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Molecular cloning and antibacterial activity of hepcidin from Chinese rare minnow (*Gobiocypris rarus*)

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ABSTRACT

Background: Hepcidins, a kind of cysteine-rich antimicrobial peptides, play important roles in host immunological processes and iron regulation, which have been identified from several fish species. The rare minnow (*Gobiocypris rarus*), an endemic cyprinid fish in China, has been used extensively as model animal in laboratory. However, little is known about its hepcidin. Here, we report the cloning and characterization of a hepcidin gene from the liver of Chinese rare minnow.
Results: The full-length cDNA of rare minnow hepcidin is 662 bp, which contains an ORF of 273 bp encoding a prepropeptide of 90 amino acid residues. The predicted prepropeptide contains three domains: a signal peptide of 24 amino acids, a prodomain of 41 amino acids, and a mature peptide of 25 amino acids. Sequence alignment showed eight conserved cysteine residues in the mature peptide, which formed four disulfide bonds in spatial structure. The deduced structure of mature peptide showed a high degree of homology to the human hepcidin. Phylogenetic analysis showed that it had a close relationship with zebrafish hepcidin, and clustered in a clade with these from Cyprinidae. Synthetic peptide of rare minnow hepcidin could inhibit the growth of Gram positive bacterium *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli* and *Aeromonas hydrophila*.
Conclusion: These results suggested that rare minnow hepcidin had typical structure of hepcidins and antibacterial activity. It could participate in innate immune response as an antibacterial agent and be used as antibiotic substance.

1. Introduction

Antimicrobial peptides (AMPs) which are widely distributed in nature constitute important components of the host innate immune system [1]. AMPs play important roles in protecting host against various microbes including bacteria, fungi, protozoa, and viruses [2]. Hepcidin, a specific AMP, has been identified from mammals, amphibians, reptiles, birds, and fishes [3,4]. It plays an important role in iron homeostasis and innate immunity [5]. Mature hepcidins containing 20–25 amino acids compose of highly disulfide-bonded (cysteine-rich) β-sheets in secondary structure. Thus, conserved cysteines in hepcidin have functional roles in its spatial conformation [6].

In teleost, hepcidins have been identified from more than 20 species, such as hybrid striped bass [7], winter flounder, Atlantic salmon [6], Japanese flounder [8], red sea bream [9], tilapia [10], turbot [11], black porgy [12,13], large yellow croaker [14], medaka [15], orange-spotted grouper [16,17], mud loach [18], miiuy croaker [19], half-smooth tongue sole [20], blunt snout bream [21], common carp [22], blotched snakehead [23], convict cichlid [24], and so on. It was mainly synthesized in liver, although smaller amounts have been detected in other tissues such as the spleen, intestine, head kidney, and skin [9]. Purified hepcidin, synthetic hepcidin peptide, and recombinant hepcidin all have antibacterial activity against Gram-positive (G+) and Gram-negative (G-) bacteria [13,14,22]. It has been reported that medaka hepcidin can inhibit the growth of the G+ bacteria *Corynebacterium glutamicum*, *Staphylococcus aureus* and the G- bacteria *Escherichia coli*, *Aeromonas hydrophila*, and *Pseudomonas stutzeri* [25]. Synthetic hepcidin peptides of orange spotted grouper can delay the growth of G- bacterium *Vibrio vulnificus* and the G+ bacterium *S. aureus* [16]. Purified hepcidin peptide of large yellow croaker exhibited strong antibacterial activity against marine vibrios [26]. In addition, previous reports have shown that synthetic hepcidin peptides can inhibit virus replication [16,27]. All these indicated the important roles of hepcidin in fish innate immune system.

The rare minnow (*Gobiocypris rarus*), an endemic cyprinid fish in China, has been used extensively as model animal in research about toxicology, fish pathology, developmental biology,

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Table 1
GenBank accession numbers of hepcidins from different species used in this study.

Gene	Species	Accession number
Hepcidin 1 isoform 1	*D. rerio*	NP_991146.1
Hepcidin 1 isoform 2		NP_001276723.1
Hepcidin isoform 1	*Cyprinus carpio*	AFR23077.1
Hepcidin isoform 2		AFY23859.1
Hepcidin isoform 3		AGO64769.1
Hepcidin	*Misgurnus mizolepis*	AEM60424.1
Hepcidin	*Megalobrama amblycephala*	AFO84706.1
Hepcidin	*Schizothorax richardsonii*	AHB79194.1
Hepcidin isoform 1	*H. molitrix*	AGZ15358.1
Hepcidin isoform 2		AGT19511.1
Hepcidin	*Systomus sarana*	CAZ68137.1
Hepcidin	*Scophthalmus maximus*	AAX92670.1
Hepcidin 1	*Psetta maxima*	CAJ34592.1
Hepcidin isoform 1	*Hypophthalmichthys nobilis*	AHB64461.1
Hepcidin isoform 2		AHB64462.1
Hepcidin	*C. idella*	AEZ51835.1
Hepcidin 1	*Maylandia zebra*	XP_004551096.1
Hepcidin isoform 1	*Oreochromis niloticus*	XP_003450578.1
Hepcidin isoform 2		XP_003453536.1
Hepcidin 1	*Neolamprologus brichardi*	XP_006791782.1
Hepcidin	*Alphestes immaculatus*	AER00227.1
Hepcidin-1	*Micropterus salmoides*	ACD13023.1
Hepcidin-1	*Plecoglossus altivelis*	CBL59464.1
Hepcidin	*Larimichthys crocea*	ABC18307.1
Hepcidin-1	*Micropterus dolomieu*	ACD13025.1
Hepcidin-1 isoform 1	*Salmo salar*	NP_001134321.1
Hepcidin-1 isoform 2		ACI69335.1
Hepcidin	*Eleginops maclovinus*	ABY84822.1
Hepcidin-1	*Pagrus auriga*	BAH03285.1
Hepcidin isoform 2	*Epinephelus moara*	ADY16662.1
Hepcidin isoform 3		ADY16663.1
Hepcidin isoform 5		ADY16665.1
Hepcidin	*Pogonophryne scotti*	ABY84821.1
Hepcidin isoform 1	*Oncorhynchus mykiss*	CDQ60733.1
Hepcidin isoform 2		CDQ60734.1
Hepcidin isoform 3		ADU85830.1
Hepcidin isoform 1	*Notothenia angustata*	ABY84825.1
Hepcidin isoform 2		ABY84832.1
Hepcidin	*Takifugu rubripes*	XP_003965681.1
Hepcidin	*Morone chrysops*	AAM28440.1
Hepcidin	*Tor putitora*	AGM90578.1
Hepcidin isoform 1	*Paralichthys olivaceus*	BAE06233.1
Hepcidin isoform 2		BAE06235.1
Hepcidin isoform 3		AAT01563.1
Hepcidin	*C. semilaevis*	AFK93414.1
Hepcidin isoform 1	*I. punctatus*	NP_001188323.1
Hepcidin isoform 2		NP_001187130.1
Hepcidin isoform 1	*Oryzias melastigma*	AEG78327.1
Hepcidin isoform 2		ADM83600.1
Hepcidin	*Solea senegalensis*	BAG69595.1
Hepcidin isoform 1	*Miichthys miiuy*	AEK98541.1
Hepcidin isoform 2		AEK98542.1
Hepcidin isoform 3	*Lates calcarifer*	ADU87111.1
Hepcidin isoform 2		AEO51037.1
Hepcidin isoform 1		AEO51036.1
Hepcidin	*Poecilia formosa*	XP_007553878.1
Hepcidin	*Channa maculata*	AFN73128.1
Hepcidin	*Xiphophorus maculatus*	XP_005808614.1
Hepcidin 1	*Xenopus* (*Silurana*) *tropicalis*	NP_001090729.1
Hepcidin	*Chlorophthalmus bicornis*	AFQ32274.1
Hepcidin	*Crocodylus siamensis*	ADA68357.1
Hepcidin	*Amatitlania nigrofasciata*	AHF46363.1
Hepcidin isoform 1	*Gadus morhua*	ACA42769.1
Hepcidin isoform 2		ACA42770.1
Hepcidin isoform 1	*Lycodichthys dearborni*	ABY84842.1
Hepcidin isoform 2		ABY84843.1
Hepcidin	*Tachysurus fulvidraco*	ABX46065.1
Hepcidin isoform 1	*Monopterus albus*	ADK79123.1
Hepcidin isoform 2		ACU26539.1
Hepcidin-1	*Lepisosteus oculatus*	XP_006641712.1
Hepcidin	*Oryzias latipes*	XP_004078365.1
Hepcidin 2	*Astyanax mexicanus*	XP_007239985.1
Hepcidin	*Ictalurus furcatus*	AAX39714.1
Hepcidin	*Zanclus cornutus*	AFQ32275.1
Hepcidin	*Tarsius syrichta*	XP_008050131.1
Hepcidin-1	*Latimeria chalumnae*	XP_005995729.1
Hepcidin	*Homo sapiens*	P81172.2
Hepcidin	*Mus musculus*	NP_115930.1
Hepcidin	*Rare minnow*	

and genetics [28,29]. In this study, a hepcidin gene was cloned from the rare minnow. Antibacterial activity of its synthetic peptide was determined.

2. Materials and methods

2.1. Bacterial strains and fish

Rare minnow (*G. rarus*), with an average weight of 0.58 ± 0.06 g, were obtained from Institute of Hydrobiology, Chinese Academy of Sciences. They were cultured at 25 ± 1°C with 12 h/12 h dark/light cycle. *A. hydrophila* (strain GIM 1.172; Guangdong Microbiology Culture Center) was grown in nutrient agar at 30°C. *S. aureus* (ATCC6538) and *E. coli* (strain GIM1.571; Guangdong Microbiology Culture Center) were grown in LB medium at 37°C.

2.2. RNA extraction and cDNA cloning

For RNA extraction, liver tissue was collected from three individuals to provide enough RNA. Total RNA was extracted with Trizol reagent (Invitrogen) following the manufacturer's protocol.

For RT-PCR, primers (F: 5′-ACAGCAGRHDSARGATGAGCATCA-3′/R: 5′-TTTRCAGCART ATCCRCAGCCTTT-3′) were designed based on homologue sequences of other fish hepcidin genes. cDNA synthesis was performed using M-MLV Reverse Transcriptase (Promega) as described previously [30]. PCR products were purified and ligated into pMD18-T vector (TaKaRa). Three clones were sequenced to obtain the cDNA sequence.

5′-RACE and 3′-RACE with gene specific primers (5′GSP: 5′-AACT CTGGAGGTTGGTCTTCTCCCG-3′/3′GSP: 5′-GTTTAAAACGGGTATAAA TGCAGGC-3′) were performed to obtain the full length sequence of rare minnow hepcidin. SMART RACE cDNA Amplification Kit (Clontech, USA) was used in this step. The PCR used the following conditions: 5 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min; followed by 25 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min. PCR products were ligated into pMD18-T vector (TaKaRa, Japan) and sequenced.

2.3. Sequence analysis and model structure building

Amino acid sequences of hepcidin from other species were retrieved from the GenBank (NCBI). The accession numbers were collected in Table 1. Nucleotide sequences and deduced amino acid sequences were analyzed using the EditSeq program (DNASTAR, USA). Multiple sequence alignments were conducted using the Clustal × 1.83 program. Sequence identities were calculated using the Clusta W method in the MegAlign program. Neighbor-joining phylogenetic trees were constructed using the Poisson correction models with 1000 bootstrap replicates in MEGA 6.0 [31].

The structural model of rare minnow hepcidin was constructed by SWISS-MODEL [32]. The NMR structure of human hepcidin (PDB accession number: 2KEF) was used as template [33]. The molecular graphics system PyMOL was used to render figure.

2.4. Synthesis of hepcidin peptides

The putatively mature peptide of rare minnow hepcidin (QSHISLCRYCCKCCRNKGCGYCCK F) was chemically synthesized by Sangon Biotech (Shanghai, China). Crude peptides were extracted, lyophilized, and then purified by high-performance liquid chromatography (HPLC). The molecular mass and purity of the purified peptide were verified by mass spectroscopy. The molecular mass was 2903 Da. The purity was greater than 95%. Synthetic peptide was reconstituted in phosphate buffered saline (pH 7.4) for the experiments.

2.5. Antibacterial activity assay

Antibacterial activity assay was performed by microtitre broth dilution method [34]. Briefly, 50 μL of test bacteria strains was diluted to approximately 1 × 106 CFU/mL, and then treated with 50 μL of serial diluted peptides in 96-well microplates, while phosphate buffered saline (pH 7.4) was used as a control. The bacteria growth was determined by measuring the absorbance at 600 nm (OD600). OD600 values at initial and after incubation for 12 h at their optimal growth temperature were measured and recorded, respectively. OD600 was corrected by initial values. Survival rates were calculated as the cell density in the presence of hepcidin peptides to the cell density of control. Assay was performed three times. All data are presented as mean ± S.D.

3. Results

3.1. Molecular characterization

The determined cDNA sequence of rare minnow hepcidin consisted of 662 nucleotides (nt) (Fig. 1). It contains a 5′ untranslated region (UTR) of 28 nt, a complete open reading frame (ORF) of 273 nt, and a 3′ UTR of 361 nt. The deduced protein was composed of 90 amino acids which was preprohepcidin. Complete cDNA and deduced amino acid sequence have been deposited into GenBank under accession number KP054296.

Amino acid sequence analysis showed that rare minnow hepcidin consisted of a signal peptide, a propeptide, and a mature peptide. The first 24 amino acids formed its signal peptide with a putative cleavage site between Ala24 and Val25. The last 25 amino acids (positions 66–90) formed its mature peptide. The motif "59GFFRTKR65" behind the mature peptide was predicted to be the cleavage site of furin-like endoproteases. A putative ATCUN motif was found in the N-terminal of the mature peptide (66QSH68, Fig. 1).

3.2. Sequence alignment and phylogenetic analysis

Sequence alignment showed that hepcidins from different species shared high similarities in C-terminal regions (amino acids of the mature peptide). In the mature peptide, eight cysteine residues and a glycine residue were found in all aligned sequences (Fig. 2). The deduced amino acid sequence of rare minnow hepcidin had the highest similarity (82.2%) with silver carp (*Hypophthalmichthys molitrix*) and grass carp (*Ctenopharyngodon idella*), and the lowest similarity (21.7%) with human (*Homo sapiens*). Diversities were mainly acquired from the signal peptide and propeptide regions. The mature peptides, especially these from teleosts, were highly conserved.

A phylogenetic tree was constructed based on amino acid sequence of hepcidins from teleosts and other species (Fig. 3). The rare minnow hepcidin was in a branch position with that from *Danio rerio*. Hepcidins from fish species of family Cyprinidae were clustered in a clade. Almost all of the hepcidins were clustered based on its species classification, except that from Sciaenidae, Cichlidae, Latidae, and Nototheniidae.

3.3. Model structure

The 3-D structure of rare minnow hepcidin was predicted by the SWISS-MODEL program using the human hepcidin structure (PDB: 2KEF) as template. Results showed that four disulfide bridges were formed among the eight conserved cysteines (Fig. 4a). On the whole, the peptide appeared a distorted β-sheet shape with a hairpin loop. Fig. 4a also showed that the β-hairpin structure curled with a turn, which created a convex and concave surface on each side of the β-sheet (Fig. 4b).

3.4. Antibacterial activity

The antibacterial activity of the synthetic peptide against *E. coli*, *A. hydrophila*, and *S. aureus* was determined by measuring their growth delay. As shown in Fig. 5, growth of the three bacteria strains was all delayed by this peptide in a dose dependent manner.

4. Discussion

In this study, a hepcidin gene was cloned and identified from rare minnow. Deduced amino acid sequence of the rare minnow hepcidin contains 90 amino acids (aa), including a 25-aa C-terminal mature peptide. BLAST analysis showed that the full length of hepcidins differed in different fish species. For example, *Tor putitora*

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AGAAGACCAACCTCCAGAGTTACAGAAG ATG AAG TGC GCA CAC GTG GCT CTC ACC GCT GCA GTC GTC ATC  70
                              M   K   C   A   H   V   A   L   T   A   A   V   V   I   14
GCA TGC GTC TGC ATC CTC CAG GCC GCA GCC GTT CCC TTC CCA CAG GAG CAG GAT GAG CAT CAT  133
 A   C   V   C   I   L   Q   A   A   A   V   P   F   P   Q   E   Q   D   E   H   H   35
GTG GAG AGG GAA ACA CAC CAG GAG AAC GAG CAC TTC ACA GAA ACC GCA CAG GAA CAA ACA AAC  196
 V   E   R   E   T   H   Q   E   N   E   H   F   T   E   T   A   Q   E   Q   T   N   56
CCT CTG GGA TTT TTC AGG ACA AAA CGT CAA AGC CAC ATT TCC CTG TGC AGA TAC TGC TGC AAG  259
 P   L   G   F   F   R   T   K   R   Q   S   H   I   S   L   C   R   Y   C   C   K   77
TGC TGT CGC AAC AAA GGC TGT GGA TAT TGC TGT AAA TTC TGA TCCCAATGGAGTGCGCTTGGGAAAAGAG 329
 C   C   R   N   K   G   C   G   Y   C   C   K   F   -                               90
TTTCTTAGAAACATTAACTTATTTGGAATTTTGTCTTCAGAATCCCTGCCGGAATGATTTTCCCTGTCGCACCATGTTGTTTAA 413
AACGGGTATAAATGCAGGCTGTGTGTTCATGCATTCTGCTTTGTAAAAGTTGCAGCTACTGAAATCTAACTGACAATATGAGGT 497
GAACTCACTCTGTTATGACTTTAAATATTTTATACATGTATATATATATTTTATGCCTGTGTACATAGACATGTTAGACCAGTG 581
AATGTGTAGGATAATGAAACAACATTTTTAATAAAATGATTTTGAAATGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   662
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Fig. 1. Nucleotide and deduced amino acid sequences of rare minnow hepcidin. The deduced amino acid sequence is reported in one-letter code. The signal peptide is shown in gray shade. The motif "GFFRTKR" that contains cleavage site is indicated by underline. The putative ATCUN motif (QSH) is indicated by black box.

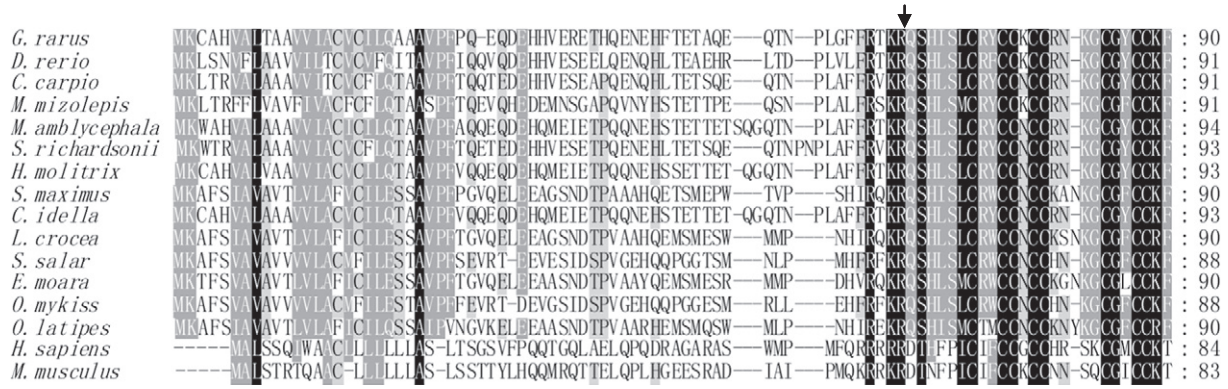

Fig. 2. Multiple amino acid sequence alignment of hepcidins. The first amino acid of mature peptide was indicated by an arrow. The highly conserved residues in all sequences are indicated over a black background. GenBank accession numbers of the sequences used in this figure were collected in Table 1.

possesses a hepcidin of 78 aa, while *Cynoglossus semilaevis* and *Ictalurus punctatus* have a hepcidin of 96 aa [3,20,35]. However, the mature peptide of fish hepcidins was highly conserved (Fig. 2), which indicated their conserved functions in fish species.

Sequence analysis and alignment showed that a putative ATCUN motif was located in rare minnow hepcidin (66QSH68, the N-terminal of the mature peptide) and was conserved in fish hepcidins. Recently, this motif was proved in trout hepcidin [4].

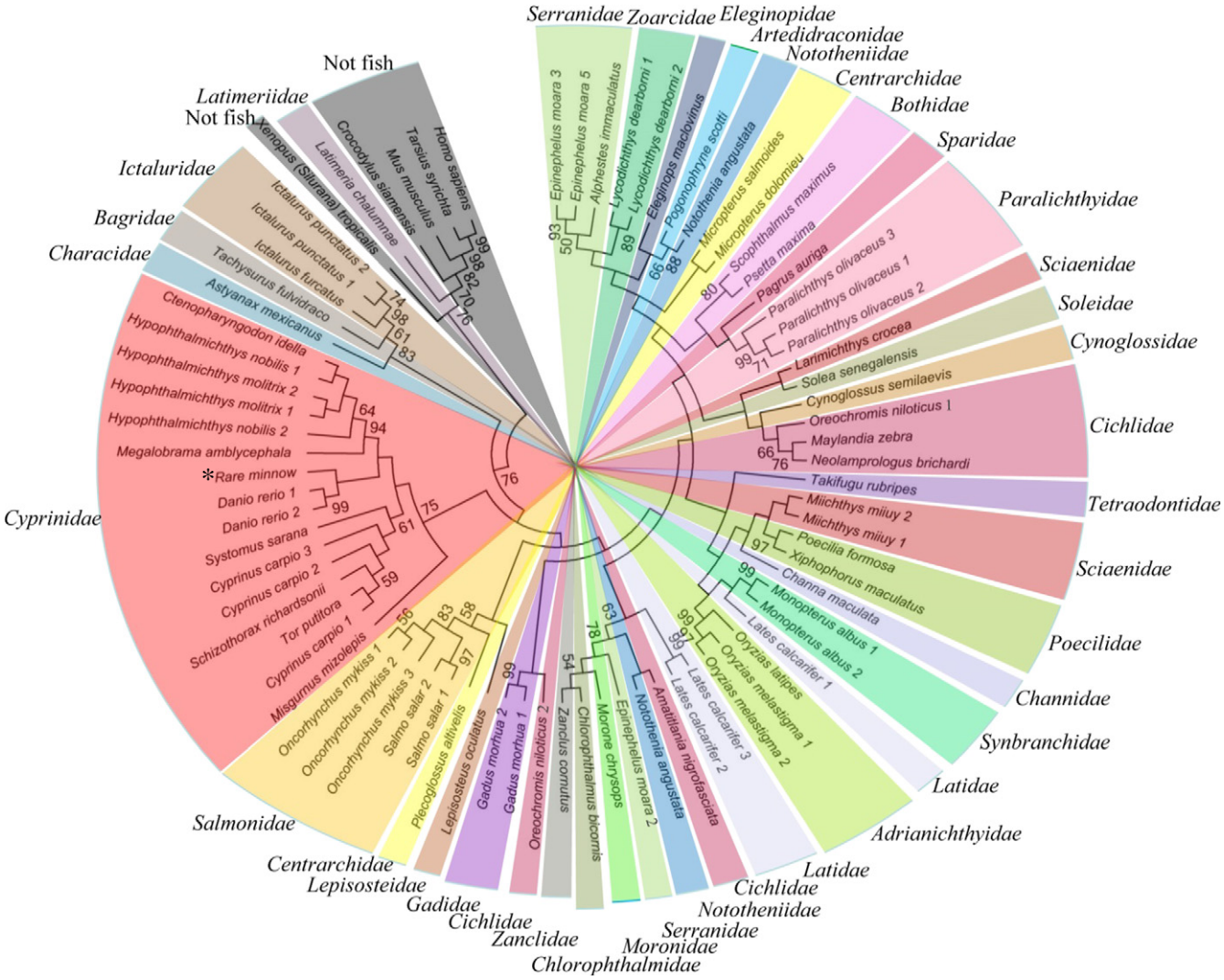

Fig. 3. Phylogenetic analysis of hepcidins from teleost and other vertebrates. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 6.0. The numbers given are frequencies (%) at which a given branch appeared in 1000 bootstrap replications. The hepcidin of rare minnow was indicated with asterisk. Organisms were marked with different colors based on its family name. The GenBank accession numbers were collected in Table 1.

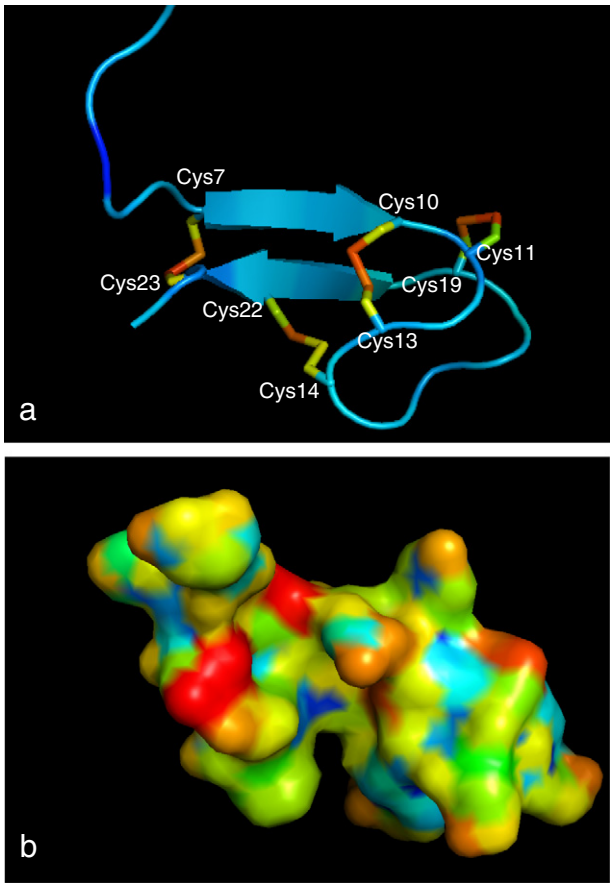

Fig. 4. Model structure of the rare minnow hepcidin. (a) Ribbon model structure of rare minnow hepcidin was constructed using the human hepcidin solution NMR revisited structure (PDB: 2KEF) as template by SWISS-MODEL program tool, and then rendering with PyMol program. Disulfide bridges between the conserved cysteine residues were colored in yellow and red. (b) The surface of the predicted structure displayed by PyMol program.

It has been showed that trout hepcidin with this motif could induce DNA hydrolysis in the presence of Cu2 +. Sequence alignment also showed that eight conserved cysteines existed in all analyzed fish

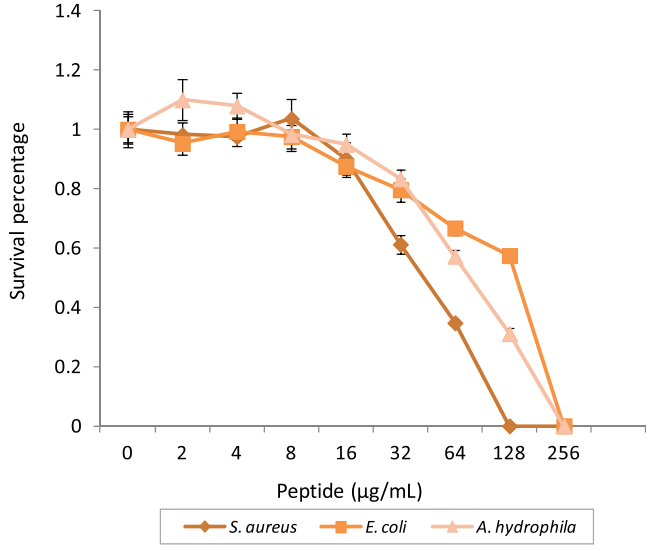

Fig. 5. Antimicrobial activity of rare minnow hepcidin. Three different bacterial strains (*S. aureus*, *E. coli*, and *A. hydrophila*) were used in this assay. The error bars represent the standard deviation from three independent experiments.

hepcidins. The conserved cysteines participate in the formation of disulfide bonds, which are involved in the conformation of the characteristic antiparallel β-sheet in human hepcidin [36]. However, hepcidins with six or four cysteines are also found in teleost [37]. To further analyze the cysteines and spatial structure of rare minnow hepcidin, we constructed its three dimensional structure using the SWISS-MODEL program. As shown in Fig. 4, four disulfide bridges formed between these eight conserved cysteines, which were consistent with the human hepcidin. The model structure of rare minnow hepcidin has the same characteristics of human hepcidin, such as the hairpin structure in distorted β-sheets, the two antiparallel strands, and the convex and concave surfaces.

Phylogenetic analysis showed that rare minnow hepcidin was clustered in a clade with that from fish species of Cyprinidae. It is in line with the taxonomic status of the rare minnow, which belongs to the family Cyprinidae. There were also some exceptions in the phylogenetic tree. For example, hepcidins from species of Sciaenidae, Cichlidae, Latidae, and Nototheniidae were not clustered in a clade (Fig. 3). It indicated the diversity of hepcidins in fish species. More than one kind of hepcidin existed in a species. Two types have been identified in Japanese flounder [8] and three have been found in tilapia [10]. Further researches have revealed that there are differences among different types of hepcidins in a fish, such as in tissue distribution and antibacterial activity. For fish species of Cyprinidae, information about the types of hepcidins is little. Whether other types of hepcidins exist in the rare minnow needs further research.

Fish hepcidins can inhibit bacteria, fungi, and virus. In this study, the synthetic peptide of rare minnow hepcidin could delay the growth of G+ bacterium *S. aureus* and G- bacteria *E. coli* and *A. hydrophila*, which is consistent with the synthetic hepcidin from marine fish (*Pseudosciaena crocea*). It has antimicrobial activity against several bacteria, such as *A. hydrophila*, *E. coli*, *Vibrio prahaemoloyticus*, *S. aureus*, *Micrococcus luteus*, and *Bacillus subtilis* [14]. It has been demonstrated that the disulfide bridges were essential for antibacterial activity of human hepcidin [38]. However, two types of trout hepcidin (reduced and oxidized) all could inhibit the growth of *E. coli* and *Piscirickettsia salmonis*, and the oxidized peptide is more effective than the one reduced [4]. It seems that there are differences between human hepcidin and fish hepcidin. In our study, significant antimicrobial activity needed a high peptide concentration. The antimicrobial activity of reduced and oxidized rare minnow hepcidin needs further research. In addition, it seems like the synthetic peptide of rare minnow hepcidin was more effective against the G+ bacterium. This phenomenon was also observed in other fish hepcidins. For example, the minimal inhibitory concentration of synthetic hepcidin from *P. crocea* is 3–6 μM to S. aureus and 12–24 μM to *E. coli* [14]. Purified hepcidin from large yellow croaker is more effective against *S. aureus* (area of inhibition was 47.12 mm^2) than *E. coli* (area of inhibition was 34.56 mm2) [26]. These could be resulted from the divergence of hepcidins and bacteria clones. Trout hepcidin could translocate across the bacteria cell membrane to exert its antimicrobial activity [4]. The mechanism for the antibacterial activity of rare minnow hepcidin needs further research.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] Hancock R, Brown KL, Mookherjee N. Host defense peptides from invertebrates — emerging antimicrobial strategies. Immunobiology 2006;211:315–22. http://dx.doi.org/10.1016/j.imbio.2005.10.017.

[2] Zhang G, Sunkara LT. Avian antimicrobial host defense peptides: From biology to therapeutic applications. Pharmaceuticals 2014;7:220–47. http://dx.doi.org/10.3390/ph7030220.

[3] Shi J, Camus AC. Hepcidins in amphibians and fishes: Antimicrobial peptides or iron-regulatory hormones? Dev Comp Immunol 2006;30:746–55. http://dx.doi.org/10.1016/j.dci.2005.10.009.

[4] Álvarez CA, Guzmán F, Cárdenas C, Marshall SH, Mercado L. Antimicrobial activity of trout hepcidin. Fish Shellfish Immunol 2014;41:93–101. http://dx.doi.org/10.1016/j.fsi.2014.04.013.

[5] Falzacappa MVV, Muckenthaler MU. Hepcidin: Iron-hormone and antimicrobial peptide. Gene 2005;364:37–44. http://dx.doi.org/10.1016/j.gene.2005.07.020.

[6] Douglas SE, Gallant JW, Liebscher RS, Dacanay A, Tsoi SCM. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev Comp Immunol 2003;27:589–601. http://dx.doi.org/10.1016/S0145-305X(03)00036-3.

[7] Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, et al. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur J Biochem 2002;269:2232–7. http://dx.doi.org/10.1046/j.1432-1033.2002.02881.x.

[8] Hirono I, Hwang JY, Ono Y, Kurobe T, Ohira T, Nozaki R, et al. Two different types of hepcidins from the Japanese flounder *Paralichthys olivaceus*. FEBS J 2005;272: 5257–64. http://dx.doi.org/10.1111/j.1742-4658.2005.04922.x.

[9] Chen SL, Xu MY, Ji XS, Yu GC, Liu Y. Cloning, characterization, and expression analysis of hepcidin gene from red sea bream (*Chrysophrys major*). Antimicrob Agents Chemother 2005;49:1608–12. http://dx.doi.org/10.1128/AAC.49.4.1608-1612.2005.

[10] Huang PH, Chen JY, Kuo CM. Three different hepcidins from tilapia, *Oreochromis mossambicus*: Analysis of their expressions and biological functions. Mol Immunol 2007;44:1922–34. http://dx.doi.org/10.1016/j.molimm.2006.09.031.

[11] Chen SL, Li W, Meng L, Sha ZX, Wang ZJ, Ren GC. Molecular cloning and expression analysis of a hepcidin antimicrobial peptide gene from turbot (*Scophthalmus maximus*). Fish Shellfish Immunol 2007;22:172–81. http://dx.doi.org/10.1016/j.fsi.2006.04.004.

[12] Yang M, Wang KJ, Chen JH, Qu HD, Li SJ. Genomic organization and tissue specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B). Fish Shellfish Immunol 2007;23:1060–71. http://dx.doi.org/10.1016/j.fsi.2007.04.011.

[13] Yang M, Chen B, Cai JJ, Peng H, Cai L, Yuan JJ, et al. Molecular characterization of hepcidin AS-hepc2 and AS-hepc6 in black porgy (*Acanthopagrus schlegelii*): Expression pattern responded to bacterial challenge and *in vitro* antimicrobial activity. Comp Biochem Physiol B Biochem Mol Biol 2011;158:155–63. http://dx.doi.org/10.1016/j.cbpb.2010.11.003.

[14] Wang KJ, Cai JJ, Cai L, Qu HD, Yang M, Zhang M. Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide. Peptides 2009;30:638–46. http://dx.doi.org/10.1016/j.peptides.2008.12.014.

[15] Bo J, Cai L, Xu JH, Wang KJ, Au DWT. The marine medaka *Oryzias melastigma* — a potential marine fish model for innate immune study. Mar Pollut Bull 2011;63: 267–76. http://dx.doi.org/10.1016/j.marpolbul.2011.05.014.

[16] Zhou JG, Wei JG, Xu D, Cui HC, Yan Y, Ou-Yang ZL, et al. Molecular cloning and characterization of two novel hepcidins from orange-spotted grouper, *Epinephelus coioides*. Fish Shellfish Immunol 2011;30:559–68. http://dx.doi.org/10.1016/j.fsi.2010.11.021.

[17] Qu H, Chen B, Peng H, Wang K. Molecular cloning, recombinant expression, and antimicrobial activity of EC-hepcidin3, a new four-cysteine hepcidin isoform from *Epinephelus coioides*. Biosci Biotechnol Biochem 2013;77:103–10. http://dx.doi.org/10.1271/bbb.120600.

[18] Nam YK, Cho YS, Lee SY, Kim BS, Kim DS. Molecular characterization of hepcidin gene from mud loach (*Misgurnus mizolepis*; Cypriniformes). Fish Shellfish Immunol 2011;31:1251–8. http://dx.doi.org/10.1016/j.fsi.2011.09.007.

[19] Xu T, Sun Y, Shi G, Wang R. Miiuy croaker hepcidin gene and comparative analyses reveal evidence for positive selection. PLoS ONE 2012;7:e35449. http://dx.doi.org/10.1371/journal.pone.0035449.

[20] Wang Y, Liu X, Ma L, Yu Y, Yu H, Mohammed S, et al. Identification and characterization of a hepcidin from half-smooth tongue sole *Cynoglossus semilaevis*. Fish Shellfish Immunol 2012;33:213–9. http://dx.doi.org/10.1016/j.fsi.2012.04.011.

[21] Liang T, Ji W, Zhang GR, Wei KJ, Feng K, Wang WM, et al. Molecular cloning and expression analysis of liver-expressed antimicrobial peptide 1 (LEAP-1) and LEAP-2 genes in the blunt snout bream (*Megalobrama amblycephala*). Fish Shellfish Immunol 2013;35:553–63. http://dx.doi.org/10.1016/j.fsi.2013.05.021.

[22] Li H, Zhang F, Guo H, Zhu Y, Yuan J, Yang G, et al. Molecular characterization of hepcidin gene in common carp (*Cyprinus carpio* L.) and its expression pattern responding to bacterial challenge. Fish Shellfish Immunol 2013;35:1030–8. http://dx.doi.org/10.1016/j.fsi.2013.07.001.

[23] Gong LC, Wang H, Deng L. Molecular characterization, phylogeny and expression of a hepcidin gene in the blotched snakehead *Channa maculata*. Dev Comp Immunol 2014;44:1–11. http://dx.doi.org/10.1016/j.dci.2013.11.007.

[24] Chi JR, Liao LS, Wang RG, Jhu CS, Wu JL, Hu SY. Molecular cloning and functional characterization of the hepcidin gene from the convict cichlid (*Amatitlania nigrofasciata*) and its expression pattern in response to lipopolysaccharide challenge. Fish Physiol Biochem 2014;41:449–61. http://dx.doi.org/10.1007/s10695-014-9996-6.

[25] Cai L, Cai JJ, Liu HP, Fan DQ, Peng H, Wang KJ. Recombinant medaka (*Oryzias melastigmus*) pro-hepcidin: Multifunctional characterization. Comp Biochem Physiol B Biochem Mol Biol 2012;161:140–7. http://dx.doi.org/10.1016/j.cbpb.2011.10.006.

[26] Zhang J, Yan Q, Ji R, Zou W, Guo G. Isolation and characterization of a hepcidin peptide from the head kidney of large yellow croaker, *Pseudosciaena crocea*. Fish Shellfish Immunol 2009;26:864–70. http://dx.doi.org/10.1016/j.fsi.2009.03.014.

[27] Wang YD, Kung CW, Chen JY. Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1–5 against nervous necrosis virus in medaka. Peptides 2010;31:1026–33. http://dx.doi.org/10.1016/j.peptides.2010.02.025.

[28] He Y, Li R, Wang J, Blanchet S, Lek S. Morphological variation among wild populations of Chinese rare minnow (*Gobiocypris rarus*): Deciphering the role of evolutionary processes. Zool Sci 2013;30:475–83. http://dx.doi.org/10.2108/zsj.30.475.

[29] Liu Y, Yuan C, Chen S, Zheng Y, Zhang Y, Gao J, et al. Global and *cyp19a1a* gene specific DNA methylation in gonads of adult rare minnow *Gobiocypris rarus* under bisphenol A exposure. Aquat Toxicol 2014;156:10–6. http://dx.doi.org/10.1016/j.aquatox.2014.07.017.

[30] Jin JY, Zhou L, Wang Y, Li Z, Zhao JG, Zhang QY, et al. Antibacterial and antiviral roles of a fish β-defensin expressed both in pituitary and testis. PLoS ONE 2010;5:e12883. http://dx.doi.org/10.1371/journal.pone.0012883.

[31] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9. http://dx.doi.org/10.1093/molbev/mst197.

[32] Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, et al. SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 2014;42:252–8. http://dx.doi.org/10.1093/nar/gku340.

[33] Jordan JB, Poppe L, Haniu M, Arvedson T, Syed R, Li V, et al. Hepcidin revisited, disulfide connectivity, dynamics, and structure. J Biol Chem 2009;284:24155–67. http://dx.doi.org/10.1074/jbc.M109.017764.

[34] Ericksen B, Wu Z, Lu W, Lehrer RI. Antibacterial activity and specificity of the six human α-defensins. Antimicrob Agents Chemother 2005;49:269–75. http://dx.doi.org/10.1128/AAC.49.1.269-275.2005.

[35] Hu X, Camus AC, Aono S, Morrison EE, Dennis J, Nusbaum KE, et al. Channel catfish hepcidin expression in infection and anemia. Comp Immunol Microbiol Infect Dis 2007;30:55–69. http://dx.doi.org/10.1016/j.cimid.2006.10.004.

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

[37] Xu Q, Cheng CHC, Hu P, Ye H, Chen Z, Cao L. Adaptive evolution of hepcidin genes in antarctic notothenioid fishes. Mol Biol Evol 2008;25:1099–112. http://dx.doi.org/10.1093/molbev/msn056.

[38] Hocquellet A, Senechal C, Garbay B. Importance of the disulfide bridges in the antibacterial activity of human hepcidin. Peptides 2012;36:303–7. http://dx.doi.org/10.1016/j.peptides.2012.06.001.