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Short communication

Chondrostean sturgeon hepcidin: An evolutionary link between teleost and tetrapod hepcidins

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ABSTRACT

Hepcidin, a cysteine-rich antimicrobial peptide (AMP), plays key roles as a regulatory hormone in iron homeostasis, providing a link between iron metabolism and innate immunity. Unlike many other AMPs displaying a high degree of sequence variability among closely related organisms, hepcidin is highly conserved from teleosts to mammals. However, little is known about the early ancestry of hepcidins in the vertebrate lineage. Here, we first report potential a prototype hepcidin from the Siberian sturgeon *Acipenser baerii*, a primitive chondrostean species. The *A. baerii* hepcidin (*Ab*HAMP) gene showed a tripartite exon-intron organization, which encoded a precursor protein comprised of three structural signatures containing eight cysteine residues, a common structure in vertebrate hepcidin genes and proteins. mRNA expression by iron-overloading and bacterial infection and antibacterial activity revealed that *Ab*HAMP might play a role in iron metabolism regulator in the liver, and in direct and/or indirect host immune response in the kidney against invading pathogen. Comparison of gene and protein sequences reveled that *Ab*HAMP possesses intermediate characteristics between tetrapodian and teleostean hepcidins (HAMP1s). Phylogenetically, *Ab*HAMP had a closer genetic affiliation to tetrapodian orthologs than to teleostean orthologs, suggesting that the structures of this chondrostean hepcidin may closely reflect the structures of an evolutionarily ancestral form that might have evolved into extant hepcidins in tetrapods and teleosts, respectively. Based on the identification of hepcidin from the chondrostean group, the emergence of the common ancestral hepcidin should be traced back to in early Osteichthyes: no later than sarcopterygian (lobe-finned fishes) – actinopterygian (ray-finned fishes) split.

1. Introduction

Antimicrobial peptides (AMPs), which exist in all eukaryotic organisms, are evolutionarily ancient molecules that play an important role in the innate immune response against pathogenic invasions [1,2]. Among these peptides, hepcidin (originally named LEAP-1, liver-expressed antimicrobial peptide) was first identified in human plasma ultrafiltrates and urine [3,4] and, since then, hepcidins have been characterized from a number of animals belonging to a wide array of taxonomic positions including tetrapods and teleosts [5–8]. Unlike many other AMPs, which usually display a high degree of sequence variability even between/among closely related species, hepcidins are known to be remarkably conserved from fish to humans [2,9,10]. In recent, increasing number of evidence has demonstrated that hepcidin is a key regulator of iron homeostasis, providing a functional link between iron metabolism and innate immunity [11,12].

To date, all known fish hepcidins share common structural features with human ortholog, essentially with regard to an antiparallel β-sheet separated by a hairpin loop stabilized by intramolecular disulfide bonds with 4, 6, 7, or 8 cysteine residues [13–15]. Furthermore, on the genomic level, hepcidin gene is highly conserved in all tetrapods and teleosts, consisting three exons interrupted by two introns, which encode a prepropeptide comprised of three structural signatures; a signal peptide, a prodomain, and a mature peptide [6]. Although many teleost fish species have been reported to possess multiple paralogous copies of hepcidin gene. However, they could be classified into two distinct functional hepcidin types; hepcidin type 1 (HAMP1, ortholog of mammalian hepcidin) is mainly involved in the regulation of iron metabolism and hepcidin type 2 (HAMP2) is believed to hold a role in antimicrobial function [10,16–18]. Although tissue-specific expression

Abbreviations: RP-HPLC, reverse-phase high-performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; ORF, open reading frame; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-qPCR, real-time quantitative PCR; UTR, untranslated region

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Table 1
A list of nucleotide sequences of the primers used in this study.

Primer	Sequence (5'→3')	Usage
Abhamp-gsp1-F	GCAATTACAGAGCGACTCAAAACAAACGGC	cDNA and gDNA
Abhamp-gsp1-R	AGTAACTTCTTTTATTTCATATAGTTTTAG	cloning
Abhamp-gsp2-F	ACCAGAGGCCTGGGACACATCG	3′ RACE
Abhamp-gsp2-R	CGACAGCAGTAGCCACAGCCC	5′ RACE
Abhamp-qPCR-F	GACAGAAGACCAGGATAGAC	RT-qPCR
Abhamp-qPCR-R	TCAAAGACGATGTGTCCCAG	
*Ab*18sRNA-qPCR-F	ATACAGGACTCTTTCGAGGC	
*Ab*18sRNA-qPCR-R	CTCAGTTAAGAGCATCGAGG	
Abhamp-BamHI-Met-F	AGCTCGGATCCATGCAAAGCCACTTCCCAATCTG	Recombinant production
Abhamp-XhoI-R	TTGCACTCGAGTTAAGTCCGACAGCAGTAGCC	

profiles for teleostean HAMP1 and HAMP2 have been reported to be variable among fish species in details, the principal organ responsible for their mRNA expression is the liver in most cases, as accordant with liver-predominant expression pattern in tetrapods [7,19–21]. Thus, in the context of structural, functional, and transcriptional orthology in the vertebrate lineage, it has been hypothesized that the function of hepcidin as an iron-regulatory hormone in modern vertebrates has evolved from an ancient hepcidin executing AMP function [22]. However, little is known about the ancestry of hepcidins that reflect its evolutionary history to provide a functional link between iron homeostasis and innate immunity. Such a gap in our knowledge leads to need to investigate hepcidins from primitive vertebrate members, which will help build the early evolutionary repertoire for hepcidins in vertebrates.

Chondrostean sturgeon (Acipenseriformes), which is considered as a member of a "living fossil" group, is an extant ancient fish that phylogenetically occupies the most basal position below the teleostean clade in ray-finned fish (Actinopterygii) lineage [23]. Due to their unique evolutionary position with an extremely slow rate of molecular evolution, sturgeons have been given much attention as an attractive model system to study evolution and functional diversification of vertebrate genes and proteins [24–27]. Accordingly, mining hepcidin sequences from sturgeon is of importance to provide unique insights into the evolutionary history of vertebrate hepcidin with a particular emphasis on the divergence of its primordial function. Recently, liver-expressed antimicrobial peptide-2 genes (*LEAP-2s*) have been identified from sturgeon species with the proposed contribution to innate immunity in chondrostean species [28]. However, nothing is known about the molecular identity of hepcidins from sturgeon species. It is of interest, therefore, to identify hepcidin from sturgeons, which would be helpful for getting further insights into the link between two functions (the iron regulation and antimicrobial activity) in tetrapodian and teleostean groups.

Here we report the identification of hepcidin (*Ab*HAMP) from the Siberian sturgeon, *Acipenser baerii*, as exemplified by sturgeon (Acipenseriformes) hepcidins. Genomic DNA and cDNA encoding *Ab*HAMP preproprotein were cloned and sequenced, and evolutionary significance of the chondrostean hepcidin was hypothesized with comparative sequence analysis and reconstruction of the phylogenetic tree in the representative vertebrate lineage. In order to understand the potential involvements of the chondrostean hepcidin in iron metabolism and innate immune response, mRNA expression levels of *Ab*HAMP were analyzed in various tissues and in response to different stimuli including bacterial challenge and iron overload. Furthermore, the antibacterial activity of mature *Ab*HAMP forming four intramolecular disulfide bonds, which was produced in a heterologous bacterial-expression system, was examined against several bacteria species *in vitro*.

2. Materials and methods

2.1. Fish and ethic statement

The Siberian sturgeon, *Acipenser baerii,* used in this study was a laboratory-bred stock that were maintained in Experimental Fish Culture Station, of Pukyong National University (Busan, Korea). The fish were reared with water recycling culture system at 18–20 °C, and fed a commercial diet (Millennium plus; Woosungfeed Co., Daejeon, Korea) with a daily feeding rate of 1% of body weight. This study was approved by the Animal Ethics Committee of Pukyong National University (Approval #201818) and performed according to the guideline for the care and use of laboratory animals.

2.2. Molecular cloning of full-length cDNA and genomic A. baerii hepcidin (AbHAMP) genes

To identify *Ab*HAMP gene, we first searched the Transcriptome Shotgun Assembly (TSA) of Acipenseriformes (taxid:7899) in National Center for Biotechnology Information (NCBI) GenBank database with the BLAST (tblastn) program using human hepcidin (accession no. MK024329) [3] and multiple hepcidin isoforms described previously [19,29] as query sequences. Queries for hepcidin resulted in three TSA contigs (accession no. GGWJ01045492, GGWJ01049542, and GGEUL01015297) in the Atlantic sturgeon *A. oxyrinchus*, which contained a hepcidin motif in the deduced peptide sequence. These three TSA contigs were re-aligned to the TSA (taxid:7899), retrieving two more TSA contigs (accession no. GETX01012667 and GETX01090512) in the Chinese sturgeon *A. sinensis*. Then all TSA contigs obtained were aligned to Sequence Read Archive (SRA) database for *A. baerii* available at the NCBI under the accession number SRX2426785 run SRR5114774 [30]. Combining these BLAST results from *A. oxyrinchus*, *A. sinensis*, and *A. baerii*, primers were designed to enable PCR amplification of the cDNA encoding *Ab*HAMP protein (Table 1). cDNA encoding a putative *Ab*HAMP precursor protein was amplified from the liver cDNA with a primer pair (*Abhamp*-gsp1-F and -R). In addition, rapid-amplification of cDNA end (RACE) was performed using SMARTer® RACE 5′/3′ Kit according to manufacturer's instructions to obtain a complete the full-length cDNA sequence of *Ab*HAMP precursor protein. Based on the cDNA sequence, a genomic gene segment was isolated from the fin genomic DNA. The amplified PCR products were introduced into pGEM-T-easy vector (Promega, Madison, WI, USA) and sequenced at both directions. The full-length cDNA sequence obtained were translated into protein sequence using ExPASy (http://web.expasy.org/translate/). Putative cleavage sites for a signal peptide and for a mature peptide in the translated protein sequence were analyzed using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/). Theoretical isoelectric points (*p*Is) of peptides/proteins were computed using ExPASy *p*I/Mw tool (https://web.expasy.org/compute_pi/).

2.3. Sequence alignment and phylogenetic analysis

The hepcidin amino acid (AA) sequences from representative vertebrates used for multiple sequence alignment and phylogenetic analysis were retrieved from the NCBI GenBank database (Supplementary Table S1). Multiple sequence alignment of *Ab*HAMP precursor protein with vertebrate orthologs was conducted using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and refined manually. A phylogenetic tree was constructed using Maximum-Likelihood (ML) method with Jones-Taylor-Thornton (JTT) model as a substitution model, Nearest-Neighbor-Interchange heuristic model, and complete deletion of gap using MEGA6 program [31]. The evolutionary distance was measured as the proportion of difference (*p*-distance) between sequences. The confidence of branch topology was accessed with 1000 bootstrap replications.

2.4. Expression analysis of AbHAMP mRNA

To analyze the basal expression of *Ab*HAMP mRNA, eleven tissues (i.e., brain, eye, fin, immature gonad, gill, heart, intestine, kidney, liver, muscle, and spleen) were surgically removed from six individual *A. baerii* juveniles [10-month-old; average body weight (BW) = 782.5 ± 65.4 g and average total length (TL) = 63.8 ± 4.9 cm]. To investigate transcriptional regulation of *Ab*HAMP gene in response to different stimuli, *A. baerii* fingerlings (3-month-old; BW = 22.1 ± 4.1 g and TL = 18.5 ± 2.2 cm) were used. For the bacterial challenge, individuals (n = 4) were intraperitoneally injected with gram-negative bacteria *Aeromonas hydrophila* KCTC2358 and *Vibrio harveyi* ATCC14126 or gram-positive bacterium *Lactococcus garvieae* ATCC49156 with two bacterial doses, 2×10^7 and 2×10^8 CFU/g BW. Bacterial suspensions were prepared with sterile phosphate-buffered saline (PBS) and injection volume was 200 μL. A non-challenged control group (n = 4) was prepared with the injection of equal volume of PBS. After bacterial injection, each group was assigned into one of four tanks each containing 300 L of 1-μm-filtered ground water at 20 °C. At 24 h post-injection (hpi), fish were euthanized with 1000 ppm of MS-222, and three tissues (liver, kidney and spleen) were surgically removed from each individual. Tissues were immediately frozen on dry ice and stored at −85 °C until used. For the iron overload, individuals (n = 3) were given an intraperitoneal injection of PBS-resuspended iron-dextran (Sigma-Aldrich, St. Louis, MO) at dose levels of 0.2 mg and 1 mg/g BW. Non-stimulated control group was prepared with the injection of PBS (200 μL). Injected fish were incubated at 20 °C as above, and tissues (liver, kidney, and spleen) were obtained at 24 hpi.

Total RNA and cDNA were prepared using RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) and Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) analysis was performed in triplicate using the LightCycler 480 Real-Time PCR system and LightCycler 480 SYBR green I master (Roche Applied Science, Germany) as described previously [29]. The *A. baerii* 18s ribosomal RNA (AY904463.1) was used as a normalization control for RT-qPCR assay [32]. Melt curve analysis was performed to ensure product specificity over the temperature range of 65–90 °C. The sequences of primers used in RT-qPCR are listed in Table 1. The levels of gene expression were calculated from the threshold cycle according to the $2^{-\Delta\Delta CT}$ method [33]. The data were shown as the mean ± standard deviation. Multiple comparisons were performed with one-way analysis of variance (ANOVA) with Bonferroni's multiple range post-hoc analyses using GraphPad Prism software version 7.0 for Windows (GraphPad Software, CA, USA).

2.5. Antibacterial activity of AbHAMP

The antibacterial activity of the predicted mature *Ab*HAMP was determined according to a slightly modified version of previously reported methods [34]. Briefly, sequence coding the mature *Ab*HAMP with Met codon (ATG) at immediately upstream of the N terminus was cloned into a laboratory modified vector, pET28a-thioredoxin A (*TrxA*) fusion vector (see primer sequences in Table 1). The constructed vector was transformed into *Escherichia coli* BL21(DE3) cells (Novagen, WI, USA) and the mature *Ab*HAMP with His6-tagged TrxA fusion protein was overexpressed with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C for 4 h. Overexpressed recombinant protein was obtained from an insoluble fraction of the bacterial cell lysate and then dissolved in 1 x phosphate buffer saline containing 8 M urea and 5 mM imidazole. The His6-tagged recombinant protein was purified according to a general protocol for His-tagged protein affinity chromatography using nickel-nitrilotriacetic acid resin (Novagen). The purified recombinant protein was dialyzed with 5% acetic acid and lyophilized. The Met residue at the N terminus of mature *Ab*HAMP was subjected to cyanogen bromide (CNBr) cleavage (10 mg/mL) by dissolving the lyophilized protein in 50% formic acid for 8 h in darkness at 25 °C. The cleavage reaction was terminated with adding 10 volume of water, followed by lyophilization. A peak predicted to be the mature *Ab*HAMP was purified through two steps of reverse-phase high-performance liquid chromatography (RP-HPLC) and its molecular mass and three AA sequence of N-terminus were determined by a nanoscale liquid chromatography tandem mass spectrometry (nano LC-MS/MS) system (maXis; Bruker Daltonics, Bermen, German) and an automated N-terminal AA gas-phase sequencer (Simadzu Co.), respectively. To measure antibacterial activity, we employed ultrasensitive radial diffusion assay (URDA) a previously described [34]. Bacterial strains used in this study were the gram-positive bacteria *Bacillus subtilis* KCTC1021, *L. garvieae* ATCC49156, and *Staphylococcus aureus* RN4220, and the gram-negative bacteria *E. coli* D31, *A. hydrophila* KCTC2358, and *V. harveyi* ATCC14126. Synthetic piscidin 1, an α-helical AMP isolated from hybrid striped bass, was used as a positive control [35].

3. Results

3.1. Molecular cloning and characterization of AbHAMP precursor protein

The full-length cDNA sequence encoding *Ab*HAMP precursor protein was cloned and sequenced from the liver cDNA using pairs of primers (Table 1). The cDNA sequence was submitted to the NCBI GenBank database (accession number: MK024329) and the nucleotide and the deduced AA sequence of *Ab*HAMP protein were shown in Fig. 1 and Supplementary Figs. S1 and S2. The full-length cDNA sequence comprised 1066 bp, starting with a 5′-untranslated region (UTR) of 222 bp, followed by an open reading frame (ORF) of 270 bp including a TAG stop codon, and a 3′-UTR of 574 bp containing a polyadenylation consensus sequence (AATAAA) located at 15 bp upstream of a poly (A+) tail. The ORF of *Ab*HAMP gene encoded an 89-residue prepropeptide that was composed of a 21-residue N-terminal signal peptide, which was predicted to be cleaved between Ser^{21} and Ser^{22} (ASS/SV) by signal peptidase using SignalP 4.1, followed by two peptide fragments: a 43-residue prodomain (Ser^{22}-Arg^{64}) with typical RX(K/R)R motif for propeptide convertases and a 25-residue mature *Ab*HAMP containing eight conserved Cys residues that are likely to form four disulfide bonds. The theoretical *p*I values for the signal peptide, the proprotein, and the mature peptide were 8.16, 6.76 (5.01 for prodomain), and 8.51, respectively (Fig. 1). Comparison of *Ab*HAMP AA sequence with other putative sturgeon hepcidin proteins from TSA contigs revealed a striking degree of conservation in the Chinese sturgeon *A. sinensis* and the Atlantic sturgeon *A. oxyrinchus* with identities of 97.8% and 94.4%, respectively, suggesting the functional equivalence of these hepcidins in sturgeon species (Fig. 1).

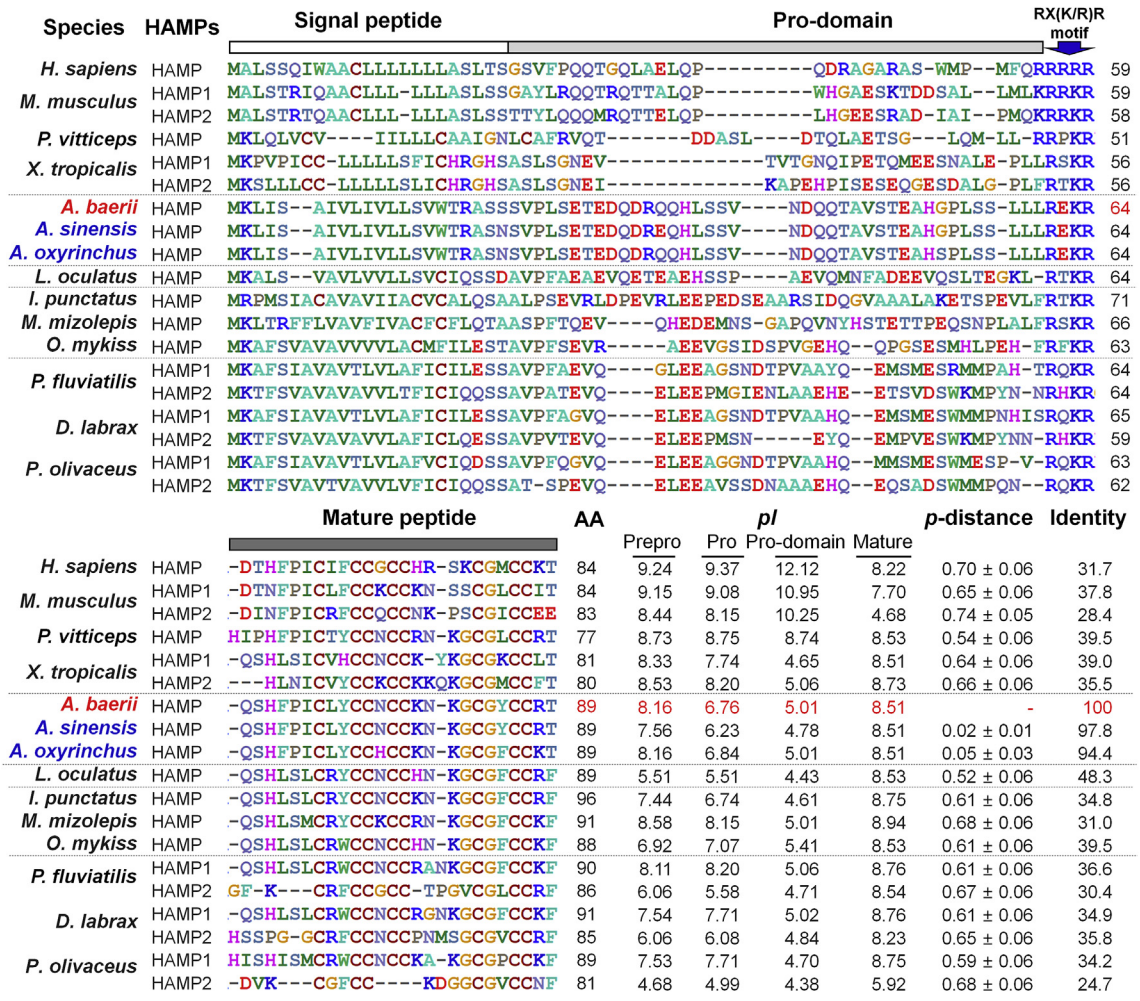

Fig. 1. Multiple sequence alignment of *Ab*HAMP protein with other hepcidin protein in tetrapod and fish. The signal peptide, the prodomain, and the mature hepcidin regions of *Ab*HAMP protein illustrate above the sequence. The theoretical isoelectric points (*p*Is), the identity, and the *p*-distance were computed using ExPASy *p*I/Mw tool, Clustal omega, and MEGA6, respectively.

3.2. Sequence comparison

To investigate the sequence similarity of sturgeon hepcidins with other hepcidins, we aligned the sequence of *Ab*HAMP protein with orthologs from representative tetrapods and fishes (Fig. 1). The multiple sequence alignment revealed that sturgeon hepcidins shared conservative AA residues in multiple positions with other vertebrate hepcidin preproproteins (Fig. 1). In particular, the RX(K/R)R motif for the cleavage site of propeptide convertase to produce a mature hepcidin is clearly conserved in all the hepcidin sequences aligned. The highest identity was observed with a putative hepcidin in the spotted gar (*Lepisosteus oculatus*), which belongs to holostean occupying a basal position in the neopterygian lineage. The range of sequence identities and proportion of AA sites difference (*p*-distance) to tetrapod and teleost hepcidins were comparable with sturgeon hepcidins: identity ranges were 28.4–39.5% (to tetrapods) and 24.7–39.5% (to teleosts) and *p*-distance ranges were 0.54 ± 0.06 to 0.74 ± 0.05 (to tetrapods) and 0.59 ± 0.06 to 0.68 ± 0.06 (to teleosts) (Fig. 1). Furthermore, in the mature peptide region, sturgeon hepcidins shared a number of conserved features with tetrapod and fish hepcidins. Eight Cys residues predicted to form four intramolecular disulfide bonds (except for *Paralichthys olivaceus* HAMP2 possessing six Cys residues) and a Gly residue between the sixth and the seventh Cys residue were clearly conserved in all the hepcidins aligned. Sturgeon hepcidins represented some intermediate characters between tetrapodian and teleostean orthologs. In N-terminal AA sequence prior to the first Cys residue, the first three AA residues (QSH-) of sturgeon hepcidins were identical to fish HAMP1s (except for *P. olivaceus* exhibiting HISH-) and amphibian HAMP1, while the following three AA residues (-FPI-) of sturgeon hepcidins were identically observed in human, mouse, and reptile hepcidins. In addition, unlike all the other fish hepcidins that had Phe as the last AA residue at C-terminus, sturgeon hepcidins shared the conserved Thr residue with most tetrapodian orthologs. Sturgeon preprohepcidin and mature hepcidins were positively charged, which was similar with tetrapod hepcidins and teleost HAMP1s. Prodomain derived from sturgeon prohepcidin had a negative charge similar to fish and amphibian orthologs, while amniotes had a strongly positively charged prodomain. However, for prohepcidins, taxonomic group-dependent pattern of *p*I values was not clearly found.

3.3. Genomic organization of AbHAMP gene

Genomic DNA sequence of the *Ab*HAMP gene represented a tripartite exon-intron organization (accession number: MK024330), which has been known as a common structure in most previously known vertebrate hepcidin genes (Fig. 2) [36]. In the *Ab*HAMP gene, exon-1 (81 bp encoding 27-residues), exon-2 (84 bp; 28-residues), and exon-3 (105 bp including stop codon; 34-residues) were intervened by two introns (591 bp of intron-1 and 1875 bp of intron-2) (Fig. 2 and Supplementary Fig. S2). The canonical splicing recognition sequence GT/AG was conserved at each exon-intron junction. In addition, the *Ab*HAMP gene contained two simple sequence repeats (SSRs) in the

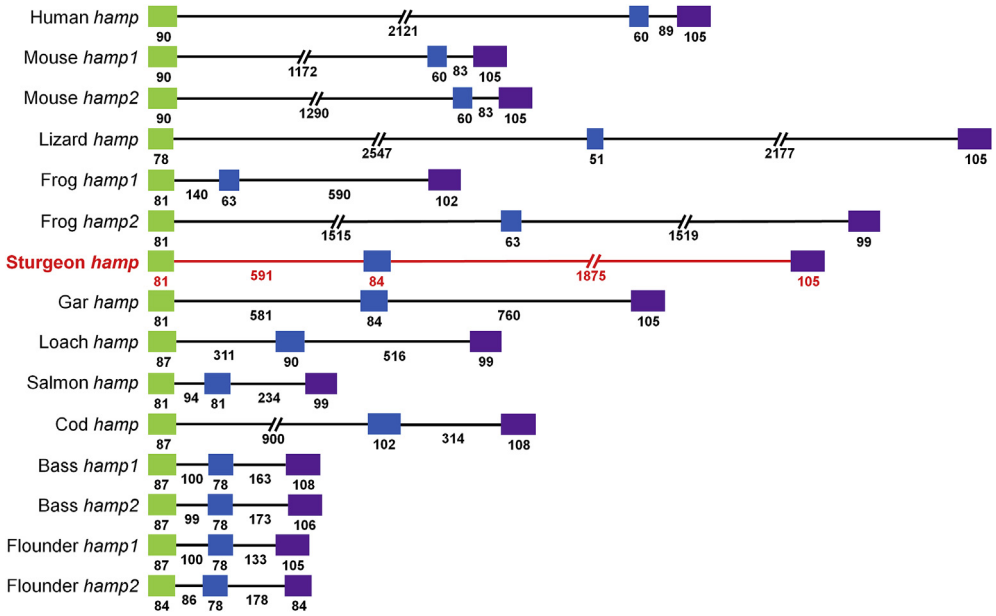

Fig. 2. Comparative view of genomic organization of *Ab*HAMP gene with other tetrapod hepcidins and teleost hepcidins. Exons are shown as boxes, and introns as solid lines, with sizes in base pairs indicated below.

intron-2: $(TC)_{32}$ and $(GT)_{24}$ dinucleotide repeats, suggesting the need of testing the intra- and interindividual polymorphisms of these SSRs (Supplementary Fig. S2). Although the tripartite architecture was strikingly conserved in all the hepcidin genes studied, the numbers of amino acid residues encoded in each exon and each intron were not identical among orthologs (Fig. 2). Particularly, the size of the second exon (84 bp) in the *Ab*HAMP gene, which was similar to those of teleostean hepcidins, was larger than those of tetrapodian orthologs. Moreover, the *Ab*HAMP intron-1 (591 bp) was smaller than intron-2 (1875 bp), and the ratio in length of intron 1 to intron 2 was similar to those observed in teleostean hepcidins. On the other hand, total length of the *Ab*HAMP introns was larger than those of teleostean orthologs.

3.4. Phylogenetic analysis

To access the evolutionary relationship of hepcidins in vertebrate lineage, 39 hepcidin precursor protein sequences [6 from tetrapods, 3 from chondrosteans (identified from this study), 1 from holostean, and 29 from teleosts] were subjected to phylogenetic analysis based on the reconstruction of ML tree (Fig. 3). The reconstructed ML tree revealed that hepcidins were clustered into two main clades each supported by 70% of bootstrap confidence value. The first main clade comprised HAMP1s and HAMP2s from neopterygian fishes. Within this neopterygian clade, a monotypic holostean (*L. oculatus*) was placed at the basal position, followed by various teleostean subclades. Internal branching patterns in the teleostean lineage were generally in agreement with known taxonomic appraisals including the separations of early teleostean groups such as ostariophysian and protacanthopterygian groups from the recently evolved teleostean cluster (i.e., acanthopterygian group). In the acanthopterygian lineage, HAMP2s were resolved as a monophyletic group. On the other hand, the second main clade was made of sarcopterygian tetrapod hepcidins and chondrostean hepcidins. Within this clade, the *A. baerii* formed a strong monophyletic clade with two other *Acipenser* species, and then the chondrostean clade was phylogenetically affiliated to the monophyletic tetrapodian clade consisting of mammalian, reptile and amphibian subclades, suggesting that chondrostean HAMPs have emerged earlier than the separation between tetrapodian and neopterygian hepcidins (Fig. 3).

3.5. Expression analysis of AbHAMP mRNA

The basal expression levels of *Ab*HAMP mRNA was quantified in eleven tissues of *A. baerii* juveniles, including brain, eye, fin, immature gonad, gill, heart, intestine, kidney, liver, muscle, and spleen, using RT-qPCR. The results showed that remarkably high expression level ($p < 0.0001$) of *Ab*HAMP mRNAs was detected in the liver, while the expressions in other tissues were only minute compared to hepatic expression level (Fig. 4A). During bacterial challenge, expression levels of the hepcidin mRNAs were strikingly increased in the kidney by gram-negative bacteria (*A. hydrophila* and *V. harveyi*) at 24 hpi, as increasing bacterial doses (Fig. 4B). However, the *Ab*HAMP mRNA expressions in the liver and spleen were not increased during bacterial challenge (Fig. 4B). After the injection of iron-dextran, the expression levels of the *Ab*HAMP mRNAs were significantly increased in the liver and the spleen, in which higher concentration of iron-dextran resulted in higher amount of induction in both tissues. However, there was no significant increase of *Ab*HAMP mRNAs in the kidney (Fig. 4C). Collectively, expression assays in this study indicated that liver is the main organ responsible for the hepcidin-mediated iron regulation while kidney would be involved in the immune response of this chondrostean species.

3.6. Antibacterial activity of recombinant mature AbHAMP peptide

In order to assess antibacterial activity of mature *Ab*HAMP, we preferentially produced a recombinant protein containing the predicted mature *Ab*HAMP with Met residue at N-terminus, which was attached to His6-tagged TrxA fusion protein, using a heterologous bacterial-expression system (Supplementary Figs. S3A and B). The produced recombinant protein was purified by affinity chromatography (Supplementary Fig. S3C) and the Met residue between the Hig6-tagged TrxA protein and the mature *Ab*HAMP was cleaved by CNBr. Finally, a single absorbance peak was obtained from the cleaved mixture through two steps of RP-HPLC purification (Supplementary Fig. S3D). The molecular mass and three AA sequence of N-terminus of the purified peptide were determined as 2841.1 Da ($M+H^+$) and QSH-, respectively (Supplementary Figs. S3E and F). Although assessment of the patterning of the disulfide linkages in the recombinant mature *Ab*HAMP should be studied further, this result indicates that the eight cysteine residues in the mature *Ab*HAMP formed four intramolecular disulfide bonds.

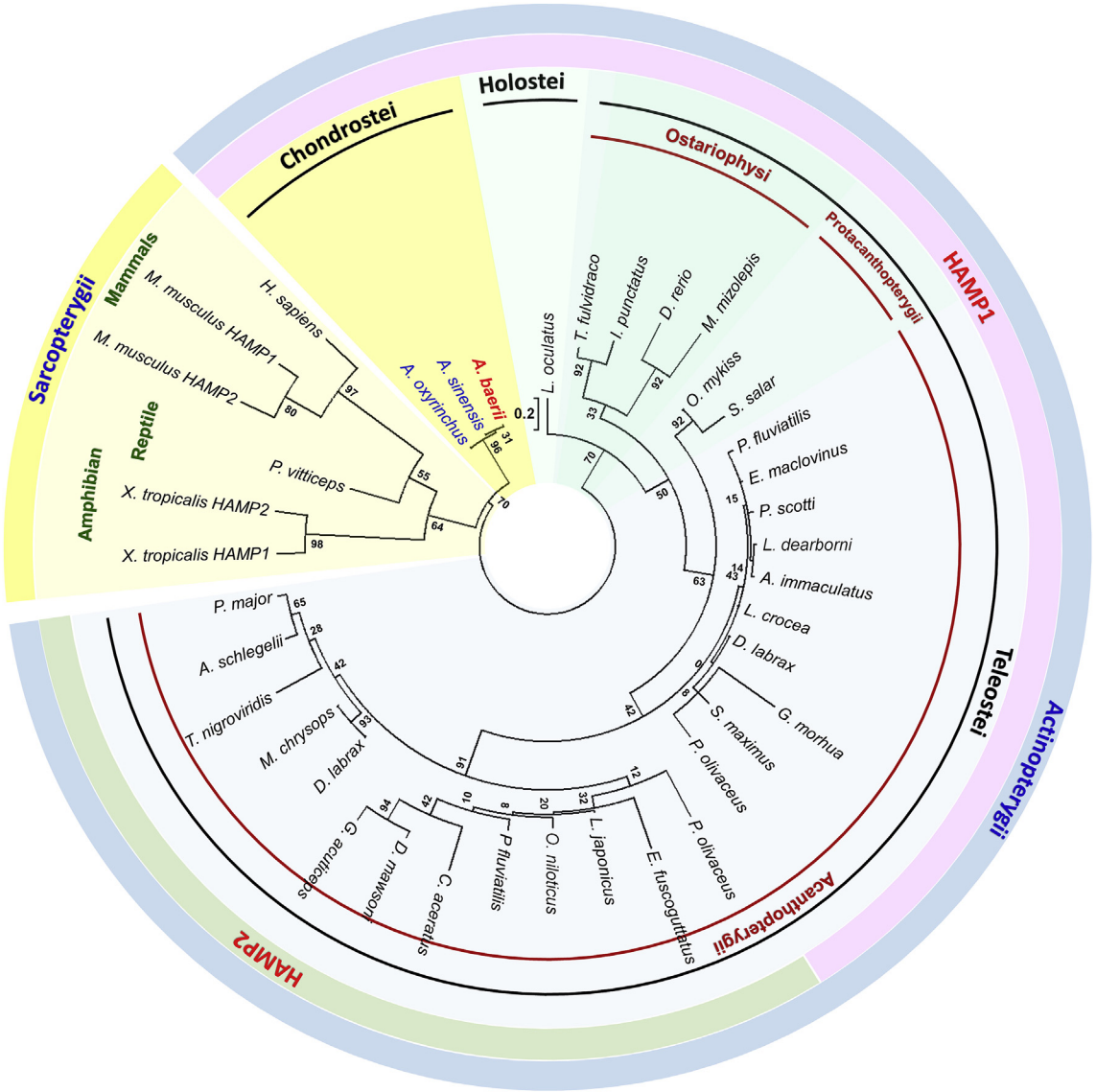

Fig. 3. Phylogenetic analysis of full-length hepcidin proteins from sturgeons with other tetrapods and fishes. Phylogenetic tree was constructed by ML method based on the JTT matrix-based model using MEGA6 [31]. The confidence in the phylogenetic tree branch topology was accessed with bootstrap using 1000 replications. The analysis involved 39 amino acid sequences listed in Supplementary Table S2.

Antibacterial activity was tested using 5 and 10 μg of the mature *Ab*HAMP against several bacterial strains using URDA method [34]. The mature *Ab*HAMP showed antibacterial activity against gram-positive bacteria including *B. subtilis* and *S. aureus* and gram-negative bacterium *E. coli* (Fig. 4D). However, the antibacterial activity of the mature *Ab*HAMP was not or barely detectable on gram-positive bacterium *L. garvieae* and gram-negative bacteria including *A. hydrophila* and *V. harveyi* (Fig. 4D), which were used in the bacterial infection experiment to investigate the expression levels of *Ab*HAMP mRNA, suggesting *Ab*HAMP may play a role in host immune responses to invading pathogens, directly and/or indirectly.

4. Discussion

Most tetrapods possess only one copy of the hepcidin gene with dual roles in both iron metabolism and immune response. Only exceptions are mouse and amphibian hepcidins, in which two mouse hepcidins evolved from a very recent duplication event, whereas two amphibian hepcidins evolved from an ancestral gene duplication [37,38]. Moreover, many teleost fishes present one, two, or multiple copies of hepcidin genes, which is attributed to genome duplications and positive Darwinian selection, suggesting that different hepcidins are involved in different functions [18,19,36,39,40]. These varying gene copies between species contribute to difficulties elucidating when and how hepcidin evolved from an AMP to an iron-regulatory hormone. For this reason, typing the prototype hepcidin has been long challenged as an important issue for elucidating the evolutionary path of hepcidin in vertebrate lineage.

We here report a potential prototype hepcidin gene in the Siberian sturgeon *A. baerii* (Acipenseriformes) that have not experienced the third round of whole-genome duplication (3R or teleost-specific genome duplication) and have the extremely slow rate of molecular evolution [26,27]. The *Ab*HAMP gene showed a high degree of conservation in its overall genomic architecture compared to its tetrapodian and teleostean orthologs. A closer look into the length of exon-2 and the ratio of intron length indicated that *Ab*HAMP gene is more similar to teleostean orthologs, which have short intron-1 and a long intron-2 (except for cod HAMP). Meanwhile, the *Ab*HAMP gene possessed longer total intron length than any of previously known teleost hepcidin genes. Although the functional roles of hepcidin introns have

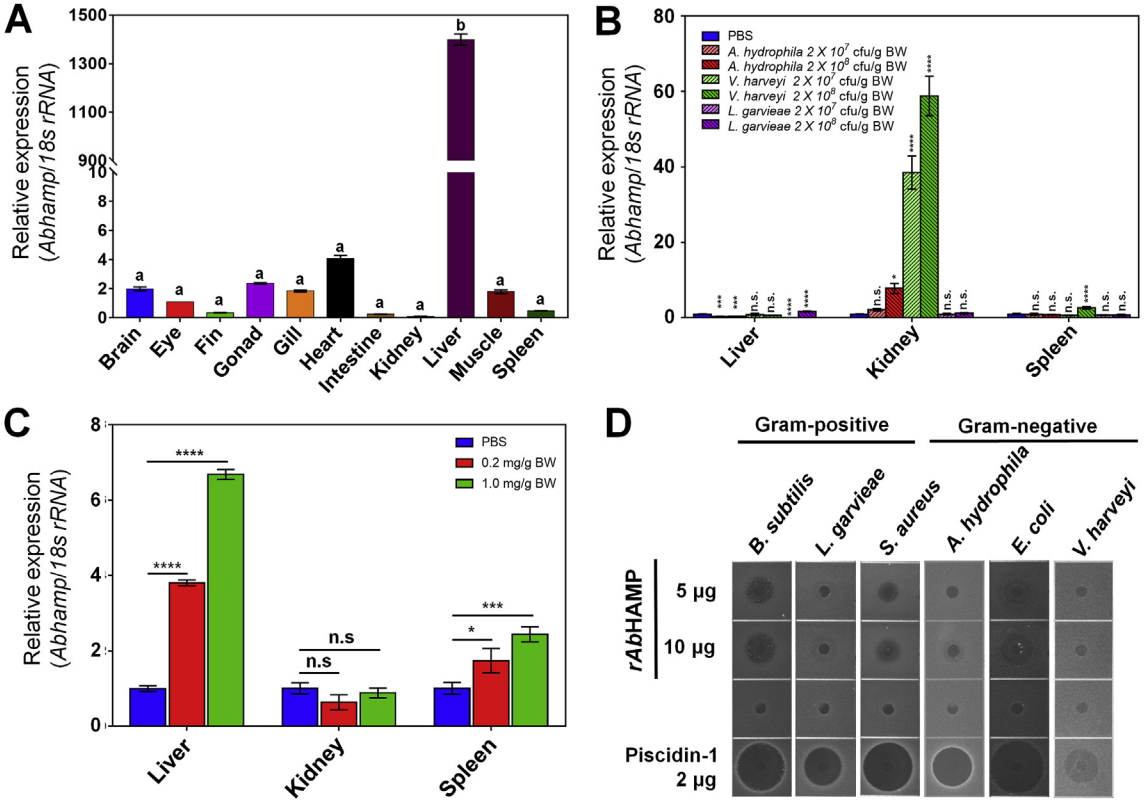

Fig. 4. Expression analysis of *Ab*HAMP mRNA and antibacterial activity of the mature *Ab*HAMP peptide. (A) The basal expression of the *Ab*HAMP mRNA in eleven tissues (i.e., brain, eye, fin, gonad, gill, heart, intestine, kidney, liver, muscle, and spleen). Means denoted by the same letter did not differ significantly ($p > 0.05$) while different letters (a and b) at the top of the bars indicate statistically significant difference ($p < 0.0001$) between tissues. Relative expression of *Ab*HAMP mRNA in three different tissues (liver, kidney, and spleen) (B) in response to *A. hydrophila*, *V. barveyi*, and *L. garvieae* challenge with two bacteria doses, 2×10^7 and 2×10^8 CFU/g BW, and (C) in response to iron-dextran injection with two concentrations, 0.2 mg and 1 mg/g BW, at 24 h post-injection (hpi). Data are expressed as mean ± SEM. Expression levels of *Ab*HAMP mRNA were normalized to those of 18s rRNA. Means denoted by "n.s." (no significance) or the asterisks (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$) at the top of the bars indicate statistically significant differences between control (PBS injection) and different stimuli (bacteria and iron-dextran injection). All statistical significances of *Ab*HAMP mRNA expression were determined by one-way ANOVA followed by Bonferroni's multiple range test. (D) The antibacterial activity of the *Ab*HAMP was tested by URDA method in the amount of 5 and 10 μg, and its activity was compared with 2 μg of piscidin-1.

not been clearly elucidated, there are two main models to explain the intron size variation in genome; 1) introns in highly expressed genes are substantially shorter than those in genes that are expressed at low levels [41] and 2) longer introns and long intergenic regions preferentially occur in tissue-specific genes because they allow chromatin-mediated gene suppression and complex regulation [42]. Accordingly, introns in hepcidins may potentially play a role in gene evolution and expression regulation. The *Ab*HAMP gene exhibits highly liver-predominant pattern of its basal expression and also its iron regulatory role takes place mainly in the liver, which is consistent with previous studies on expression pattern of hepcidins in tetrapods including mammals and amphibian [4,37,38]. The induced expression of *Ab*HAMP mRNAs by bacterial challenge was largely exclusive in the kidney which is known as an important hematopoietic organ for immunity in teleost [43]. Although a high increase in *Ab*HAMP expression in the kidney during infection was clearly evident, however, this seemingly highly increased *Ab*HAMP expression (around 60 times increase by 2 x 10^8 CFU/g BW *V. harveyi* infection) is relatively very minute compared to tremendously high basal expression observed in the liver. Apart from hepatocytes in liver that is the main organ of hepcidin production, other cell types in non-hepatic organs express hepcidin mRNA at a much lower level in mammals [44,45]. The explicit role of hepcidins produced in non-hepatic organs is still unclear, however, hepcidins could have a role in local regulation of iron fluxes and could be involved in the intervention of pathogenesis. The *Ab*HAMP mRNA expression in the kidney, therefore, might be related to control over local iron fluxes, leading to iron retention in the kidney, a major hematopoietic organ, which eventually limit iron mobilization and availability for pathogen growth in the early stages of infection. Therefore, gene organization and expression patterns observed with *Ab*HAMP implies that the chondrostean hepcidin can be regarded as an early version of hepcidin laid in evolutionary changeover toward the acquisition of novel function (iron-regulation) or the complementary loss of gene subfunction (antimicrobial activity) in tetrapod and teleost groups.

Comparison of the structure of hepcidins revealed that the *A. baerii* hepcidin protein share highly conserved features with tetrapod and fish hepcidins. The predicted mature *Ab*HAMP resulting from the cleavage of the RX(K/R)R motif by the propeptide convertase furin [46] contains the eight Cys residues that are likely to form one vicinal and three interstrand disulfide bonds, which are known to provide mature hepcidin the structural integrity, which is important for proper antimicrobial activity [47,48]. The predicted *p*I of mature *A. baerii* hepcidin was positively charged (*p*I = 8.51), similar to other hepcidins that exhibited antimicrobial activity *in vitro* [48,49]. Studies on truncated variants of human and trout hepcidins revealed the N-terminal AA sequence do not affect their antimicrobial activity [48,49], suggesting cationic property and conformation of disulfide bonds are important to their antimicrobial activity. With respect to the structural analyses for antimicrobial activity and transcriptional expression level analysis, the mature *Ab*HAMP is expected to exert an antibacterial activity against invading pathogens. However, while the heterologously produced mature *Ab*HAMP with four intramolecular disulfide bonds showed antibacterial activity against some bacteria, little or no *in vitro* antimicrobial activity was observed against bacterial strains that were used

in immune challenge. This is most likely related to the *in vitro* nature of the antibacterial activity assay. As mentioned above, hepcidin could get involved in the defense against pathogenic bacteria in an indirect manner, especially through the regulation of iron metabolism, suggesting demonstration of antibacterial activity *in vivo* [18,36]. Previous studies have demonstrated that N-terminal six residues prior to the first Cys residue in the mature hepcidin, DT(H/N)FPI- in human HAMP and mouse HAMP1 and QSHLSL-in zebrafish hepcidin, are essentially important for its iron regulatory activity [50,51]. The N-terminal QSHFPI-sequence of *Ab*HAMP articulates intermediate characteristics between teleostean/amphibian HAMP1s (i.e., QSH-) and amniote (human, mouse, and lizard) HAMPs (i.e., -FPI-). Study on three-dimensional modeling revealed mouse HAMP2 a considerable loss of electrostatic potential by two glutamic acid residues at the C-terminus (see the sequence of mouse HAMPs in Fig. 3), which can reflect the mechanism that two mouse hepcidins are similarly regulated by iron, nevertheless, mouse HAMP2 does not appear to have a role in iron metabolism [52]. Together with mRNA expression data, the structural comparison antibacterial activity assay demonstrates that *Ab*HAMP possesses dual functions: regulation of iron metabolism mainly in the liver like tetrapods and innate immune response in the kidney, a major immune-relevant organ in teleosts, although its iron-regulatory action and antimicrobial activity should be further validated in detail.

Phylogenetic relationship between chondrostean hepcidin with other vertebrate orthologs was addressed. A previous phylogenetic study has proposed that teleost hepcidins are classified into two clades (HAMP1 and HAMP2) – each teleost has only one copy of HAMP1 gene as an ortholog counterpart of mammalian HAMP, while the other multiple copies are always categorized into HAMP2 class [10]. More recently, another previous phylogenetic analysis has revisited that all teleost hepcidins could be resolved as a clade of a sister clade of tetrapod hepcidins [53]. Our phylogenetic analysis refined these two previous phylogenetic hypotheses, suggesting vertebrate hepcidins are divided into two major clades, tetrapod-like hepcidins and fish hepcidins. The tetrapod-like hepcidins include amphibian, reptile, and mammalian hepcidins with a phylogenetic affiliation to chondrostean hepcidins. The fish hepcidins (i.e., neopterygian hepcidins) are further subdivided into two clades, holostean hepcidins and teleostean hepcidins. Within a teleostean group, acanthopterygians have acquired additional HAMP2 copies in a taxa/lineage-dependent manner potentially through the positive Darwinian selection and/or gene duplications [39,40]. Taken together, phylogenetic analysis in this study suggests that sturgeon hepcidins reflect the form of an early hepcidin with dual functions, which might have diverged to extant tetrapod and fish (i.e., neopterygian) hepcidins. Because the prototypic form of early hepcidin with dual functions is identified from the chondrostean group that is regarded as the most basal clade in the actinopterygian lineage, the ancestral origin of hepcidin might be proposed to have emerged in early osteichthyans. Previously, it was hypothesized ancestral antimicrobial hepcidin-like gene would have been recruited for iron regulation first in mammals [52], which has been already challenged by considerable claims that many teleostean HAMP1s also possess potential iron regulatory function. However, from this study, the acquisition of hepcidin-mediated iron regulatory function in vertebrate lineage should be further dated back to no later than actinopterygian (ray-finned fish) –sarcopterygian (lobe-finned) split. To gain further insight into ancestral origin and early evolutionary history of hepcidin, deciphering hepcidin (and hepcidin-like) sequences from other extant primitive species in Vertebrata phylum including chondrichthyans and agnathans would be valuable in future study.

In this study, *Ab*HAMP gene was cloned, and its structural characteristics, expression profiles, and antibacterial activities were analyzed. The experimental data collectively demonstrate that *Ab*HAMP gene encodes potentially the first prototype hepcidin that has retained ancestral characteristics with unique structural motifs represented in tetrapod hepcidins and/or teleost hepcidins. Sequence characterizations in parallel to mRNA expression and antibacterial activity assays suggest *Ab*HAMP could be involved in both iron metabolism regulation and host immune system. Phylogenetic analysis suggests *Ab*HAMP occupies an evolutionarily intermediate position in the hepcidin evolution repertoire from fish to mammals. Collectively, the sturgeon hepcidins may reflect the early version of hepcidin carrying both iron regulatory activity and antimicrobial activity that might have evolved to present hepcidin forms found in both extant tetrapodian and neopterygian descendants. Results from this study provide useful information not only to bridge gaps in our knowledge on the early evolutionary repertoire of hepcidin in vertebrates but also to gain better insights into the innate immunity of this commercially important chondrostean fish species.

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Appendix A. Supplementary data

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