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Pengonadins: A penaeid shrimp-specific antimicrobial peptide family related to anti-lipopolysaccharide factors

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Crustacean - Invertebrate immunity - Host defense peptide - Antilipopolysaccharide factor - Antimicrobial activity

ABSTRACT

Anti-lipopolysaccharide factors (ALFs) are antimicrobial peptides (AMPs) defined by a central β -hairpin stabilized by two cysteine residues. Recently, ALF-related molecules containing three or four cysteine residues were identified in penaeid shrimp, but their characterization remained unexplored. Here, we describe these molecules as pengonadins, based on their taxonomic restriction, molecular features, and predominant expression in gonads. Comprehensive mining of sequence databases revealed that pengonadins are exclusive to penaeid shrimp and form a distinct AMP family, separated from canonical ALFs and classified into two Types (I and II). Transcriptomic analyses that pengonadins are expressed in gonads, dynamic expression during ovarian maturation, a role in reproductive immunity. Genomic analyses revealed up to six tandemly duplicated pengonadin genes per species, with conserved chromosomal organization across penaeids. Their genomic distribution and synteny analyses indicate that pengonadins originated from an ancestral ALF gene and diversified prior to penaeid shrimp speciation. Functionally, a synthetic peptide based on a Type II pengonadin from the Pacific white shrimp *Penaeus vannamei* exhibited antifungal activity, suggesting specialized roles beyond antibacterial defense. Together, our findings identified pengonadins as a novel family of penaeid AMPs with unique molecular features, reproductive tissue-biased expression, and a likely origin via gene duplication and diversification from an ALF ancestor.

1. Introduction

Antimicrobial peptides (AMPs) are classically described as diverse cationic, amphipathic peptides with low molecular weight [1]. These molecules have a broad spectrum of activity, acting against Gram-positive and Gram-negative bacteria, filamentous fungi, yeasts and, to a lesser extent, viruses. Although their primary function was initially described as acting against infectious pathogens, several AMPs have other biological functions, such as tissue regeneration, cell proliferation and protease inhibition [2]. Furthermore, AMPs reproductive-related organs [3,4]. They can be produced in reproductive tracts for microbial control or transferred during mating to protect during fertilization. Interestingly, the interaction with reproductive-related AMPs during mating can as a possible mechanism of cryptic female choice, showing the multifunctionality of these molecules [3,5–7]. The transfer of AMPs can also be carried out from the reproductive tract of arthropods to their offspring, where *Ceratitidis capitata* females can coat their eggs with AMPs as they are laid [8].

Of the twelve gene-encoded AMP families currently recognized in crustaceans, anti-lipoplysaccharide factors (ALFs) stand out for their molecular and transcriptional diversity, as well as for their broad and potent spectrum of antimicrobial activity [9].

Table 1

Antimicrobial activity spectrum of synthetic peptides based on *Penaeus vannamei* pengonadin segments flanked by cysteine residues.

Strain	pengonadin-I ₄₈		pengonadin-I ₅₀	
	MIC	MFC	MIC	MFC
Gram-positive bacteria				
<i>Corynebacterium stationis</i> CIP 101282	na	na	na	na
<i>Microbacterium maritypicum</i> CIP 105733	na	na	na	na
<i>Staphylococcus aureus</i> SG511	na	na	na	na
Gram-negative bacteria				
<i>Escherichia coli</i> SBS 363	na	na	na	na
<i>Vibrio harveyi</i> ATCC 14126	na	na	na	na
<i>Vibrio parahaemolyticus</i> IOC 18950	na	na	na	na
Filamentous fungi				
<i>Fusarium oxysporum</i> MUCL 909	na	na	na	na
<i>Penicillium</i> sp. Yeasts	na	na	na	na
<i>Candida albicans</i> 12A (MDM8)	na	na	20–40	na
<i>Rhodotorula mucilaginosa</i>	na	na	2.5–5	2.5–5

MIC values are reported in micromoles per liter (μM) and refer to the minimum concentration required to achieve 100 % growth inhibition. MFC values (μM) refer to the minimum concentration required to kill 100 % of the fungi. na stands for not active at the tested concentrations (up to 40 μM).

They are amphipathic peptides with a hydrophobic N-terminal region and two conserved cysteine residues that stabilize a central β -hairpin [10,11]. This central β -hairpin charged amino acids involved in the recognition and binding of microbial structures, such as lipopolysaccharides (LPS) from Gram-negative, lipoteichoic acid (LTA) from Gram-positive, and β -glucans from fungi [12]. Notably, synthetic peptides inspired by the central β -hairpin exhibit biological properties similar to mature ALFs [13–17].

In penaeid shrimp, ALFs form a multigene family of seven members (Groups A-G), biochemical characteristics, gene expression distribution, and biological activities [9]. Shrimp ALFs are encoded by different genes and are simultaneously transcribed in the circulating hemocytes of a single individual [13,18]. This AMP family includes both cationic (Groups B, C, and F) and anionic (Groups A, D, E, and G) members, displaying a broad spectrum of biological properties. These properties encompass antibacterial, antifungal, and antiviral activities, as well as the recognition and binding of microbial cell wall components, and the regulation and control of the host microbiome [9].

In a previous study, we identified ALF-related sequences in penaeid shrimp containing three and four cysteine residues [13]. Here, we investigated the diversity of these peptides in terms of sequence, gene expression, and antimicrobial activity. We showed that these peptides comprise a novel family of gene-encoded AMPs that evolved from a common ancestor of the ALF genes. These peptides are divided into two subgroups (Types I and II) and are taxonomically restricted to penaeid shrimp (suborder Dendrobranchiata). Unlike classic ALFs, these peptides are not primarily produced by hemocytes, nor are they induced in response to microbial challenge. Instead, these genes are primarily expressed in the gonads and show increased expression during gonad maturation. Thus, we named this gene-encoded AMP family pengonadins.

2. Materials and methods

2.1. Database searches, sequence data analysis and phylogeny

An ALF-related sequence (GenBank: XM_027362708) was used as a query to search for new sequences with the tBLASTx tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) [19]. The search was performed against NCBI annotated (nt-nr) and non-annotated (Expressed Sequence Tags/EST, Transcriptome Shotgun Assembly/TSA and Whole Genome Shotgun Contigs/WGS) databases. Retrieved sequences were used to search for new sequences until no new ones were found. Only full-length coding sequences (CDS) with e-value $>1 \times 10^5$ were collected.

Retrieved nucleotide sequences were translated using the ExPASy Translate Tool (<https://web.expasy.org/translate/>). Deduced amino acid sequences were checked for signal peptide using the SignalP software (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>) [20], and biochemical properties were analyzed with ExPASy ProParam tool (<https://web.expasy.org/protparam/>) [21]. Deduced mature peptides were aligned using MAFFT (https://mafft.cbrc.jp/alignment/server/in_dex.html) [22] and the phylogenetic tree using all retrieved unique pengonadin sequences was generated with the Maximum Likelihood method (1000 bootstraps) using the MEGA 12 software [23]. Additionally, a Bayesian phylogenetic inference was performed with

pengonadins, ALFs from penaeid shrimp, and ALFs from horseshoe crabs as an external group. For that, phylogenetic analysis was conducted in MrBayes (<https://nbisweden.github.io/MrBayes/index.html>) [24] using a mixed amino acid substitution model with gamma-distributed rate variation. The Markov Chain Monte Carlo (MCMC) analysis was run for 1,000,000 generations with four chains, sampling every 500 generations and discarding the first 25 % as burn-in to generate a consensus tree with posterior probabilities.

Threedimensional (3D) structures of mature pengonadins (Type I GenBank: GDUV01012518; Type II GenBank: GDTK01016426) were predicted using AlphaFold 3 software (<https://alphafold.ebi.ac.uk/>) [25,26]. Their structures were aligned with the ALF-B 3D structure (PDB: 2JOB), previously determined by X-ray crystallography [10] for comparison.

2.2. Gene structure, chromosome mapping and collinearity relationships

The structure of pengonadin genes was assessed by the comparison between genomic and transcriptomic sequences. For that, the sequences were aligned using MAFFT to determine number and size of exons, introns and untranslated regions (UTR), and plotted using TBtools [27]. The total number of genes and their distribution in chromosomes of *Penaeus vannamei* (NCBI Assembly: GCF_042767895) [28], *Penaeus monodon* (NCBI Assembly: GCF_015228065) [29] and *Penaeus chinensis* (NCBI Assembly: GCF_019202785) [30] were manually checked and genetic maps were plotted using MG2C (http://mg2c.iask.in/mg2c_v2.1/) [31]. Distribution of ALFs was determined in the same genomes for comparison. Syntenic relationships of pengonadins and ALFs among *P. vannamei*, *P. monodon* and *P. chinensis* chromosomes were determined using MCScanX [32] with default parameters in TBtools [27].

2.3. In silico transcriptome analysis

Transcriptomic analyses were performed using publicly available data from Sequence Read Archive (SRA) NCBI databases (<https://www.ncbi.nlm.nih.gov/sra>). Only mRNA-selected Illumina raw reads from penaeid shrimp were used. Tissue distribution of gene expression analyses was evaluated in hemocytes, lymphoid organ, gills, hepatopancreas, stomach, muscle, eyestalk, ovary and testis from *P. vannamei* and *P. monodon* (NCBI Bioprojects: PRJNA420111, PRJNA413606, PRJNA252793, PRJNA285934, PRJNA255337, PRJNA413606, PRJNA255337, PRJNA713898, PRJNA713898, PRJNA713898 and PRJNA421400) [33–39]. Gene expression was also in *P. vannamei* and *P. monodon* female gonad tissues at different stages of maturation after eyestalk ablation (NCBI Bioprojects: PRJNA285934 and PRJNA542029) [39,40] and during shrimp development (NCBI Bioprojects: PRJNA253518 and PRJNA421400) [37,41].

The transcriptional profile of ALFs and pengonadins was assessed by using the RSEM package [42]. Read quality was assessed using FastQC (v0.11.9 - <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), any adaptor sequences or bases with a quality score below 20 were removed using Trimmomatic (v0.39) [43] keeping a minimum read length of 75 bp (leading:20; trailing:20; slidingwindow:4:20; minlen:75). Gene expression analysis was performed using RSEM software package (v1.3.3) [42] with STAR (v2.7.11a) [44] as the mapping software. Filtered reads were mapped to the annotated genomes of *P. vannamei* and *P. monodon* (GenBank: GCF_042767895 and GCF_015228065). Counts were normalized to transcripts per million (TPM) using RSEM, transformed to $\log_2(\text{TPM} + 1)$, and plotted in R using ggplot2 [45].

2.4. Peptide synthesis

Peptides were synthesized in a Liberty Blue automated microwave peptide synthesizer (CEM) using Fmoc-protected amino acids as previously described [13]. Synthetic peptides were based on the amino acid segment flanked by the cysteine residues of two pengonadin members from *P. vannamei*: pengonadin-I₄₈₋₈₃ (CEFSVTPKSKDWMPYHESNFSCPWTNIVGEATGRC; MW: 4123.57 Da; pI: 4.90) and pengonadin-II₅₀₋₈₄ (CKIEVEHRFSHWTVYHQCTFTCDHWPNTGKASSRC; MW: 4308.85 Da; pI: 7.91). The peptides were purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on an XBridge™ BEH C18 column (Waters), and their molecular masses were determined using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry on an LCMS-2020 ESI-MS (Shimadzu).

2.5. Antibacterial and antifungal assays

The antimicrobial assays were performed as previously described [13]. *In vitro* activity of synthetic peptides was performed against the Gram-positive bacteria *Corynebacterium stationis* CIP 101282, *Microbacterium maritypicum* CIP 105733 and *Staphylococcus aureus* SG511, the Gram-negative bacteria *Escherichia coli* SBS 363, *Vibrio harveyi* ATCC 14126 and *Vibrio parahaemolyticus* IOC 18950, the filamentous fungi *Fusarium oxysporum* MUCL 909 and *Penicillium* sp., and the yeasts *Candida albicans* 12A (MDM8) and *Rhodotorula mucilaginosa*.

Minimum inhibitory concentration (MIC) values of synthetic peptides were determined by the liquid growth inhibition assay in duplicate, as previously described [45]. Briefly, microorganisms in liquid media were diluted 1:100 in specific culture media for activity assays and, afterwards, tested with different concentrations of synthetic peptide (final concentration ranging from 2.5 to 40 μM). Poor Broth (PB: 1 % peptone, 1 % NaCl, pH 7.2) and PB supplemented with 0.5 M NaCl (PB–NaCl) were used as culture media for standard bacteria and *Vibrio* strains, respectively. The PB–NaCl medium was supplemented with 20 mM KCl, 5 mM MgSO₄ and 1.5 mM CaCl₂ to be used as culture medium for *M. maritypicum*. Diluted potato dextrose broth (v:v; 1:2) (Kasvi, Sao ~ Jos'e dos Pinhais, PR, Brazil) and Sabouraud media (1 % peptone, 4 % glucose, pH 5.6) were used for filamentous fungi and yeasts, respectively. Microbial growth was monitored spectrophotometrically ($\lambda = 595 \text{ nm}$) and hyphae formation observed in an inverted microscope. MIC values are expressed as the lowest concentration of synthetic peptide that inhibits growth by 100 %. When microbial growth inhibition was observed, cultures were plated in nutrient agar media for 48 h to determine the bactericidal or fungicidal activity of synthetic peptides.

3. Results

3.1. Pengonadins, a novel gene-coded AMP family exclusive to penaeid shrimp

In a previous study [13], we identified ALF-related sequences in penaeid shrimp containing three and four cysteine residues. To investigate the diversity of these peptides and understand their relationship with classic ALFs, we performed an exhaustive *in silico* screening approach, followed by molecular

characterization and phylogenetic reconstruction. Our data mining approach identified 47 ALF-related sequences (complete CDS) in annotated and non-annotated databases. All sequences were identified exclusively in penaeid shrimp, highlighting that these AMPs are restricted to this taxon. Based on their taxonomic restriction, molecular features, and gene expression patterns (see section 3.3), we named these ALF-related molecules as pengonadins.

The predicted pengonadin amino acid sequences consist of a 20-residue signal peptide followed by a mature peptide of 106–124 residues. Their molecular weights range from 12.22 to 14.65 kDa, and their isoelectric points (pI) range from 5.12 to 10.02 (Table S1). Alignment and phylogenetic analysis of the pengonadin mature peptides revealed two distinct groups, classified as Type I and Type II based on specific molecular signatures (Fig. 1A). Type I mature peptides range from 107 to 124 residues, with molecular weights between 12.39 and 14.65 kDa ($pI = 5.8–10.02$). In contrast, Type II mature peptides are shorter, ranging from 106 to 108 residues, with molecular weights of 12.22–12.77 kDa ($pI = 5.12–9.78$). Type I pengonadins were identified in *P. vannamei*, *P. monodon*, *Penaeus japonicus* and *Penaeus semisulcatus*, while Type II pengonadins were found in *P. vannamei*, *P. monodon*, *P. chinensis*, *Penaeus indicus* and *Penaeus merguensis*.

Notably, in addition to the two conserved cysteine residues characteristic of ALFs, pengonadins contained one or two extra cysteine residues. All pengonadins displayed one additional conserved cysteine residue in the C-terminal region, while some sequences displayed another extra cysteine residue within the classic ALF β -hairpin structure (Fig. 1A). This extra β -hairpin cysteine residue is commonly found in Type II pengonadins but is mostly absent in Type I. *In silico* 3D structure predictions indicate that pengonadins share a similar tertiary structure with ALFs, featuring three α -helices packed against a four-stranded β -sheet (Fig. 1B). While the two conserved cysteine residues form a disulfide bond flanking the β -hairpin, the additional cysteine residues do not appear to form disulfide bonds, suggesting that their thiol groups might be involved in other molecular interactions.

To explore the phylogenetic relationships between ALFs and pengonadins, we conducted a phylogenetic reconstruction using all pengonadin sequences identified in this study, along with ALF sequences from penaeid shrimp and ALFs from horseshoe crabs used as an external group (Table S1). The Bayesian phylogenetic analysis revealed a clear distinction between penaeid shrimp ALFs and pengonadins (Fig. 1C). Specifically, the penaeid shrimp ALF clade grouped sequences corresponding to the seven groups previously described (ALFs A to G), while all pengonadins sequences (Types I and II) clustered into a distinct, separate clade (Fig. 1C).

3.2. Pengonadins comprise a diverse multigene family in penaeid shrimp

The genomic distribution and structure of pengonadin genes were analyzed using chromosome-level genome assemblies available in public databases. Our *in silico* analysis indicates that pengonadins form a multigene family of gene-encoded AMPs, with multiple gene copies present in each genome (Fig. 2A). In *P. vannamei*, five pengonadins genes were identified: two encoding Type I peptides (LOC113807192 and LOC113807191) and three encoding Type II peptides (LOC113807189, LOC113807190 and LOC113807194). In *P. chinensis*, only two Type II genes (LOC125043344 and LOC125040762) were detected, with no Type I genes present. In *P. monodon*, three pengonadin genes were found: two Type I (LOC119578797 and LOC119578362) and one Type II (LOC119578796) (Table S1). Despite differences in sequence and classification, all pengonadin genes share a conserved structure comprising three exons spaced by two introns (Fig. 2A). The first exon contains the 5'-UTR, the leader sequence and the N-terminal portion of the mature peptide containing the first α -helix. The second exon encodes four stranded β -sheets, including the central β -hairpin containing the two cysteine residues involved in the disulfide bond, and the extra cysteine residue when present. The third exon contains the last two α -helices and the conserved cysteine residue in the C-terminal region and the 3'-UTR.

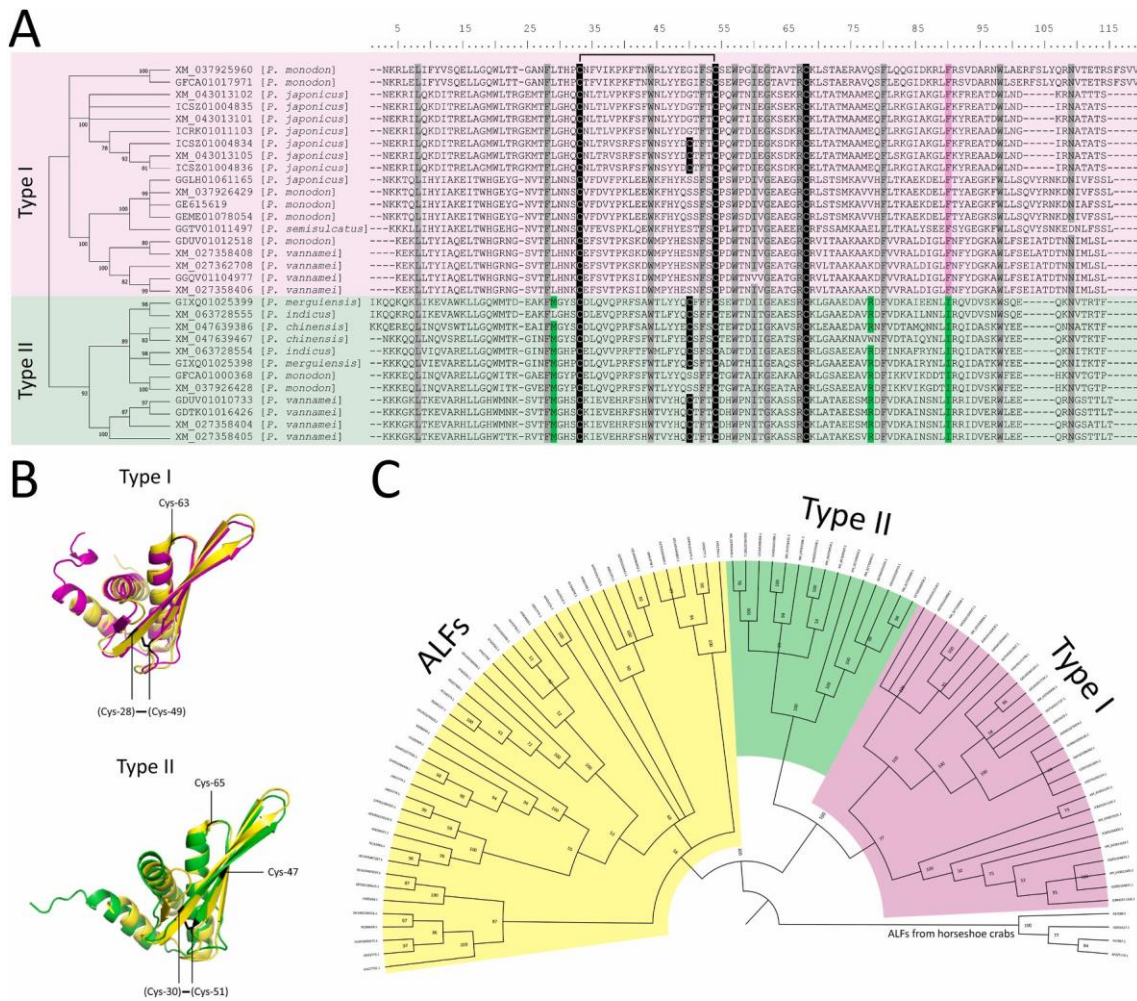


Fig. 1. Molecular, structural and phylogenetic characterization of pengonadins. **(A)** Maximum likelihood tree (left) and multiple alignments (right) of pengonadin Types. The molecular signature of all pengonadins is highlighted in grey, while Types I and II molecular signatures are highlighted in purple and green, respectively. All cysteine residues are in black, while the conserved disulfide bond is indicated on top of linked cysteines. Branches with bootstrap values lower than 50% were collapsed. **(B)** Predicted tertiary structures of Type I (purple) and Type II (green) pengonadins aligned with the tertiary structure of an ALF from Group B (yellow) (ALFPm3; PDB: 2J0B). All cysteine residues are displayed in black and cysteine residues linked by a disulfide bond are in parenthesis. **(C)** Bayesian phylogenetic tree of pengonadin Types I (purple) and II (green), penaeid shrimp ALFs (yellow) and horseshoe crabs ALFs. Branches with posterior probabilities lower than 50% were collapsed. The list of the pengonadin and ALF sequences included in analyses (annotations, sequences and GenBank accession numbers) is provided in [Table S1](#). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

All pengonadin genes are distributed in tandem in the available genomes of penaeid shrimp ([Fig. 2A](#)). We found that the two Type I genes are followed by the three Type II genes on chromosome 10 of *P. vannamei*, and the Type II gene is followed by two Type I genes on chromosome 11 of *P. monodon*. The two Type II genes of *P. chinensis* are tandemly distributed on chromosome 1. Interestingly, the pengonadin genes are followed by two or three ALF gene copies ([Fig. 2A](#)). Chromosomes containing *pengonadins* and/or *ALFs* were compared in search for collinear blocks and synteny between genes. Gene synteny analyses revealed that *pengonadins* and *ALFs* are within a collinear block in the three penaeid shrimp species analyzed ([Fig. 2B](#)). Similarly, other *ALFs* are also in collinear blocks, but different chromosomes. Taken together, the patterns of gene arrangement and synteny indicate that the gene diversification of Type I and Type II pengonadins occurred through gene duplication events before the speciation of penaeid shrimp.

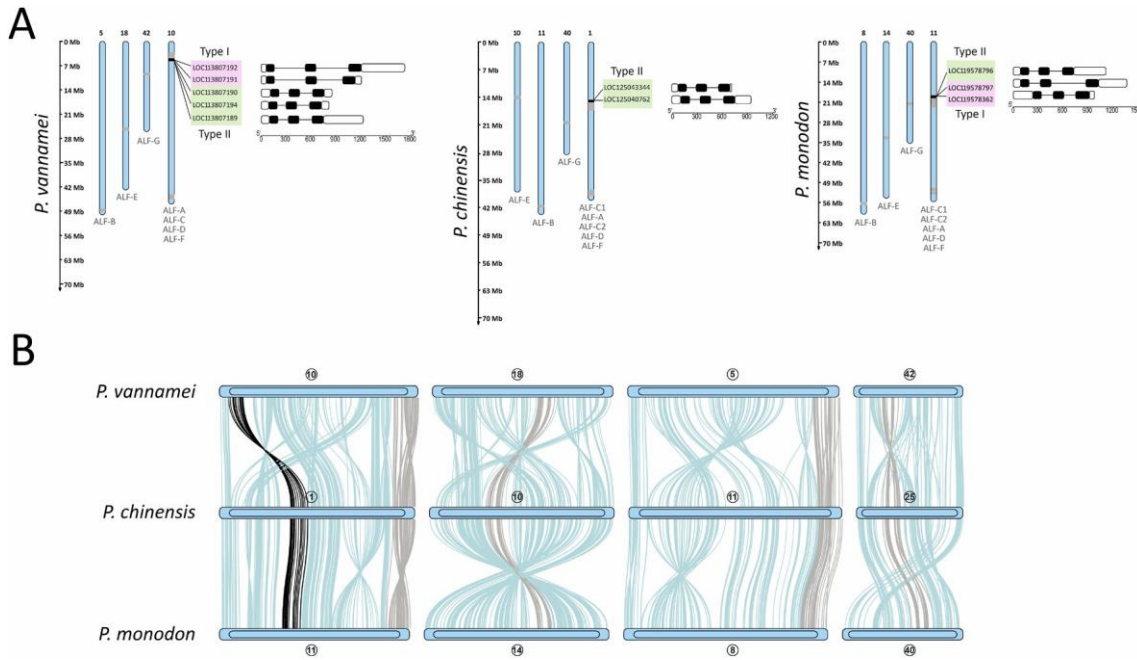
3.3. Pengonadin transcripts are primarily expressed in mature ovaries

To gain insight into their transcriptional profile, we analyzed the gene expression of ALFs and pengonadins in different tissues of *P. vannamei* and *P. monodon* using publicly available transcriptomic databases. To minimize biases from differences in library preparation and sequencing protocols, we compared transcript levels within the same sample only. Gene expression values were normalized as $\log_2(\text{TPM} + 1)$, enabling comparison between genes within each sample by accounting for gene length and sequencing depth. We first evaluated the expression of ALFs and pengonadins in nine different tissues: hemocytes, lymphoid organ, gills, hepatopancreas, stomach, muscle, eyestalk, ovary, and testis ([Fig. 3A](#)). In both species, ALFs showed consistently higher expression levels than pengonadins across nearly all tissues. In both species, ALFs showed consistently higher expression levels than pengonadins across nearly all tissues ([Fig. 3A](#)). Remarkably, pengonadin transcripts were expressed at low levels in general and were nearly undetectable in circulating hemocytes, the main AMP expression site in crustaceans ([Fig. 3A](#)). We also investigated pengonadin expression in hemocyte libraries upon viral (White spot syndrome virus/ WSSV) and bacterial (*Vibrio*

sp.) challenges, but no expression was detected. Despite the low levels, pengonadin transcripts were identified in lymphoid organ, hepatopancreas, stomach, muscle, eyestalk, ovary, and testis (Fig. 3A).

To explore the potential involvement of pengonadins in gonad development, we analyzed the expression of ALFs and pengonadins during ovarian maturation induced by eyestalk ablation (Fig. 3B).

Fig. 2. Pengonadin and ALF genes distribution, structure and syntenic relationships across *Penaeus vannamei*, *Penaeus chinensis* and *Penaeus monodon* genomes. (A) Numbered chromosomes showing pengonadin and ALF genes as, respectively, black and grey rectangles within chromosomes, and schematic representation of structure of genes in base pairs. Black and white boxes of gene structures represent, respectively, UTR and CDS regions, and lines represent introns. Types I and II pengonadins loci are highlighted in purple and green, respectively. Only chromosomes containing pengonadin and ALF genes are shown. Genes of ALFs were written under chromosomes according to their order in the given chromosome. (B) *P. vannamei*, *P. chinensis* and *P. monodon* numbered chromosomes and the syntenic relationships of their genes, which are represented by lines. Collinear blocks containing ALF genes are highlighted in grey and collinear blocks containing pengonadin and ALF genes are highlighted in black. Only chromosomes containing pengonadin and ALF genes are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).



In *P. vannamei*, four of the five pengonadin genes were expressed across all ovary libraries, and their transcript levels increased following eyestalk ablation. In *P. monodon*, pengonadin transcripts were not detected in immature ovaries; however, all pengonadin genes were expressed in mature ovaries collected nine days after ablation. Notably, our transcriptomic analysis suggests a trend of increasing pengonadin expression during ovary maturation, while ALF expression remains unchanged.

Lastly, given the expression of pengonadins in mature ovaries, we investigated their transcriptional profile during shrimp development. We analyzed gene expression levels of ALFs and pengonadins across five developmental stages: embryo, nauplius, zoeae, mysis, and post larvae (Fig. 3C). Surprisingly, pengonadin transcript levels were mostly absent in embryos (Fig. 3C). In fact, pengonadins were mostly absent in all *P. vannamei* stages analyzed, while in *P. monodon*, pengonadin-I transcripts were detected starting from the zoeae stage (Fig. 3C).

Overall, our transcriptomic analyses highlight a clear divergence in the expression profiles of ALFs and pengonadins. While ALFs are broadly and consistently expressed across tissues and developmental stages, pengonadins exhibit limited expression, with notable enrichment in mature ovaries.

3.4. Type II pengonadin-inspired peptides show yeast antimicrobial activity

To explore the functional properties of pengonadins, we assessed the antimicrobial activity of synthetic peptides derived from *P. vannamei* pengonadins, representing both Type I and Type II sequences. The peptides were designed based on amino acid segments that include all cysteine residues. The synthetic pengonadin-I₅₀₋₈₄ showed *in vitro* antifungal activity (Table 1). It inhibited the growth of the yeasts *Candida albicans* (MIC 20–40 μM) and *Rhodotorula mucilaginosa* (MIC 2.5–5 μM) and also exhibited fungicidal activity against *R. mucilaginosa* (MFC 2.5–5 μM), a yeast species isolated from the digestive tract of *P. vannamei* (Table 1). Interestingly, the peptide did not exhibit antimicrobial activity against the Gram-positive and Gram-negative bacteria and filamentous fungi tested. Moreover, the synthetic pengonadin-I₄₈₋₈₃ did not show *in vitro* activity against the tested microorganisms.

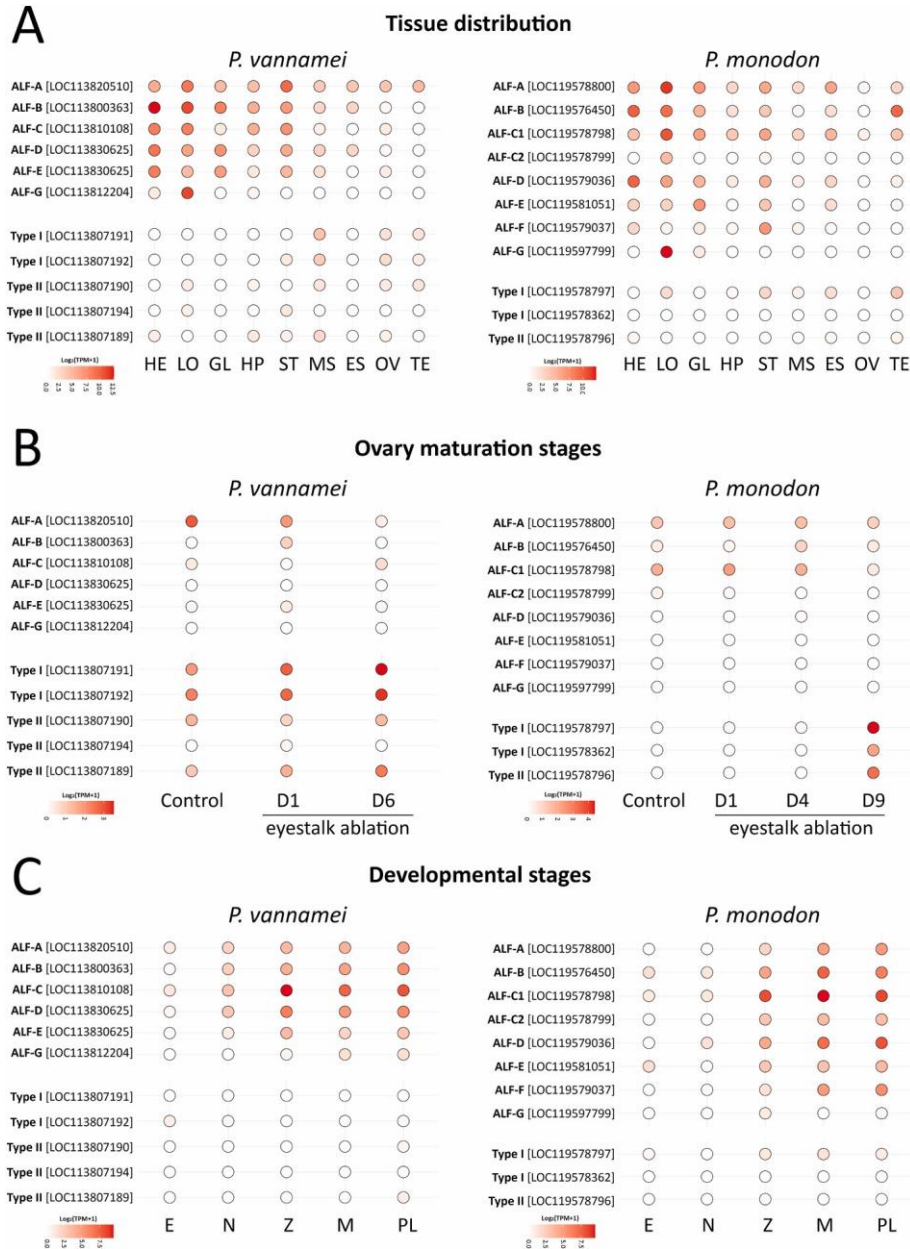
4. Discussion

Here, we identified and characterized a novel gene-encoded AMP family in penaeid shrimp, named pengonadins. Derived from ALFs, pengonadins are structurally distinct, possessing three or four cysteines, and appear to be exclusive to penaeid shrimp species. Phylogenetic analysis and sequence signatures allowed us to classify them into two distinct types, Type I and Type II. The transcriptional profile of these molecules indicated that pengonadins are mainly

expressed in the gonads, and have their expression induced during gonad maturation. Considering their taxonomic restriction, molecular characteristics, and transcriptional profile, we coined the term "pengonadin" to refer to this AMP family, reflecting both their phylogenetic origin and their functional and tissue-specific divergence from classical ALFs.

Tandem duplication events are crucial for gene family expansion, allowing for gene differentiation and adaptation to environmental changes [46]. In addition to enabling the evolution of new gene functions, gene duplication can generate functional redundancy, affecting transcriptional levels and host susceptibility to infectious diseases. [46, 47]. Here, we determined that both Type I and Type II pengonadins are encoded by multiple gene copies in the genomes of *P. vannamei*, *P. monodon*, and *P. chinensis*, which may be associated with functional redundancy and the physiological relevance of this family. The presence of both types across different penaeid species, along with conserved gene synteny, indicates that these duplication and diversification events likely occurred prior to the divergence of penaeid shrimp, paralleling the evolutionary history observed in ALF gene groups [13].

Fig. 3. Gene expression of *Penaeus vannamei* and *Penaeus monodon* pengonadins and ALFs. (A-C) Gene expression is shown as circles based on log₂(TPM+1) normalized using RSEM, where genes loci are within brackets. (A) Gene expression patterns in nine tissues of shrimp: hemocytes (HE), lymphoid organ (LO), gills (GL), hepatopancreas (HP), stomach (ST), muscle (MS), eyestalk (ES), ovary (OV) and testis (TE) tissue distribution. (B) Gene expression in ovaries after different periods (D1: day 1; D4: day 4; D6: day 6; D9: day 9) of eyestalk ablation. (C) Gene expression during five shrimp development stages: embryo (E), nauplius (N), zoeae (Z), mysis (M) and postlarvae (PL).



The expansion and genomic arrangement of the pengonadin family support the hypothesis that these peptides may have acquired important physiological roles, possibly including reproductive or immunological functions, sustained through both redundancy and diversification.

A previously identified pengonadin from *P. japonicus* was named MjALF-E1 due to the presence of the characteristic β -hairpin identified in ALFs [18]. However, we have previously proposed that such variants represent ALF-related molecules that originated from an ancestral ALF gene within penaeid shrimp [9]. Here, we provide further phylogenetic evidence revealing that pengonadins and classical ALFs form distinct clades, supporting their divergence from a common ancestor. Furthermore, we found additional taxonomic, genomic and structural evidence to reinforce the evolutionary history of pengonadins. *In silico* results showed that, unlike ALFs, pengonadins are restricted to penaeid shrimp, suggesting that they emerged within this lineage. Interestingly, ALFs and pengonadins are tandemly distributed in the same collinear blocks in the genomes of *P. vannamei*, *P. monodon* and *P. chinensis*, implying a shared evolutionary origin. Moreover, pengonadins and ALFs share the same genomic structure, tertiary structure and the classical β -hairpin [13]. Altogether, it is possible to infer that pengonadins and ALFs originated from gene duplication events from the same ancestral gene. However, a full phylogenetic analysis including other crustacean ALFs must be performed to elucidate these β -hairpin molecules.

Gene expression analysis revealed that pengonadins from *P. vannamei* and *P. monodon* exhibit a different transcriptional profile from most crustacean AMPs. Classically, crustacean AMPs, including ALFs, are predominantly or exclusively expressed in circulating hemocytes [9,13,15,48]. In contrast, pengonadin transcripts were found primarily in gonads, with minimal or no expression in hemocytes. The expression of AMPs exclusively in gonads has been described in different species of arthropods, suggesting a role in reproductive immunity [49–53]. For instance, andropins are found exclusively in the ejaculatory duct of *Drosophila melanogaster* [49], whereas some ceratotoxins are expressed exclusively in female reproductive accessory glands of the medfly *Ceratitis capitata* [50]. Moreover, some AMPs are found in both sexes, but with higher expression in one type of gonad, such as Type I crustins in crabs [54], thaumatins in beetles [53] and scygonadins, ALF-related molecules exclusively found in crabs from the *Scylla* genus [5,55–57].

Scygonadins are predominantly expressed in the ejaculatory ducts of pre-mating male crabs, especially during the mating season [5,54–56]. After mating, transcript levels decrease in males but increase in the spermatheca of post-mating females, suggesting a possible transfer of the peptide to provide antimicrobial protection during fertilization [5, 55,56]. Scygonadin transcripts are also more abundant in embryos and mature adults than in larval or juvenile stages, supporting a dual role in development and reproductive immunity [5,54]. These examples underscore the functional importance of AMPs in protecting reproductive tissues, regulating microbial communities in the reproductive tract, and safeguarding against pathogens during oviposition [5,51,52,57]. Based on our findings, it can be hypothesized that while classical ALFs function primarily in systemic immune defense, pengonadins may have evolved to protect specific epithelial barriers, particularly those associated with the reproductive system in penaeid shrimp.

In vitro antimicrobial assays of MjALF-E1 (Type I pengonadin from *P. japonicus*) showed that this peptide exhibits low antimicrobial activity, being active mainly against Gram-positive bacteria [18]. Similarly, our results demonstrate that the synthetic peptide derived from a Type II pengonadin segment limited antimicrobial activity; however, its activity is directed against yeasts rather than bacteria. Although a limited spectrum, antifungal activity is important for the immunity of crustaceans, especially during initial developmental stages [58]. It is important to note that *in vitro* activity of AMPs does not always reflect *in vivo* activity, since they may act to protect against pathogens to they do not show *in vitro* activity [59]. Therefore, it would be important to perform *in vivo* studies to evaluate the activity of pengonadins broadly. Alternatively, their low activity could be indicative of additional biological functions. A growing body of evidence shows that many AMPs, including those in crustaceans, perform roles beyond microbial defense, such as wound healing, angiogenesis, cell differentiation, immunoregulation and proteinase inhibition [4,9,60]. Peptides primarily involved in these processes but exhibiting residual antimicrobial activity have been increasingly documented in crustaceans [60].

Given their predominant expression in gonads, pengonadins may contribute not only to reproductive tract immunity but also to key physiological processes such as gonadal maturation or fertilization. Supporting this hypothesis, scygonadins, structurally related ALF-like molecules, show low antimicrobial activity, but they are likely to play a role in the sperm acrosome reaction of *Scylla paramamosain* [5,61]. Thus, the possibility that pengonadins have evolved away from classical antimicrobial roles toward specialized functions in reproduction or development should be carefully considered in future studies.

5. Conclusions

In conclusion, this study introduced pengonadins, a novel family of gene-encoded AMPs found exclusively in penaeid shrimp and predominantly expressed in the gonads. Genomic analyses suggested that pengonadins originated from an ancestral ALF gene through tandem duplication events that occurred before the diversification of classical ALFs, while preserving similar gene architecture and tertiary structure. Phylogenetic and structural evidence supported the classification of pengonadins into two types, Type I and Type II, which likely emerged through additional gene duplication events prior to penaeid shrimp speciation. The transcriptional profile of pengonadins, marked by low expression in immune tissues and induction during ovarian maturation, along with their limited antimicrobial activity, points toward a functional divergence from the canonical immune role of AMPs. That pengonadins may have evolved specialized roles, potentially linked to reproductive immunity or other physiological processes associated with gonadal development.

Data availability

Data used for transcriptomic analyses are available on Sequence Read Archive (SRA) NCBI (<https://www.ncbi.nlm.nih.gov/sra>) under the Bioprojects PRJNA420111, PRJNA413606, PRJNA252793, PRJNA285934, PRJNA255337, PRJNA413606, PRJNA255337, PRJNA713898, PRJNA713898, PRJNA713898, PRJNA285934, PRJNA542029, PRJNA253518 and PRJNA421400.

Author's statement

All authors confirm that this manuscript has not been published elsewhere and is not under consideration by any other journal. We have approved the manuscript and agreed with its submission to Fish and Shellfish Immunology.

CRedit authorship contribution statement

Gabriel Machado Matos: Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. Cassio Bar´cellos Herv´e: Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. Fanny Guzman: Methodology, Writing – review & editing. Paulina Schmitt: Data curation, Writing – review & editing, Funding acquisition. Rafael Diego Rosa: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

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