Probiotics & Antimicro. Prot. (2013) 5:1–7
DOI 10.1007/s12602-012-9120-0

A Novel Isoform of the Hepatic Antimicrobial Peptide, Hepcidin (Hepc-CB1), from a Deep-Sea Fish, the Spinyjaw Greeneye *Chlorophthalmus bicornis* (Norman, 1939): Molecular Characterisation and Phylogeny

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Published online: 25 November 2012

Abstract Hepcidin is cysteine-rich short peptide of innate immune system of fishes, equipped to perform prevention and proliferation of invading pathogens like bacteria and viruses by limiting iron availability and activating intracellular cascades. Hepcidins are diverse in teleost fishes, due to the varied aquatic environments including exposure to pathogens, oxygenation and iron concentration. In the present study, we report a 87-amino acid (aa) preprohepcidin (Hepc-CB1) with a signal peptide of 24 aa, a prodomain of 39 aa and a bioactive mature peptide of 24 aa from the gill mRNA transcripts of the deep-sea fish spinyjaw greeneye, *Chlorophthalmus bicornis*. Molecular characterisation and phylogenetic analysis categorised the peptide to HAMP2-like group with a mature peptide of 2.53 kDa; a net positive charge (+3) and capacity to form β-hairpin-like structure configured by 8 conserved cysteines. The present work provides new insight into the mass gene duplication events and adaptive evolution of hepcidin isoforms with respect to environmental influences and positive Darwinian selection. This work reports a novel hepcidin isoform under the group HAMP2 from a non-acanthopterygian deep-sea fish, *C. bicornis*.

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Keywords Antimicrobial peptide · Spinyjaw · *Chlorophthalmus bicornis* · HAMP · Hepcidin

Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved key effectors of innate immunity produced by nearly all organisms, from bacteria to plants and animals including fishes which represent the transition form between species depending only on innate immunity and species highly rely upon adaptive immunity [1, 2]. Generally, they are cationic small peptides of size less than 10 kDa (15–45 aa) with hydrophilic as well as hydrophobic residues, which account for their specificity towards prokaryotic cell membrane, its disintegration and there by the death of the microorganisms [3, 4]. Since the discovery of the first AMP, cecropin from insects [5], more than thousands of AMPs have been reported [6]. Based on the three-dimensional structure, amino acid composition and mode of action, AMPs have been classified under several headings, in which cysteine-rich peptides stabilised by disulphide bonds formed the largest group [7], which include defensins, tachyplesins, protegrins and hepcidins [8].

The first report of hepcidin was from human blood ultrafiltrate [9] as liver-expressed antimicrobial peptide (LEAP-1). Later, it was isolated from human urine and was named as hepatic antimicrobial peptide (HAMP) [10]. Since then more than 68 hepcidin-like AMPs have been reported [11], bass hepcidin was the first isolated non-human vertebrate hepcidin from striped hybrid bass, *Morone chrysops* × *M. saxatilis* [12]. Multiple copies of hepcidin isoforms were identified from many fishes including, *Scophthalmus maximus* [13], *Acanthopagrus schlegelii* [14], *Lates*

calcarifer [15], *Alphestes immaculatus* [16], *Pagrus auriga* [17], *Pseudosciaena crocea* [18], *Sparus aurata* [19], *Dicentrarchus labrax* [20], *Paralichthys olivaceus* [21], *Gadus morhua* [8], *Oreochromis mossambicus* [22] and Antarctic notothenioid fishes [23]. Hepcidins are diverse in teleost fishes, due to the diversity of aquatic environments, that is, exposure to pathogens, oxygenation and iron concentration [23]. Apart from antimicrobial properties, hepcidin exhibits pivotal roles in immunomodulation and iron homeostasis [24, 25]. It also has anticancerous [26] and antiviral [27] properties. They are evolutionarily conserved with a capacity to mobilise shortly after infection and act rapidly to neutralise a broad range of microbes.

Though a number of hepcidins have been reported from various fishes, no HAMP2-like hepcidin sequences were detected from non-acanthopterygian fishes so far. In the present study, we report a novel HAMP2 peptide sequence from a non-acanthopterygian deep-sea fish spinyjaw greeneye (*Chlorophthalmus bicornis*, Chlorophthalmidae), its molecular characterisation and phylogenetic analysis.

Materials and Methods

Sample Collection

Live samples of *C. bicornis* were collected from a depth of 500 m from Andaman coast using a high-speed demersal trawl (HSDT) net operated on-board FORV *Sagar Sampada* (Ministry of Earth Sciences, Govt. of India) during Cruise No. 292. They were killed humanely, gills were carefully dissected out immediately after the death of the animal and preserved in 100 % methanol at the Biological Laboratory on-board the Research Vessel.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from the preserved tissue with TRI® Reagent (sigma) in accordance with the manufacturer's instructions. Purity and quality of the RNA was analysed on 0.8 % agarose gel, and the total purified RNA was quantified using spectrophotometer (A_{260}:A_{280}). Only those RNAs having an absorbance ratio greater than 1.8 were used for the present work. The first-strand cDNA synthesis was carried out by reverse transcription in a 20 µl reaction mixture containing 5 µg total RNA, 1× RT buffer, 2 mM dNTPs, 2 mM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of MMLV 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′-gatcatgttcgagaccttcaacac-3′, R: 5′-cgatggtgatgacctgtccgtc-3′) for the control gene β-actin.

PCR Amplification

The PCR amplification of cDNA from *C. bicornis* were performed in a 25 µl reaction volume containing 1× 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 F: 5′-cgaagcagtcaaaccctcctaagatg-3′, R: 5′-gaacctgcagcagacaccacatccg-3′ [28] 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, 68 °C for 30 s and a final extension at 68 °C for 10 min. The amplicons were analysed by electrophoresis in 1.5 % agarose gel in TBE buffer, stained with SYBR® safe and visualised under UV light. 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 sequence were performed using BLASTn and BLASTp algorithm of the National Centre for Biotechnological Information (http://www.ncbi.nlm.nih.gov/blast). Predeposited preprohepcidin sequences were retrieved from NCBI and multi-aligned using ClustalW and GeneDoc computer programmes. A rooted phylogenetic tree was constructed using MEGA 5.05 by neighbor-joining (NJ) method with complete deletion of gaps and subjected to 1,000 iterations of bootstrap. The cleavage site for the signal peptide was predicted using SignalP software (http://www.cbs.dtu.dk/services/SignalP), and the motif RX (K/R) R, typical of the propeptide convertase, was identified by MEGA 5.05 [17]. The physicochemical properties of preprohepcidin, its signal peptide, propeptide and the mature peptide were characterised separately using the ProtParam tool (http://cn.expasy.org/cgi-bin/protparam) as well as antimicrobial peptide database (APD) (http://aps.unmc.edu/AP/main.php) prediction. The structural modelling of the mature peptide region of Hepc-CB1 was carried out with the software ViewerLite 4.2, with the PDB data generated by SWISS-MODEL server using the crystal structure of hybrid white-striped bass hepcidin (PDB ID: 1S6W) as template.

Results

A 294-bp fragment cDNA having an ORF of 87 amino acids (Fig. 1) was obtained from the mRNA of gill tissue of

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atgaagactttcagggttacagtagcagtggccgtcatgctcacctttatttgtattcag
 M  K  T  F  R  V  T  V  A  V  A  V  M  L  T  F  I  C  I  Q
gagagctctgctggtccagtctctgaagtacaagagctggaggagccaatgaacaatgac
 E  S  S  A | G  P  V  S  E  V  Q  E  L  E  E  P  M  N  N  D
aatccagttgttgtgcatgaagagatgtcagaagaatcctggaagatgccgtataacaga
 N  P  V  V  V  H  E  E  M  S  E  E  S  W  K  M  P  Y  N  R
cagaagcgtaaccctgctggctgtaggttttgctgtggttgctgtcccaacatgcgcgga
 Q  K  R  N  P  A  G  C  R  F  C  C  G  C  C  P  N  M  R  G
Tgtggtgtctgctgcaagttctga
 C  G  V  C  C  K  F  *
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Fig. 1 Nucleotide and deduced amino acid sequences of HAMP2 isoform from the gill mRNA transcripts of *C. bicornis*. The cleavage site of 24-amino acid signal peptide is marked between Ala[24] and GLy[25] , and the region is showed as *underlined*. The mature active peptide is indicated in *bold letters* followed by stop codon which is denoted by an *asterisk*

C. bicornis by RT-PCR. BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptide belonged to hepcidin super family and will be referred as Hepc-CB1 here after. The obtained nucleotide sequences and deduced amino acid sequences were deposited in GenBank database (GenBank Accession number: JX163299). SignalP software predicted the cleavage site for signal peptidases between Ala[24] and Gly[25] resulting in a 24-amino acid signal peptide. Multiple alignments (Fig. 2) identified the motif RX (K/R) R, after which, is the cleavage site for propeptide convertase typical of hepcidin. This cleavage of the propeptide would result in a mature peptide of 24 amino acids and a prodomain of 39 amino acids. ScanProsite recognised a cysteine-rich region between amino acids 68 and 85, which forms the C-terminal region of mature peptide. The mature peptide of *C. bicornis* was found to be cationic with a net charge of +3, whereas the prepropeptide (−1) and propeptide (−5) were found to be anionic. The 24-amino acid mature hepcidin was found to have 2.53 kDa and a theoretical isoelectric point (pI) of 8.54 as predicted by ProtParam. APD predicts its hydrophobic potential as 54 %, which is contributed by amino acids Val (1) Phe (2) Cys (8) Met (1) and Ala (1). As per the similarity searches using BLAST algorithm, the 87-amino acid Hepc-CB1 showed 81 % similarity with hepcidin 2 precursor of *Acanthopagrus schlegelii* (AS-hepc2) (**AY669377.2**) and 80 % similarity with hepcidin-like precursor of *Pagrus major* (**AAS66305.1**). The 24-amino acid Hepc-CB1 mature peptide differs from the mature peptide of AS-hepc2 sequence only by two amino acids. The N-terminal amino acids Ser[65] and C-terminal Arg[87] of AS-hepc2 were replaced by the amino acids Asp[64] and Lys[86] in Hepc-CB1, respectively (http://aps.unmc.edu/AP/main.php). The structural model created by ViewerLite 4.2 exhibits β-hairpin-like structure framed of two anti-parallel beta sheets (Fig. 3) stabilised by four disulphide bonds formed in the following pattern, Cys[68]–Cys[85], Cys[71]–Cys[84], Cys[72]–Cys[81] and Cys[74]–Cys[75]. The β1 sheet composed of amino acids from Arg[69] to Cys[71], and β2 sheets composed of amino acids Gly[82] to Cys[84].

The phylogenetic tree constructed by neighbor-joining method shaped a tree having seven sub clusters within a main cluster and a small separate cluster (Fig. 4) formed by

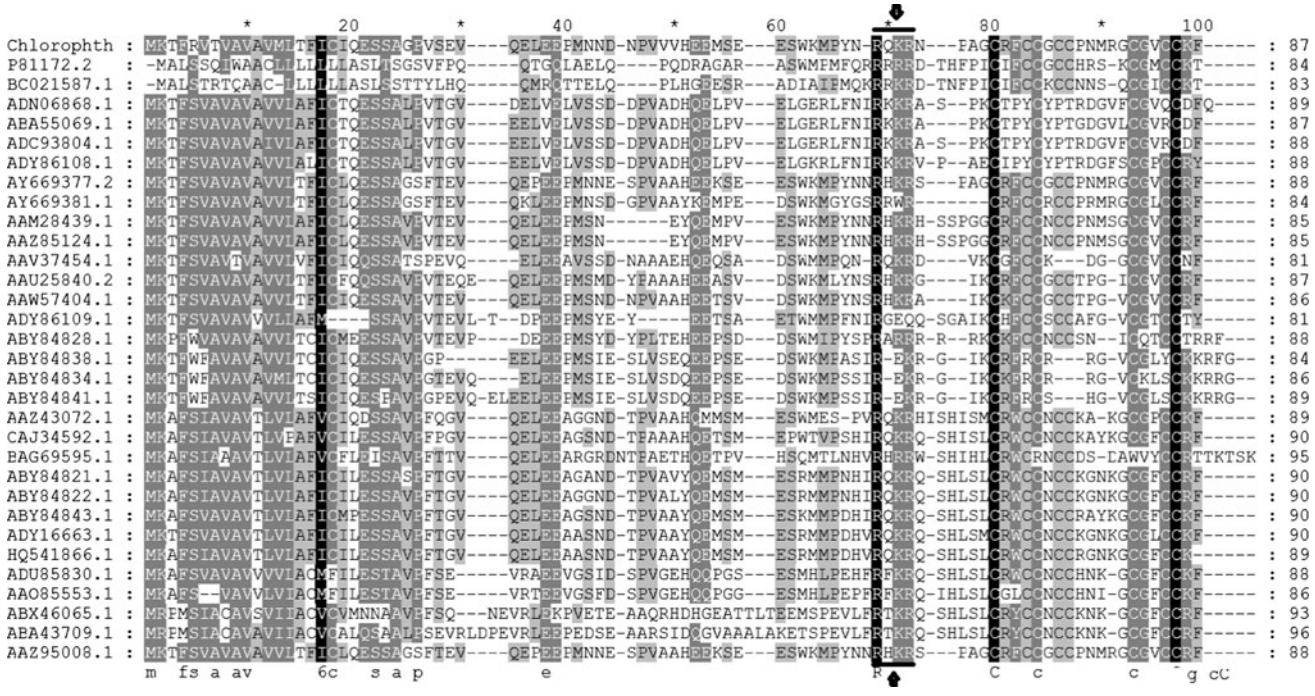

Fig. 2 ClustalW multiple alignment of amino acid sequences of *C. bicornis* with hepcidin-like antimicrobial peptide sequences of fishes and mammals obtained using GeneDoc programme version 2.7.0. The predicted cleavage site of propeptide convertase (where the RX (K/R) R motif ends) is marked with *arrows*

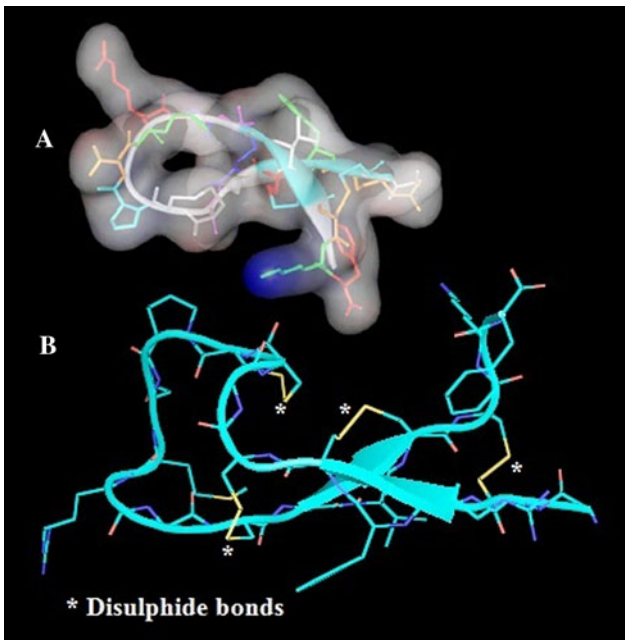

Fig. 3 Three-dimensional structure of the mature peptide of *C. bicornis* Hepc-CB1 was created with the software ViewerLite 4.2 using the PDB data generated by SWISS-MODEL server. The crystal structure of hybrid white-striped bass hepcidin (PDB ID: 1S6W) was used as template for the data generation. 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*

mammalian sequences. The main cluster could be subdivided into two groups, HAMP1 and HAMP2. The first group, HAMP1, composed of both acanthopterygian and non-acanthopterygian fishes, while only acanthopterygian fishes framed the second group HAMP2. As portrayed by the phylogenetic tree, the *C. bicornis* Hepc-CB1 belongs to HAMP2 class and closely related to antimicrobial peptides from perciform fish, *A. schlegelii*.

Discussion

Hepcidin is one of the most studied antimicrobial peptides from fishes [16, 29], mainly because of its physiological relevance as antimicrobial peptide and iron-regulating hormone [25]. Iron is an essential nutrient for bacterial growth, and its overload increases the susceptibility to intracellular and blood pathogens [30, 31]. In order to limit pathogenic invasion and its multiplication, host immune system has specific mechanisms of withholding iron from microbes. This includes the production of iron-binding proteins, reducing dietary iron assimilation and increasing hepatic production of haemoglobin and hemin scavengers [10]. Hepcidin can directly bind to iron-binding proteins like ferroprotein, which leads to its internalisation and degradation resulting in limited availability of iron for bacterial growth [15, 32]. The iron regulatory activity of hepcidin thereby further enhances its antimicrobial activity. Though the first report of hepcidin was from mammals, adaptive evolution of hepcidin gene is prominent in fishes, since mammals except mice possess a single copy of HAMP1 gene and fishes more than two copies of hepcidin genes. These multiples always fell in HAMP2 class, which were reported only in acanthopterygian fishes [11]. In repudiation with this statement, we report a novel 87 residue hepcidin prepropeptide from the mRNA transcripts of gill tissue of a non-acanthopterygian fish *C. bicornis*, belonging to the order Aulopiformes [33].

The antimicrobial peptide hepcidin is generally synthesised as a prepropeptide. Proteolytic cleavage of the signal peptide and prodomain from the prepropeptide yields the bioactive mature peptide [9, 34, 35]. Highly conserved cysteine residues in the mature peptide regions form the core domain signature, which serves as the disulphide-bridged back bone of the β-hairpin-like structures. The structural organisation and bonding pattern of Hepc-CB1 are similar to the earlier reported hepcidins of *A. schlegelli* [14], humans [36] and bass [37]. The predicted cleavage site of signal peptide between Ala^{24} and Gly^{25} of *C. bicornis* Hepc-CB1 is exactly similar to that of the cleavage sites of *A. schlegelli* hepc-2 and hepc-6 (Fig. 1). Net positive charge of +3, amphipathic nature (Hydrophobic index 54 %), a predicted molecular weight of 2.53 kDa and the formation of β-sheet-like structure by the C-terminal-conserved cysteines, in the light of available literature, confirm *C. bicornis* Hepc-CB1 as an antimicrobial peptide. The replacements of N-terminal polar hydrophilic serine of *A. schlegelli* hepc-2 by hydrophilic asparagine and the C-terminal positively charged arginine by similarly charged lysine in *C. bicornis* Hepc-CB1 do not make a prominent difference in the property of the peptide. However, further characterisation of the peptide with regard to its antimicrobial activity needs to be carried out with synthetic or recombinant peptide in order to ascertain the activity of the peptide. The reason for this high diversity of mature peptide region in fishes could be due to synonymous substitution of amino acids and positive Darwinian selection [38].

Phylogenetic analysis revealed the position of *C. bicornis* Hepc-CB1 to HAMP2 group. The N-terminal region of the mature peptide of HAMP1 significantly differs from that of the HAMP2 in having, either the mammalian motif (DTHFP) or fish motif (QSHLS), which is essential for ferroprotein internalisation. The prepropeptide, propeptide and mature peptide of HAMP1 is cationic. However, for HAMP2 fish sequence, though the mature peptide is cationic, the prepropeptide and propeptide are anionic [11, 15]. The

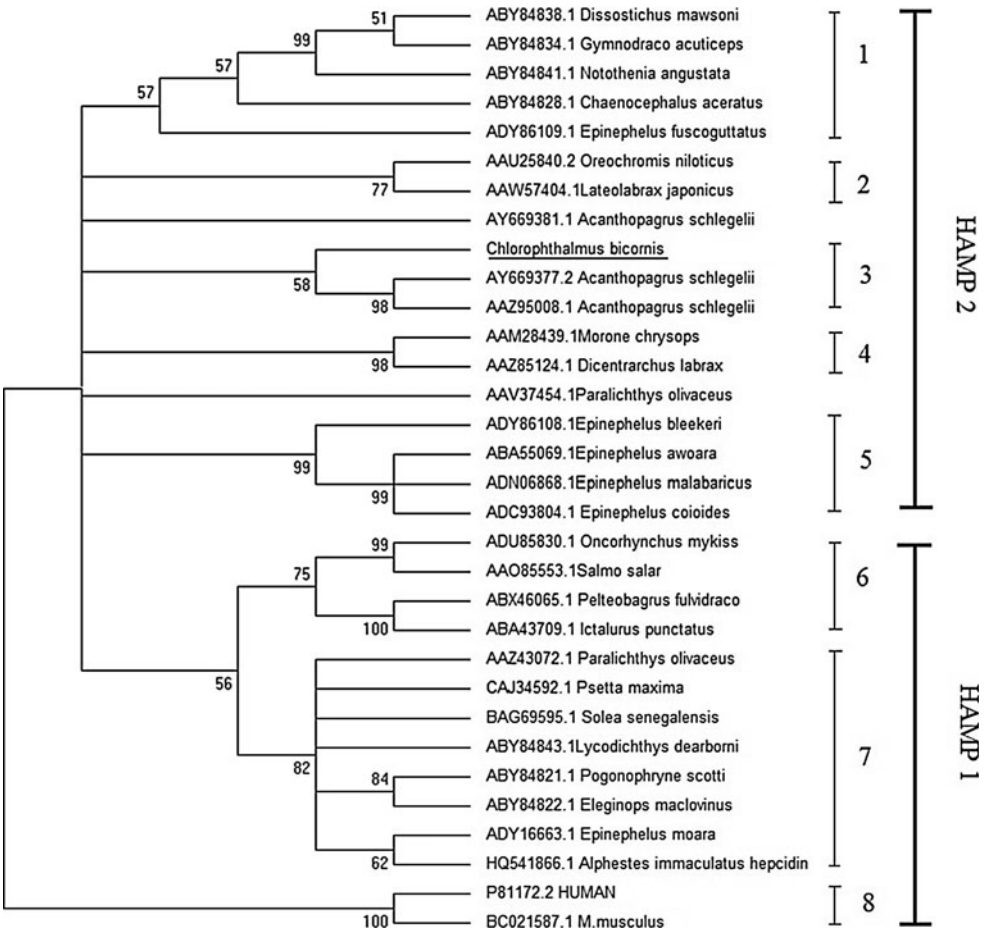

Fig. 4 A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating the phylogenetic relationship between *C. bicornis* hepcidin and other reported hepcidin-like antimicrobial peptides of fishes and mammals (out group). *Numbers on the branch* indicates the percentage of 1,000 bootstrap samples

absence of QSHLS/DTHFP motif in the N-terminal region of the mature peptide, anionic nature of prepropeptide and propeptide further confirmed the identity of *C. bicornis* as HAMP2. Duplication of HAMP genes and retention of both HAMP1- and HAMP2-like lineages in acanthopterygian fishes and Antarctic notothenioid fishes could be favoured by the radiation of teleosts to different marine and brackish water environments and positive Darwinian selection [17, 23, 38]. The extreme habitats of the deep sea might have produced fascinating evolutionary events [33] like gene duplication and conservation of HAMP2-like genes in non-acanthopterygian fishes also. More investigation is required in this context to get a clear picture of the molecular evolution and functional diversification of HAMP2-like genes in deep-sea fishes, which is living in an environment where heterotrophy dominates.

Conclusion

This is the first report of a novel HAMP2-like peptide from a non-acanthopterygian fish, which may provide useful information to the distribution of HAMP2-like genes in non-acanthopterygian fishes, its molecular evolution and phylogenetic relationships. High similarity of *C. bicornis* Hepc-CB1 with other hepcidins of proven antimicrobial activity and its physicochemical properties in agreement with those of traditional antimicrobial peptides strongly endorse it to be an antimicrobial peptide. Further studies on the antimicrobial activity of Hepc-CB1 as a synthetic or recombinant peptide would reveal the potentials of this new hepcidin isoform as a possible therapeutant in aquaculture/medicine.

Acknowledgments The authors are grateful to the Centre for Marine Living Resources and Ecology (CMLRE) and Ministry of Earth Sciences (MoES), Govt. of India, for the research grant (MoES/10-MLR/2/2007) and scientific/technical support for the work. Thanks are due to the crew of the research vessel *FORV Sagar Samapda* for their wholehearted support during the sample collection and Mr. Vinu Jacob, Research scholar, Department of Marine biology Microbiology and Biochemistry, CUSAT, Kochi-16, for the assistance in the taxonomic identification of the fish.

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