

Cloning and organisation analysis of a hepcidin-like gene and cDNA from Japan sea bass, *Lateolabrax japonicus*

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Abstract

A hepcidin gene was amplified from the liver of *Lateolabrax japonicus* challenged with a mixed bacterial suspension. Using RT-PCR and RACE, a full length cDNA sequence of the hepcidin like antimicrobial peptide was determined (GenBank accession number AY604195). The complete hepcidin cDNA Hpc2 is 581 bases and contains an ORF of 258 bases with a coding capacity of 86 amino acids. The deduced amino acid sequence, which shares eight cysteines at the identical conserved positions, and gene organisation are conserved between Japan sea bass and other fish species. The predicted molecular weight of the peptide is 9.4 kDa. The 3'-untranslated region is composed of 225 bp with a polyadenylation signal AATAAA sequence appearing at 189 nt and the poly(A) tail at 212 nt downstream of stop codon TGA. The predicted signal peptide cleavage site of its deduced peptide is between codons 24 and 25. Japan sea bass hepcidin-like genomic DNA hpc2 sequence including upstream and downstream regions (GenBank accession number AY864813) was composed of two introns and three exons. The cloned 173-bp upstream sequence of Japan sea bass hepcidin-like gene contains putative regulatory elements and several binding motifs for transcription factors. High homologies with hepcidin cDNAs and peptides of white bass (*Morone chrysops*), human and other fish were shown. Hpc2 of *Lateolabrax japonicus* is a new member of the hepcidin gene family.

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1. Introduction

Since the first report of hepcidin antimicrobial peptides isolated from human blood ultrafiltrate [1] and urine [2], a series of studies on characterisation and expression of the hepcidins were performed in mammals [3] and fish. Mature peptide of hepcidin was isolated from the hybrid striped bass [4]. Identification and expression analysis of winter flounder, Atlantic salmon [5] and zebrafish [6] hepcidins were carried out, and hepcidin peptides from EST databases were also predicted in various fish species including medaka, rainbow trout [7], winter flounder [8], long-jawed mud-sucker [9] and Atlantic salmon [5] and so on, which means that the hepcidin antimicrobial peptides are widespread among fish. The hepcidin peptides that share eight cysteines at conserved positions with a distinctive cysteine bridge

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structure are unique among antimicrobial peptides [1–4]. Cysteine-rich antimicrobial peptides of the defensin family have been detected in the fat body of insects and the haemolymph of molluscs [10–13]. Other than antimicrobial function, hepcidin takes part in iron metabolism and relates to disorders in iron homeostasis resulting in iron deficiency or overload [14–18].

Here we present sequences of Japan sea bass (*Lateolabrax japonicus*) hepcidin-like genomic DNA (*hepc2*) and cDNA (*Hepc2*) amplified from the liver, and compare their deduced amino acid sequences with hepcidins of other fish species and mammals. Determination of the complete hepcidin cDNA and genomic DNA of Japan sea bass will be the basis for construction of recombinant hepcidin and for further study of its transcriptional regulation.

2. Materials and methods

2.1. Total RNA extraction and generation of full-length cDNA

Healthy Japan sea bass fingerlings (20–30 g) were raised in a mimic-natural environment for 3 days before challenge. The fingerlings were immersed for 5 min in a mixed suspension of *Staphylococcus aureus*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Micrococcus lysodeikticus* (approximately 10^8 cfu each). The challenged fingerlings were randomly selected, and killed 24 h post-challenge. Each individual liver (approximately 100 mg each) was immediately collected and frozen for RNA isolation. Total RNA was extracted using Trizol reagent (Invitrogen), and was used for RT-PCR, 5'- and 3'-RACE cDNA synthesis with primers designed from the complete published cDNA sequence of *Morone chrysops* hepcidin (GenBank accession number AF394246). The reverse transcription reaction was performed using primer A1(5'GAACCTGCAGCAGACACCACATCCG3') and AMV reverse transcriptase (Promega), and was incubated at 42 °C for 60 min. The PCR reaction was performed using reverse transcription product, primers A1 and S1(5'CGAAGCAGTCAAACCCTCCTAAGATG3'), Taq DNA polymerase (Promega). The amplification conditions were: 2 min at 94 °C; 30 cycles of 40 s at 94 °C, 45 s at 60 °C (annealing temperature), 60 s at 72 °C; then 5 min at 72 °C for further extension. The RACE reactions were performed using total RNA, primers S1 and A1 and the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. RT-PCR, 5'- and 3'-RACE products were purified from an agarose gel using a QiaQuick gel purification kit (Qiagen) and were ligated into the T/A cloning vector pMD18-T (TaKaRa) and transformed into competent *Escherichia coli* ER1647 (TaKaRa). Three randomly selected clones were identified as positive clones using restriction endonuclease *Xba*I and *Pst*I (TaKaRa) and were sequenced at least twice by Invitrogen Biotechnology Co., Ltd (Shanghai, China).

2.2. Genomic DNA (gDNA) extraction and generation of full-length gDNA

Total genomic DNA was prepared from the liver of Japan sea bass using MiniBEST Animal Tissue Genomic DNA Extraction Kit Ver.2.0 (TaKaRa). LA PCR™ in vitro Cloning Kit (TaKaRa) was used to amplify the gDNA. Total gDNA was digested with *Eco*RI according to the manufacturer's recommendations, then the digested gDNAs were ligated with *Eco*RI Cassettes provided by the kit. Six primers based on the hepcidin-like cDNA isolated in the present paper were designed, Cassette Primers C1 and C2 were provided by TaKaRa in the kit and the others are marked on Fig. 1. The diluted ligation product was used as template DNA and was heated at 94 °C for 10 min. Two PCR reactions were carried out with two different primer pairs, LH2gDs1 + Cassette Primer C1 and Cassette Primer C1 + LH2gDx1, respectively. The amplification scheme was designed as 35 cycles for 2 min at 61 °C (for LH2gDs1 + Cassette Primer C1) and at 55 °C (for Cassette Primer C1 + LH2gDx1) (annealing temperature), 5 min at 72 °C; 8 min at 72 °C for further extension. The amplification product was diluted 100 times and was used as template for nested PCR to obtain introns and 3' and 5' flanking sequences, by three reactions separately with three different primer pairs, LH2gDs2 + LH2gDx2, LH2gDs2 + Cassette Primer C2, Cassette Primer C2 + LH2gDx2. The amplification conditions were 1.5 min at 94 °C (pre-denaturing temperature); 35 cycles of 50 s at 94 °C, 60 s at 60 °C (annealing temperature), 3 min at 72 °C; 5 min at 72 °C for further extension. The purification, cloning and sequencing of amplification products were performed as above.

2.3. Computer analysis

The gene and predicted protein sequences were analysed by means of GeneTool1.0 Lite, DNASTar5.0 (to perform the multiple alignments and to calculate the percent identity with the Clustal W program of MegAlign) and

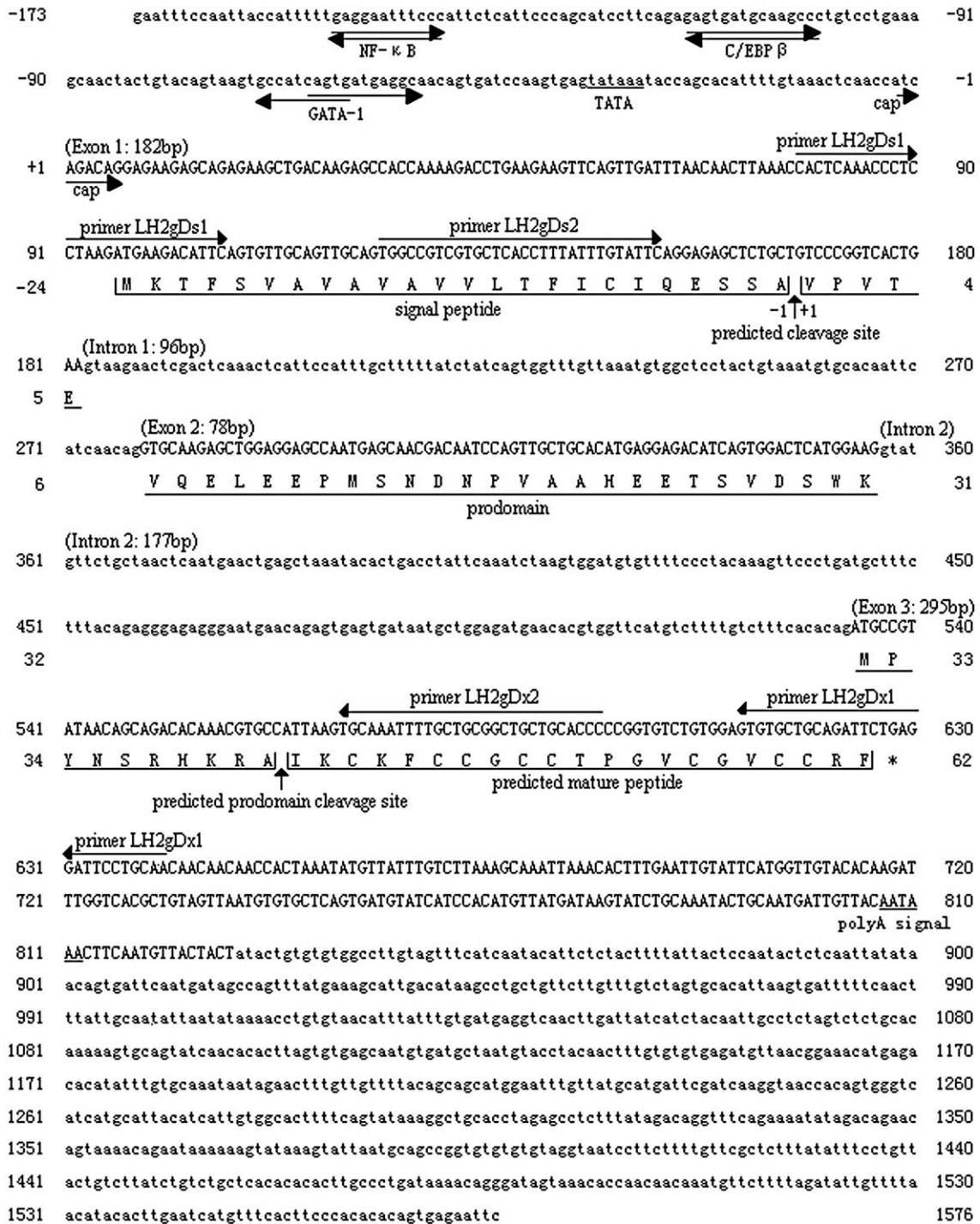


Fig. 1. Hepcidin-like genomic DNA, cDNA and the predicted amino acid sequence of Japan sea bass. Numbering of the genomic sequence is relative to the transcription start site. Location of putative transcription factor binding sites is indicated by an arrow. The TATA box and polyadenylation signal are underlined. Exons are shown in capital letters and introns in small letters. Three exons and a polyadenylation tail, which is not shown, constitute the cDNA sequence. The predicted peptide sequences are translated below the coding sequence. The stop codon is indicated by an asterisk. Primer binding sites are shown with arrows (5' to 3'). The organisation of the predicted peptide domains (signal peptide, prodomain and mature peptide) shown by the amino acid sequence is enclosed by an underlined bar. The predicted cleavage sites of the signal peptide and prodomain are also shown with arrows (genomic DNA, GenBank accession number AY864813; cDNA, GenBank accession number AY604195).

ClustalX1.83 (to bootstrap the neighbor-joining phylogenetic tree). Homology searches were performed using BLASTn and BLASTp by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Putative transcription factor binding sites were predicted by TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The cleavage site for the signal peptide was predicted by the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP>).

3. Results and discussion

A full length hepcidin-like cDNA Hepc2 was amplified from the liver of *Lateolabrax japonicus* challenged with a mixed bacteria suspension, using RT-PCR and RACE with specific primers S1 and A1. The complete cDNA Hepc2 (GenBank accession number AY604195) is 581 bases and contains an ORF of 258 bases with a coding capacity of 86 amino acids. The predicted molecular weight of the protein is 9.4 kDa. The 3' non-coding region is composed of 225 bp with a polyadenylation signal AATAAA sequence appearing at 189 nt and the poly(A) tail at 212 nt downstream of stop codon TGA. The predicted signal peptide cleavage site of its deduced protein is between codon 24 and 25 (Fig. 1).

The deduced amino acid sequence from Japan sea bass cDNA Hepc2 was highly similar to hepcidins of other fish species and mammals, which share eight cysteines at the identical conserved position (Fig. 2). Alanine (A) and valine (V) residues abound in the hepcidin signal peptide part of fish, but leucine-rich residues are in the signal peptide part of

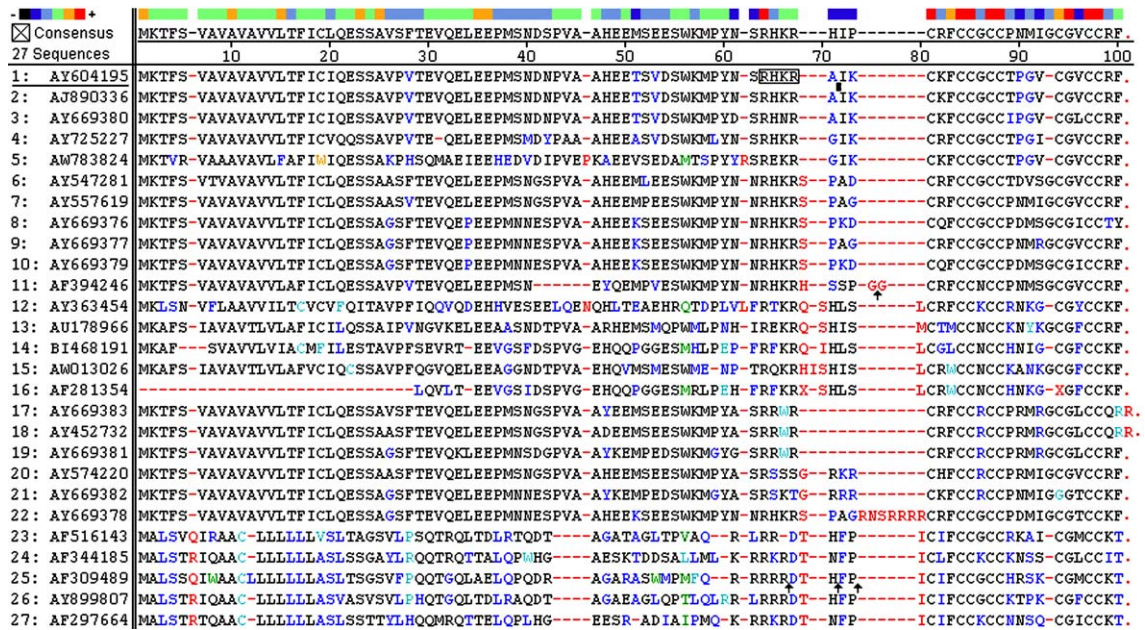


Fig. 2. Comparison of amino acid sequence deduced from *Lateolabrax japonicus* Hepc2 cDNA with some similar peptide sequences. There are 7–8 cysteine residues in the C-terminal. They are a rather conserved domain in the mature peptides. The amino-acid sequence deduced from *Lateolabrax japonicus* Hepc2 (accession number AY604195) is underlined, and the predicted prodomain cleavage site usually after RHKR enclosed in a black frame is indicated by the black spot (such as A–I). The cleavage sites for mature peptides of *Morone chrysops* and *Homo sapiens* are shown with the black arrows. Other similar peptides were predicted. *Lateolabrax japonicus* hepcidin-like Hepc1–2 (accession numbers AY547281, AY604195), *Acanthopagrus schlegelii* hepcidin-like 1-7 precursor (accession numbers AY669376, AY669377, AY669378, AY669379, AY669380, AY669381, AY669382), *Pagrus major* hepcidin-like 1–4 (accession numbers AY452732, AY669383, AY574220, AY557619), *Morone chrysops* hepcidin precursor (accession number AF394246), *Scophthalmus maximus* hepcidin-like precursor (accession number AJ890336), *Danio rerio* preprohepcidin (accession number AY363454), *Salmo salar* hepcidin (accession number B1468191), *Oreochromis niloticus* hepcidin-like precursor (accession number AY725227), *Oncorhynchus mykiss* putative hepcidin antibacterial peptide (accession number AF281354), *Mus musculus* prohepcidin (accession number AF297664), *Homo sapiens* prohepcidin (accession number AF309489), *Rattus norvegicus* prohepcidin (accession number AF344185), *Sus scrofa* hepcidin (accession number AF516143), *Canis familiaris* hepcidin precursor (accession number AY899807), *Oryzias latipes* unnamed peptide (accession number AU178966), *Pseudopleuronectes americanus* unnamed peptide (accession number AW013026), *Gillichthys mirabilis* unnamed peptide (accession number AW783824).

mammalian hepcidins. The tetrapeptide Arg-His-Lys-Arg (R-H-K-R) is highly conserved in front of the prodomain cleavage site among most fish species (Fig. 2). This motif is composed of positively charged residues. The interactive domain of proteins that make inactive prohepcidin become active hepcidin probably consists of negatively charged amino acid residues. By analogy to human hepcidin [2], white bass hepcidin [4] and zebrafish hepcidin [6], the predicted prodomain cleavage site is probably between Ala⁴¹ and Ile⁴². Its precise prodomain cleavage site has not been studied yet. The predicted mature hepcidin, composed of 21 amino acid residues with a MW of 2232.51 Da and pI of 8.13, is relatively conserved.

Homologies with hepcidin or hepcidin-like proteins of mammals and other fish species were analysed. The highest identity of amino acid sequence deduced from Japan sea bass Hepc2 was 100.0% with turbot (*Scophthalmus maximus*) hepcidin-like precursor (CAI65387), and cDNA identity between them was 62.0%. The identity with white bass (*Morone chrysops*) hepcidin precursor (AAM28440) was 74.1%, and their cDNA identity was 74.2%. Turbot hepcidin-like precursor cDNA (AJ890336) was incompletely published without a polyadenylation signal AATAAA sequence and was highly different from Japan sea bass Hepc2 cDNA in 5' and 3' UTRs. Phylogenetic analysis of the hepcidin-like family indicated that two clusters were present: mammalian and fish hepcidins (bootstrap value >95%). The deduced amino acid sequence from Japan sea bass cDNA Hepc2 was in a branch position with published turbot and black sea bream hepcidin-like peptides (Fig. 3).

Japan sea bass hepcidin-like genomic DNA hepc2 sequence including the hepcidin-like gene, upstream and downstream regions was determined (GenBank accession number AY864813, Fig. 1). The hepcidin-like gene of Japan sea bass is composed of two introns and three exons (Fig. 1). The first exon contains the 5' UTR, the signal peptide and part of the prodomain. The prodomain extends from exon 1 to exon 3 which also encodes the mature peptide and the 3' UTR. The organisations with three exons and two introns of the known hepcidin genes are quite conservative in humans, mice and fish [2–6]. Introns of the Japan sea bass hepcidin-like gene are similar to the corresponding introns of the white bass hepcidin gene, but the first and the second introns of the Japan sea bass hepcidin-like genes (96 bp and 177 bp) are respectively much shorter than the first introns of zebrafish, human and murine hepcidin genes (hepcidin1 624 bp or hepcidin2 446 bp, 2121 bp and 1172 bp, respectively) and than the second intron of zebrafish

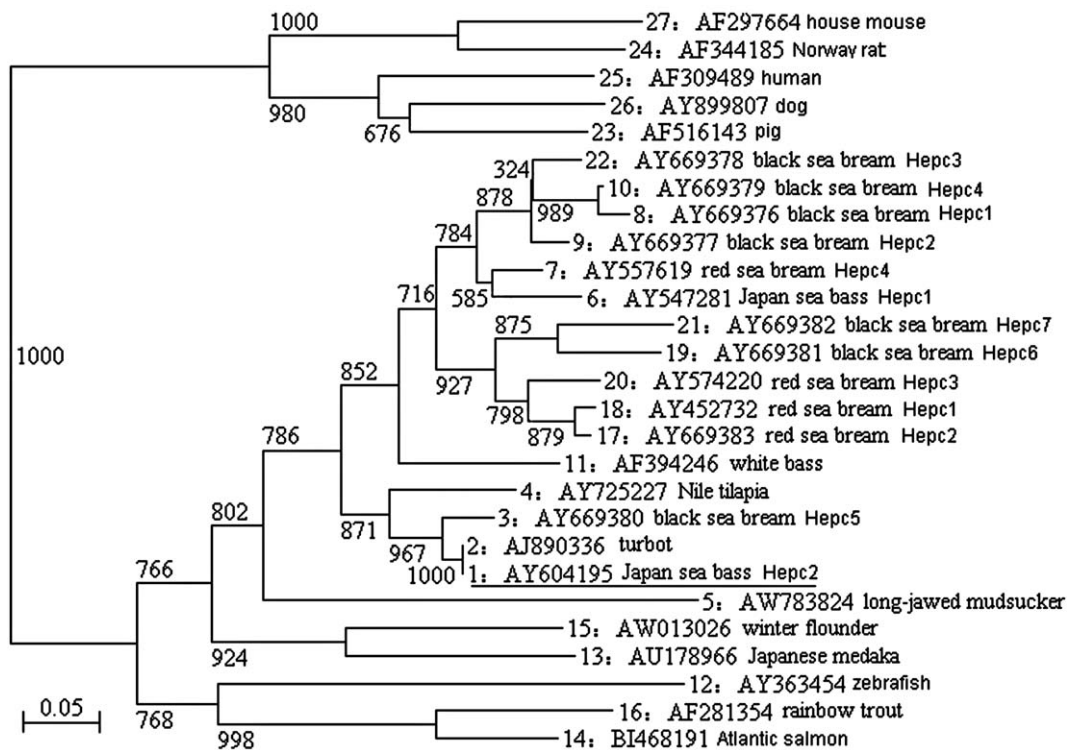


Fig. 3. Phylogenetic analysis of deduced amino acid sequences from *Lateolabrax japonicus* Hepc2 cDNA and other hepcidin precursor peptides with ClustalX1.83. Numbers next to the branches indicate bootstrap value from 1000 replicates.

hepcidin gene (hepcidin1 1553 bp or hepcidin2 1556 bp). The cloned 173-bp upstream sequence of Japan sea bass hepcidin-like gene hepc2 contains putative regulatory elements and several binding motifs for transcription factors. A TATA box is at position –35 nt, and putative binding sites for C/EBP β and NF- κ B appear at positions –115 nt and –152 nt upstream from the transcriptional start site, respectively (Fig. 1). The transcription factor C/EBP β and NF- κ B are important transcription factors. C/EBP β , which is regulated by interactions of cytokines and protein kinases, takes part in transcription of acute phase response genes [19], and NF- κ B plays an important role in the Toll signalling pathway and defense system [20]. They were found in the upstream region of white bass [4], zebrafish [6], human and murine hepcidin genes [3].

Antimicrobial peptides of fish are thought to exist in gills, gastrointestinal tract and skin [4] to prevent bacteria from penetrating into the circulatory system. Hepcidin peptides were primarily isolated from human urine and plasma ultrafiltrate [1] or gills of bass [4], but it was found that they were expressed highly in the liver of human, mice and bass [2–4]. Those observations suggested that the hepcidin peptides synthesised in the liver were transported to gills in fish or distant sites in humans or animals through the circulation. This proposed induction and expression mechanism of hepcidin peptides is different from the other known classes of antimicrobial peptides. Hepcidin also limits intestinal iron absorption as a signal [21]. Its expression is affected by hypoxia and inflammation [22,23] and decreases in haemochromatosis patients [18]. Because hepcidin can depress iron absorption, iron-deficiency anaemia often occurs as a secondary effect of bacterial infection in the clinic. Earlier observations on the induction of hepcidin in two unrelated physiopathological situations indicated that hepcidin not only have an antimicrobial function like other known antimicrobial peptides, but may have other unique functions. However, the mechanisms of hepcidin regulation remained unknown until recently transcription factors, such as C/EBP α , were shown to be involved in an iron-mediated regulation of hepcidin gene expression [24]. This current study presenting the complete cDNA and DNA sequences of Japan sea bass hepcidin-like peptide will facilitate the analysis of the 5'-flanking region of the Japan sea bass hepcidin gene to identify putative binding sites for liver-enriched transcription factors. Subsequent studies are now focused on understanding the phenomenon of predominant expression of the hepcidin gene in the liver and mechanisms of its regulation during various physiopathological situations.

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