematic illustration of the primary structure of the invertebrate crustins. (I) Type I crustin (*C. maenas* Car*Cm*; CAD20734), (II) type IIa crustin (*P. monodon* Crus*Pm*1; ACQ66004) and type IIb crustin (*P. monodon* Crus-like*Pm*; EF654658), (III) type III crustin (*P. monodon* SWD*Pm*2; EU623980), (IV) type IV crustin (*F. chinensis Fc*DWD; ACY64754) and (V) type V crustin (*Acromyrmex echinatior Aecrustin*; EGI58835).

vertebrate and invertebrate WAP-domain containing proteins (Fig. 6). In group I, the tree could be broadly sub-classified into two major subgroups of the (a) type I crustins of crab, crayfish, prawn and lobster and the type V crustins of hymenopteran (insect) and (b) the type II crustins (II-a and II-b) of shrimps. However, the type III and IV crustins were clustered into group II that was composed of the secretory leucocyte protease inhibitor (SLPI), Elafin and Waprin families in the phylogenetic tree. The tree topologies revealed the evolutionary relationships of crustin families and other WAP domain containing proteins from vertebrates and

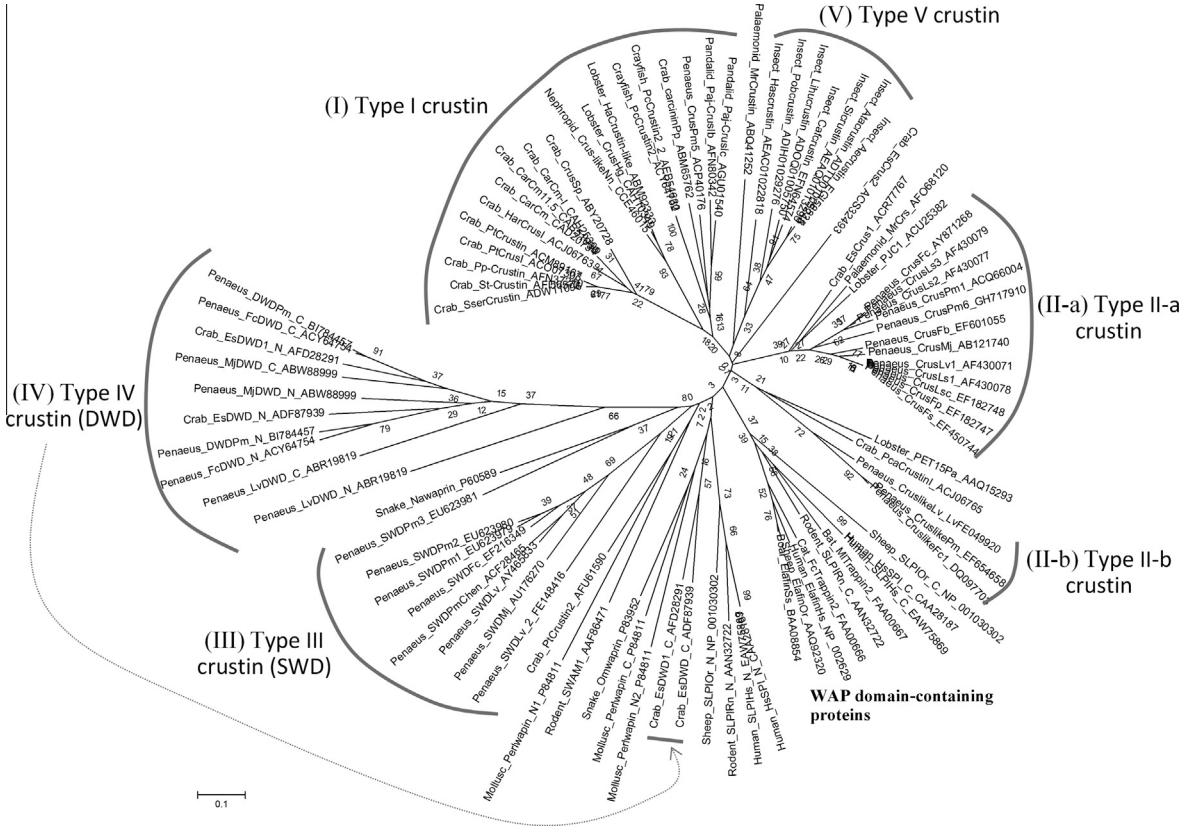

Fig. 6. Phylogenetic analysis of the WAP domains of invertebrate crustins and WAP domain containing proteins. Neighbor-Joining distance-based phylogenetic analysis of the type I, II, III, IV and V crustins from invertebrates and WAP domain containing proteins from invertebrates and vertebrates. Bootstrap analysis (1000 replications) values are shown at each node. Sequences of the crustin genes were obtained from the NCBI GenBank database.

invertebrates and indicated that crustins have evolved from a common ancestor and have likely undergone divergent evolution. Note that members of these crustin families are present in different invertebrate species.

Generally, WAP domain-containing proteins in animals display highly diverse biological functions in the immune system, including antimicrobial and proteinase inhibitory activities. Several studies have reported that crustins show different expression patterns and also vary in response to different environmental stimuli, such as the microorganism species, microbial cell wall components and temperature (Smith, 2011; Smith et al., 2008; Tassanakajon et al., 2010). Additionally, studies on the function of crustins suggest that they play a role as AMPs against Gram-positive bacteria (type I, II and III crustins) and Gram-negative bacteria (type IIb and some type III crustins) and as proteinase inhibitors (type III and IV crustins) in invertebrate immunity.

4.1. Type I crustins

Generally, type I crustins are only active against Gram-positive bacteria and are also present mainly in crabs. The carcinin, isolated from hemocytes of the green crab *C. maenas*, is the first native crustin. The carcinin *Cm*1 of the green crab only exhibited specific antibacterial activity against Gram-positive or salt-tolerant bacteria (Relf et al., 1999). In the mud crab, *Scylla paramamosain*, the recombinant crustin protein (rCrus*Sp*) could inhibit the growth of Gram-positive bacteria but had no inhibitory activity towards Gram-negative bacteria (Imjongjirak et al., 2009). In the spider crab, *Hyas araneus*, the native crustins (Cru*Ha*1 and 2) showed antimicrobial activities mainly against Gram-positive bacteria with only low activities against Gram-negative bacteria and yeasts (Sperstad et al., 2009). In the crayfish *Pacifastacus leniusculus*, both r*Pl*crustin1 and r*Pl*crustin2 inhibited the growth of only one Gram-positive bacterium (Donpudsa et al., 2010). In the Chinese mitten crab *Eriocheir sinensis*, rCrus*Es* could inhibit the growth of Gram-positive bacteria, but showed no significant activity against Gram-negative bacteria (Mu et al., 2010). Recently, a crustin (Crus*Es*2) from the crab *E. sinensis* was identified as a novel type III crustin that contains only a C-terminal single WAP domain with anti-Gram-positive bacterial activity (Mu et al., 2011). However, after re-analysis of the Crus*Es*2 sequences (GQ200833), we found that two mutations at the conserved cysteine residues normally involved in disulfide bond formation were present in the N-terminal cysteine-rich domain. Therefore, the placement of Crus*Es*2 as a member of either the type I or III crustin awaits further characterization.

To date, little is known about the biological activities and molecular functions of type I crustin. However, based on the molecular phylogenetic tree (Fig. 6), crustins of several species from crabs, crayfish and lobsters are clustered in same branches on the basis of the similarity of their amino acid sequences to those of type I crustins. These data imply that these crustins may have similar Gram-positive bactericidal activity in the immune defense system of crustaceans.

4.2. Type II crustins

Type II crustins are the most abundant type of crustin families and show a very diverse mixture of isoforms (Smith et al., 2008; Tassanakajon et al., 2010). Phylogenetic and alignment analyses revealed that type II crustin could be divided into two subgroups, type IIa crustins and type IIb crustins (Crustin-like peptides that differ from Type IIa in their peptide length between the cysteine-rich domain and thye single WAP domain) (Amparyup et al., 2008a; Tassanakajon et al., 2010). In type IIa crustins, crustin*Pm*1 is the major crustin isoform in the shrimp *Penaeus monodon* and exhibited a bactericidal activity against only Gram-positive bacteria (Supungul et al., 2008; Krusong et al., 2012). In type IIb crustins, the rCrus-like*Pm* of *P. monodon* showed strong antimicrobial activity towards both Gram-positive and Gram-negative bacteria (Amparyup et al., 2008a; Krusong et al., 2012). In addition, two recombinant peptides (*P. monodon* Crus*Pm*5 and *Fenneropenaeus chinensis* Crus-like*Fc*1) showed *in vitro* antimicrobial activities against Gram-positive bacteria but not against Gram-negative bacteria (Vatanavicharn et al., 2009; Zhang et al., 2007). In contrast, an *in vivo* study using gene silencing of crustin (type IIa crustin) in the shrimp *Litopenaeus vannamei* caused an increased susceptibility to infection with the Gram-negative bacteria *Vibrio penaeicida*, but not the fungus *Fusarium oxysporum* (Shockey et al., 2009), suggesting a potential immune role against Gram-negative bacteria.

A comparative *in vitro* study of the antimicrobial properties of rcrustin*Pm*1 (type IIa) and rCrus-like*Pm* (type IIb) indicated that both crustins could bind to both Gram-positive and Gram-negative bacteria and also to microbial cell wall components of LPS and lipoteiochoic acid (LTA). However, quantitative binding and bacterial agglutination assays showed that rcrustin*Pm*1 bound more tightly to the LTA of Gram-positive bacteria than to the LPS of Gram-negative bacteria and induced agglutination of Gram-positive bacteria but not Gram-negative bacteria. In contrast, rCrus-like*Pm* could bind LPS and LTA with similar affinities and induce bacterial agglutination of Gram-negative bacteria, suggesting that the antimicrobial activity of the type IIa and type IIb crustins mainly relies on their ability to agglutinate bacterial cells and to disrupt the physiochemical properties of the microbial cell surface (Krusong et al., 2012).

The variation in the antimicrobial activities of crustins may arise from the differences in the type and/or isoform (amino acid sequence). Recently, the adaptive evolution of type IIa and type IIb crustins in crustaceans revealed the pervasive role of positive codon selection in the WAP domain, suggested that those few amino acid substitutions might play a crucial role in the successful host adaptation to a diverse array of bacterial pathogens (Padhi, 2012). Nevertheless, further studies to elucidate the function of the likely positively selected sites are still required.

4.3. Type III crustins

According to the NJ-based phylogenetic tree (Fig. 6), the type III crustins are grouped into the second group (group II) of WAP proteins that is also composed of the type IV crustins, SLPI and Elafin with proteinase inhibitory activity. In shrimps, type III crustin (rSWD*Pm*2 of *P. monodon*) possesses a strong antimicrobial activity against Gram-positive bacteria and exhibits a proteinase inhibition activity against subtilisin A (Amparyup et al., 2008b). In *F. chinensis*, rSWD*Fc* had antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi and also had inhibitory activity against subtilisin A, proteinase K and trypsin (Jia et al., 2008). Both SWD*Pm*2 and SWD*Fc* have the typical characteristics of type III crustins with an N-terminal proline and arginine-rich region and a C-terminal WAP domain. However, two crustins (atypical type III crustins) from the shrimp *Marsupenaeus japonicus* and the crayfish *Procambarus clarkii* contain only a single WAP domain and the proline and arginine-rich region is absent. These crustins have only proteinase inhibitory activities against subtilisin A, proteinase K and bacteria-secreted proteases, but no antimicrobial activity despite the fact that they could bind to several Gram-negative and Gram-positive bacteria. Accordingly, the proline and arginine-rich region of type-III crustins may be responsible for their antimicrobial activity (Du et al., 2010; Jiang et al., 2013), but the full biological role of this region requires further investigation.

4.4. Type IV crustins

The putative type IV crustins (DWD-containing proteins) contain two WAP domains in the mature peptide. To date, type IV crustins have only been found in crustaceans, and specifically one isoform in shrimps (Chen et al., 2008; Du et al., 2009; Jiménez-Vega and Vargas-Albores, 2007; Suthianthong et al., 2011) and two isoforms in crabs (Li et al., 2012, 2013). The NJ-based phylogenetic analysis of the WAP proteins showed that the N- and C-terminal WAP domains of the type IV crustins are closely related to the WAP domain of type III crustins (SWD) (Fig. 6). Both WAP domains of type IV crustins share only a limited sequence similarity within and between species. However, two conserved motifs (KXGXCP and CXXP motif) found in the WAP domain of most vertebrate WAPs (Ranganathan et al., 1999) are also found in the C-terminal WAP domains of the type IV crustins in the crab, but are absent in the N-terminal WAP domains of the crab and in both WAP domains in the shrimp type IV crustins.

With respect to the biological activities, the shrimp type IV crustins exhibit proteinase inhibitory activities but not antimicrobial activities, where the crab type IV crustins exhibit both proteinase inhibitory and antibacterial growth. In the shrimp *M. japonicus*, r*Mj*DWD can inhibit bacterial proteinases (Chen et al., 2008), whilst in the shrimp *F. chinensis* the mature DWD (*Fc*DWD), DWD domain, N-terminal WAP domain (WAP1) and C-terminal WAP domain (WAP2) did not exhibit any antibacterial activity. However, *Fc*DWD and WAP2 bind to both Gram-negative bacteria and Gram-positive bacteria, but WAP1 only binds to Gram-positive bacteria. In addition, *Fc*DWD and WAP1 exhibited proteinase inhibitory activity against secretory bacterial proteinases, but WAP2 had no activity (Du et al., 2009). In *P. monodon*, *Pm*DWD has no antibacterial activity and no inhibitory activity against commercial proteinase, but inhibited the proteinase activity of crude extracts of shrimp lymphoid organs and bacterial proteinases. Mutation of the putative proteinase inhibitory region (Phe70 to Arg), conferred inhibitory activity against subtilisin A. The proteinase inhibitory activities of shrimp DWDs suggest that they are involved in anti-proteinase response(s) to microbial invasion (Suthianthong et al., 2011). In the crab *E. sinensis*, two isoforms of DWD with a 60% amino acid similarity have been reported (Li et al., 2012, 2013). Isoform-A (*Es*DWD; GU002539) displayed antimicrobial activities against Gram-negative bacteria and yeasts (Li et al., 2012), whilst isoform-B (*Es*-DWD1; JX101865) exhibited both antimicrobial and proteinase inhibitory activities and also agglutination activities and can bind to fungal and bacteria cells (Li et al., 2013).

The different role of type IV crustins from shrimps and crabs in their antimicrobial properties needs to be studied further, including the mechanisms of their antimicrobial activities. However, the currently available data suggest that type IV crustins play an important role in the crustacean immunity against microbial infection, possibly through proteinase inhibitory activity and/or antimicrobial activity.

4.5. Type V crustins

The putative type V crustins were first discovered in the ant genome. They share a consensus cysteine-rich domain at the N-terminus and a single WAP domain at the C-terminus. However, type V crustins contain an extra aromatic amino acid-rich region located between the cysteine-rich and WAP domains. Based on the sequence analysis of insect genomes (Zhang and Zhu, 2012), type V crustins are also found in several species of Hymenoptera, including ants (*Atta cephalotes*, *A. echinatior*, *Camponotus floridanus*, *Harpegnathos saltator*, *Pogonomyrmex barbatus*, *Solenopsis invicta* and *Linepithema humile*), bees (*Bombus impatiens*, *B. terrestris* and *Megachile rotundata*) and the parasitoid wasp (*Nasonia vitripennis*), but not in the honeybee *Apis mellifera*. Furthermore, homologs of type V crustins are not found in crustaceans as well as other invertebrate species. The NJ-based phylogenetic tree indicates that type V crustins are more closely related to type I crustins than to the other crustin types (Fig. 6), which suggested that type V crustins may have evolved from an ancestral type I crustin by an insertion of an aromatic amino acid-rich region (Zhang and Zhu, 2012). Nevertheless, the biological roles of these putative type V crustin genes have not yet been fully elucidated.

5. Anti-lipopolysaccaride factor (ALF)

Anti-LPS factor (ALF) is a family of AMPs identified in horseshoe crabs and subsequently in many crustaceans, including shrimps, lobsters, crayfish and crabs (Afsal et al., 2012; Arockiaraj et al., 2014; Liu et al., 2010, 2011, 2012a,b,c, 2013, 2014; Ren et al., 2012a,b; Rosa et al., 2013; Sun et al., 2011; Tassanakajon et al., 2010; Yue et al., 2010; Zhu et al., 2014). ALFs are composed of 114–124 amino acid residues with a short signal peptide sequence of 16–26 residues. The molecular mass of the mature peptide is about 11 kDa. According to their p*I* ALFs were thought to be cationic peptides, but growing evidence has revealed the existence of anionic ALFs (Rosa et al., 2013; Zhang et al., 2010). Typically, ALFs are highly hydrophobic at the N-terminal region and contain a conserved three-dimensional structure, consisting of three α–helices packed against a four-stranded β-sheet. ALFs are amphipathic peptides containing a LPS-binding domain (LPS-BD), which is a β-hairpin structure linked by a conserved disulfide bridge. This stable disulfide loop harbors either a highly conserved cluster of positively charged (Lys and Arg) residues in the cationic ALFs or negatively charged (Glu and Asp) and hydrophobic residues in the anionic ALFs. Only the structures of LALF and ALF*Pm*3 have been resolved (Hoess et al., 1993, Yang et al., 2009). Based on the NMR structure of ALF*Pm*3 (a cationic ALF), it can recognize LPS, a

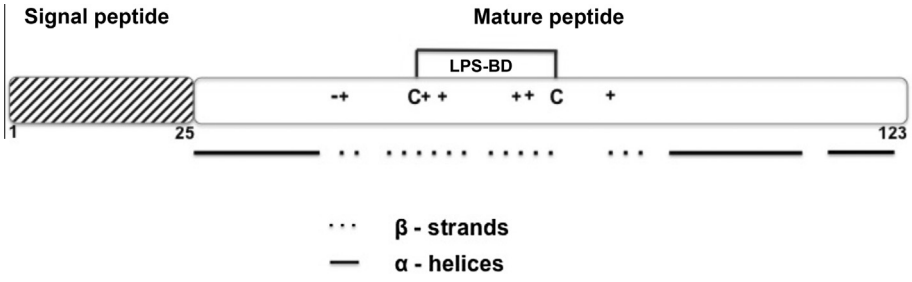

Fig. 7. Schematic illustration of the primary structure of ALF. Based on the resolved 3D-structure of ALF*Pm*3, ALF contains three α-helices (solid lines) and a four-stranded β-sheet (dashed line). ALF has a signal peptide of about 25 amino acid residues and a mature peptide of 98 amino acid residues. The signature of ALF is the cluster of positive charged amino acids (+) within the disulfide bridge stabilized LPS-BD and the positive charged residues within the flanking β-strands that are responsible for LPS recognition.

Table 1
ALFs from GenBank database and the corresponding p*I* of mature peptides and the LPS binding domain (LPS-BD).

Name	Organisms	GenBank	Theoretical p*I* of mature peptide	Theoretical p*I* of LPS-BD
LvALF1a	Litopenaeus vannamei	EW713395	7.09	9.39
LvALF1b	L. vannamei	FE109538	7.09	9.39
LvALF1c	L. vannamei	FE183080	7.09	9.39
LvALF1d	L. vannamei	FE092417	7.09	9.39
LvALF1e	L. vannamei	FE109539	7.09	9.39
LvALF2	L. vannamei	FE087264	8.06	9.84
LvALF2	L. vannamei	EW713396	7.77	9.85
LvALFB1a	L. vannamei	ABB22833	10.09	10.04
LvALFB1b	L. vannamei	ABB22835	10.09	10.04
LvALFB1c	L. vannamei	ACT21197	10.09	10.04
LvALFB2a	L. vannamei	ABB22832	10.17	10.04
LvALFB2b	L. vannamei	ABB22836	10.17	10.04
LvALFB2c	L. vannamei	ABB22831	10.17	10.04
LvALFC1	L. vannamei	FE153599	9.5	10.32
LvALFC10	L. vannamei	FE052210	9.18	10.32
LvALFC11	L. vannamei	FE088625	9.5	10.32
LvALFC2a	L. vannamei	FE176556	9.5	10.32
LvALFC2b	L. vannamei	FE176555	9.5	10.32
LvALFC3a	L. vannamei	FE058235	9.5	10.32
LvALFC3b	L. vannamei	FE155445	9.5	10.32
LvALFC4	L. vannamei	FE079082	9.5	10.32
LvALFC5	L. vannamei	FE088301	9.5	10.32
LvALFC6	L. vannamei	FE078559	9.5	10.32
LvALFC7	L. vannamei	FE079755	9.5	10.32
LvALFC8	L. vannamei	FE105941	9.77	10.95
LvALFC9	L. vannamei	FE090668	9.18	10.32
LvALFD1	L. vannamei	FE151634	5.58	4.03
LvALFD2	L. vannamei	FE152534	5.58	4.03
LvALFD3	L. vannamei	FE110967	5.58	4.03
LvALFD4a	L. vannamei	FE115964	5.58	4.03
LvALFD4b	L. vannamei	FE098450	5.58	4.03
LvALFD4c	L. vannamei	FE156649	5.58	4.03
LvALFD4d	L. vannamei	FE155982	5.58	4.03
LvALFD4e	L. vannamei	FE152063	5.58	4.03
LvALFD4f	L. vannamei	FE116643	5.58	4.03
LvALFD4g	L. vannamei	FE115660	5.58	4.03
ALFPm1	Penaeus monodon	ABP73290	6.07	9.39
ALFPm2a	P. monodon	ABP73291	6.46	9.39
ALFPm2b	P. monodon	GW993385	6.46	9.39
ALFPm3	P. monodon	ABP73292	9.95	9.93
ALFPm3a	P. monodon	ABP73289	9.95	9.93
ALFPm3b	P. monodon	AEW91438	9.95	9.93
ALFPm3c	P. monodon	ACC86067	9.95	9.93
ALFPm3d	P. monodon	AEW91477	9.95	9.93
ALFPm3e	P. monodon	ADC32520	9.7	9.83
ALFPm6a	P. monodon	ADM21460	9.77	10.32
ALFPm6b	P. monodon	DW678002	9.77	10.32
ALFPm6c	P. monodon	HO000126	9.77	10.32
ALFPm6d	P. monodon	AER45468	9.77	10.32
ALFPm6e	P. monodon	GO080476	9.77	10.32
LsALFB1	Litopenaeus stylirostris	KC346373	10.24	10.04
LsALFD1	L. stylirostris	AAY33769	6.1	4.37
LscALF	Litopenaeus schmitti	ABJ90465	10.18	10.04
LseALFD1	Litopenaeus setiferus	BE846661	4.53	4.37
ALFFc	Fenneropenaeus chinensis	AAX63831	10.29	9.93
ALFFpau	Farfantepenaeus paulensis	ABQ96193	10.17	10.04
FiALF1	Fenneropenaeus indicus	ADE27980	10.09	9.93
FiALF2	F. indicus	ADK94454	10.18	10.04
MjALF1	Marsupenaeus japonicus	BAE92940	7.98	9.84
MjALF2	M. japonicus	BAH22585	5.88	9.39
MoALF	Macrobrachium olfersii	ABY20736	9.56	9.7
MrALF1	Macrobrachium rosenbergii	AEP84102.1	10.02	10.04
MrALF2	M. rosenbergii	ADI80707.1	9.1	9.31
MrALF3	M. rosenbergii	ADI80708.1	6.33	9.14
MrALF4	M. rosenbergii	ACG60660.2	9.34	9.59
MrALF5	M. rosenbergii	JQ412135	5.08	9.84
MrALF6	M. rosenbergii	JQ412136	6.33	9.14
MrALF7	M. rosenbergii	JQ412137	9.61	9.24
HaALF1	Homarus americanus	ACC94268	10.63	10.95
HaALF2	Homarus americanus	ACC94269	9.19	9.85
PtALF	Portunus trituberculatus	ACM89169	10.17	10.54
PtALF1	P. trituberculatus	ADU25042.1	9.46	10.54
PtALF2	P. trituberculatus	ADU25043.1	9.69	10.54
PtALF3	P. trituberculatus	ACS45385.2	10.17	10.54

(*continued on next page*)

Table 1 (*continued*)

Name	Organisms	GenBank	Theoretical p*I* of mature peptide	Theoretical p*I* of LPS-BD
PtALF4	P. trituberculatus	AFA42332	9.07	8.04
PtALF5	P. trituberculatus	AFA42333	8.74	8.96
PtALF6	P. trituberculatus	AFA42334	5.79	8.95
PtALF7	P. trituberculatus	AFA42335	10.24	10.21
SpALF1	Scylla paramamosain	ABP96981	9.95	10.56
SpALF2	S. paramamosain	ADT71677	6.78	8.03
SpALF3	S. paramamosain	AFI43796	10.35	10.56
SpALF4	S. paramamosain	AHB62419	6.93	6.74
SsALF1	Scylla serrata	ADW11095.2	9.48	10.56
SsALF2	S. serrata	ACH87655	9.78	10.31
EsALF1	Eriocheir sinensis	ABG82027	8.79	9.84
EsALF2	E. sinensis	ACY25186	4.96	8.86
EsALF3	E. sinensis	ADZ46233	9.51	9.9
PcALF1	Procambarus clarkii	ADX60063	8.79	10.09
PlALF	Pacifastacus leniusculus	ABQ12866.1	9.2	9.14
TtALF	Tachypleus tridentatus	AAK00651	9.8	10.15
LpALF	Limulus polyphemus	P07086	10.09	10.4

major cell wall component of Gram-negative bacteria, through seven amino acid residues; six positively-charged residues and one negatively-charged residue located in the LPS-BD and in the flanking β-strands (Fig. 7).

In penaeid shrimps, ALFs have been classified into the four groups (A–D), according to the sequences and range of calculated isoelectric points (p*I*), of highly cationic (Group B), cationic (Group C), anionic and cationic (Group A) and very anionic (Group D) groups (Rosa et al., 2013). The theoretical p*I* of the mature peptide and LPS-BD of ALFs ranges from 5.35–10.35 and 4.37–10.95, respectively (Table 1). Cationic LPS-BDs have been found in both basic and anionic ALFs while anionic LPS-BDs have been found in anionic ALFs only.

The activity of ALFs relies on the binding activity with pathogens, which is mediated mainly via a positively charged cluster within the LPS-BD. Amino acid sequence comparison of the LPS-BD of the anionic ALFs with those of the cationic ALFs revealed the likely relationship between the antimicrobial activity and LPS-BD p*I*. The antimicrobial activity of highly cationic ALFs against Gram-negative bacteria was first characterized for the *Limulus polyphemus* ALF, LALF (or *Lp*ALF) (Morita et al. 1985). Highly cationic ALFs such as ALF*Pm*3 from *P. monodon* exhibit a broad spectrum of activities against Gram-negative and Gram-positive bacteria, fungi and virus (Carriel-Gomes et al., 2007; de-la-Re-Vega et al., 2008; Liu et al., 2006, 2012a; Somboonwiwat et al., 2005; Tharntada et al., 2009). Cationic ALFs, such as PcALF1, SpALF1 and SpALF2, and anionic ALFs, such as EsALF2, PtALF6, MrALF and SpALF4, have been shown to exhibit a lower strength antibacterial activity against Gram-negative and Gram-positive bacteria and in some cases only against Gram-negative bacteria (Liu et al., 2013, 2014; Sun et al., 2011; Zhang et al., 2010; Zhu et al., 2014). On the other hand, very anionic ALFs (shrimp ALF group D) have an impaired LPS-BD and are devoid of antimicrobial activity (Rosa et al., 2013). The absence of a positively charged amino acid cluster in the anionic LPS-BD causes the deficient LPS binding ability leading to the lack of antimicrobial activity of the ALF. Therefore, we noted here that the more positively charged amino acids in the LPS-BD, the better the antimicrobial activity of the ALF.

The mechanism of how ALFs perform their antimicrobial activity involves the binding of ALFs to microbial components, such as LPS, LTA and β-glucan (Rosa et al., 2013; Somboonwiwat et al., 2008; Sun et al., 2011). Their high affinity to LPS corresponded to the high antibacterial activity of ALFs on Gram-negative bacteria. It has been shown that ALF*Pm*3 treatment disrupts the inner- and outer-membrane integrity of Gram-negative bacteria as a result of inducing pore formation in the bacterial cell membrane (Jaree et al., 2012). However, SpALF1 and SpALF2 from *Scylla paramanosain* have no effect of the membrane integrity of *E. coli*, even for those SpALFs that are also active against *E. coli* (Liu et al., 2012a).

Th high sequence diversity of ALFs within species can be used to synergistically improve their antimicrobial defenses against microbial infection. Other than the direct killing of microbes, *Mr*ALF in the plasma and hemocytes at a lower concentration than the minimum inhibitory concentration functions as an opsonin, enhancing the phagocytic activity of hemocytes (Liu et al., 2014).

For most ALFs identified so far, their genomic organization contains three exons interrupted by two introns of a diverse length and the LPS-BD located in the exon 2 (Afsal et al., 2011; Imjongjirak et al., 2011; Liu et al., 2012b; Ren et al., 2012b). The exception is that of *Pt*ALF5 where it contains two exons interrupted by one intron and the LPS-BD located in the exon 1 (Liu et al., 2012c), and in ALF*Pm*3 that contains four exons interrupted by three introns (Tharntada et al., 2008).

Phylogenetic analysis of the mature ALF peptides revealed the relationship within and between species (Fig. 8), where ALFs can be divided into three clades. Based on the NJ tree topology, species-specific duplication causing gene orthologues were observed; for example, *Pt*ALF6 and *Sp*ALF2, *Pt*ALF5 and *Sp*ALF4, *Lse*ALFD1 and *Ls*ALFD1, *Sp*ALF and *Ss*ALF1, *Mo*ALF and *Mr*ALF4, and *Tt*ALF and *Lp*ALF. However, the evolutionary analysis of the ALF genes in crustaceans showed no evidence of positive selection but rather is likely to be by purifying selection (Ren et al., 2012a,b) except in the *M. rosenbergii* ALF gene members, which include most *M. rosenbergii* ALF genes (*MrALF2*, *MrALF3*, *MrALF5*, *MrALF6* and *MrALF7*) (Ren et al., 2012b). Like other AMPs, such as penaeidin in penaeid shrimps (Padhi et al., 2007), crustin in decapods (Padhi, 2012), and myticin-C in *M. galloprovincialis* (Padhi and Verghese, 2008), their aqueous environment enriched with pathogenic microorganisms may also induce adaptive evolution of ALFs in accord with the Red Queen hypothesis.

As stated above, several isoforms of ALF have been identified in many species. The gene expression of any particular ALF isoform is tissue specific but sometimes ALF transcripts are found in many tissues. Overall ALFs are mainly expressed in the hemocytes, which are the major immune tissues of crustaceans (Arockiaraj et al., 2014; Liu et al., 2012a,c; Nagoshi et al., 2006; Ponprateep et al., 2012). Also some ALF isoforms are predominantly expressed in other immune-related tissues, such as the gills (Liu et al., 2013), lymphoid organ, intestine and hepatopancreas. Some ALFs are expressed in several tissues at different levels (Mekata et al., 2010; Ponprateep et al., 2012). ALFs that act as an effector of the innate immune system by inhibiting the growth and killing bacteria

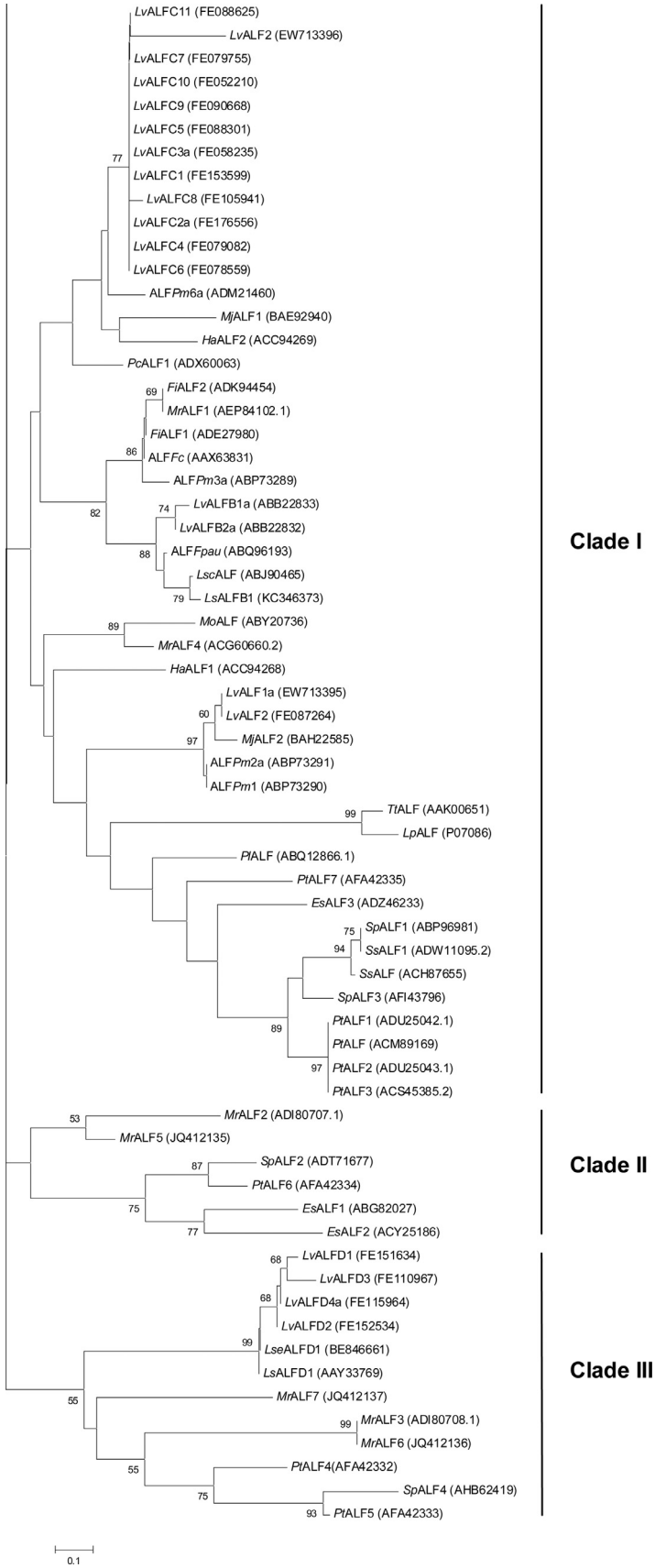

Fig. 8. Phylogenetic analysis of ALFs from horseshoe crabs and crustaceans. Neighbor-Joining distance-based phylogenetic analysis of the ALFs from horsehoe crabs and crustaceans. Bootstrap analysis (1000 replications) values are shown at each node if above 50%. Sequence names and accession numbers are indicated in the trees and Table 1.

or inhibiting viral replication, how a rapid response, in terms of the change in transcript expression level, expression of ALF transcripts responds rapidly upon pathogen infections or immune stimulations by LPS, LTA and β-glucan injections. It has been reported that as the expression of ALF*Pm*3 transcripts are up-regulated by *Vibrio harveyi* infection, the production of ALF gene product is also sharply induced and is released from the granular of hemocytes to the site of infection (Somboonwiwat et al., 2008).

Acknowledgements

We thank Dr. Irene Söderhäll for the opportunity to write this article. Our work is supported by grants from the Thailand Research Fund (TRF Senior Scholar number RTA5580008). The support from Chulalongkorn University under the Ratchadaphisek Somphot Endowment to the Center of Excellence for Molecular Biology and Genomics of Shrimp is also acknowledged.

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