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Molecular Characterisation and Phylogenetic Analysis of a Novel Isoform of Hepatic Antimicrobial Peptide, Hepcidin (Zc-hepc1), from the Coral Fish Moorish idol, *Zanclus cornutus* (Linnaeus, 1758)

E. R. Chaithanya · Rosamma Philip · Naveen Sathyan · P. R. Anil Kumar · Sherine Sonia Cubelio · I. S. Bright Singh

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Abstract Hepcidin is a family of short cysteine-rich antimicrobial peptides (AMPs) participating in various physiological functions with inevitable role in host immune responses. Present study deals with identification and characterisation of a novel hepcidin isoform from coral fish *Zanclus cornutus*. The 81 amino acid (aa) preprohepcidin obtained from *Z. cornutus* consists of a hydrophobic aa rich 22 mer signal peptide, a highly variable proregion of 35 aa and a bioactive mature peptide with 8 conserved cysteine residues which contribute to the disulphide back bone. The mature hepcidin, Zc-hepc1 has a theoretical isoelectric point of 7.46, a predicted molecular weight of 2.43 kDa and a net positive charge of +1. Phylogenetic analysis grouped *Z. cornutus* hepcidin with HAMP2 group hepcidins confirming the divergent evolution of hepcidin-like peptide in fishes. Zc-hepc1 can attain a β-hairpin-like structure with two antiparallel β-sheets. This is the first report of an AMP from the coral fish *Z. cornutus*.

E. R. Chaithanya · R. Philip (✉) · N. Sathyan · P. R. Anil Kumar
Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, Kerala, India
e-mail: rosammap@gmail.com; rose@cusat.ac.in

S. S. Cubelio
Centre for Marine Living Resources and Ecology, Ministry of Earth Sciences, Government of India, Kochi 682037, Kerala, India

I. S. Bright Singh
National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, Kerala, India

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Introduction

Fishes are considered as a transition form between organisms which possess only innate immunity and organisms which depend profoundly on acquired immunity. Though they possess adaptive and specific immune responses, the innate parameters are at the forefront because of the feeble and delayed responses of the specific immune system [1, 2]. In fishes, the primary immune barriers against invading microbes such as skin secretions and mucus are known to contain several bacteriostatic and bactericidal compounds including antimicrobial peptides (AMPs). AMPs are vital effector molecules of humoral components in fish innate immune system. They are specific, broad spectrum endogenous antibiotics produced by almost all organisms ranging from prokaryotes to mammals [3]. Despite their structural diversity, the specificity towards the prokaryotic cell membrane and relative non-toxicity towards mammalian cells make them promising candidates as novel therapeutic agents [4, 5]. The unique mode of action of AMPs, which is comparatively irresistible, such as destabilisation of cell membrane and subsequent osmotic imbalance mediated lysis as a sum total of the amphipathic nature and net positive charge opens their way to the warfront against multidrug resistant bacteria [6, 7].

A number of AMPs have been reported from several fishes. These include hepcidins from bony fishes [8–10], pleurocidins from winter flounder and allied fishes [11] and piscidins from bass family [12] besides, histone derived peptides [13–16], defensins [17, 18] and cathelicidins [19, 20]. Hepcidins represent short cysteine-rich peptides of multiple physiological functions, primarily identified as

liver expressed antimicrobial peptide (LEAP-1) from the human blood ultra-filtrate [21], now synonymously called as hepatic antimicrobial peptide or HAMP [22]. Hepcidin-like peptides are widely distributed among members of the subphylum vertebrata with representatives in all classes including fishes [23–25]. In fishes, HAMPs are transcription products of multiple genes of two main lineages, that is, HAMP1 and HAMP2. HAMP1-like peptides are distributed in all groups of fishes and are an orthologue of mammalian HAMP while HAMP2-like group is reported only from acanthopterygian fishes [23] with a single exception [24]. These two groups of peptides differ mainly in the overall cationicity and the presence or absence of the presumed iron binding motif QSHLS/DTHFP.

Since the isolation and characterisation of first non-human vertebrate hepcidin from striped hybrid sea bass [25], a number of hepcidins have been isolated and characterised from various fishes, but there were no reports of any AMPs from the coral fish Moorish idol (*Zanclus cornutus*). It is one of the most popular coral fishes inhabiting the coral reefs of Indo West Pacific. Despite their superficial similarity with the butterfly fishes, Moorish idol is the sole extant fish of the family Zanclidae. It is one of the most beautiful aquarium fishes with graceful movements and notorious for its short lifespan in aquaria. Better understanding of immune parameters of the fish may definitely help to improve its health and survival in artificial environment. In the present study, we report a novel HAMP2-like sequence from the tropical coral fish Moorish idol, its molecular characterisation and phylogenetic analysis. Though a number of hepcidin isoforms have been reported from different taxonomic groups of fishes, this is the first report of HAMP from coral fishes.

Materials and Methods

Sample Collection

Live Moorish idol was obtained as a by-catch from 30 m depth zone off Cochin coast during daily fishing cruise of *ORV Sagar Sakthi* (Central Institute of Fishery Technology, Kochi). The live specimen was brought to the laboratory and maintained in natural sea water at 25 °C for a week. The fish appeared to be apparently healthy with regular movements and feeding.

Total RNA Extraction and Reverse Transcription

The fish was killed humanely, and the gills were dissected out and homogenised (10 mg) in TRI reagent (Sigma). Total RNA was extracted from the samples preserved in TRI® reagent following the manufacturer's instruction. The purity and quality of RNA was tested on 0.8 % agarose gel. Single stranded cDNA was synthesised from good quality RNA (RNAs having an absorbance ratio of $\geq$1.8 at A_{260}/A_{280}) using reverse transcription in a 20 µl reaction mixture containing 5 µg total RNA, 1× RT buffer, 2 mM dNTP, 2 mM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of M-MLV Reverse transcriptase (Fermentas, Inc.). The reaction was carried out at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min. The efficacy of the reverse transcription reaction was tested using primers (F-5′-GATCATGTTC GAGACCTTCAACAC-3′, R-5′-CGATGGTGATGACCT GTCCGTC-3′) for the control gene β-actin.

PCR Amplification and cDNA Cloning

The PCR amplification of hepcidin-like AMP sequence from cDNA of Moorish idol was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The primers HF (5′-CGAAGCAGTCAAACC CTC CTAAGATG-3′) and HR (5′-GAACCTGCAGCA GACACCACATCCG-3′) [26] were used for the amplification. The PCR condition involved an initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. The PCR products were analysed by electrophoresis in 1.5 % agarose gel in TBE buffer, stained with SYBR® Safe and visualised under UV light using the Gel Doc XR system and the quantity one programme (Bio-Rad).

The purified PCR products were ligated into the pTZ57R/T easy clone vector and transformed using competent *Escherichia coli* cells, JM107 as per manufacturer's protocols (InsTAclone™ PCR Cloning Kit, Fermentas Inc.). Transformed bacteria were cultured in Luria–Bertani agar plates containing ampicillin, IPTG and X-Gal at 37 °C for 24 h, and the recombinant clones with the insert were selected by blue–white screening. The white colonies were selected and patched on to fresh ampicillin containing plates and screened using vector specific primers, M13 F (5′-GTAAAACGACGGCCAG-3′) and M13 R (5′-CAG GAAACAGCTATGAC-3′) and sequence specific primers, HF and HR. For M13 primers, the thermal profile used was 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. The PCR products were purified and sequenced with an ABI Prism Sequencing kit (Big-Dye Terminator Cycle) at SciGenom, India.

Sequence Analysis and Molecular Characterisation

The sequences were analysed, trimmed and assembled using GeneTool software. The cDNA-based gene

sequences were translated using Expert Protein Analysis System (http://au.expasy.org/). Homology searches of nucleotide sequence as well as the deduced amino acid (aa) sequence were performed using BLASTn and BLASTp algorithm of the National Centre for Biotechnological Information (http://www.ncbi.nlm.nih.gov/blast). The processing sites of signal peptide and the propeptide convertase were identified using the ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/). Physico-chemical properties and peptide characteristics were analysed using ProtParam Tool (http://cn.expasy.org/cgi-bin/protparam) as well as antimicrobial peptide data base (APD) (http://aps.unmc.edu/AP/main.php). The secondary structure of the mature peptide was generated by SWISS-MODEL server using the crystal structure of hybrid white striped bass hepcidin (PDB ID: 1S6W) as template, and the pdb data generated was used to predict the spatial structure and bonding patterns of the mature peptide using the software, ViewerLite version 4.2.

The relevant preprohepcidin sequences of fishes as well as representatives from other vertebrate classes were retrieved from GenBank and multialigned using ClustalW and GeneDoc computer programmes. Phylogenetic analysis was carried out using MEGA version 5.05, and the phylogenetic tree was constructed by neighbour joining (NJ) method with complete deletion of gaps and subjected to 1,000 repetitions of bootstrap. A distance matrix was also constructed to analyse the evolutionary distance of *Z. cornutus* hepcidin isoform to previously reported hepcidins.

Results

A 267 bp fragment cDNA with a complete coding sequence of 81 aas (Fig. 1) was obtained from the gill mRNA transcripts of Moorish idol via RT-PCR. BLAST analysis of the nucleotide and deduced aa sequence revealed that the peptide belonged to hepcidin super family. The obtained nucleotide and deduced aa sequences were deposited in GenBank database (ID: JX163300). Similarity searches using BLAST algorithm indicated that the Moorish idol preprohepcidin showed 70 % similarity with hepcidin-like precursor of *Pagrus major* (AAS66305.1), 69 % similarity with hepcidin 2 (AAU00795.1) and 67 % similarity with hepcidin 4 (AAU00797.1) precursors of *Acanthopagrus schlegelii*. ProP 1.0 predicted the cleavage sites for signal peptidase and propeptide convertase positioned between Ala^{22} and Val^{23}; Arg^{57} and Ser^{58} after the motif RLKR, resulting in a signal peptide of 22 aa, prodomain of 35 aa and mature peptide of 24 aa with eight conserved cysteine residues (Fig. 2). Alignment of the 24 aa mature peptide, here after called as Zc-hepc1 with APD, revealed that its identity with AS-hepc2 (AP01696) as 83.33 %, Striped white bass hepcidin (AP00302) as 70.83 % and hepcidin TH1–5 (AP00808) as 62.5 %. Peptide characterisation using ProtParam and APD softwares indicated that the mature peptide, Zc-hepc1, showed highest theoretical isoelectric point (p*I*) (7.46) followed by preprohepcidin (4.89) and propeptide (4.38). Zc-hepc1 has a predicted molecular weight of 2.43 kDa, a net positive charge of +1 and an overall hydrophobic index of 58 %. Protean programme of DNASTAR Lasergene 10 Core Suite predicted a concentration of 244 mg/ml Zc-hepc1 for an absorbance of 1 OD at 280 nm, and 1 μg of the peptide would contain 410.5 pmol. The structural models created by ViewerLite 4.2 exhibit β-hairpin-like structure for Zc-hepc1 (Fig. 3), consisting of two antiparallel beta sheets strengthened by four disulphide bonds structured in the following pattern, Cys^{62}–Cys^{79}, Cys^{65}–Cys^{78}, Cys^{66}–Cys^{75} and Cys^{68}–Cys^{69}. The aas Lys^{63}, Phe^{64} and Cys^{65} constituted β1 sheet, while the aas Gly^{76}, Val^{77} and Cys^{78} framed β2 sheet. The phylogenetic tree constructed based on the alignment of *Z. cornutus* hepcidin with previously reported hepcidins composed of two main clusters (Fig. 4). Cluster 1 is framed entirely of fish hepcidins, whereas cluster two represents higher vertebrate hepcidins and form an out group to fishes. The main cluster, cluster 1, could be clearly demarcated into HAMP1 and HAMP2-like groups. The HAMP2-like cluster could again be subdivided into three small clusters, viz., (1) a

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Atgaagacattcagtgttgcagtggccgtcgtgctcacctttatttgccttcagcagagctctgct
 M  K  T  F  S  V  A  V  A  V  V  L  T  F  I  C  L  Q  Q  S  S  A
gtcccagtcactgaaggggaagatccagaggtgccaatggtggatgtatatgaagaggttccagtg
 V  P  V  T  E  G  E  D  P  E  V  P  M  V  D  V  Y  E  E  V  P  V
gagtcgtggaagatgccgtataacaacagacttaagcgcagtgctgctggctgtaaattttgctgc
 E  S  W  K  M  P  Y  N  N  R  L  K  R  S  A  A  G  C  K  F  C  C
ggttgctgtcctgacatgaacggatgtggtgtctgctgcaggttctga
 G  C  C  P  D  M  N  G  C  G  V  C  C  R  F  *
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Fig. 1 Nucleotide and deduced amino acid sequence of the hepcidin isoform from the gill mRNA transcripts of *Z. cornutus* (GenBank ID: **JX163300**). *Bold underlined* portion specifies the 22 amino acid signal peptide. The bioactive mature peptide is highlighted in *grey*, followed by the stop codon indicated with *asterisk*

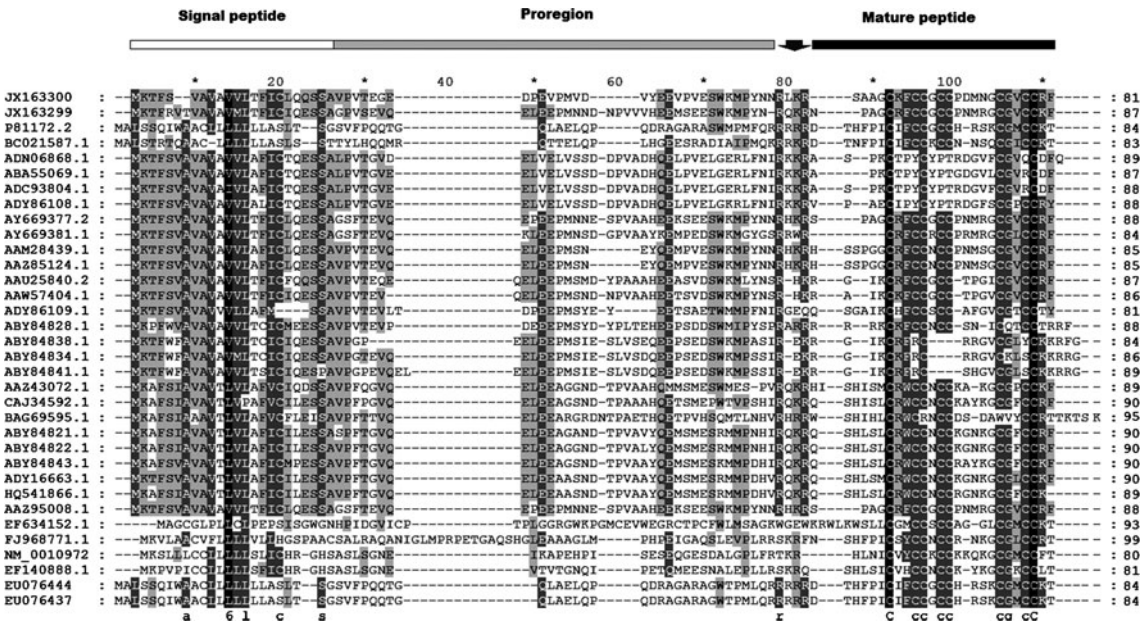

Fig. 2 ClustalW Multiple alignment of *Z. cornutus* hepcidin with other reported fish and non-fish vertebrate hepcidins created using GeneDoc version 2.7.0. The four level shading indicates different degrees of conservation. The signal peptide and proregion followed by mature peptide are indicated in the increasing order of colour intensity. The RXK/RR motif which is typical of the propeptide convertase is marked with *bold down arrow*

cluster of hepcidin isoforms from *Acanthopagrus schlegelli*, (2) a cluster of the genus *Epinephelus* and (3) a final cluster by Antarctic notothenioid fishes. According to the distance matrix calculated using MEGA 5.05, *Z. cornutus* hepcidin was more closely related to the hepcidin isoforms of *Morone chrysops* and *Dicentrarchus labrax* followed by the hepcidins of *Lateolabrax japonicus*, *A. schlegelli* and *Oreochromis niloticus*. Hepcidin of *Solea senegalensis, Paralichthys olivaceus and Psetta maxima* represents the most distant lineage of *Z. cornutus* hepcidin.

Discussion

Hepcidin is an acute-phase protein, endogenously synthesised as an inactive precursor and is known to participate in host defence of almost all vertebrates. It has been reported to exhibit microbicidal activity against gram positive (*Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis, Corynebacterium glutamicum*, *Streptococcus epidermidis, Bacillus cereus*) as well as gram negative bacteria (*Aeromonas hydrophila, Escherichia coli, Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio harveyi*) and fungi (*Aspergillus niger, Fusarium graminearum* and *F. solani*), and the activity could be detected down to micromolar levels (up to 1.5 μM) [27]. Hepcidin gene expression is regulated positively by proteins responsible for hereditary hemochromatosis and bone morphogenetic proteins (BMPs) through Sma and Mad-related family (SMAD) signalling pathway. There are also reports of an additional regulatory mechanism in fishes which is induced by inflammatory cytokines, IL(6) through JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) [28, 29]. Though the expression of hepcidin genes could be detected in all fish tissues, liver is presumed to be the major site of hepcidin synthesis [32].

The 81 aa preprohepcidin obtained from the gill mRNA transcripts of *Z. cornutus* showed the hallmarks of fish hepcidin with a more or less conserved leader signal peptide and a mature bioactive peptide preceded by a variable proregion owing to the execution of destined functions. A 22 aa signal peptide rich in hydrophobic aas valine and alanine preceded by a N terminal lysine [25] with typical signal peptidase cleavage site between Ala^{22} and Val^{23} was observed in *Z. cornutus*. The signal peptide of hepcidin from *Z. cornutus* was found to be similar to the signal peptide of most of the reported hepcidins, but differ from them in the total number of aas. It has a 22 aa signal peptide, whereas the other hepcidins reported from most of the fishes have 24 aas. Hepcidin reported from Cod also have a 22 aa signal peptide [30], but it differs greatly from *Z. cornutus* in aa composition.

Cleavage of the signal peptide would result in the formation of a prohepcidin which is exported to the lumen of endoplasmic reticulum. Here, the prohepcidin undergo a second cleavage probably by the action of furin and related propeptide convertases such as PC5, PC7 and PACE4 [31, 32], resulting in a mature bioactive peptide. These are

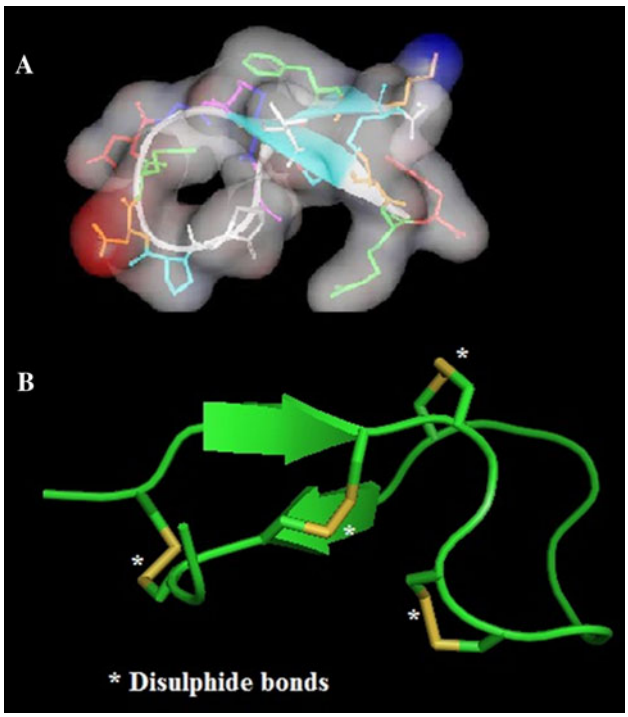

Fig. 3 Spatial structure of Zc-hepc1 created with the software ViewerLite version 4.2 using the pdb data generated by SWISS-MODEL server. The *crystal structure* of hybrid white striped bass hepcidin (PBD ID: 1S6W) was used as template for the data generation. The spatial structure (**a**) and the diagrammatic representation of the β-hairpin structure (**b**) are presented in figure. The disulphide bonds which stabilise β-hairpin are highlighted with *asterisks*

serine proteases which play crucial role in the maturation of prohepcidin in mammals [32]. The cleavage site in hepcidins is generally preceded by a conserved RXK/RR motif, and its presence was also observed in *Z. cornutus* hepcidin. Generally, the function of propeptide region is to assist the transit of AMP through the subcellular compartments and to protect the cellular mechanism of host against toxic effects of mature peptide [33]. The release of mature peptide from propeptide region of hepcidin is pretty fast, unlike other cysteine-rich AMPs such as defensins, indicating the efficiency of hepcidin in immune response [33].

Hepcidins contain a conserved core domain signature with 8 cysteine residues forming the disulphide backbone of β-hairpin [34], and the case was found to be same for Zc-hepc1. Hepcidins of varying size (20–31 aa) have been reported from several fishes irrespective of their evolutionary position and ecological niches [35, 36]. Zc-hepc1 differs from its most similar counterpart AS-hepc2 by 4 aas [36]. The aas Pro^{66}, Arg^{70}, Asn^{78} and Arg^{80} of the AS-hepc2 are replaced by the aas Ala^{59}, Lys^{63}, Asp^{71} and Asn^{73} in Zc-hepc1, resulting in a difference of 16.67 %. This diversity in the relatively conserved mature peptide region could be because of synonymous to non-synonymous substitution and positive Darwinian selection mediated adaptive evolution of hepcidins with respect to environmental cues [37, 38]. Such adaptive evolution of hepcidin could also be observed in various groups of Antarctic notothenioid fishes where a novel type of hepcidin isoform with only four cysteine residues could be observed. The evolution of hepcidins in Antarctic notothenioid fishes could be attributed to cold oxygen rich water and agrees with the hypothesis of environmental basis of hepcidin evolution [38].

The measure of cationicity is an important parameter in the determination of antimicrobial properties mediated by electrostatic attraction and subsequent cell lysis. Unlike previously reported hepcidins, the observed theoretical p*I* of Zc-hepc1 is far less from the average p*I* of fish hepcidins and comparatively less than that of the lowest recorded p*I* of 7.70 for turbot hepcidin 2 [39]. The replacement of neutrally charged Asn by negatively charged Asp and positively charged Arg by neutrally charged Asn could be the reason for the reduced theoretical p*I* and cationicity (7.46 and +1) of the Zc-hepc1. Difference in these physicochemical properties from reported hepcidins make Zc-hepc1 a unique hepcidin isoform with novel characters. Theoretical p*I* of Zc-hepc1 lies in the margin of acidic and basic natures. However, evidences approve participation of such hepcidin isoforms as in Atlantic salmon and turbot (p*I* 7.73 and 7.70, respectively) in pathogen-induced defence in teleost fishes [8, 39]. A similar up-regulation of hepcidin mRNA transcripts when compared to the control gene β-actin could be observed in *Z. cornutus* also. This up-regulated expression might be the result of some pathogenic invasion which is not visible externally. The secondary structure and bonding pattern (C^1–C^8, C^2–C^7, C^3–C^6 and C^4–C^5) of Zc-hepc1 were similar to the hepcidins reported from human, sea bass and black porgy [34, 36, 40]. The vicinal disulphide bonds formed at the hairpin turn could be the possible decisive domain for biological activity of the molecule [41]. Conservation of basic structure with modified physicochemical properties from lower vertebrates to higher vertebrates signifies the evolution of iron regulatory peptide from a protein ancestor whose general purpose was to participate in immune defence mechanism [42].

Phylogenetic analysis of the *Z. cornutus* hepcidin with previously reported vertebrate hepcidin discloses orthologous origin of fish and non-fish vertebrate hepcidins. Hepcidin gene evolution and diversification is more prominent in fishes [23] than any other vertebrates due to the varied ecological niches to which they adapt and highly dynamic and austere external environments. The subcluster formed by Antarctic fishes is a clear indication of environment-based convergent evolution of hepcidin genes. Two main lineages of HAMP gene, HAMP1 and HAMP2-like

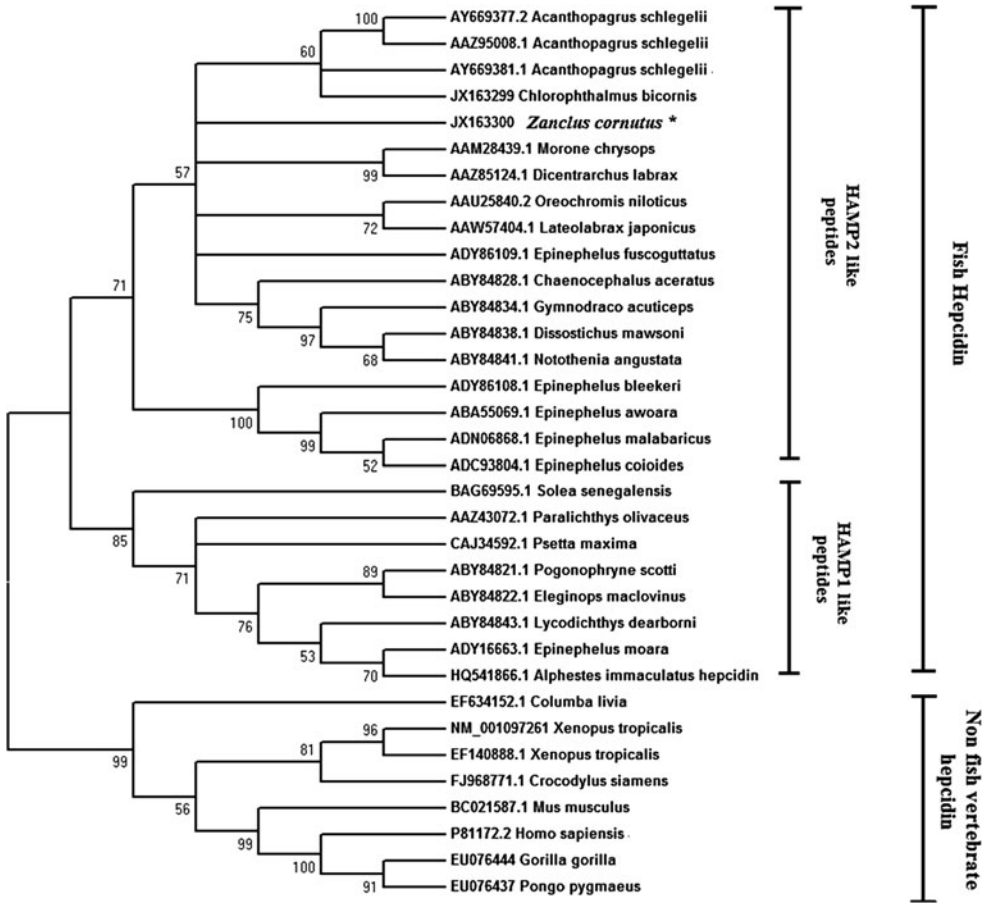

Fig. 4 A bootstrapped neighbour joining tree constructed using MEGA version 5.05 illustrating the phylogenetic relationship between *Z. cornutus* hepcidin and other reported hepcidin-like antimicrobial peptides of fishes and non-fish vertebrates. *Numbers* on the branch indicate the percentage of 1,000 bootstrap samples

group of peptides were observed in fishes [23], and it is clearly evident from the phylogenetic tree. The subdivisions of HAMP2-like group are either genus specific or environment-based evolution of hepcidin isoforms. Phylogenetic analysis confirmed the identity of *Z. cornutus* hepcidin as HAMP2-like lineage and is placed in a midway between two groups of perciform fishes, the sea bream and sea bass. This type of grouping may not necessarily be because of the direct evolutionary relationships of *Z. cornutus* with these fishes, but may be due to the absence of hepcidins that connect these groups together.

The hepcidin isoform of *Z. cornutus* is definitely a new subtype of HAMP2-like lineage. The exact biological function of the peptide is yet to be revealed. However, previous reports of up-regulation of low cationic hepcidin transcripts during pathogenic invasion suggest defensive role of these peptides in host immune responses [8, 39]. The HAMP2-like group is generally considered as AMPs and HAMP1-like group as an iron regulating hormone as well as antimicrobial in function. However, even without the iron regulatory motif, sea bass hepcidin transcripts were found to be up-regulated with iron overload [43]. Recently, Na^+ dependent active transport of hepcidin across the cell membrane has been reported, and it was the first report of an AMP that is being transported actively [44]. Though hepcidin is one of the most studied iron regulatory antimicrobial peptide, exact physiological function of all hepcidin isoforms are yet to be understood.

Conclusion

Hepcidin is a cysteine-rich antimicrobial peptide of multiple physiological functions. It has never been identified from coral fishes, and hence, the study provides first ever report of hepcidin isoform from a coral fish. The 81 aa preprohepcidin obtained from the gill mRNA transcripts of *Z. cornutus* could be considered as a new isoform of hepcidin belonging to HAMP2-like group of peptides. Since it is a novel isoform, the exact physiological function of the peptide is still a matter of ambiguity, but further characterisation of the Zc-hepc1 will throw light on the exact

physiological role of the peptide. Information on the immune system/responses of the fish will definitely help in adoption of prophylactic and therapeutic measures and improving the survival of fishes in culture environments. Moreover, this HAMP would definitely be a valuable addition to the field of medicine as well as aquaculture.

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Conflict of interest The authors declare that they have no conflict of interest.

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