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Molecular characterization and expression analysis of hepc1 and hepc2 in three tilapia species collected from Lake Manzala

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Abstract

Background: Antimicrobial peptides were examined as an evolutionarily preserved component of the innate immune response and identified as a vital first-line defense against a broad spectrum of pathogens in fish. The objective of this study was to investigate the molecular characterization and expression analysis of hepc1 and hepc2 in three strains of tilapia species infected naturally in Lake Manzala.

Results: The results revealed that the alignment of nucleotide sequences including cDNA and deduced amino acid sequences showed that hepcidin 1 in *Sarotherodon galilaeus* has four genotypes due to SNPs in codon 34^{Gln} (CAG/Leu (CTG)) and codon 36^{Glu} (GAA/GAG). Regarding hepcidin 2 gene, different genotypes were detected in *Tilapia zillii* and *Sarotherodon galilaeus* due to SNPs in codons 19^{Met} (ATG/Ile (ATT)), 57^{Pro} (CCA/Ser (TCA)), and codon 14^{Leu} (CTT / CTC). Hepcidins 1 and 2 coding region sequences in three tilapia species deposited to GeneBank and phylogenetic analysis indicated that tilapia species are more similar to each other and closely related to Sea perch. On the other hand, the expression levels of hepc1 and hepc2 genes were over-expressed in different tilapia tissue species (hepc1 in *Sarotherodon galilaeus* and hepc2 in *Tilapia zillii*).

Conclusions: The results concluded that the hepcidin 1 and 2 genes showed constitutive expressions in most of tested tissues and have a very similar three-dimensional structure as well as mature peptides which mean that these genes are highly conserved within the species examined.

Keywords: Tilapia, RT-PCR, Sequencing, Antimicrobial peptides, Hepcidin

Introduction

Fish have developed several defense mechanisms against waterborne pathogens with one of the first lines of defense consisting relatively small peptides called antimicrobial peptides (AMPs) (Neves et al. 2015). Antimicrobial peptides are identified as a positively charged molecules with short amino acid chains which play crucial roles in host defense against pathogens through antimicrobial actions or immunomodulatory functions (Jenssen et al. 2006). Among these peptides, a cysteine-rich molecule isolated from human urine named hepcidin (Hep) is based on its hepatic expression and antibacterial effect in vitro (Falzacappa and Muckenthaler

2005). Hepcidin has a dual function as a key regulator of iron metabolism and as a defense peptide (Ganz 2003). Fish hepcidin was first identified and isolated from the hybrid striped bass (Hirono et al. 2005), and since then, hepcidins have been identified in at least 37 fish species (Masso-Silva and Diamond 2014). In fish and mammals, hepcidin genes consist of three exons and two introns which encode a pre-pro peptide, an acidic propiece, and a mature peptide (Douglas et al. 2003). The pre-pro peptides contain a furin cleavage site, and the mature peptide contains eight conserved cysteines and a conserved glycine residue (Asmamaw 2016).

Fish hepcidin are highly expressed in the liver and kidney while they are less expressed in the heart, brain, skin, intestine, blood cells, spleen, and stomach and unnoticeable in muscle (Asmamaw 2016). Earlier studies

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found that the expression of hepcidin is upregulated during infection, inflammation, and iron overload and predominantly reduced under certain conditions like anemia and hypoxia (Ganz 2007). The development of hepcidins are assorted in teleost fishes as it is influenced by the diversity of aquatic environments in which it is found (Lee et al. 2012). It has been found that the two hepcidin peptides identified in teleost fish show a degree of sub functionalization of its functions, where hepcidin 1 (HAMP1) more implicated in iron metabolism regulation and hepcidin 2 (HAMP2) mainly performing an antimicrobial role (Neves et al. 2015). The AMP production go through a main pathway by the recognition of pathogen-associated molecular patterns (PAMPs) through receptors (PRRs), such as Toll-like receptors (TLRs), exciting a cascade of intracellular signaling involving MyD88, TRAF6, IRAK1, and IKK that promotes the activation and translocation of NF- κ B to the nucleus where it transcriptionally stimulates AMPs and other effector molecules (Campoverdea et al. 2017).

In fact, the structures and sequences of hepcidin genes are highly preserved between mammals and fish (Hsieh et al. 2010). According to previous studies on different fish species, two types of hepcidins have been identified in turbot (*Scophthalmus maximus*), using Matras with approximately 50% sequence identity; the synthesized form of these peptides showed apparent bactericidal activities against both Gram-positive and Gram-negative bacteria in a dose-dependent manner (Jain et al. 2015). Concerning the analysis results of genomic characterization and gene expression of four hepcidin genes in the red-banded seabream (*Pagrus auriga*), it was apparent that although hepcidin antimicrobial peptides (HAMP) might play an important role against bacterial infections, it also has additional functions in iron regulation and embryogenesis in fish (Martin-Antonio et al. 2009). Former study by Pridgeon (2012) demonstrated relative transcriptional levels of AMHP, using quantitative polymerase chain reaction (QPCR), in response to *Edwardsiella ictaluri* infection. After identification and characterization of AMHP in *miuuy croaker*, Xu et al. (2012) concluded that the expression level of these peptides was highest in the liver and an upregulation of transcription was seen in the intestine, spleen, and kidney in response to pathogenic bacteria, *Vibrio anguillarum*, which implied that HAMP has a vital role as a first line of defense against invading pathogens.

The objective of this study was to investigate the molecular characterization and expression analysis of hepc1 and hepc2 in three strains of tilapia species infected naturally in Lake Manzala.

Materials and methods

One hundred and ten fishes were collected from Lake Manzala in Egypt.

a. Collection of samples

Tissue samples were obtained from normal and naturally infected fish in Lake Manzala. The selected tissues were gonads, spleen, head kidneys, and liver. The fish was kept on ice, while the tissues were kept in Hank's Balanced Salt Solution (HBSS), then they were homogenized and the PeqGold TriFast™ (or Trisure or Tripure) (stabilization solution) was added. After this addition, the RNA was not degraded by Rnase and the samples could be stored at -80°C indefinitely. Individual fish were dissected, and these samples were collected for bacteriological examination, and isolation of some types of bacteria was attempted according to Noga (1996).

b. RNA isolation and cDNA synthesis

Total RNA was isolated from several tissues (gills, kidney, liver, and spleen) of *Oreochromis niloticus*, *Sarotherodon galilaeus*, and *Tilapia zillii*. Tissue samples were separately ground quickly in liquid nitrogen and transferred to 1 mL TRIzol solution (cat#15596-026, Invitrogen, Germany) to isolate RNA following the manufacturer's instructions. RNA was quantified and checked for purity using a NanoDrop (Thermo Scientific, Waltham, MA, USA). Integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Extracted RNA was stored in a -80°C freezer until used as template for reverse transcription PCR (RT-PCR). One microliter of RNA isolated from several tissues was reverse transcribed into cDNA in a total volume of 20 μl using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) according to the manufacturer's instructions.

c. Primer design and PCR optimization

The specific primers for the antimicrobial peptide genes were designed using known sequences of Teleost AMPs using the methodology of the homology cloning. Eleven pairs of primers were designed using the primer3 online software (<http://frodo.wi.mit.edu/primer3/>), based on the NCBI reference sequences. Accession numbers of the genes for which the primers are designed are listed in Table 1. They are aimed to amplify fish antimicrobial peptides messenger RNAs (mRNAs) using PCR. All the primers pairs were designed to amplify full coding sequence of the target antimicrobial peptide mRNA. The designed primers to amplify hepcidin mRNA in *Oreochromis niloticus*, *Tilapia zillii*, and *Sarotherodon galilaeus* based on the conserved region in hepcidin mRNA of bony fish. The primers were synthesized by HVD life sciences GMBH, Germany. Designed

Table 1 The designed primer sequences used for PCR amplification and RT-PCR in tilapia species

Gene name	Forward sequence	Sequence and RT-PCR	NCBI reference sequence
Hepcidin 1	5'-ACCAGGAGAAAGAGTCAAAC-3' 5'-ACAGTATAATCTCACACATTGTC-3'	Sequence	XM_003456839.1
Hepcidin 2	5'-TCTTCAAAGGGTACAACAAC-3' 5'-AGAAAGAGTCAGACAATAAGC-3'	Sequence	XM_003450530.4
Hepcidin 1	5'-CAG CAG CAC ATG AGG AGG CAT-3' 5'-ACC TGC AGC AAA CTC CAC AGA-3'	RT-PCR	MH651353.1
Hepcidin 2	5'-AGA GCA ATG ACA CTC CAG TTG-3' 5'-AGA TGG CTC TGA CGC TTT TG-3'	RT-PCR	MH651359.1
β-Actin	5'-CGG AAT CCA CGA AAC CAC CTA-3' 5'-TTG CTG ATC CAC ATC TGC TGG-3'	RT-PCR	AB037865.1

primers were tested in order to determine its optimum annealing temperature that amplifies the target cDNA segment.

d. Polymerase chain reaction (PCR) amplification

PCR amplification of cDNA was performed according to the method described by Ausubel et al. (1990). The optimized PCR cycles were differed according to the gene under study. The PCR cycle started with initial denaturation of 94 °C for 5 min, then followed by 35 cycles (three steps) as: 94 °C for 30 s, annealing temperature for 45 s. with variable temperatures according to the gene under study, then extension at 72 °C for 1 min and finally one step for final extension 72 °C for 5 min. The annealing temperatures were as follows: hepcidin 1 (53 °C) and hepcidin 2 (55 °C). For the PCR optimization and detection of the PCR success, some of the PCR products were subject to run horizontally on agarose electrophoresis and stained with ethidium bromide.

e. Sequencing, alignment analysis and amino acid translation

Prior to sending the PCR products to the company for sequencing, the PCR products were purified using GeneJET *Gel Extraction Kit* from Thermo scientific Company according to the manufacturer's instructions. PCR products from two genes (hepcidin 1 and hepcidin 2) of three tilapia fish species were sent for sequencing in Macrogen Company (South Korea).

Statistical analysis

Sequence analysis was performed by multiple alignments using CLUSTAL-W program (Thompson et al. 1994). Protein translations of CDs sequences were carried out using open reading frame finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) while modeling of hepcidin protein was determined by Phyre2 software (<http://www.sbg.bio.ic.ac.uk/phyre2/>). For the analysis of potential cleavage site of the signal peptide, SignalP Software was used

(<http://www.cbs.dtu.dk/services/SignalP/>). The alignment of the amino acid sequences of hepcidin proteins was done using Clustal X. The phylogenetic tree was drawn by the neighbor-joining method (Saitou and Nei 1987) and analyzed with MEGA-3 (Kumar et al. 2018).

Quantitative evaluation of hepcidin 1 and 2 mRNA by real-time PCR (qPCR)

StepOne™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to determine the tilapia's cDNA copy number. Sequencing results of different hepcidin genes were used to design gene specific primers, for qPCR, by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). β-Actin gene used as internal control to correct for differences in total cDNA input between samples. PCR reactions were set up in 25 μL reaction mixtures containing 12.5 μL × 1 SYBR® Premix Ex Taq™ (TaKaRa, Biotech. Co. Ltd.), 0.5 μL 0.2 μM sense primer, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water, and 5 μL of cDNA template. The qPCR conditions were 95.0 °C for 10 min followed by 40 cycles of 95.0 °C for 15 s and 60.0 °C for 1 min. The relative amount of the genes of interest was calculated by the comparative threshold cycle (CT) method with efficiency correction, using the mean PCR efficiency for each amplicon (Ruijter et al. 2009). The sequences of QPCR primers are listed in Table 1.

Results

Naturally infected tilapia

Three strains of fish including *O. niloticus*, *T. zillii*, and *S. galilaeus* were naturally infected with bacteria in the Lake Manzala. The *O. niloticus* strain was infected with *Aeromonas hydrophila* but *T. zillii* and *S. galilaeus* were infected by *Pseudomonas luteola*.

Polymerase chain reaction (PCR)

Hepcidin 1 gene-specific primers amplified a product of 264 bp and hepcidin 2 gene-specific primers amplified a product of 273 bp (Fig. 1).

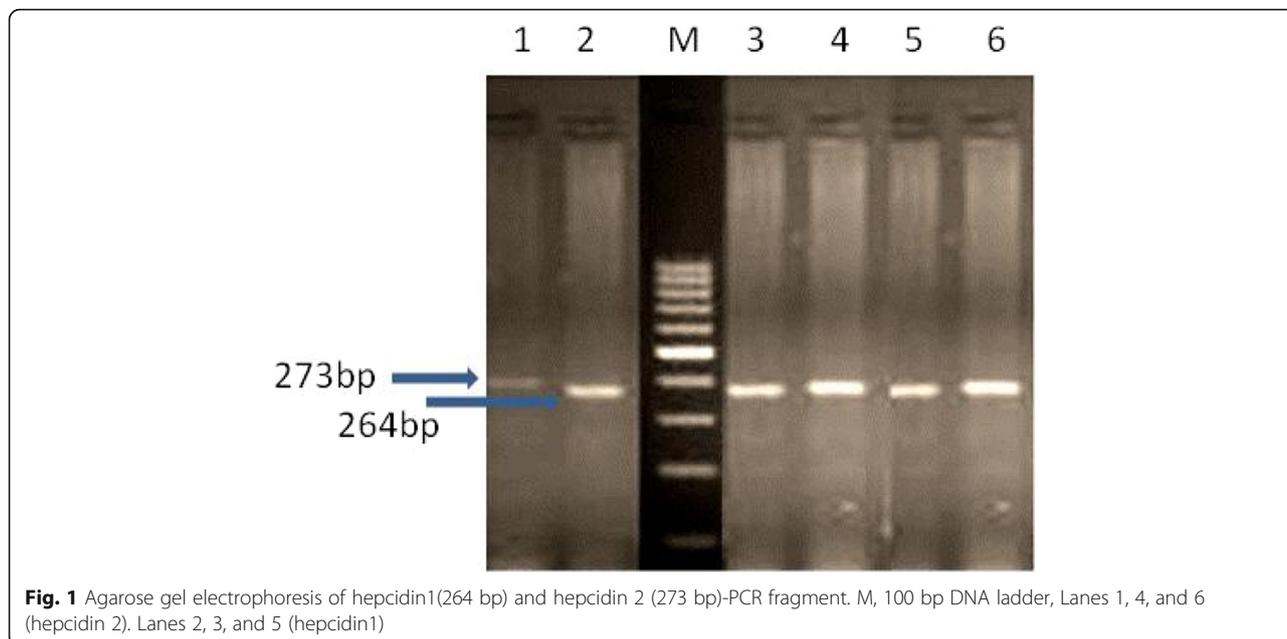


Fig. 1 Agarose gel electrophoresis of hepcidin1(264 bp) and hepcidin 2 (273 bp)-PCR fragment. M, 100 bp DNA ladder, Lanes 1, 4, and 6 (hepcidin 2). Lanes 2, 3, and 5 (hepcidin1)

Characterization of hepcidin 1 coding region sequences in *O. niloticus*, *T. zillii*, and *S. galilaeus*

The *O. niloticus*, *T. zillii*, and *S. galilaeus* hepcidin 1 coding region (CDs) were sequenced, and their sequences have been deposited to GeneBank with accession numbers of (MH651353, MH651354, and MH651358), respectively. CD regions of *T. zillii* and *S. galilaeus* have the same length (264 bp) while *O. niloticus* has 258-bp CD length with a deletion portion (AGCAGG) when aligned with *T. zillii* and *S. galilaeus* CDs (Fig. 2). Moreover, two SNPs are determined among these species at nt 36 (C/G) and at nt 240 (A/C).

Four genotypes are determined in *S. galilaeus* species due to the presence of SNPs at nt 101 W(A/T) and nt 108 R (G/A) while no different genotypes are detected in *O. niloticus* and *T. zillii* species (Fig. 2).

The deduced amino acid sequences for hepcidin 1 CDs of *T. zillii* and *S. galilaeus* consists of 87 amino acids. One SNP at nt 101 which presents in codon 34 (CAG/CTG) in *S. galilaeus* species is translated glutamine (Q) into leucine (L) while the other SNP at nt 108 which correspond to codon 36 (GAA/GAG) is silent mutation as in Fig. 2a, b. On the other hand, the amino acid sequence for hepcidin 1 CD of *O. niloticus* species



Fig. 2 Alignment of CD regions of *O. niloticus*, *T. zillii*, and *S. galilaeus* species shows two SNPs at nt 36 (C/G) and at nt 240 (A/C) in red box and deletion portion of *O. niloticus* species in bold and underlined while different genotypes of *S. galilaeus* species in blue box



Fig. 4 Alignment of deduced amino acid sequences of *O. niloticus*, *T. zillii*, and *S. galilaeus* hepcidin 1 gene with those of turbot_S.maximu (AY994074), *Homo sapiens* (BC020612), white bass (AAM28440), Atlantic salmon (BI468191), Canis lupus familiaris (AY899807), *Mus musculus* (BC021587), black seabream (AAU00801), red seabream (AAR28076), pig (NP_999282), sea perch (AAT09138), Japanese flounder (BAE06234), winter flounder (AW013026), channel catfish (AAX39713), blue catfish (AAX39714), and zebrafish (P61516)

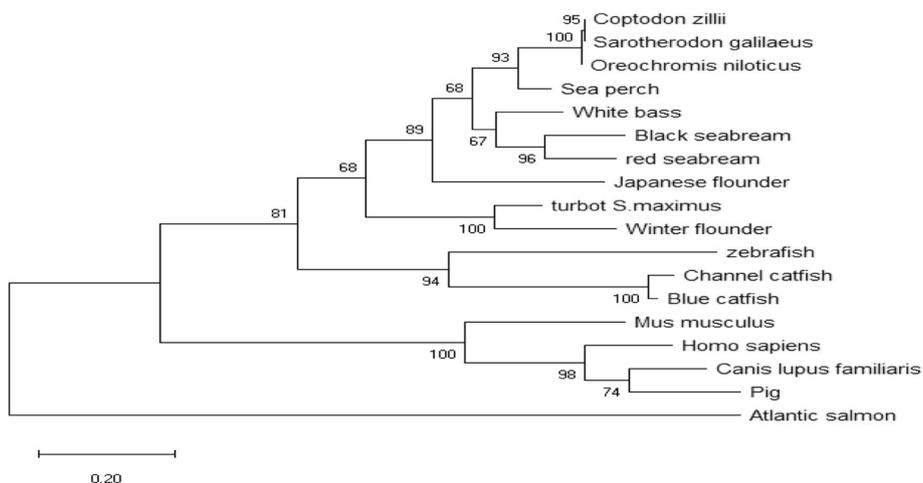


Fig. 5 Phylogenetic analysis of *O. niloticus*, *T. zillii*, and *S. galilaeus* hepcidin 1 gene and other species. Constructions are formed on the basis of the homology sequences calculated from the complete amino acid sequence of hepcidin genes. The tree is constructed using a neighbor-joining method. The scale is 0.20 which refers to percentage of divergence

<i>O. niloticus</i>	TCTTCAAGGGTACAACTTAA <u>CCATTTAAACCCCGACCAAATGAAGGCATT</u> CAGCA	60
<i>S. galilaeus</i>	TCTTCAAGGGTACAACTTAA <u>CCATTTAAACCCCGACCAAATGAAGGCATT</u> CAGCA	60
<i>T. zillii</i>	TCTTCAAGGGTACAACTTAA <u>CCATTTAAACCCCGACCAAATGAAGGCATT</u> CAGCA	60
<i>O. niloticus</i>	TTGCAGTTGCAGTGCACACTCGTGCCTGSCCTTTATTTCATGCTGGAGAGCCTCTGCCATCC	120
<i>S. galilaeus</i>	TTGCAGTTGCAGTGCACACTCGTGCCTGSCCTTTATTTCATGCTGGAGAGCCTCTGCCATCC	120
<i>T. zillii</i>	TTGCAGTTGCAGTGCACACTCGTGCCTGSCCTTTATTTCATGCTGGAGAGCCTCTGCCATCC	120
<i>O. niloticus</i>	CATTTCCTGGGGTGCAGAGCCGAGGAGGCAGGGAGCAATGCACTCCAGTTGTGGCC	180
<i>S. galilaeus</i>	CATTTCCTGGGGTGCAGAGCCGAGGAGGCAGGGAGCAATGCACTCCAGTTGTGGCC	180
<i>T. zillii</i>	CATTTCCTGGGGTGCAGAGCCGAGGAGGCAGGGAGCAATGCACTCCAGTTGTGGCC	180
<i>O. niloticus</i>	ATCAAGAGATGTCAGCGGAGTCAATCAATGATGCAAAATCACATCAGGCAAAGCGTCA	240
<i>S. galilaeus</i>	ATCAAGAGATGTCAGCGGAGTCAATCAATGATGCAAAATCACATCAGGCAAAGCGTCA	240
<i>T. zillii</i>	ATCAAGAGATGTCAGCGGAGTCAATCAATGATGCAAAATCACATCAGGCAAAGCGTCA	240
<i>O. niloticus</i>	GCCATCTTTCCTTGTGCGCTGGTGTGCAATTGCTGCGGAAACAAAGGGCTGCGGCT	300
<i>S. galilaeus</i>	GCCATCTTTCCTTGTGCGCTGGTGTGCAATTGCTGCGGAAACAAAGGGCTGCGGCT	300
<i>T. zillii</i>	GCCATCTTTCCTTGTGCGCTGGTGTGCAATTGCTGCGGAAACAAAGGGCTGCGGCT	300
<i>O. niloticus</i>	TCTGCTCAGGTTCTAGGATTCCCGCAACAGCCAAAATATTAATTTATTATGCTTAT	360
<i>S. galilaeus</i>	TCTGCTCAGGTTCTAGGATTCCCGCAACAGCCAAAATATTAATTTATTATGCTTAT	360
<i>T. zillii</i>	TCTGCTCAGGTTCTAGGATTCCCGCAACAGCCAAAATATTAATTTATTATGCTTAT	360
<i>O. niloticus</i>	TGCTGACTCTTTCT	375
<i>S. galilaeus</i>	TGCTGACTCTTTCT	375
<i>T. zillii</i>	TGCTGACTCTTTCT	375

Fig. 6 cDNA sequence of hepcidin 2 gene. The coding regions start codon ATG and termination codon TGA are both bold and underlined. The alignment of nucleotide sequences of *O. niloticus*, *T. zillii*, and *S. galilaeus* shows two SNPs at nt 252 (T/C) and nt 310 (G/A) (in red box) and different genotypes for each species (in blue box)

genotype detected for *O. niloticus* has methionine (M) at codon 19 (ATG) as in Fig. 7e.

Sequence alignment and phylogenetic analysis

The deduced amino acid sequences of hepcidin 2 in *O. niloticus*, *T. zillii*, and *S. galilaeus* have been aligned with protein sequences of turbot_S.maximus (AY994074), *Homo sapiens* (BC0 C0 20612), white bass (AAM28440), Atlantic salmon (BI468191), *Canis lupus familiaris* (AY 89980897), *Mus musculus* (BC021587), black seabream (AAU00801), red seabream (AAR28076), pig (N P_ P_999282), sea perch (AAT09138), Japanese flounder (BAE06234), winter flounder (AW W1013026), channel catfish (AAX39713), Blue catfish (AAX39714), zebrafish (P61516) as in Fig. 8. It is obvious that *O. niloticus*, *T. zillii*, and *S. galilaeus* hepcidin 2 gene is more similar to each other and closely related to sea perch than other species (Fig. 9).

Structural model analysis of hepcidin 1 and hepcidin 2 and signal peptide detection

Three-dimensional structure of hepcidin 1 and hepcidin 2 shows it to be an amphipathic molecule composed of two distorted antiparallel β -sheets separated by a hairpin loop, containing a vicinal disulfide bond (disulfide bond between adjacent cysteines), and stabilized by three inter- β -sheet disulfide bonds which remarkably similar

among *O. niloticus*, *T. zillii*, and *S. galilaeus* species as shown in Fig. 10a and b. On the other hand, the signal peptide is detected from 1 to 24 amino acids with high probability (0.9753 and 0.9968, respectively) and mature protein starts at position 25 amino acid (Fig. 11a, b).

Gene expression results

Expression of antimicrobial peptide gene hepcidin 1 (H1) in *O. niloticus*, *T. zillii*, and *S. galilaeus* naturally infected with bacteria in the Lake Manzala

The expression levels of the antimicrobial peptide gene hepcidin 1 (H1) in several tissues of *O. niloticus*, *T. zillii*, and *S. galilaeus* were quantified by real-time RT-PCR (Fig. 12). The results revealed that expression levels of h1 gene were increased in tissues of *S. galilaeus* compared with those of *O. niloticus* and *T. zillii*. Moreover, the lowest expression levels of h1 gene were observed in tissues of the *T. zillii*. Additionally, the highest expression levels were observed in the gills and spleen tissues of all fish strains compared with kidney and liver tissues.

Expression of antimicrobial peptide gene hepcidin 2 (H2) in *O. niloticus*, *T. zillii*, and *S. galilaeus* naturally infected with bacteria in the Lake Manzala

The expression levels of the antimicrobial peptide gene hepcidin 2 (H2) in several tissues of *O. niloticus*, *T. zillii*, and *S. galilaeus* were quantified by real-time RT-PCR

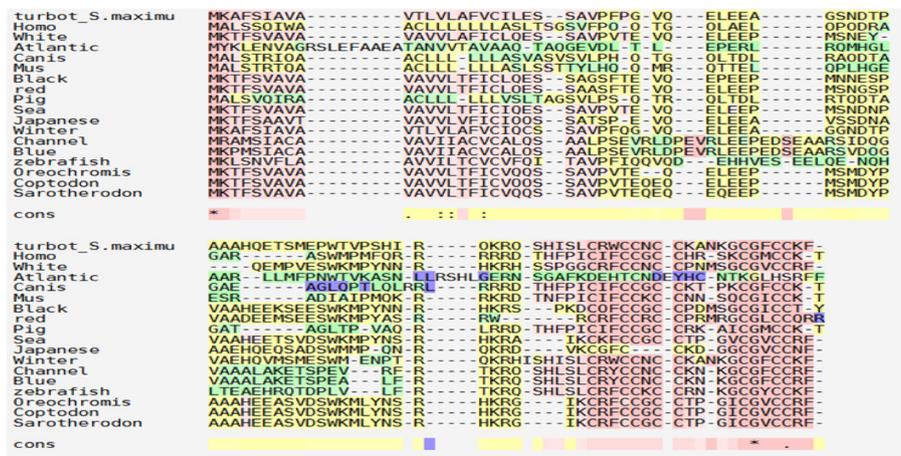


Fig. 8 Alignment of deduced amino acid sequences of *O. niloticus*, *T. zillii*, and *S. galilaeus* hepcidin 2 gene with those of *turbot_S.maximus* (AY994074), *Homo sapiens* (BC020612), white bass (AAM28440), Atlantic salmon (BI468191), *Canis lupus familiaris* (AY899807), *mus musculus* (BC021587), black seabream (AAU00801), red seabream (AAR28076), pig (NP_999282), sea perch (AAT09138), Japanese flounder (BAE06234), winter flounder (AW013026), channel catfish(AAX39713), blue catfish (AAX39714), and zebrafish (P61516)

represented identity > 50% and > 80% (Bian et al. 2019). Parallel to our results, a study by Bao et al. (2006) reported that the sequence alignment of channel and blue catfish hepc2 differ in two amino acids at two positions: (a) at 28, the channel catfish hepc2 has a proline residue while the blue catfish hepc2 has a serine residue, (b) at 32, the channel catfish hepc2 has an arginine residue while the Blue catfish hepc2 has a proline residue. The two hepc2 cDNAs of channel and blue catfish had 25 single-nucleotide polymorphisms. So far, Neves et al. (2015) detected high number of SNPs in the sequence of mature

peptide (~ 7 SNPs) compared with the remainder of prepropeptide (only 1 SNP) of hamp2 gene in sea bass. Furthermore, alignment of the two types of hepcidin in sea bass exhibited differences in the amino acids between both gene sequences of Hamp1 and Hamp2. However, the sequence of the mature peptides exhibited unchanged eight cysteine residues sequences at the conserved positions.

The action mechanism of how sequence differences in the SNPs formation is that it could be possible during life generation of the fish, various type of deletion or insertion might be occurred in the introns of the hepcidin

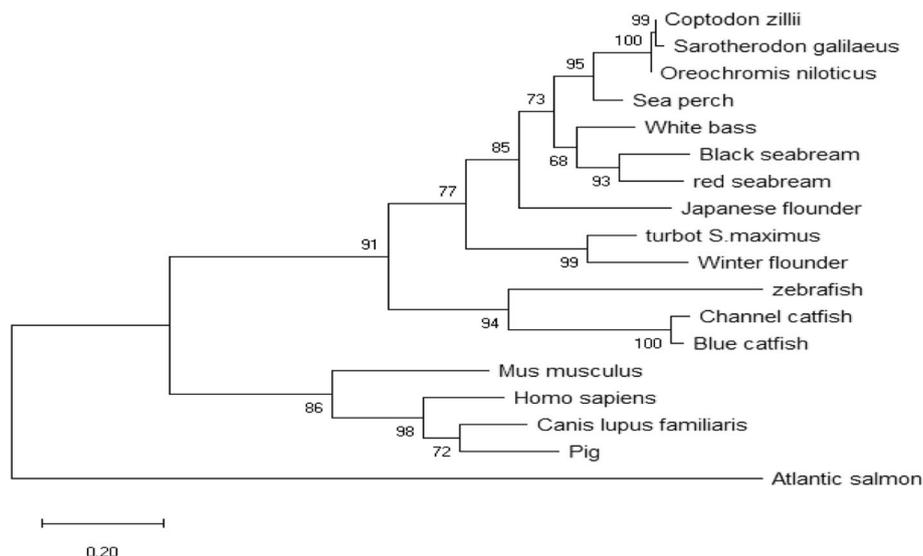
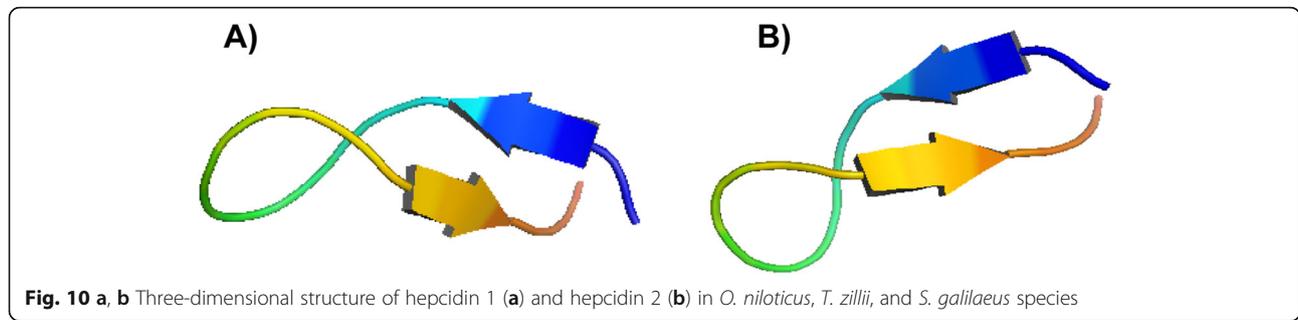


Fig. 9 Phylogenetic analysis of *O. niloticus*, *T. zillii*, *S. galilaeus* hepcidin 2 gene and other species. Constructions are formed on the basis of the homology sequences calculated from the complete amino acid sequence of hepcidin genes. The tree is constructed using a neighbor-joining method. The scale is 0.20 which refers to percentage of divergence

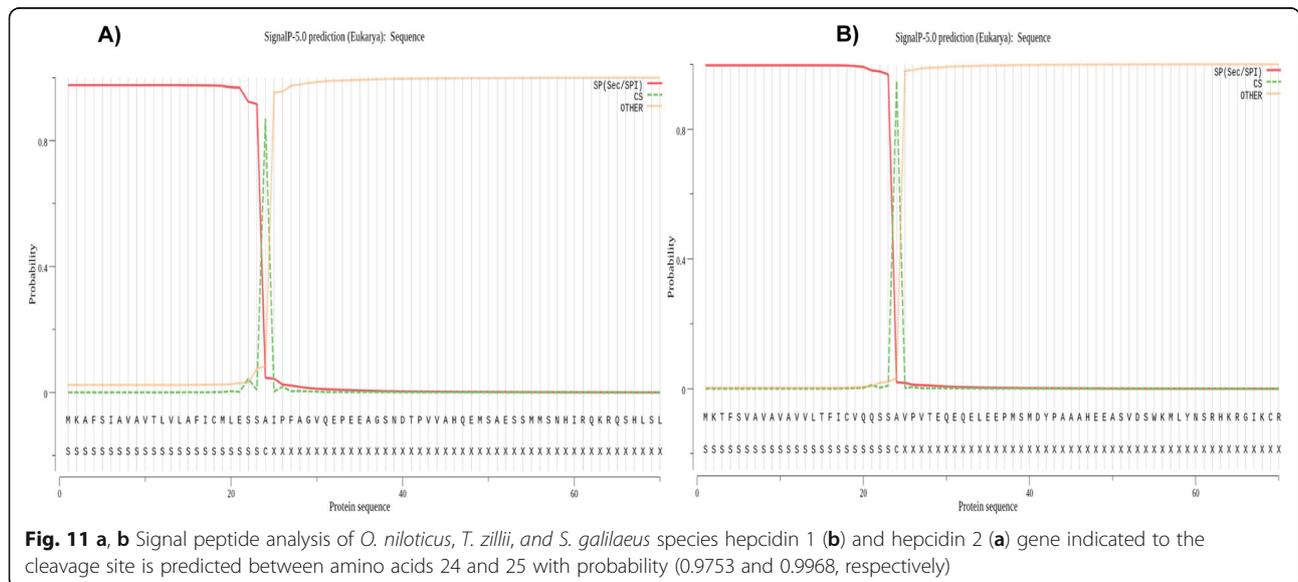


sequences. However, molecular analysis in fish exhibited that a slight differences are occurred because the sequences of hamp1 and hamp2 genes are remain conserved.

The deduced amino acid sequences for hepcidin 1 CDs of *T. zillii* and *S. galilaeus* consists of 87 amino acids for *O. niloticus* 85 aa due to deletion portion in mature peptide while those for hepcidin 2 CDs encodes a peptide of 90aa in three tilapia species. Previous work by Bao et al. (2006) reported that hepcidin 2 of the blue catfish and channel catfish shared similar features as it encodes a peptide of 94 aa. The results of numerous studies conducted by Wang et al. (2012), Hu et al. (2007), and Shi and Camus (2005) showed that a hepcidin of 78 aa and 96aa were detected in *Tor putitora*, *Ictalurus punctatus*, *Cynoglossus semilaevis*, respectively. As well as, results by Neves et al. (2015) detected that hepc1 and hepc2 genes of teleost fish encode 91aa and 85aa, respectively. The miyu croaker HAMP was predicted to encode of 89 amino acids (Xu et al. 2012) which in consistent with results of Silva et al. (2011),

where *A. immaculatus* prepro hepcidin is composed of 90 aa. It was concluded that the conserved functions of fish hepcidin is due to the conservation of their mature peptide.

Phylogenetic analysis indicated that translated protein of hepcidins CDs of the species examined are organized as clusters and very related to sea perch while so far from two or more hepcidins of winter flounder, Atlantic salmon, and mouse. Lauth et al. (2005) had shown that fish hepcidins in bass and tilapia species were characterized by cysteine-rich peptides and a central model of four conserved disulfide bridges. In addition, a study by Huang et al. (2007) revealed that tilapia hepcidin (2–3) situated in a group with Japanese flounder hepcidin-JF1, 2 and tilapia hepcidin 2–2 while tilapia hepcidin1–5 is closest to white bass and black porgy which suggest that the three tilapia hepcidins may have different functions. Based on results recorded by Silva et al. (2011), HAMP-1of *A. Immaculatus* had a sequence similarity of 96% with Serranid fish and *Epinephelus moara* and grouped with *A. immaculatus* in the same cluster. According to



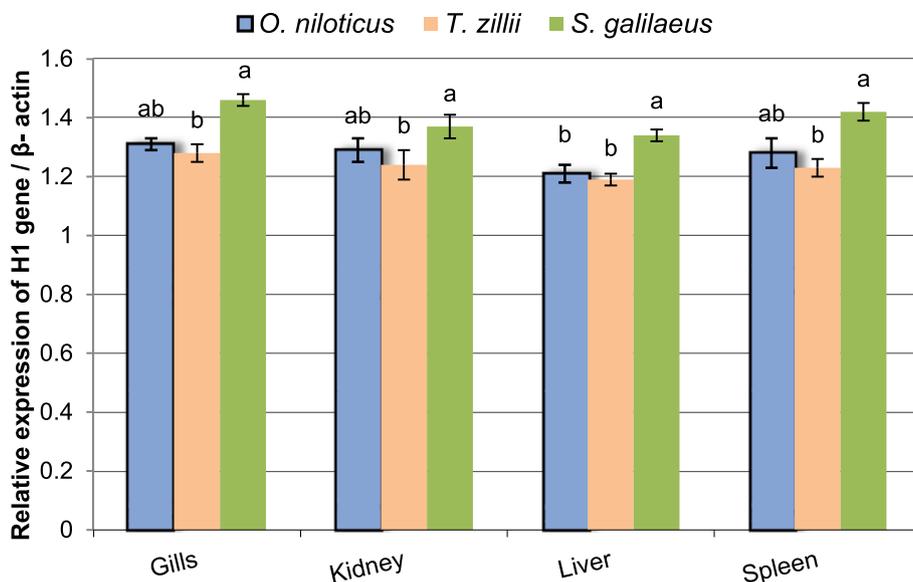


Fig. 12 Expression levels of some antimicrobial peptide gene hepcidin 1 (H1) in different tissues of *O. niloticus*, *T. zillii*, and *S. galilaeus*. Data are presented as mean \pm SEM. ^{a,b} followed by different superscripts are significantly different ($P \leq 0.05$)

results reported by Neves et al. (2015), phylogenetic analysis cluster fish hepcidins 1& 2 of Perciformes and Pleuronectiformes together separated from other vertebrate hepcidins. Additionally, Campoverde et al. (2017) found that the phylogenetic analysis revealed that meager hep2 sequence was more similar to that of *Larimichthys crocea*. Even though, there are antithesis opinions suggesting that progression and divergence of hepcidin antimicrobial peptide is connected with their surrounding habitats and pathogens.

This study reported that the three-dimensional structure of hepcidin 1 and hepcidin 2 shows it to be an amphipathic molecule composed of two distorted anti-parallel β -sheets separated by a hairpin loop, containing a vicinal disulfide bond (disulfide bond between adjacent cysteines), and stabilized by three inter- β -sheet disulfide bonds and signal peptide is detected from 1 to 4 amino acids which remarkably similar among *O. niloticus*, *T. zillii*, and *S. galilaeus* species. Xu et al. (2008) demonstrated that lower vertebrates have very similar

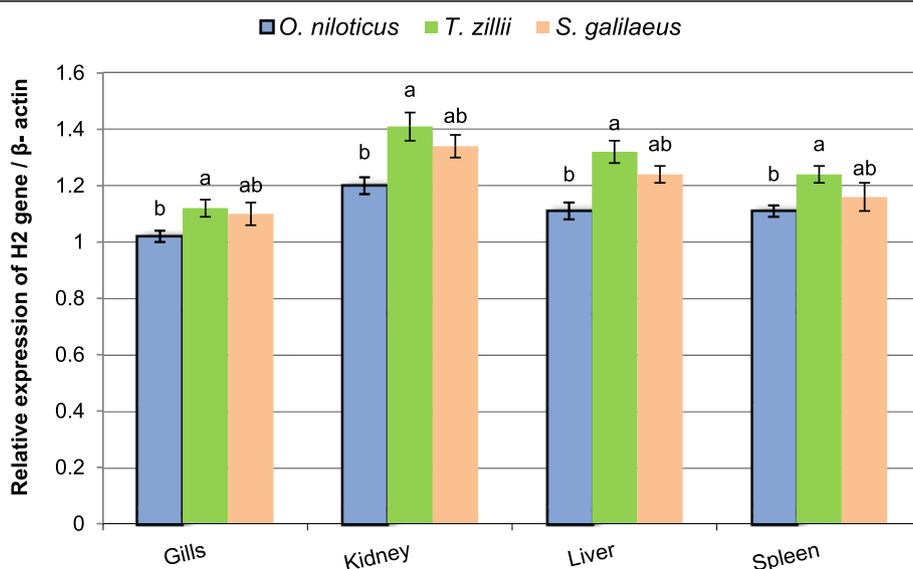


Fig. 13 Expression levels of some antimicrobial peptide gene hepcidin 2 (H2) in different tissues of *O. niloticus*, *T. zillii* and *S. galilaeus*. Data are presented as mean \pm SEM. ^{a,b} followed by different superscripts are significantly different ($P \leq 0.05$)

structures of hepcidin homologs (20-25aa) with eight highly conserved cysteines and four intramolecular disulfide bonds, giving hepcidin a hairpin structure. Tilapia fish *Oreochromis mossambicus* (Huang et al. 2007) and *schizothorax richardsonii* (Gray) (Chaturvedi et al. 2014) presented the same results. Previous studies by Zhang et al. (2009), Wang et al. (2010), and Chen et al. (2009) demonstrated that hepcidin hairpin structure with cationic residual act against fungi, bacteria, tumor cells, and viruses. Hepcidin hairpin-like structure are considered to be essential for its activity (Neves et al. 2015).

Regarding gene expression results, this study found that expression levels of H1 and H2 genes were increased in gills, kidney, and liver tissues of *O. niloticus*, *T. zillii*, and *S. galilaeus* naturally infected with bacteria in the Lake Manzala. However, the expression levels of H1 and H2 genes were decreased in spleen tissues. In the same line with our findings, several studies found that in many fish species and other vertebrates (Krause et al. 2000; Park et al. 2001; Zhou et al. 2011; Li et al. 2013; Chen et al. 2007; Rodrigues et al. 2006), both sea bass H1 and H2 genes were abundantly expressed in the liver. Additionally, they also reported that the expression levels of H1 and H2 genes were very low in the spleen. Hepcidin expression has been reported to have an important role in macrophages, lymphocytes, and monocytes but further analysis will be required to determine the exact cells responsible for this expression (Costa et al. 2011; Sow et al. 2007; Zhang and Rovin 2010; Pinto et al. 2010). The higher antimicrobial potential of H1 and H2 genes were observed in this study with over-expression in gills. That could be explained because these tissues are in constant contact with the surrounding environment, many times a doorway to microbial infection.

The present study found that strain of fish including *O. niloticus*, *T. zillii*, and *S. galilaeus* were naturally infected with bacteria in the Lake Manzala. The *O. niloticus* strain was infected with *Aeromonas hydrophila* but *T. zillii* and *S. galilaeus* were infected by *Pseudomonas luteola*. Several studies reported that both H1 and H2 genes were upregulated in fish with bacterial infections (Hirono et al. 2005; Huang et al. 2007). A significant induction of both sea bass H1 and H2 gene types was observed after experimental bacterial challenge with *P. damsela* spp. *piscicida*.

Additionally, it has been reported that H1 and H2 genes may have antimicrobial activity even so the initial pathogen density is very low (4.3 × 10³ CFU/ml) (Zhang et al. 2014). Moreover, H1 and H2 peptides manifested strong antimicrobial activities against Gram-positive bacteria. Further studies will be required to better understand the relevance of each type of hepcidin genes and to gain insight into their antimicrobial properties.

Conclusions

The results concluded that the hepcidin 1 and 2 genes showed constitutive expressions in most of tested tissues and have a very similar three-dimensional structure as well as mature peptides which mean that these genes are highly conserved within the species examined.

Abbreviations

Hepc1: Hepcidin 1; Hepc2: Hepcidin 2; *T. zillii*: *Tilapia zillii*; *S. galilaeus*: *Sarotherodon galilaeus*; *O. niloticus*: *Oreochromis niloticus*; HAMP1: Hepcidin Antimicrobial peptide 1; HAMP2: Hepcidin Antimicrobial peptide 2; cDNA: Complementary DNA; AMPs: Antimicrobial peptides; β-actin: Beta actin; CD: Coding region; aa: Amino acid; mRNA: Messenger RNA; RT-PCR: Real time –polymerase chain reaction

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Authors' contributions

KFM contributed her idea, designed the experiments, co-wrote the paper, and supervised the research. HAMAK performed the experiments and co-wrote the paper. DMM performed the experiments and co-wrote the paper. MMA designed the primers and performed the experiments. NMO performed the bioinformatic and statistical analyses. WKB Khalil performed the experiments and co-wrote the paper. Hassanane M. S supervised the research. All authors contributed to the study design, laboratory analysis, and writing of the manuscript. All authors revised the final manuscript and approved it.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article

Ethics approval and consent to participate

The study was approved by the ethical committee of the National Research Centre

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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