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Identification and characterization of antimicrobial peptide genes in *Clarias gariepinus* and *Chelon ramada*

Karima F Mahrous*, Dalia M Mabrouk§, Mohamad M Aboelenin§, Heba AM Abd El kader and Mohamed S Hassanane

Cell Biology Dept., Division of Genetic Engineering and Biotechnology Research- National Research Centre, 33 El Bohouth St., Dokki, Giza, P.O.12622, Egypt.

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Abstract

Antimicrobial peptides (AMPs) are small molecular weight proteins that play an important role in the innate immune response against pathogenic invasions. In the present study, the coding sequences (CDs) of four AMPs were characterized and analyzed. NK-lysin (Cgnkl) and hepcidin (Cghep) coding sequences were obtained from *Clarias gariepinus* (North African catfish), while two hepcidin paralogs (Crhep1 and Crhep2) coding sequences were identified from *Chelon ramada* (thin-lipped mullet). Cgnkl coding sequence consists of 128 amino acids with predicted signal peptide cleavage site between codons 17 and 18 and six conserved cysteine residues that are held together by three disulfide bonds. On the other hand, Cghep, Crhep1, Crhep2 coding sequences consist of 91, 85 and 90 amino acids, respectively, and show the similar predicted signal cleavage site between codons 24 and 25 as well as eight characteristic cysteine residues. Several synonyms and non-synonyms SNPs were detected within Cghep and Crhep1 CDs. Finally, phylogenetic and conservation analyses were carried out on the amino acid sequences of the discovered AMPs and the 3D structures of their propeptides was predicted.

Keywords: Antimicrobial peptides, Hepcidin, NK-lysin, *Clarias gariepinus, Chelon ramada*

1. Introduction

Although AMPs have been recognized in the middle of the 20th century, they have gained increasing attention in recent years. AMPs are small size peptides that have a great diversity in amino acid composition, structural organization, and mechanism of action (Bruno *et al*., 2019). They display antimicrobial activity against several microorganisms and viruses and participate in the innate host defense of each eukaryotic species (Masso-Silva and Diamond, 2014). Generally, all AMPs are able to disrupt microbes lipidic membranes to kill or inhibit proliferation (Lai and Gallo, 2009; Mahrous *et al*, 2020a; Mahrous *et al*, 2020c).

Fish is a great source of AMPs, as it mainly relies on humoral primary immune defense mechanism to succeed in the highly dynamic and challenging external environment rich with microorganisms (Sathyan *et al*., 2013). A large number of AMPs has been identified from different fish species, including hepcidin and NK-lysin.

Hepcidin, a small cysteine-rich AMP, plays an essential role in host iron metabolism regulation and immunological processes. Its amphipathic structure is similar to other AMPs, such as defensins. It has the ability to defend against pathogenic bacteria in an indirect way through decreasing iron in the plasma and extracellular fluids and, as a result, limiting its proliferation (Drakesmith & Prentice, 2012; Huang *et al*., 2019). Unlike many other AMPs displaying a high degree of sequence variability among closely related organisms, hepcidin is highly conserved from teleosts to mammals (Masso-Silva and Diamond, 2014). Most mammals present only one copy of the hepcidin gene, but in fish, multiple copies exist. Hepcidin antimicrobial peptides (HAMPs) of fish are divided into two groups, HAMP1 and HAMP2. HAMP1 is present in all fish while, HAMP2 is present only in acanthopterygian (Neves *et al*., 2017).

NK-lysin, a member of the Saposin-like protein family, is orthologous with human granulysin. It is larger (74–78 amino acids) than the classical AMPs (Kim *et al*., 2016). It is released from the natural killer cells and cytotoxic T lymphocytes (Zhou *et al*., 2016) with remarkable broad-spectrum activities against fungi (Stenger *et al*., 1998), protozoa (Lama *et al*., 2018) and bacteria (Pereiro *et al*., 2017), and tumor cells (Fan *et al*., 2016). Its sequence is rich in positively charged amino acids and the sulfide bond-forming cysteines (Wang *et al*., 2018).

Egypt is one of the top seven aquaculture producers by quantity. Its average annual production is 3.3 tons (FAO, 2017). With the continued expansion of cultured fish species, aquaculture became a key component of the animal production industry. *Chelon ramada* (Thin-lipped mullet) is a potential species for fish culture in Egypt due to its temperature tolerance, superior growth, wide salinity tolerance range and high-quality flesh (Mehanna *et al*., 2019). Also, *Clarias gariepinus* (North African catfish), is an important catfish species for aquaculture purposes due

* Corresponding author e-mail: l_fathy@yahoo.com.
§ These authors have contributed equally to this work

to its rapid growth and the high protein content (Shourbela *et al*., 2014).

In the present study, we analyze the coding sequence of Cgnkl,Cghep, Crhep1 and Crhep2. We provide the predicted conserved domains, post-transcriptional modification motifs, signal peptides cleavage sites and the propeptides 3D structure. Further, we compare their deduced amino acid sequences with orthologous peptides of other bony fish species and construct phylogenetic trees.

2. Materials and Methods

2.1. Tissue Collection

Seven North African catfish (*C. gariepinus*) and five thin-lipped mullet (*C. ramada)* fish were collected from Lake Manzala and liver tissues were isolated and stored directly in -80°C for RNA extraction.

2.2. RNA isolation and cDNA synthesis

Liver tissue was homogenized in Trizol reagent (Invitrogen, Germany) and total RNA was extracted according to the manufacturer's instructions. RNA yield and purity were measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNase-free DNase kit (Promega) was used to remove any DNA contamination. One microgram (1 µg) of RNA was reverse transcribed into cDNA using Revert Aid First-strand synthesis cDNA kit (Thermo Fisher Scientific, USA) as described previously (Sroor *et al*., 2020).

2.3. Pre-processing and de novo assembly of transcriptome data

Since some genes are tissue-specific and or inducible, we assembled RNA-seq datasets (which belong to the same or closely relates species) in order to maximize the chance of identifying the target AMPs. Moreover, assembly of RNA-seq from several datasets helps in designing primers primer pairs within the mRNA conserved regions without SNPs which may prevent amplification due to mismatching. The transcriptome raw RNA-Seq data of *C. gariepinus* (Sequence Read Archive (SRA) accession # SRX4609822- SRX4609825, ERX538457 and ERX2104803) were assembled. For *C. ramada*, the transcription raw RNA-Seq data of *Chelon labrosus* (Thicklip grey mullet; SRA accession # SRX1672957) *and Mugil cephalus* (Grey mullet; SRA accession # SRX1817285 - SRX1817288) were assembled. FastQC (Andrews, 2010) assessment reports of sequence reads were performed to evaluate read quality before pre-processing. Adapter clipping, trimming reads based on quality, and removing sequences with ambiguous bases (N) were conducted using Trimmomatic (Bolger *et al*., 2014). Bases at both ends of reads were removed within a sliding window of 10 base pairs when the average quality in this window was lower than Q20 score. To verify the integrity of the remaining raw sequence reads, FastQC was performed again. Upon completion, the quality assessed reads were then ready to be used as the input for the various assembly strategies and all subsequent analyses were conducted using clean reads. Trinity RNA-Seq de novo transcriptome assembly version 2.0.4 was run using the default parameters (Haas *et al*., 2013). Reference transcripts were generated by combining all clean reads of the sequencing data sets. To represent the assembled component from each cluster, only the longest transcript was selected to prevent redundancy. All the data processing steps were performed online depending on Galaxy project (Enis *et al*., 2018).

Geneious Software version 10 (Biomatters, Ltd., New Zealand) was used to perform batch Basic Local Alignment Search Tool (BLAST) for the assembled mRNA sequences against NCBI databases to find homologues sequences based on blastn and blastx tools. The assembled mRNA sequences found to be homologues to the target antimicrobial peptides gene (NK-Lysin and Hepcidin) were used to design specific primers (Table 1) using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast) to amplify the CDs of each gene.

2.4. Amplification and sequencing of the Coding sequence (CDs) within the target genes

The designed primers (Table 1) were synthetized by Macrogen (South Korea). Polymerase chain reaction (PCR) were performed in a 25 µl of reaction volume, which included a 1 µl of cDNA, a 50 ng of each primer, a 200 µM of each dNTP, a 2.5 µl of 10X PCR buffer and a 0·5 U of Taq DNA polymerase (Promega, Madison, WI, USA). Amplification was carried out in a thermocycler programmed as follows: an initial start separation cycle at 94°C for 2 min, 35 cycles including a denaturation step at 94°C for 30 sec, an annealing of each gene (Table 1) for 30 sec, a polymerization step at 72°C for 45 sec and a final extension cycle at 72°C for 10 minutes. The PCR products were screened by electrophoresis on a 2% agarose gel in a 0.5X of TBE buffer stained with ethidium bromide and visualized with an UV transilluminator as described previously (Mahrous *et al*., 2020b). The PCR products were purified using GeneJET *Gel Extraction* Kit (Thermo Fisher Scientific, USA) according to the manufacture instructions and sent for sequencing (Macrogen, South Korea).

2.5. Amino acids sequence analysis

The CDs of each gene were translated into amino acids sequence and analyzed using CD-Search software (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to identify the conserved domains. SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) software was used to identify the cleavage sites of the signal peptide. The amino acid sequences were scanned using MOTIF online software (https://www.genome.jp/tools/motif/) to compute the potential motifs within them. The 3D tertiary structure of the peptide (without the signal peptide) was predicted using I-TASSER server (Yang *et al*., 2015) **a**nd the secondary structure assignment was performed using STRIDE a web server (http://webclu.bio.wzw.tum.de /cgi-bin /stride/stridecgi.py). The conservation score of the amino acids within the target proteins was computed using ConSurf server (https://consurf.tau.ac.il/). UCSF Chimera software- version 1.14 (https://www.cgl.ucsf.edu/ chimera/) was used for 3D structure visualization and secondary structure assignment. MEGA-X software-version 10.16 (Kumar *et al*., 2018) was used to align the selected sequences by MUSCLE algorithm based in neighbor joining clustering method while the phylogenetic tree was constructed using Maximum Likelihood (ML) algorithm.

Table 1: The designed primer sequences used for PCR amplification

Species	Gene name		Primer sequence	Annealing temp
Clarias gariepinus	NK-lysin (*cgnkl*)	*cgnkl-F* *cgnkl-R*	AACTATCTTTCCCATCTTTAAC AGAAAAGCATCAATCAGTTC	50
Clarias gariepinus	Hepcidin (*cghep*)	*cghep-F* *cghep-R*	ACTTGCTTTTAAACGACGACTA ACGTCCCATCTCATGTCTGA	53
Chelon ramada	Hepcidin-1 (*crhep1*)	*crhep1-F* *crhep1-R*	CACAAAGATCAGGAGAAAAC GTGGTCATTTTGTCACATG	52
Chelon ramada	Hepcidin-2 (*crhep2*)	*crhep2-F* *crhep2-R*	AGAAGACCTATCAACTCTAATC GATGAAGGAAGGGTCTTTAG	53

3. Results

The total RNA was isolated from *C. gariepinus* and *C. ramada* and converted into cDNA, then the target regions within the mRNA of the studied genes were amplified using specific primers. The resultant PCR products of Crhep1, Crhep2, Cghep and Cgnkl genes had molecular weights of 520, 408, 362 and 459 bp, respectively (Figure 1A). The PCR products were purified and sequenced then the nucleotides sequences were analyzed using several bioinformatics tools to predict the biological characteristics of these peptides.

3.1. Characterization of C. gariepinus NK-lysin (Cgnkl)

The determined sequence of *C. gariepinus* NK-lysine gene complete CDs consisted of 387 nucleotides coding for 128 aa (Figure 1B). The mRNA CDs sequence was deposited into GenBank under accession number MH674388. Saposin (B) SapB domain (aa position 56–128) which is a characteristic for saposin-like proteins (*SAPLIPs*) superfamily was detected within Cgnkl peptide (Figure 2A) Four N-Myristoylation sites (MYRISTYL; 13-GSACAI-18, 34-GSLDSV-39, 54-PGACMAC-59, 71-GNNSNQ-76), a N-glycosylation site (ASN GLYCOSYLATION; 72-NNSN-75), a Casein kinase II phosphorylation site (CK2 PHOSPHO SITE; 27-SDEE-30) and a Protein kinase C phosphorylation site (PKC PHOSPHO SITE; 78-TIR-80) motifs were found in the full length peptide sequence (Figure 2B). The first 17 amino acids represented a signal peptide with a putative cleavage site between Ala17 and Ile18 (Figure 2C).

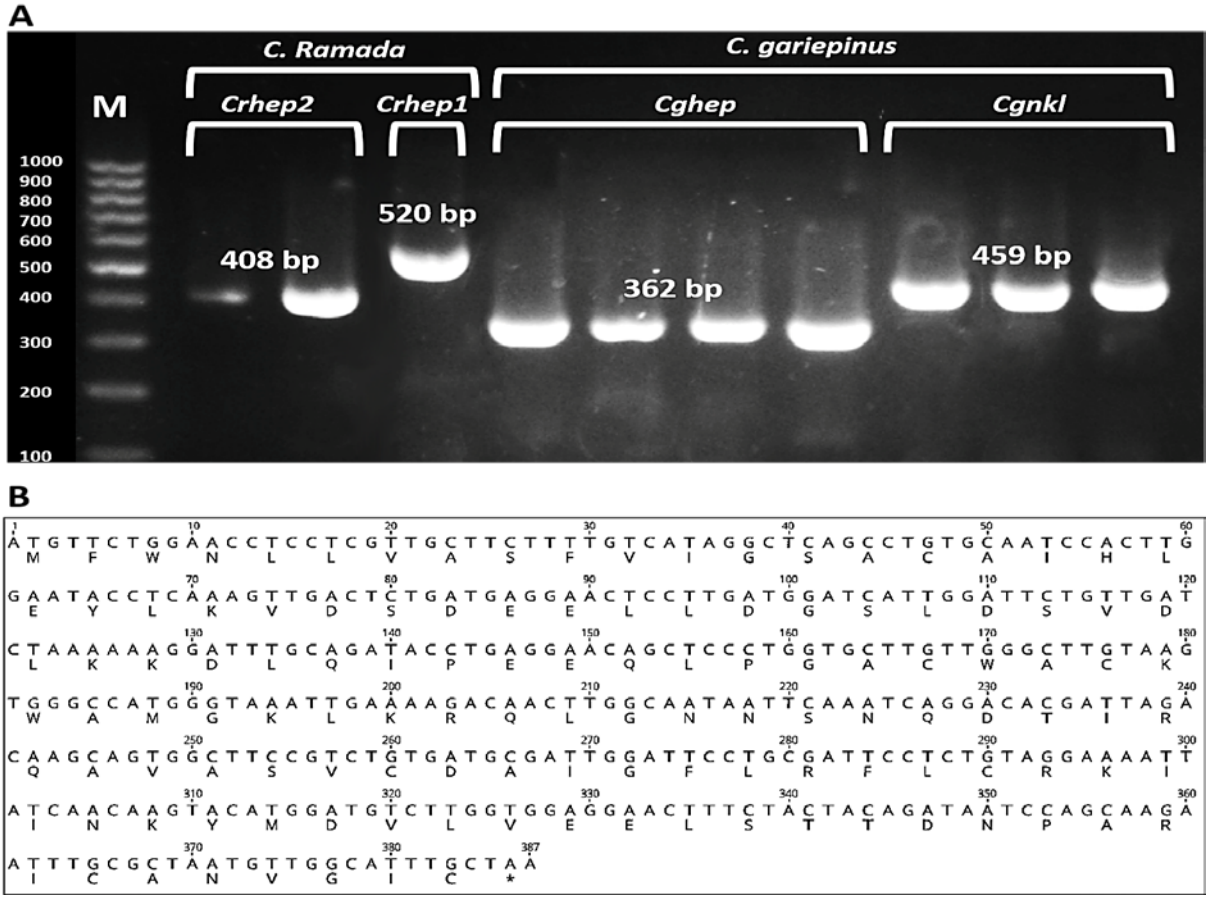


Figure 1: (A) Agarose gel electrophoresis for the PCR product of the amplified region within antimicrobial peptides Crhep1, Crhep2, Cghep and Cgnkl cDNA. M: 100 bp molecular marker (B) The nucleotides and translated amino acids sequences of Cgnkl prepropeptide.

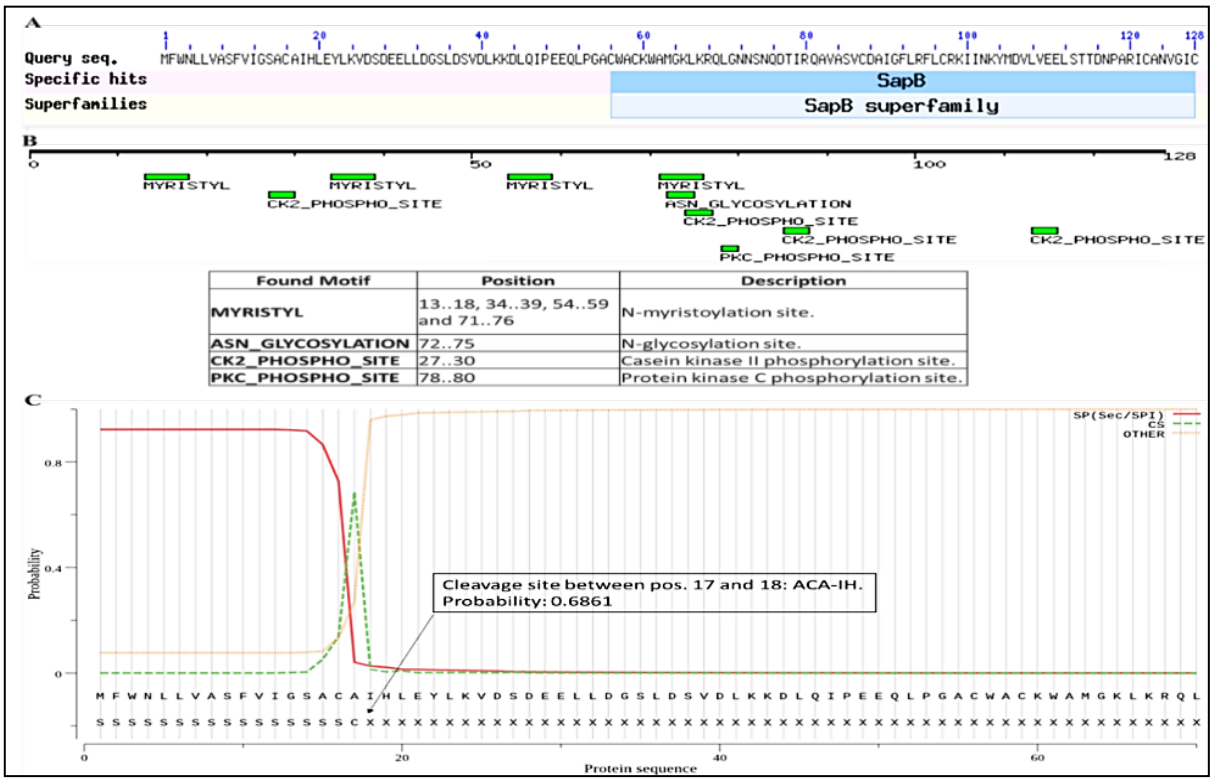


Figure 2. Predicted motifs and domains within Cgnkl prepropeptide. A) The detected conserved domain for SapB superfamily within the amino acids sequence. B) The predict motifs within the prepropeptide. C) The predicted signal peptide cleavage site. SP(Sec/SPI): standard secretory signal peptides transported by the secretory translocon and cleaved by Signal Peptidase I, CS: cleavage site, Other, Other: the sequence does not have any kind of signal peptide.

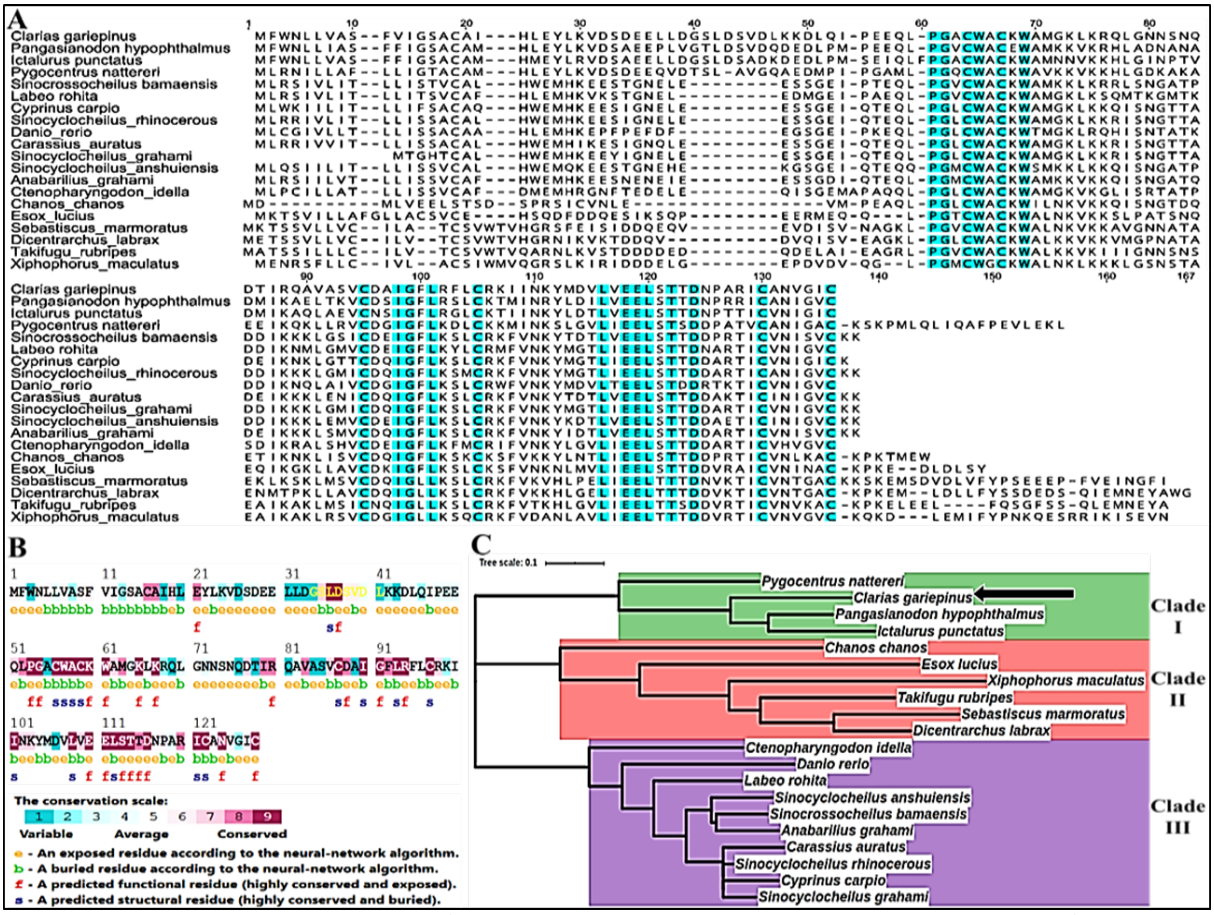


Figure 3. Phylogenetic and conservation analysis of the relationship Cgnkl and its orthologous prepropeptide in some bony fish species. (A) multiple sequence alignment (conserved residues highlighted in cyan). (B) conservation analysis. (C) phylogenetic tree.

Alignment of Cgnkl amino acid sequence with the NK-lysine prepropeptides from 19 species belong to bony fish (Figure 3A and B) displayed that 19 aa were conserved of which, 13 aa were positioned at the last 35 residues in the Cgnkl C-terminus. Moreover, 10 conserved residues could be exposed whereas the remaining 9 conserved residues might be buried. The constructed phylogenetic tree was composed of 3 main clades (Figure 3C). Cgnkl existed in clade I with *P. nattereri, P. hypophthalmus* and *I. punctatus*. The distance between *C. gariepinus* and *P. hypophthalmus* (Iridescent shark fish) NK-lysin sequences is the smallest distance and *C. gariepinus* and *X. maculatus* (Southern flatfish) NK-lysine sequences is largest distance.

The predicted 3D structure of Cgnkl propeptide was constructed of 57.7% alpha helix,7.2% beta sheet, 0.9% isolated beta bridge,25.2% turn and 9% coil. Six conserved cysteine (Cys) residues forming three disulfide bonds (Cys39-Cys111, Cys42-Cys105, and Cys70-Cys80) were detected (Figure 4). There were differences in the secondary structure assignment between the results of UCSF Chimera software which consist of (Figure left) and stride results which contain additional beta-sheets and isolated beta bridge (Figure right). These differences in the secondary structure assignment results could be explained by the fact that each tool uses a different secondary structure assignment algorithm, and the different secondary structure assignment algorithms could lead to several alterations in the assignment results (Martin *et al.*, 2005).

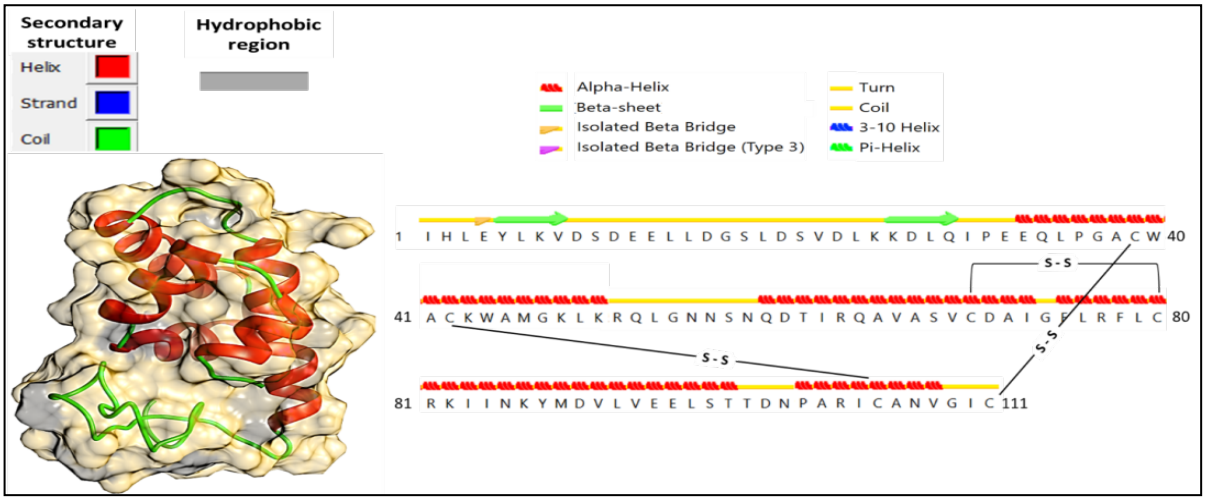


Figure 4. The predicted 3D structure of Cgnkl propeptide shows the secondary structures and molecular surface of the peptides (left) and description for the secondary structure and disulfide bonds within the peptides (right**).**

3.2. Characterization of C. gariepinus Hepcidin (Cghep)

The coding sequence of Cghep is 276 bp coding for 91 aa residues (Figure 5A). The amplified mRNA coding sequences was deposited into GenBank under accession numbers from MH674372 to MH674387. Four SNPs were identified in Cghep prepropeptide containing two synonyms SNPs (C78T and A195G) and two non-synonyms SNPs (A67G and T144G). The non-synonymous SNP (A67G) leads to threonine/alanine while the non-synonymous SNP (T144G) leads to glutamic acid/aspartic acid variation (Figure 5A and B). A segment from Phe62 to Phe91 was found to be a conserved domain belonging to hepcidin super family (Figure 6A). A Casein kinase II phosphorylation site (56-TGPE-59), a N-myristoylation site (84-GCGYCC-89),a cAMP- and cGMP-dependent protein kinase phosphorylation site (65-KRQS-68), a Microbodies C-terminal targeting signal (89-CRF-91) and Cysteine-rich region (73-CRYCCNCCKNKGCGYCC-89) motifs were found within the amino acid sequence (Figure 6B). The predicted signal peptide cleavage site is located between Ala24 and Val25, and the predicted signal peptide spanned from position 1 to 24 (Figure 6C).

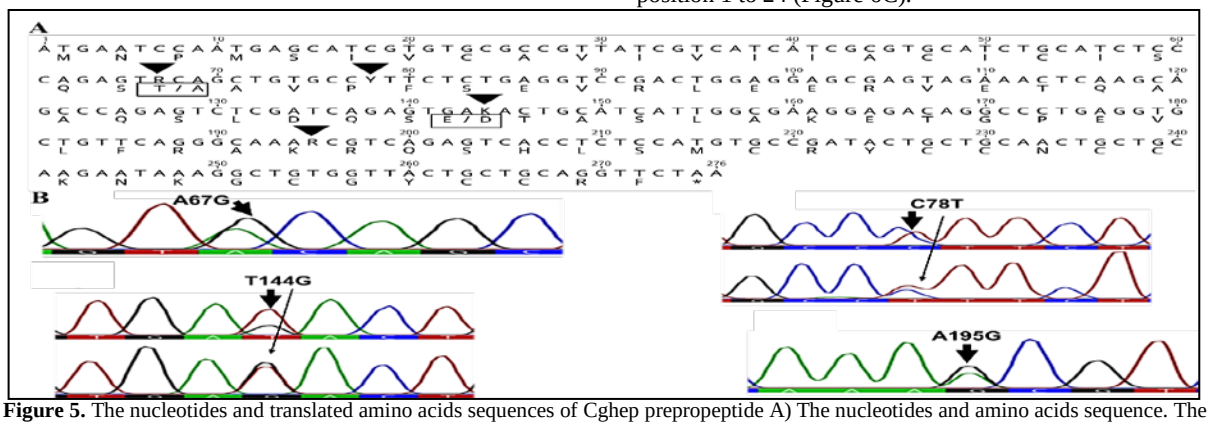
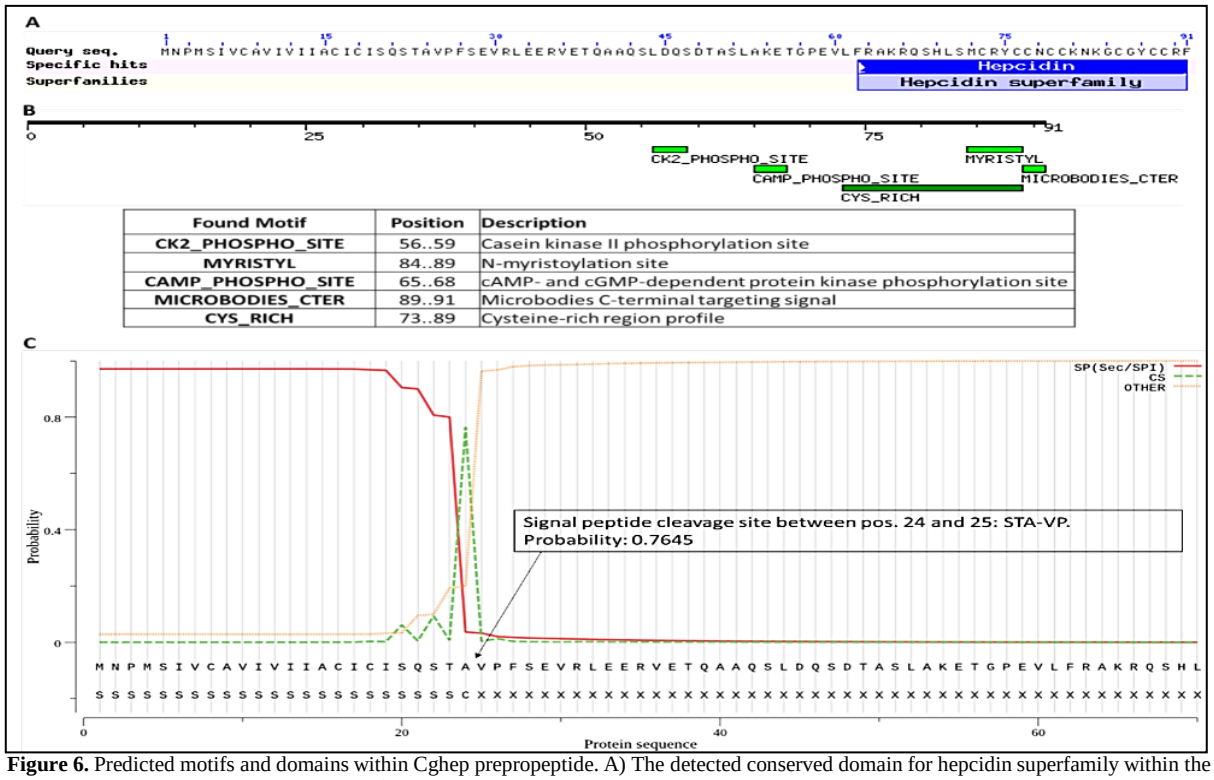


Figure 5. The nucleotides and translated amino acids sequences of Cghep prepropeptide A) The nucleotides and amino acids sequence. The solid arrows indicate the SNPs position while the boxes indicate the amino acids substitutions. R= G or A, Y=T or C and K= G or T. B) The detected SNPs within hepcidin CDs. A67G non-synonyms SNP, C78T synonyms SNP, T144G non-synonyms SNP and A195G synonyms SNP.



Found Motif	Position	Description
CK2_PHOSPHO_SITE	56..59	Casein kinase II phosphorylation site
MYRISTYL	84..89	N-myristoylation site
CAMP_PHOSPHO_SITE	65..68	cAMP- and cGMP-dependent protein kinase phosphorylation site
MICROBODIES_CTER	89..91	Microbodies C-terminal targeting signal
CYS_RICH	73..89	Cysteine-rich region profile

Figure 6. Predicted motifs and domains within Cghep prepropeptide. A) The detected conserved domain for hepcidin superfamily within the amino acids sequence. B) The predict motifs within the prepropeptide. C) The predicted signal peptide cleavage site. SP(Sec/SPI): standard secretory signal peptides transported by the secretory translocon and cleaved by Signal Peptidase I, CS: cleavage site, Other, Other: the sequence does not have any kind of signal peptide.

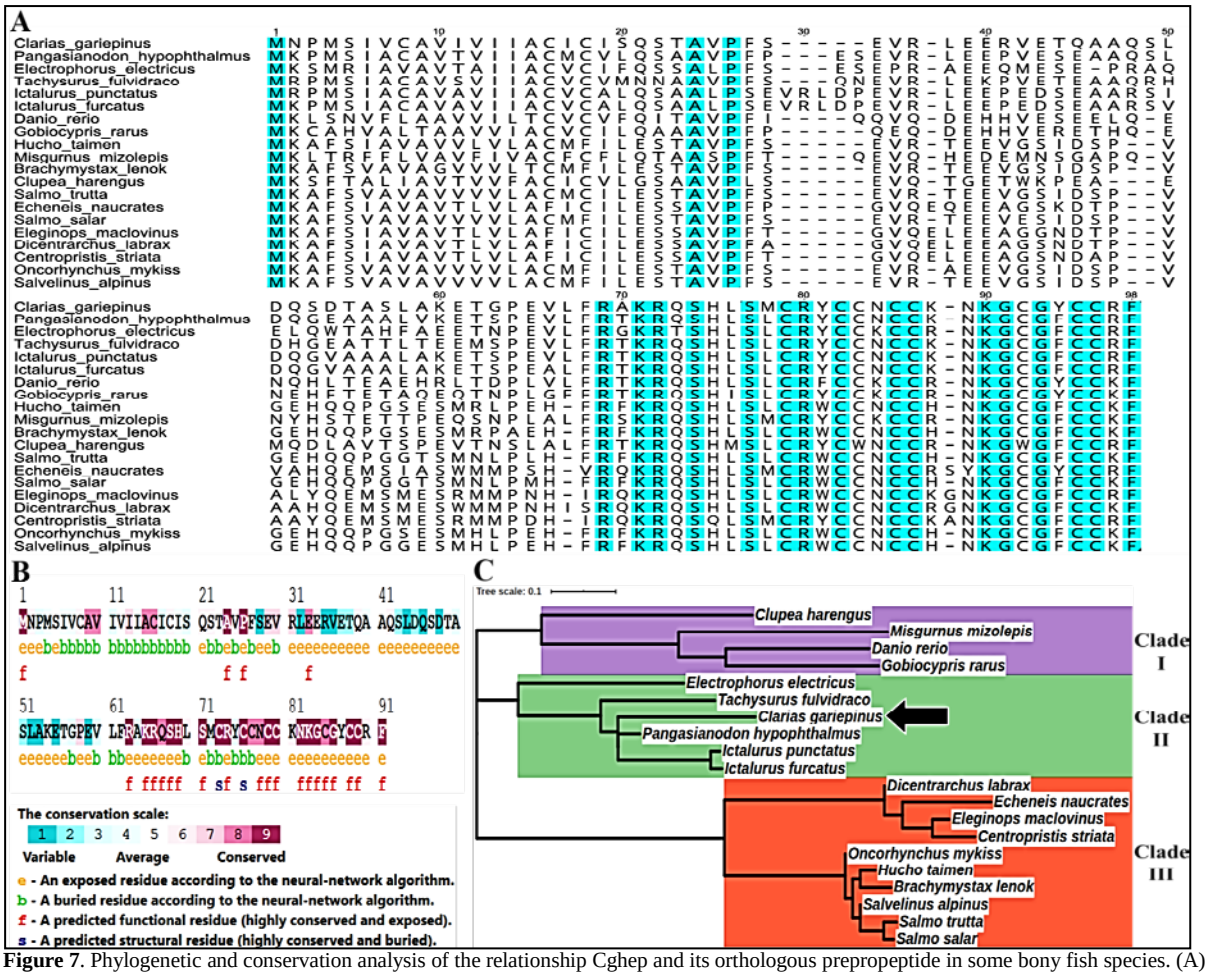


Figure 7. Phylogenetic and conservation analysis of the relationship Cghep and its orthologous prepropeptide in some bony fish species. (A) multiple sequence alignment (conserved residues highlighted in cyan). (B) conservation analysis. (C) phylogenetic tree.

Amino acid sequence alignment of Cghep with the orthologous prepropeptide from 19 bony fish species (Figure 7A and B) showed that there were 19 highly conserved amino acids among these species, and 16 amino acids among them were located within the last 29 amino acids of Cghep. 17 amino acids out of the total highly conserved amino acids were predicted to be exposed which means that they could have roles in the antimicrobial peptide function or processing while, highly conserved 2 cysteine residues were buried and could have a role in the stabilization of the hepcidin polypeptide 3D structure in these species. The phylogenetic tree of the aligned sequences (Figure 7C) showed that the tree divided to 3 main clades. Cghep is located in clade II which consists of six species. Cghep formed a subclade contains *P. hypophthalmus, I. punctatus and I. furcatus* hepcidin prepropeptides while the hepcidins from the last 2 species hepcidin are clustered together faraway from Cghep. Among the investigated species the distance between *C.gariepinus and P. hypophthalmus* (Iridescent shark fish) hepcidins is the smallest within the tree while the distance between *C. gariepinus and E. naucrates (Live sharksucker)* hepcidins in clade III *is the largest.* The predicted structure of polypeptide was constructed of 22.4% alpha helix,53.7% turn,14.9% coil and 9% 3-10 helix. Eight cysteine residues were identified in C-terminal of Cghep two residues were predicted to form one disulfide bond (Cys55-Cys56) as evident from the secondary structure (Figure 8).

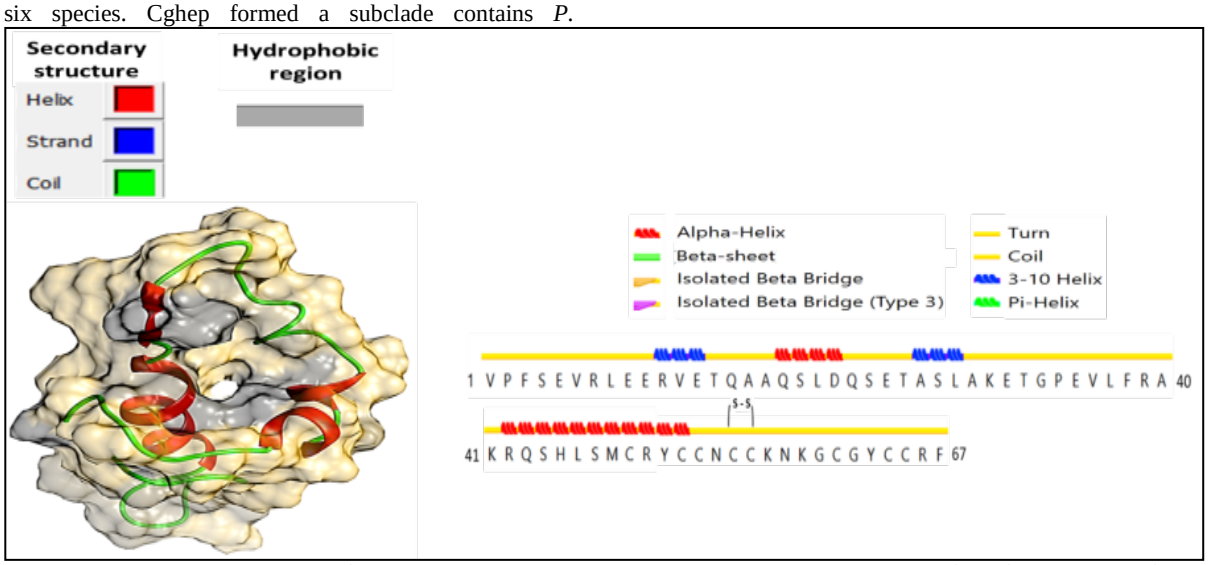


Figure 8. The predicted 3D structure of Cghep propeptide shows the secondary structures and molecular surface of the peptides (left) and description for the secondary structure and disulfide bonds within the peptides (right).

3.3. Characterization of C. ramada Hepcidin-1 (Crhep1) and Hepcidin-2 (Crhep2)

The coding sequence of Crhep1 mRNA (GenBank accession numbers from MH674362 to MH674369) consisted of 258 nucleotides (nt) coding for 85 aa (Figure 9). Three SNPs were identified in Crhep1 prepropeptide, containing one synonyms SNP (C196A C/A) and two non-synonyms SNPs (C194A and T205C). The C194A caused non-synonymous SNP alanine/aspartic acid variation while the T205C SNP caused phenylalanine/leucine variation (Figure 9A and B). Both of Crhep1 and Crhep2 have hepcidin conserved regions (which are characteristic for hepcidin superfamily members) were identified between the amino acids positions 37-85 and 38-91, respectively. A potential cleavage site for the signal peptide was predicted between Ala24 and Val25, resulting in a 24-aa signal peptide (Figure 10C). Two Crhep2 isoforms, Crhep2A and Crhep2B, were identified and deposited in GenBank under accession number MH674370 and MH674371, respectively. The nucleotide sequence of Crhep2A CDs consisted of 276 nt coding for 91 aa while Crhep2B lacked a codon coding for the amino acid number 30 (Glutamine or Q) resulting in 273 nt coding for 90 aa (Figure 13). Crhep2 prepropeptide contains a 24-aa signal peptide where its cleavage site was predicted between Ala24 and Val25 (Figure 14C). In Crhep1 prepropeptide, the ferredoxin-type iron-sulfur binding region (74-CGPGICGVC-82), Microbodies C-terminal targeting signal (83-CRF-85), Casein kinase II phosphorylation site (48-TSVD-51), and Cysteine-rich region (67-CRLCCGCCEPGICGVCC-83) motifs were found (Figure 10B). In Crhep2A prepropeptide, the cAMP- and cGMP-dependent protein kinase phosphorylation site (64-KRQS-67), Cysteine-rich region (72-CRWCCNCCRGNKGCGFCC-89), Two N-myristoylation site (81-GNKGCG-86, 84-GCGFCC-89), Microbodies C-terminal targeting signal (89-CKF-91) and motifs were found (Figure 14 B).

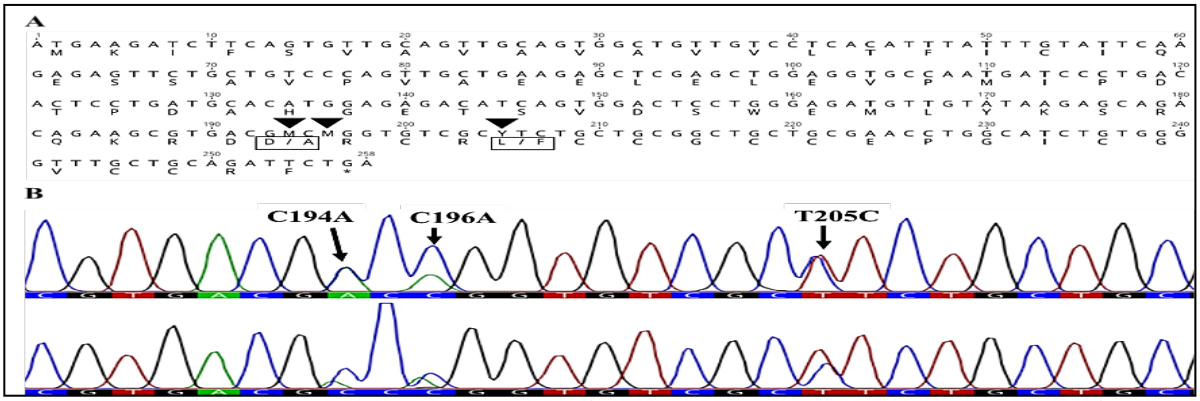
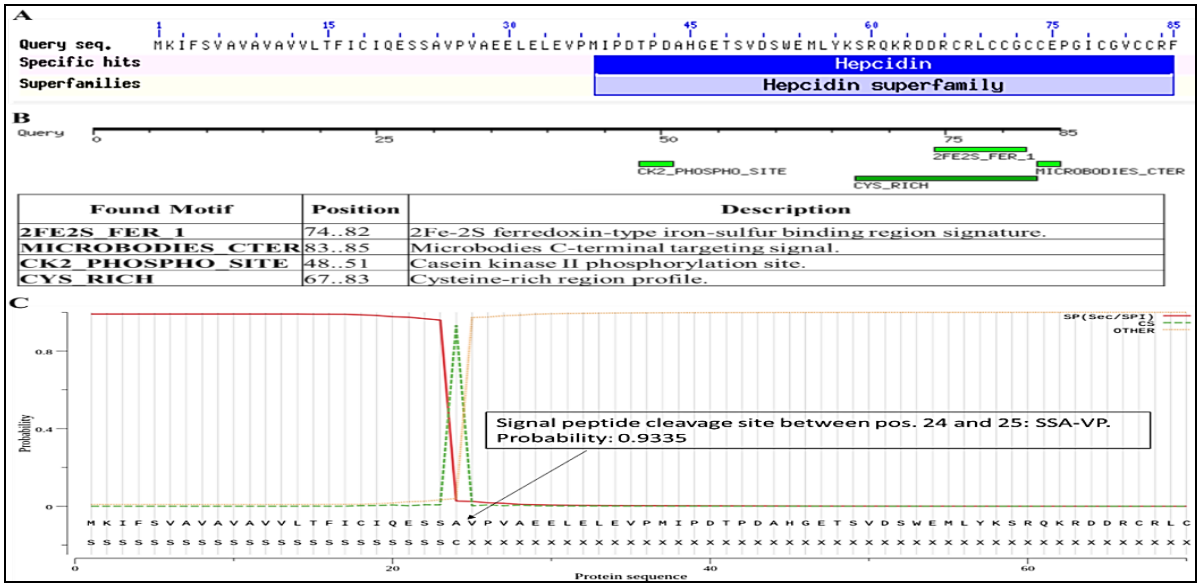


Figure 9. The nucleotides and translated amino acids sequences of Crhep1 A) The nucleotides and amino acids sequence of hepcidin-1 CDS. The solid arrows indicate the SNPs position while the boxes indicate the amino acids substitutions. M= C or A. Y=T or C. B) The detected SNPs within H1 CDS. C194A non-synonyms SNP, C196A synonyms SNP and T205C non-synonyms SNP.



Found Motif	Position	Description
2FE2S_FER_1	74..82	2Fe-2S ferredoxin-type iron-sulfur binding region signature.
MICROBODIES_CTER	83..85	Microbodies C-terminal targeting signal.
CK2_PHOSPHO_SITE	48..51	Casein kinase II phosphorylation site.
CYS_RICH	67..83	Cysteine-rich region profile.

Figure 10. Predicted motifs and domains within Crhep1 prepropeptide. A) The detected conserved domain for hepcidin superfamily within the amino acids sequence. B) The predict motifs within the prepropeptide. C) The predicted signal peptide cleavage site. SP(Sec/SPI): standard secretory signal peptides transported by the secretory translocon and cleaved by Signal Peptidase I, CS: cleavage site, Other, Other: the sequence does not have any kind of signal peptide.

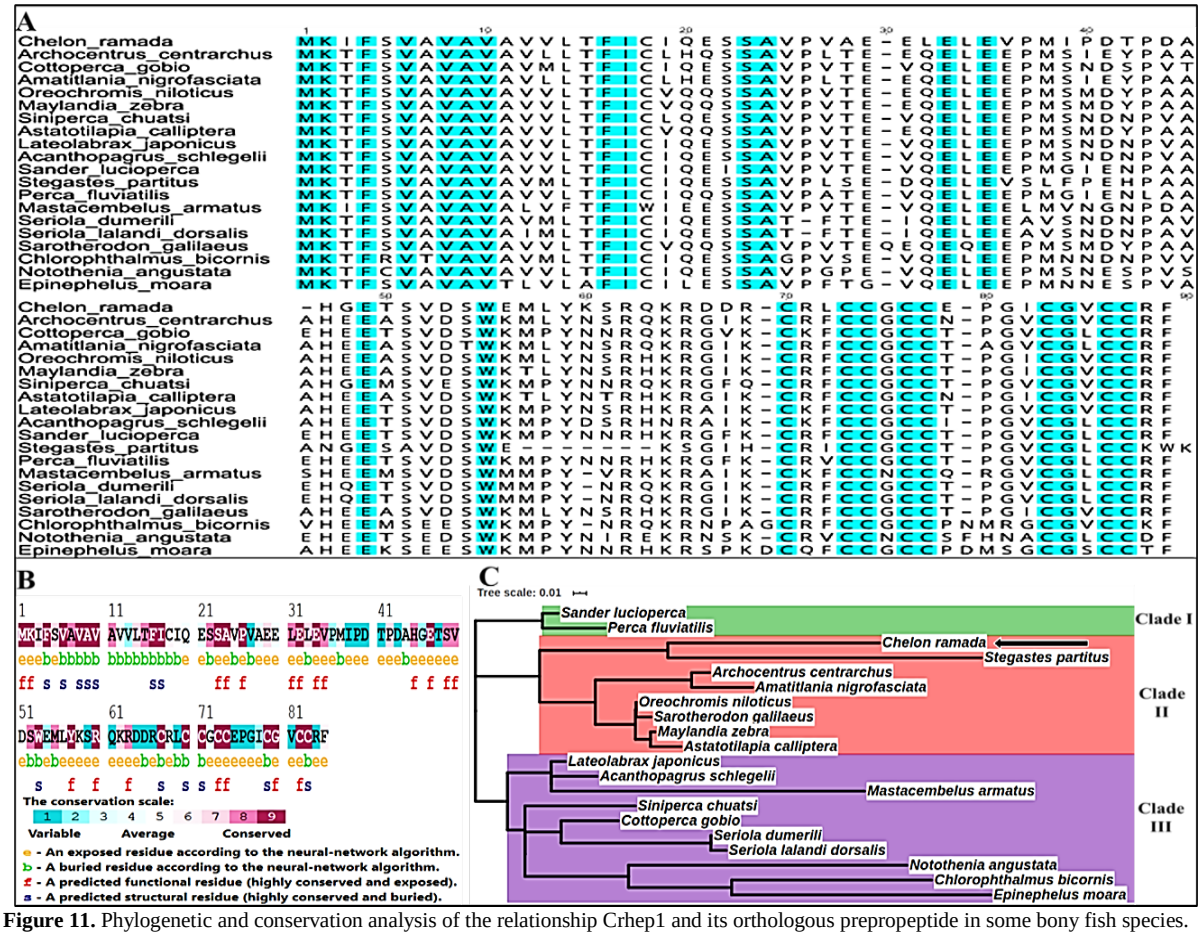


Figure 11. Phylogenetic and conservation analysis of the relationship Crhep1 and its orthologous prepropeptide in some bony fish species. (A) multiple sequence alignment (conserved residues highlighted in cyan). (B) conservation analysis. (C) phylogenetic tree.

The Crhep1 was aligned with orthologous prepropetides of 19 bony fish and about 28% from Crhep1 amino acids (24 aa) were found to be conserved among all the studied sequences (Figure 11A). Furthermore, Figure (11B) indicated that 11 conserved residues were predicted to be exposed and 13 conserved residues were predicted to be buried. The aligned sequences were used to build a phylogenetic tree which had 3 main clades (Figure 1C). Crhep1 was clustered with hepcidins from 7 species in clade II and form a subclade with *S. partitus*. The hepcidin sequences of O. niloticus (Nile tilapia) (within Clade II) and E. moara (Longtooth grouper fish) (within Clade III) had the shortest and longest distances within the tree from Crhep1 sequence. The predicted structure of the propeptide was constructed of 19.7% alpha helix, 3.3% isolated beta bridge, 52.5% turn, 13% coil and 11.5% 3-10 helix. Eight conserved cysteine residues were detected from which, four residues form two disulfide bonds (Cys43-Cys55, Cys46-Cys50) (Figure 12).

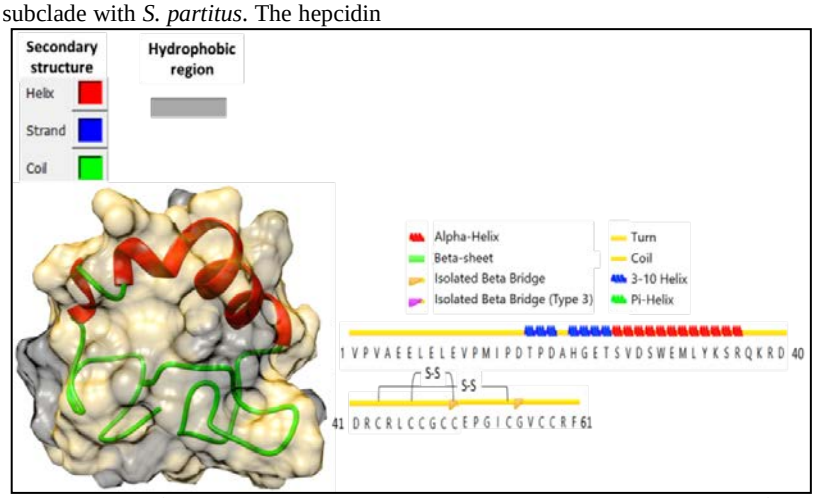


Figure 12. The predicted 3D structure of Crhep1 propeptide shows the secondary structures and molecular surface of the peptides (left) and description for the secondary structure and disulfide bonds within the peptides (right).

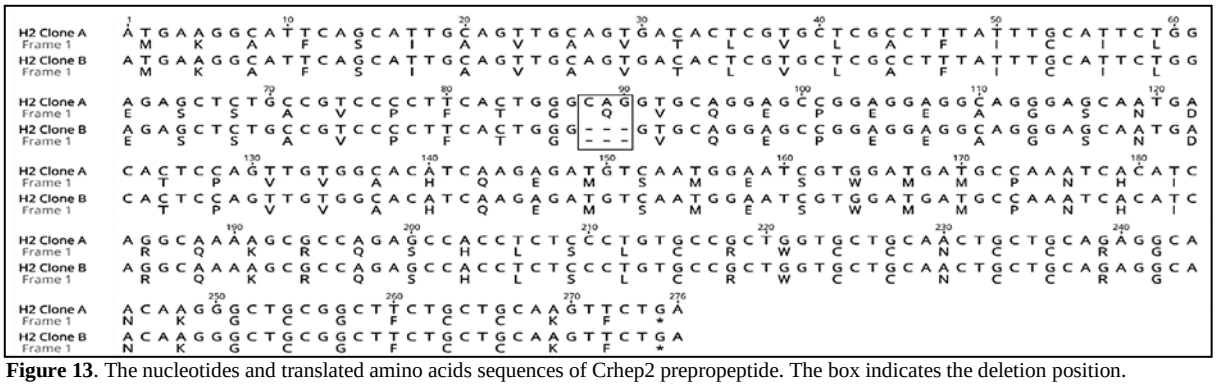
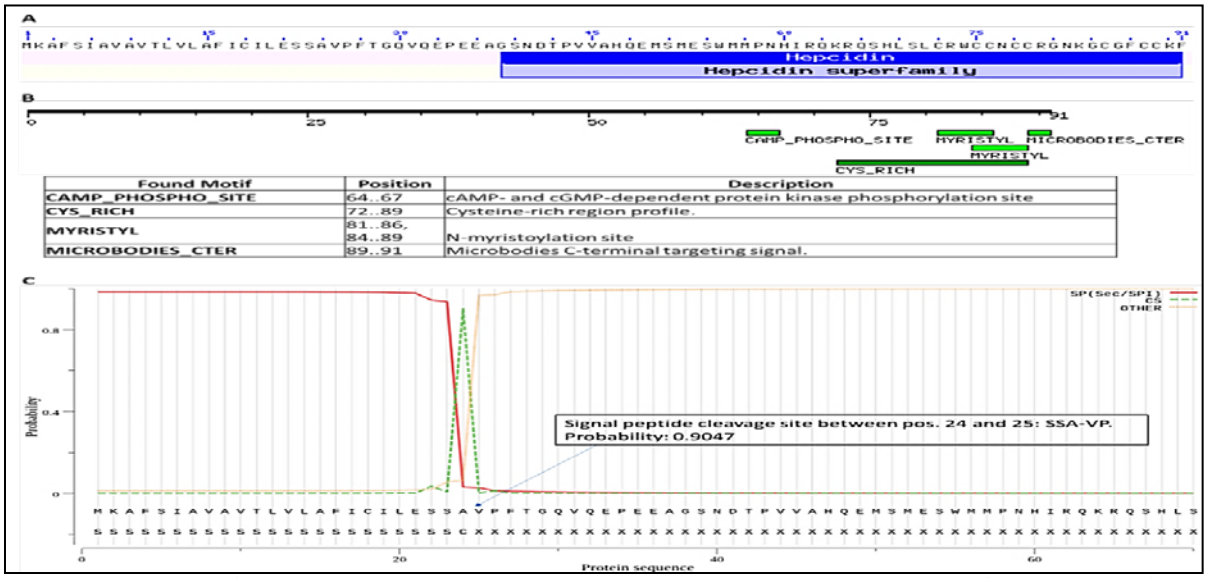


Figure 13. The nucleotides and translated amino acids sequences of Crhep2 prepropeptide. The box indicates the deletion position.



Found Motif	Position	Description
CAMP_PHOSPHO_SITE	64..67	cAMP- and cGMP-dependent protein kinase phosphorylation site
CYS_RICH	72..89	Cysteine-rich region profile.
MYRISTYL	81..86, 84..89	N-myristoylation site
MICROBODIES_CTER	89..91	Microbodies C-terminal targeting signal.

Figure 14. Predicted motifs and domains within Crhep2A prepropeptide. A) The detected conserved domain for hepcidin superfamily within the amino acids sequence. B) The predict motifs within *the* prepropeptide. C) The predicted signal peptide cleavage site. SP(Sec/SPI): standard secretory signal peptides transported by the secretory translocon and cleaved by Signal Peptidase I, CS: cleavage site, Other, Other: the sequence does not have any kind of signal peptide.

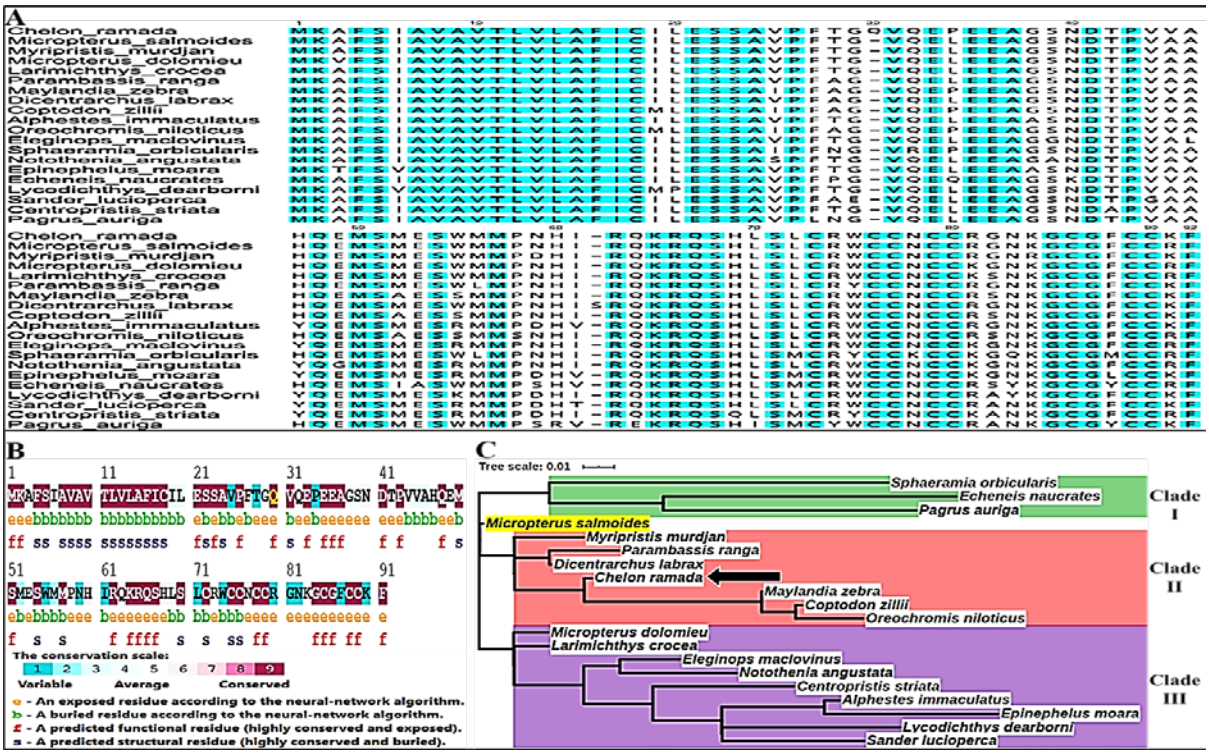


Figure 15. Phylogenetic and conservation analysis of the relationship Crhep2A and its orthologous prepropeptide in some bony fish species. (A) multiple sequence alignment (conserved residues highlighted in cyan). (B) conservation analysis. (C) phylogenetic tree.

Interestingly, about 55% (50 amino acids) of Crhep2A were highly conserved when the sequence was aligned with 19 orthologous prepropeptides from 19 bony fish species (Figure 15A and B). The 26 conserved amino acids could be exposed in the folded polypeptide, while 24 could be buried (Figure 15B). The amino acid in the position 30 (Glutamine or Q) was characteristic for Crhep2A and was not found in Crhep2B or the aligned sequences. The aligned data were used to make a phylogenetic tree and the results showed that the investigated hepcidins sequences were clustered in 3 main clades in addition to isolated branch which contained only the *M. salmoides* hepcidins (Figure 15C). Crhep2A was positioned in Clade II which contained hepcidins of 7 species. *C. ramada, M. zebra, C. zillii* and *O. niloticus* hepcidins were connected to the same node and form a subclade in which the last 3 prepropeptides grouped together. Regarding the distances between Crhep2A and the other hepcidin sequences in the tree, the *D. labrax* (European bass) and *E. moara* (Longtooth grouper fish) hepcidin sequences were estimated to have the smallest and the largest distances, respectively. The predicted structure of Crhep2A propeptide was constructed of 77% turn and 23% coil while, Crhep2B was constructed of 13.1% alpha helix,65.5% turn,14.8% coil and 6.6% 3-10 helix. Conserved eight cysteine residues were detected in both isoforms of Crhep2. In Crhep2A, four cysteine residues formed two disulfide bonds (Cys48-Cys64, Cys52-Cys61) (Figure 16A) while, in Crhep2B two cysteine residues formed one disulfide bond (Cys54-Cys60) (Figure 16B).

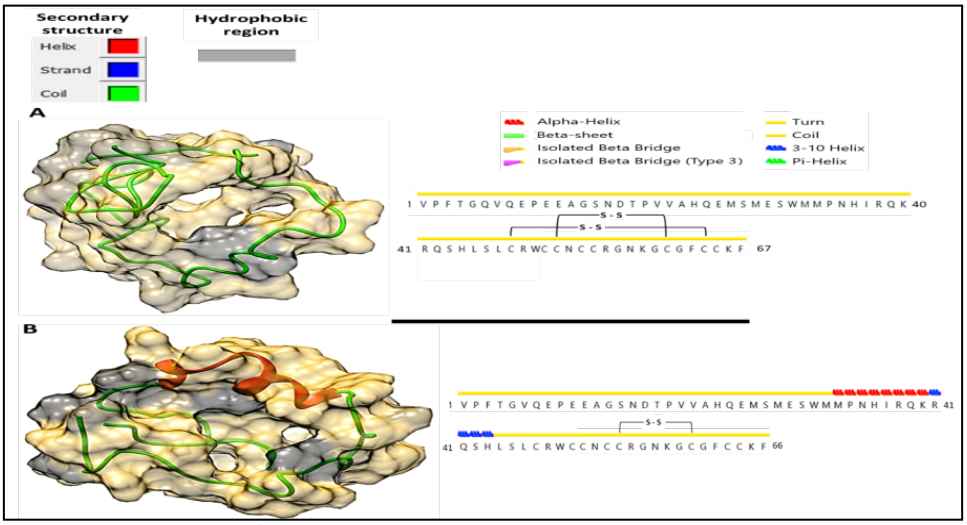


Figure 16. The predicted 3D structure of Crhep2 clone A (A) and clone B (B) propeptides shows the secondary structures and molecular surface of the peptides (left) and description for the secondary structure and disulfide bonds within the peptides (right).

4. Discussion

NK-lysin has been identified in many fish species, such as Japanese flounder (*Paralichthys olivaceus*) (Hirono *et al*., 2007), half-smooth tongue sole (*Cynoglossus semilaevis*) (Zhang *et al*., 2013), zebrafish (*Danio rerio*) (Pereiro *et al*., 2015), channel catfish (*Ictalurus punctatus*) (Wang *et al*., 2006a and 2006b), common carp (*Cyprinus carpio*) (Wang *et al*.,2018), Nile tilapia (*Oreochromis niloticus*) (Huang *et al*., 2018), turbot (*Scophthalmus maximus*) (Pereiro *et al*., 2017), Atlantic salmon (*Salmo salar*) (Acosta *et al*., 2019), and large yellow croaker (*Larimichthys crocea*) (Zhou *et al*.,2016), mudskipper (*Boleophthalmus pectinirostris*) (Ding *et al*., 2019). In contrast to the single copy gene in human, some fish have several copies of Nk-lysins, for example, zebrafish showed the highest score as it possesses four copies.

Deduced amino acid sequence of Cgnkl contains 128 aa. A signal peptide (17aa) was predicted as well as the characteristic surfactant-associated protein B (SapB) domain of the SAPLIPs (saposin-like protein family). Saposins have been reported to increase the level of intracellular ceramide, a molecule involved in the induction of apoptosis, via activating lipid-degrading enzymes, such as glucosylceramidase and sphingomyelinases. Saposin-like polypeptides are known to be greatly resistant to thermal denaturation; however, such property is abolished after reduction of the disulfide bonds (Gonzalez *et al*., 2000). One N-glycosylation site was identified in Cgnkl at 72-75aa, which is needed for the intracellular transport mechanism (Martınez *et al*., 2001). Also, four N- myristoylation sites at 13-18, 34-39, 54-59 and 71-76aa were detected. Lately, Krishnakumari *et al* (2018) indicated that myristoylation enhances antibacterial activity and modulates hemolytic activity to different extents.

The amino acid sequence of Cgnkl contains six well conserved cysteines residues that form three disulfide bonds. Cgnkl consisted of five-helices, spaced by three loops, as evident from the predicted 3D structure. As previously reported by Pereiro *et al*. (2015), this structure allows the interaction with biological membranes and the ability to altering the membrane integrity. The second and third α-helices in NK-lysin define a helix-loop-helix motif that is similar to the structural patterns of smaller anti-bacterial peptides such as bactenecin (coil-loop-coil) or protegrins (sheet-loop-sheet) (Andreu *et al*., 1999). NK-lysin is known to be extremely stable polypeptide, as its structure remains conserved after the interaction with their target cell membranes. This stability is due to its amphipathic character of 3 of its 5 helices and the disulfide bridges (Waring *et al*., 2016). Phylogenetic tree analysis showed that Cgnkl was grouped with bony fish from the same order (*siluriforms*) and was closely related to *P. hypophthalmus* (Iridescent Shark fish).

Hepcidin, a small cysteine-rich antimicrobial peptide, plays an important role in host immunological process and

iron regulation (Ke *et al*., 2015). The genomic copies of hepcidin differ notably depending on the organism. There are multiple copies of hepcidin in fish unlike most mammals which possess a single copy. It has been reported that at least five copies of hepcidin genes are found in winter flounder (Douglas *et al*., 2003) and seven copies in black porgy (Yang *et al*., 2007). In the present study, all identified hepcidins showed the characteristic features of fish hepcidins. The predicted cleavage site for the signal peptide was between Ala24 and Val25.

Fish HAMPs are divided into HAMP1 and HAMP2 groups. HAMP2 presents only in acanthopterygians, while HAMP1 presents in both acanthopterygians and non-acanthopterygians (Kim *et al*., 2019). *C. gariepinus* is a non-acanthopterygian species. Thus, the identified Cghep belongs to the HAMP1 group. The hypothetical iron regulatory sequence, QSHLS that is found in the N–terminus prior to the first Cysteine residue in the mature peptide, is not found in HAMP2 (Kim *et al*., 2019). Crhep2 lacks QSHLS motif indicating that it belongs to HAMP2 group. Consequently, Crhep2 could be more involved in iron regulation, whereas Crhep1 could be involved in the immune defense.

Sequence alignment of Cghep, Crhep1 and Crhep2 with other fish species showed a high degree of conservation, particularly the eight cysteine residues. These residues are involved in the formation of four disulfide bonds, which may stabilize hairpin-like structure. This structure is believed to be essential for proper antimicrobial and cytotoxic properties (Álvarez *et al*., 2014). Nevertheless, hepcidins of some fish have been found to have less than eight cysteine residues and still likely to be fully functional (Nemeth *et al*., 2006). Hirono *et al*. (2005) reported only six cysteine residues in Japanese Flounder (*P. olivaceus*).

Interestingly, as evident from the structure of prohepcidins identified in this study, not all of eight cysteine residues participated in formation of disulfide bonds. In Cghep and Crhep2B, only two residues formed one disulfide bond while, in Crhep1 and Crhep2A, four residues formed two bonds. Previous studies reported that Cysteine residues may serve different functions including: structural stabilization through forming stable disulfide-bonded, Metal-binding, catalytic activity and regulatory roles as they serve as sites of post translational modifications (Tu *et al*., 2004; De Domenico *et al*., 2008; Marino and Gladyshev, 2011; Marino and Gladyshev, 2012).

In Cghep, four SNPs were detected. C78T and A195G were synonyms SNPs while A67G and T144G were non-synonyms SNPs which caused threonine/alanine and glutamic acid/aspartic acid variations, respectively. In Crhep1, one synonyms SNP (C196A) and two non-synonyms SNPs (C194A and T205C) were detected. The non-synonymous SNPs C194A and T205C leads to alanine/aspartic acid and phenylalanine/leucine variations, respectively. Fernandes *et al* (2010) suggested that this genetic variation due to accelerated evolutionary rates might be directed when the host is exposed to pathogens. In our previous study, two SNPs were detected in hepcidin-1 that was identified from Nile tilapia species (*S. galilaeus*). 108 A/G was synonyms SNP and 101 A/T was non-synonyms SNP that caused Glutamine / Leucine variation. Also, one synonyms SNP (86 T/C) and one non-synonyms SNP (101 G/T) were detected in hepcidin-2. In hepcidin 2 identified from *T. Zilli*, one synonyms SNP (213 C/T) and one non-synonyms SNP (101 G/T) were found (Karima *et al*., 2020). In the present study, two Crhep2 isoforms were detected due to the absence of amino acid, Glutamine or Q, in the position 30. Pereiro *et al.* (2012) suggested that the different isoforms help fish to develop their innate immune recognition capabilities.

The post-translational modifications, such as phosphorylation, myristoylation and microbodies targeting motifs, are important for both structure and function of AMPs. They have the ability to tune peptide activities and lead to diverse structural scaffolds (Wang *et al*., 2012). Maurer-Stroh and Eisenhaber (2004) observed that N-myristoylation mediates the viral infectivity and eukaryotic infections. Recently, Latendorf *et al* (2019) demonstrated that the extent of post-translational modification and the peptide chain length are the major factors that control the antimicrobial output.

Sequence alignment with different bony fish species showed that about 55% of the deduced amino acid of Crhep2A is highly conserved. The higher variability in the amino acid sequence of Crhep1 might be an evolutionary mechanism for the recognition of a diverse range of microbes and the longer half-life with regard to Crhep2 could favor the elimination of pathogens (Pereiro *et al*., 2012). *C. ramada* hepcidins placed it with species from orders cichliformes and perciforms. Phylogenetic analysis showed that Crhep2 is highly homologous with *D. labrax* (European seabass), while Crhep1 is highly homologous with *O. niloticus* (Nile tilapia). Furthermore, the Cghep is highly homologous with *P. hypophthalmus* (Iridescent shark fish) from the same order (*Siluriformes*) and less homologous with *E. naucrates* (slender shark sucker) from order *carangiform*.

5. Conclusion

In the present study, coding sequences of Cgnkl, Cghep, Crhep1 and Crhep2 were analyzed. Cgnkl was found to possess all characteristic features of previously identified NK-lysins. The coding sequences of Cghep, Crhep1 and Crhep2 show the similar predicted signal cleavage site as well as eight characteristic cysteine residues. Further studies will be required to analyze the basal expression of the identified AMPs mRNA in different tissues.

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References

Acosta J, Roa F, Gonzalez-Chavarria I, Astuya A, Maura R, Montesino R, Munoz C, Camacho F, Saavedra P and Valenzuela A. 2019. In vitro immunomodulatory activities of peptides derived from Salmo salar NK-lysin and cathelicidin in fish cells. *Fish Shellfish Immunol*, 88:587–594. d.

Álvarez CA, Guzmán F, Cárdenas C, Marshall S H and Mercado L. 2014. Antimicrobial activity of trout hepcidin. *Fish and ShellfishImmunology*, **41**:93–101.

Andreu D, Cristina C, Charlotte L, Hans G B and Mats A. 1999. Identification of an anti-mycobacterial domain in NK-lysin and granulysin. *Biochem J* ,344: 845-849.

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Bolger AM, Lohse M and Usadel B. 2014 . Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15):2114-2120.

Bruno R, Maresca M and Canaan S. 2019. Worms' Antimicrobial Peptides. *Mar Drugs*.17:512.

De Domenico I, Nemeth E, Nelson JM, Phillips JD, Ajioka RS and Kay MS. 2008.The hepcidin-binding site on ferroportin is evolutionarily conserved. *Cell Metab*, 8:146–56. doi.org/10.1016/j.cmet.2008.07.002.

Ding FF, Li CH and Chen J. 2019. Molecular characterization of the NK-lysin in a teleost fish, Boleophthalmus pectinirostris: Antimicrobial activity and immunomodulatory activity on monocytes/macrophages**.** *Fish Shellfish Immunol*, 2:256-264. d

Douglas SE, Gallant JW, Ryan SL, Andrew D and Stephen CMT. 2003. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. *Developmental and Comparative Immunology*, 27 :589–601.

Drakesmith H and Prentice AM. 2012. Hepcidin and the iron-infection axis. *Science*, 338:768–772.

Enis A, Dannon B, Bérénice B, Marius B, Dave B, Martin Č, John C, Dave C, Nate C, Björn G,AG, Jennifer H, Vahid J, Helena R, Nicola S, Jeremy G, James T, Anton N, and Daniel B. *Nucleic Acids Research*, 46(W): W537–W544.

Fan K, Li H, Wang Z, Du W, Yin W, Sun Y and Jiang J. 2016. Expression and purification of the recombinant porcine NK-lysin in Pichia pastoris and observation of anticancer activity in vitro. *Pre. Biochem Biotechnol*, 46: 65–70.

FAO**.** 2017. Global aquaculture production dataset 1950–2015 (FishstatJ). Available at :SSSSSSSSSSSSSSSSSSSSSSSSSSSSS www.fao.org/fishery/statistics/software/fishstatj/e.

Fernandes JMO, Ruangsri J and Kiron V. 2010. Atlantic cod piscidin and its diversification through positive selection. *PloS One*, **5**: 9501.

Gonzalez C, Langdon GM, Bruix M, Galvez A, Valdivia E, Maqueda M and Rico M. 2000. Bacteriocin AS-48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin. *Proceedings of the National Academy of Sciences*, 97(21):11221-11226.

Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD and Bowden J. 2013. *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protoc*, 8:1494–512.

Hirono I, Hwang JY, Ono Y, Tomofumi K, Ohira T, Nozaki R, et al. Two different types of hepcidins from the Japanese flounder. Paralichthys olivaceus FEBS J. 2005;272:5257-64.

Hirono I, Kondo H, Koyama T, Arma NR, Hwang JY, Nozaki R, Midorikawa N and Aoki T. 2007. Characterization of Japanese flounder (Paralichthys olivaceus) NK-lysin, an antimicrobial peptide. *Fish Shellfish Immunol.* 22:567–575.

Huang T, Wei G, Bingqian W, Yuyong Z, Lili C, Zuochun Y, Cheng Z, Gefeng X. 2019. Identification and expression of the hepcidin gene from brown trout (Salmo trutta) and functional analysis of its synthetic peptide. *Fish Shellfish Immunol*, 87:243–253

Huang X, Hu B, Yang X, Gong L, Tan J, and Deng L. 2018. The putative mature peptide of piscidin-1 modulates global transcriptional profile and proliferation of splenic lymphocytes in orange-spotted grouper (*Epinephelus coioides*). *Fish Shellfish Immunol*. 86:1035–1043.

Karima FM, Dalia MM, Aboelenin MM and Heba AM. 2020. Molecular characterization and expression analysis of hepc1 and hepc2 in three tilapia species collected from Lake Manzala. *Bulletin of National Research Centre*, accepted.

Ke F, Yun W, Chuan-Shun Y and Chen X. 2015. Molecular cloning and antibacterial activity of hepcidin from Chinese rare minnow (Gobiocypris rarus). *Electronic Journal of Biotechnology,18:* 169–174.

Kim C, Eun J and Yoon K N. 2019. Chondrostean sturgeon hepcidin: An evolutionary link between teleost and tetrapod hepcidins. *Fish Shellfish Immunol*,88 :117–125.

Kim WH , Lillehoj HS and Gay CG. 2016. Using genomics to identify novel antimicrobials. *Rev Sci Tech Off Int Epiz*, 35 (1): 95-103.

Krishnakumari V , Ankeeta G , Harikrishna A and Ramakrishnan N. 2018. Effects of increasing hydrophobicity by N-terminal myristoylation on the antibacterial and hemolytic activities of the C-terminal cationic segments of human-β-defensins. *J Chem Biol Drug Des*, 92:1504–1513.

Kumar S, Stecher G, Li M, Knyaz C, and Tamura K. 2018. Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, 35:1547-1549.

Lai Y and Gallo R L. 2009. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol*, 30:131–141.

Lama R, Pereiro P, Costa MM, Encinar JA, Medina-Gali RM, Perez L, Lamas J, Leiro J, Figueras A and Novoa B. 2018. Turbot (*Scophthalmus maximus*) NK-lysin induces protection against the pathogenic parasite philasterides dicentrarchi via membrane disruption. *Fish Shellfish Immunol*,82:190–199.

Latendorf T, Ulrich G and Zhihong W. 2019. Antimicrobial Peptides (CIDAMPs) Represent a New Paradigm of Innate Defense with a Potential for Novel Anti-Infectives. *Scientific Reports, 9:3331.*

Mahrous KF, Aboelenin MM, Abd El-kader HA, Mabrouk DM, Gaafar AY, Younes AM, Mahmoud MA, Khalil WKB and Hassanane MS. 2020a. Piscidin 4: Genetic expression and comparative immunolocalization in Nile tilapia (Oreochromis niloticus) following challenge using different local bacterial strains. *Developmental & Comparative Immunology*, 112: 103777.

Mahrous KF, Aboelenin MM, Rashed MA, Abd El-Hafiez MA and Rushdi HE. 2020b. Detection of polymorphism within leptin gene in Egyptian river buffalo and predict its effects on different molecular levels. *Journal of Genetic Engineering and Biotechnology*, 18: 6.

Mahrous KF, Mabrouk DM, Aboelenin MM, Abd El-kader HA, Gaafar AY, Younes AM, Mahmoud MA, Khalil WKB and Hassanane MS. 2020c. Molecular characterization and immunohistochemical localization of tilapia piscidin 3 in response to Aeromonas hydrophila infection in Nile tilapia. *Journal of Peptide Science*, 26 (11): e3280.

Marino S. M. and Gladyshev VN. 2011. Redox biology: computational approaches to the investigation of functional cysteine residues. *Antioxid Redox Signal*, 15: 135–146

Marino S. M. and Gladyshev VN. 2012. Analysis and Functional Prediction of Reactive Cysteine Residues. *The Journal Of Biological Chemistry*, 287(7):4419–4425.

Martin J, Letellier G and Marin A .2005 Protein secondary structure assignment revisited: a detailed analysis of different assignment methods. *BMC Struct Biol*, 5: 17.

Martınez M, Irene P, Beatriz L, Enrique N, Cecilio G, Francisco Z, and Carmen A. 2001.The Role of N-Glycosylation in Transport

to the Plasma Membrane and Sorting of the Neuronal Glycine Transporter, *The Journal Of Biological Chemistry*. 276(3):2168 – 2173.

Masso-Silva JA and Diamond G. 2014. Antimicrobial peptides from fish. *Pharmaceuticals*, 7:265–310.

Maurer-Stroh S and Eisenhaber F . 2004. Myristoylation of viral and bacterialproteins. *Trends in Microbiology*,12:178–185,.

Mehanna SF, Desouky MG, Makkey AF. 2019. Some Targeted Reference Points for Thin Lip Grey Mullet Liza Ramada Management in Bardawil Lagoon, North Sinai, Egypt. *Fish Aqua J* 10:263.

Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. 2006. The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. *Blood*,107:328-333.

Neves JV, Migue F R, Ana CM, Tânia S, Maria SG and Pedro NS.2017. Rodrigues Hamp1 but not Hamp2 regulates ferroportin in fish with twofunctionally distinct hepcidin typesHepcidin is a small cysteine rich peptide. *Scientific Reports*, 7: *14793*.

Pereiro P, Figueras A and Novoa B. 2012. A novel hepcidin-like in turbot (*Scophthalmus maximus L*.) highly expressed after pathogen challenge but not after iron overload. Fish Shellfish Immunol, 32 : 879-889.

Pereiro P, Romero A, Diaz-Rosales P, Estepa A, Figueras A and Novoa B. 2017. Nucleated teleost erythrocytes play an NK-lysin-and autophagy-dependent role in antiviral immunity. *Front Immunol*. 8:1458.

Pereiro P, Varela M, Diaz-Rosales P, Romero A, Dios S, Figueras A and Novoa B. 2015. Zebrafish NK-lysins: First insights about their cellular and functional diversification. Dev. Comp. Immunol. 51:148–159.

Sathyan N, Philip R, Chaithanya ER, Kumar PR, Sanjeevan VN and Singh IS. 2013. Characterization of Histone H2A Derived Antimicrobial Peptides, Harriottins, from Sicklefin Chimaera Neoharriotta pinnata (Schnakenbeck, 1931) and Its Evolutionary Divergence with respect to CO1 and Histone H2A. *ISRN Molecular Biology*,2013:1-10.

Shourbela R. M. 1; Ashraf, M. Abd El-latif 2 and Abd-El-Azem. 2014. Induced spawning of african catfish , *clarias gariepinus* using (gnrha) combined with dopamine antagonist. *Veterinary Medical Journal*, 27:25-35.

Sroor FM, Aboelenin MM, Mahrous KF, Mahmoud K, Elwahy AHM and Abdelhamid IA. 2020. Novel 2-cyanoacrylamido-4,5,6,7-tetrahydrobenzo[b]thiophene derivatives as potent anticancer agents. *Arch Pharm (Weinheim)*, 353(10): e2000069.

Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melian A and Bogdan C. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science*, 282:121–125.

Tu, B P, and Weissman, J S. 2004. Oxidative protein folding in eukaryotes :mechanisms and consequences. *J Cell Biol*, 164: 341–346.

Wang GL, Wang MC, Liu YL, Zhang Q, Li CF, Liu PT, Li EZ, Nie P, Xie HX. 2018. Identification, expression analysis, and antibacterial activity of NK-lysin from common carp *Cyprinus carpio*. *Fish Shellfish Immunol*, 73:11–21.

Wang Q, Bao B, Wang Y, Peatman E and Liu Z, 2006b. Characterization of a NK-lysin antimicrobial peptide gene from channel catfish. *Fish Shellfish Immunol*, 20 (3):419–426.

Wang Q, Wang Y, Xu P and Liu Z. 2006a. NK-lysin of channel catfish: gene triplication, sequence variation, and expression analysis. *Mol Immunol*, 43 (10): 1676–1686.

Wang Y, Liu X, Ma L, Yu Y, Yu H, Mohammed S, Chu G, Mu L, and Zhang Q. 2012. Identification and characterization of a hepcidin from half smooth tongue sole Cynoglossus semilaevis. *Fish Shellfish Immunol*, 33: 213–219.

Waring AJ, Gupta M, Gordon LM, Fujii G and Walther FJ. 2016. Stability of an amphipathic helix-hairpin surfactant peptide in liposomes. *Biochim Biophys Acta*, 1858(12):3113–3119.

Yang M, Wang K, Chen J, Qu H and Li S. 2007. Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B). *Fish Shellfish Immunol*, 23:1060-1071.

Yang, J, Yan R, Roy A, Xu D, Poisson J and Zhang Y. 2015. The I-TASSER Suite: protein structure and function prediction. Nat. Methods, 12(1): 7-8.

Zhang M, Long H and Sun L. 2013. A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens. *Dev Comp Immunol*, 40:258–265.

Zhou QJ, Wang J, Liu M, Qiao Y, Hong WS, Su YQ, Han KH, Ke QZ and Zheng WQ. 2016. Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish *Larimichthys crocea*, 55:195-202.