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Identification and characterization of matrix metalloproteinase-13 sequence structure and expression during embryogenesis and infection in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

Matrix metalloproteinase-13 (MMP-13), referred to as collagenase-3, is a proteolytic enzyme that plays a key role in degradation and remodelling of host extracellular matrix proteins. The objective of this study was to characterize the MMP-13 gene in channel catfish, and to determine its pattern of expression in various healthy tissues and during embryogenesis. Since MMP-13 has been shown to have importance in tissue remodelling and some pathological processes, we further studied its involvement in the defense responses of catfish after bacterial infection. The channel catfish MMP-13 cDNA contains an open reading frame of 1416 bp encoding 471 amino acids. Using RT-PCR analysis, MMP-13 was widely expressed in various health tissues. Using quantitative real-time PCR analysis, expression of MMP-13 gene was up-regulated by bacterial infection. During normal embryological development, MMP-13 expression was slightly increased in the first day post-fertilization and sharply up-regulated from 1-day post-fertilization through hatching.

1. Introduction

Matrix metalloproteinases (MMPs), also called matrixins, are a large family of zinc-dependent endoproteinases. The main function of MMPs is to degrade and turnover extracellular matrix, the important cellular environment that supports and directs tissue remodelling, development, and morphogenesis [1]. MMPs not only act upon extracellular matrix, but also on non-matrix proteins such as cytokines, chemokines, and antimicrobial peptides [2]. MMPs are also extracellular enzymes that play a role in regulating cell–matrix and cell-to-cell signalling events [3,4].

MMPs are involved in numerous physiological and pathological processes, such as development, wound healing, inflammation, cell invasion, angiogenesis, and immune surveillance [5–9]. MMPs have been demonstrated to be involved in various inflammatory processes, such as regulating physical barriers, cytokines and chemokines, and further downstream leukocyte migration [10]. Specifically, MMPs participate in re-epithelialization, resolution of scar formation, and inflammation by loosening the cell–cell and cell–extracellular matrix contacts at the wound margin, activating chemokines, establishing the chemotactic gradient, extravasating the leukocytes out of the blood to the injury, degrading existing collagen fibrils, and synthesizing new fibrils [6].

MMPs, therefore, are further involved in regulation of the host immune system. A first-line of host defense against the environment is the epithelium. The epithelium acts as a barrier to the external environment, regulates inflammation, and secretes antimicrobial peptides [11]. A critical process in innate immunity includes the repair of epithelium. Many MMPs are associated with the repair of epithelium [12–14]. A few members have been demonstrated to have an indirect role in removal of bacteria. For instance, MMP-7 deficient mice have an impaired ability to clear enteric pathogens, such as *Escherichia coli* and *Salmonella typhimurium* [15].

The MMPs also play an important role in development. Zebrafish lacking specific MMPs such as MMP-2, MT1-MMP, or

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MMP-13, through embryogenesis developed morphological abnormalities during somitogenesis, organ development, and tissue architecture [16–18]. In mice, a few MMPs were found to be critical during bone and vascular remodelling as well as mammary development [19–22]. A recent report on *Tribolium* demonstrated that knock-down of certain MMPs resulted in larvae with tracheal defects and abnormal intestines [23].

Currently, 25 members of the MMP family have been reported in vertebrates [2]. All MMPs share three common domains: the predomain, which is necessary for secretion; the prodomain, which regulates the molecule's function; and the catalytic domain containing the zinc-binding site, which is responsible for substrate processing [7]. MMPs are synthesized as zymogens. After the removal of the propeptide to reveal the zinc-binding site, MMPs are activated. Based on their substrate specificity and domain organization, MMPs are classified into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and a few other ungrouped members [1].

MMP-13 is the third member in the collagenase subfamily of proteases. While MMP-13 preferentially hydrolyzes collagen type II, it has wide substrate specificity, allowing it to degrade several other forms of collagen and other extracellular matrix components.

MMP-13 is also referred to as collagenase-3, where the numbering was based on the order in which it was identified. MMP-13 was first recognized and cloned from human breast carcinoma [24]. Subsequent work showed that MMP-13 was expressed in chondrocytes of human cartilage [25]. As a collagenase-type MMP, MMP-13 hydrolyzes collagen into N-terminal and C-terminal polypeptide fragments [25,26]. MMP-13 was also found to be expressed in osteoblasts and periosteal cells during human fetal ossification [27]. Hence, MMP-13 plays an important role in the process of bone formation and remodelling during development [28], and normal remodelling of bone and cartilage during skeletal repair [29]. MMP-13 has been further shown to be expressed in various pathological conditions [26,30], during wound healing [31], and bacterial infections [32,33].

In teleosts, only limited information on the biological function of MMP-13 is available. The gene structure was identified and found capable of degrading type I collagen in rainbow trout [34]. In zebrafish, MMP-13 is required for normal embryogenesis [15]. MMP-13 expression varied during zebrafish development, with peak expression at 48 h post-fertilization. In Japanese flounder, MMP-13 was up-regulated during the course of *Edwardsiella tarda* infection [35]. No information is available on the MMP-13 gene and its function for channel catfish *Ictalurus punctatus*, the predominant aquaculture species in the United States (USDA-NASS, 2008). In this study, we identified and characterized a complete cDNA transcript and the MMP-13 gene in channel catfish, determined its pattern of expression in various healthy tissues and during embryogenesis and evaluated its involvement in the defense responses of catfish against the Gram-negative bacteria *Edwardsiella ictaluri*, the causative agent in enteric septicemia of catfish (ESC).

2. Materials and methods

2.1. Identification and sequencing of the catfish MMP-13 cDNA

A partial cDNA sequence of channel catfish MMP-13 was initially identified from an EST sequence (GenBank: CK411123) using BLAST similarity comparison. MMP-13 cDNA partial sequence was obtained by sequencing each end of the existing EST clone (AUF_IpHdk_44_p11) corresponding to this accession number. The clone was completely sequenced using a primer-walking strategy. Using BLAST analysis, the completely sequenced clone was putatively missing the 5′-end of the transcript. Therefore, 5′-rapid amplification of cDNA ends (5′-RACE) was conducted to complete the sequence of the MMP-13 transcript. RACE was conducted using the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA) following the manufacturer's instructions. The 5′-RACE amplicon was gel purified and cloned using the pGEM-T Easy cloning kit (Promega, Madison, WI). Plasmid DNA was purified and sequenced. Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3130*XL* automated sequencer (Applied Biosystems). Primers used for RACE and sequencing reactions are listed in Supplemental Table 1. Vector NTI 10 software (Invitrogen, Carlsbad, CA) was used to analyze and cluster sequences.

2.2. BAC library screening and genomic DNA sequencing

The MMP-13 gene was screened from the CHORI-212 BAC library [36] purchased from the Children's Hospital of the Oakland Research Institute. A gene-specific probe was designed from the cDNA sequence and generated by PCR using primers listed in Supplemental Table 1. The probe was labeled with ^{32}P-dCTP (Amersham Biosciences, Piscataway, NJ) by the Random Primed DNA Labeling kit (Roche Applied Science, Indianapolis, IN). After removing the unincorporated nucleotides with Sephadex® G-50 spin columns (Amersham Biosciences), the labeled probe was denatured at 95 °C for 5 min and added to hybridization tubes, which had been pre-hybridized for 2 h with 30 ml of hybridization buffer (20× SSPE, 20% SDS, 100× Denhardt's solution and 3 mg salmon sperm DNA). The hybridization was performed at 63 °C for 16 h. The filters were washed twice and exposed to X-ray film at −80 °C overnight. Positive BAC clones were identified and cultured in 2× YT medium for 20 h. BAC DNA was isolated using the R.E.A.L. Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA) and sequenced using a primer-walking method. Primers used for BAC sequencing are listed in Supplemental Table 1. Vector NTI 10 software (Invitrogen) was used to analyze and cluster sequences. BLAST searches were conducted to identify the gene. The MMP-13 cDNA and genomic DNA sequences were aligned using the Spidey program at the NCBI.

2.3. Bacterial challenge and sample preparation

The ESC challenge was conducted following established protocols, with modification [37,38]. Fish were randomly divided into different time points: 4 h control (3 pools), 4 h treatment (3 pools), 24 h control (3 pools), 24 h treatment (3 pools), 3-day control (3 pools), 3-day treatment (3 pools), 7-day control (3 pools) and 7-day treatment (3 pools). Treated group were injected with *E. ictaluri*, while control group with PBS. At each time point, 15 fish from each pool were collected, euthanized with MS-222 (300 mg/ml), and liver, spleen, intestine, and skin were collected, pooled, and immediately submerged in RNAlater solution (Invitrogen) per the manufacturer's protocol. Tissues were stored at −80 °C until RNA extraction. Samples were homogenized using a mortar and pestle under liquid nitrogen, and total RNA extracted using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the supplied protocol for tissue extraction.

To determine catfish MMP-13 gene expression in healthy channel catfish, 13 tissues were collected including brain, skin, muscle, blood, head kidney, trunk kidney, liver, spleen, intestine, stomach, gill, heart, and ovary. To determine the expression of MMP-13 occurring during the normal catfish embryogenesis, channel catfish embryos were collected at different time points: 2 h post-fertilization (hpf), 6, 12, 24, 48, 72, 96 hpf, and newly hatched. Samples from the healthy catfish and embryos were flash

frozen in liquid nitrogen immediately after collection, and stored at −80 °C until RNA isolation. TRIzol reagent (Invitrogen) was used for RNA isolation according to the manufacturer's protocol. The concentration of total RNA was quantified using a spectrophotometer (Ultrospec 1100 pro, Amersham Biosciences).

2.4. Southern blot analysis

Southern blot analysis was performed to determine the genomic copy numbers of channel catfish MMP-13 gene. Southern blot analysis was conducted using standard methods [39] and as previously described [40], with modifications. Briefly, genomic DNA was isolated from three individual channel catfish. The genomic DNA (10 μg) was digested with 10 U each of the restriction endonucleases *Eco* RI, *Hin*d III and *Pst* I (New England Biolabs, Beverly, MA). DNA was electrophoresed on a 0.8% agarose gel at 20 V for 24 h. After submersion in 0.25N HCl, the gel was denatured in buffer (1.2 M NaCl, 0.5N NaOH), and neutralized in buffer (1 M Tris–HCl at pH 7.5, 1.5 M NaCl) at 30 min intervals. The DNA from the gel was transferred to an Immobilon™ positively charged nylon membrane (Millipore, Bedford, MA) overnight using 20× SSC buffer by downward capillary transfer. DNA was fixed to the membrane by UV radiation on a UV cross-linker (Stratagene, La Jolla, CA) and the auto-cross-link setting was used. A cDNA probe specific for MMP-13 was amplified by PCR. Primers for the gene-specific probe are listed in Supplemental Table 1. The probe was labeled with ^{32}P-dCTP using the Random Primed DNA Labeling kit (Roche Applied Science). Hybridization of the probe to the membrane and detection was conducted as described above for BAC-based hybridization.

2.5. Phylogenetic analysis

A phylogenetic tree was constructed to assess the evolutionary relationships of MMP-13. The tree was constructed using the protein sequences of MMP-13 retrieved from GenBank. The tree was built using the Molecular Evolutionary Genetics Analysis (MEGA 4) package [41]. An initial neighbour-joining tree was created. Using this tree as a guide, a final minimum evolution tree was created. Settings used included Poisson correction and complete deletion of gaps and/or missing data. The topological stability of the tree was evaluated by 10 000 bootstrap replications. The MMP-13 from *Arabidopsis thaliana* (thale cress) was used as the outgroup [42].

2.6. Analysis of MMP-13 expression in healthy tissue using RT-PCR

Total RNA (1 μg) was used to create cDNA using the Super-Script™ First-Strand Synthesis Kit (Invitrogen). RT reactions were conducted in a volume of 20 μl master mixes containing the following: 1 μg DNase-I-treated RNA, 1 μl (10 mM) dNTP mix, 1 μl (40 uM) oligo dT primer, 1 μl RNase inhibitor, 1× RT reaction buffer and 50 U Super-Script™ II reverse transcriptase. Detailed procedures followed the manufacturer's protocol. Then, 1 μl of products were directly used as template for RT-PCR with the following reaction cocktail: 2 μl 10× buffer, 1 μl JumpStart *Taq* polymerase (Sigma, St. Louis, MO), 1.6 μl dNTPs, 1.6 μl (25 mM) $MgCl_2$, 1.6 μl (10 μM) gene-specific primers and 0.4 μl (10 μM) β-actin primers (Supplemental Table 1). The β-actin gene served as an internal control. Reactions were conducted in a thermocycler with the following thermal profile: denaturing at 95 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min. A final extension was performed at 72 °C for 10 min to complete the RT-PCR. Reactions were analyzed by electrophoresis on a 1% agarose gel and documented with a gel documentation system (Bio-Rad, Hercules, CA).

2.7. Gene expression of MMP-13 in liver, spleen, intestine, skin and embryo using qRT-PCR

One step quantitative real-time RT-PCR (qRT-PCR) was carried out in a LightCycler 1.0 (Roche Applied Science). Concentration and quality of total RNA was determined by spectrophotometry and by 1% agarose gel containing formaldehyde. The qRT-PCR reactions were performed in triplicate using a FastStart RNA Master SYBR Green I Reagent Kit (Roche Applied Science) following the manufacturer's protocol with modifications. Briefly, 10 μl total reactions were conducted, which includes 4.3 μl H_2O, 0.3 μl of each primer (5 μM), 0.6 μl $Mn[OAc]_2$, 3.5 μl SYBR Green, and 1 μl (100 ng) RNA template. The following cycling parameters were used: (i) reverse transcription, 20 min at 61 °C; (ii) denaturation, 30 s at 95 °C; (iii) amplification repeated 50 times, 5 s at 95 °C, 5 s at 55 °C, 20 s at 72 °C; (iv) melting curve analysis, 5 s at 95 °C, 15 s at 65 °C, then up to 95 °C at a rate of 0.1 °C/s; (v) cooling, 30 s at 40 °C. Amplicon specificity was assessed by melting curve analysis. The data were normalized to the expression of 18S rRNA based on the gene expression stability over various tissues and treatment conditions in fish [43]. Cycle threshold (Ct) values were analyzed and converted to fold differences by relative quantification using the Relative Expression Software Tool (REST 2008) [44].

3. Results

3.1. Identification and sequencing of the catfish MMP-13 cDNA

The full-length cDNA for MMP-13 was sequenced and characterized. The MMP-13 cDNA encodes a putative protein of 471 amino acids with a 5′-UTR of 27 bp and a 3′-UTR of 193 bp. A typical polyadenylation signal sequence (AATAAA) was identified 13 bp upstream of the poly-A tail. The cDNA sequence was deposited to GenBank with the accession number GU122923.

Analysis of domain regions using SMART indicated that MMP-13 consists of a signal peptide, a peptidoglycan binding domain, a ZnMc domain, and four sequential hemopexin-like domains (Fig. 1). Multiple sequence alignment using amino acid sequence of available MMP-13 genes indicated that this gene is highly conserved through evolution. As showed in Fig. 2, the zinc-dependent catalytic domain, which commonly exists in the MMPs family, was also conserved in the MMP-13 gene. The multiple sequence alignment also indicated conserved sequence characteristics of catfish MMP-13 gene, such as the highly conserved 'cysteine switch' motif PRCGXPD near the catalytic domain and the zinc-binding sequence HEXXHXXGXXH in the catalytic domain. Analysis of multiple deduced amino acid sequences aligned by ClustalW shows MMP-13 gene in channel catfish is 56–62% similar to fish species, and 49% similar to human (Table 1).

3.2. Phylogenetic analysis of MMP-13 gene

A phylogenetic tree based on amino acid sequences was constructed to analyze the evolutionary relationship between

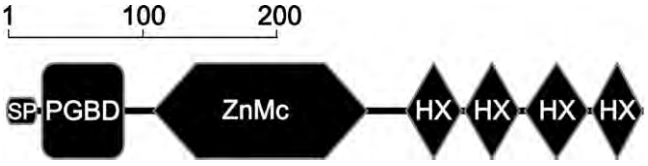

Fig. 1. Domain structure of the catfish MMP-13 gene generated by the SMART program (http://smart.embl-heidelberg.de). Different shapes represent different domains. SP represents signal peptides, PGBD represents peptidoglycan binding domain, ZnMc represents zinc-binding domain, HX represents hemopexin-like domain. The scale on the top margin is measured by amino acid number.

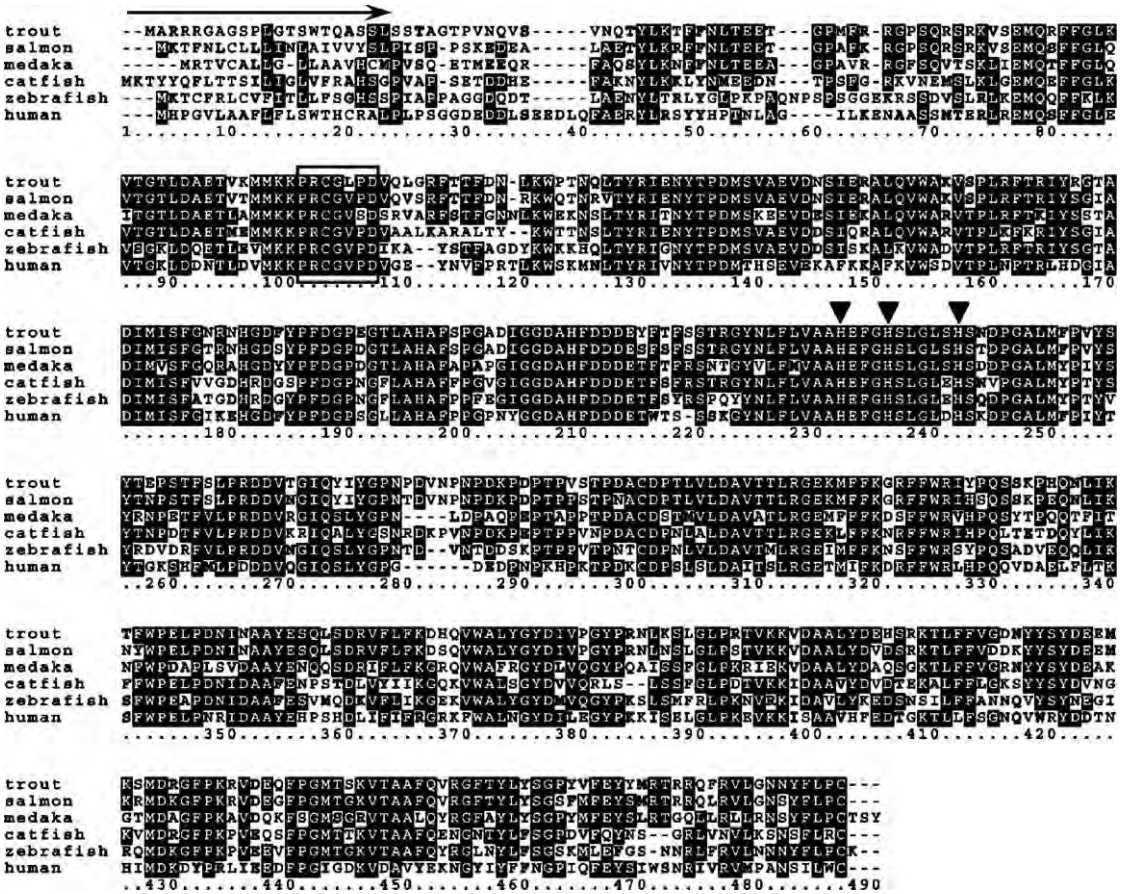

Fig. 2. Comparison of amino acid sequences of the channel catfish MMP-13 gene with MMP-13 genes from rainbow trout, Atlantic salmon, *medaka*, zebrafish and human. The signal peptides of MMP-13 gene are indicated by arrow. The "cysteine switch" motif is indicated by horizontal open box. Triangles indicate the conserved histidine residues in the catalytic domain.

catfish MMP-13 gene and other MMP-13 genes (Fig. 3). The MMP-13 gene from catfish clusters with other teleosts including zebrafish, Atlantic salmon, rainbow trout, *medaka*, and pufferfish forming a distinct clade with high bootstrap support, while the mammalian MMP-13 genes formed a separate clade. MMP-13 from frog was intermediary between the mammalian and teleost groups.

3.3. Structural analysis of the MMP-13 gene

Six BAC clones from the CHORI-212 BAC library were identified by hybridization of a high-density BAC filter to contain the MMP-13 gene. These six clones are 103_A15, 103_B14, 117_O10, 123_I04, 137_H01, and 146_F04. Clone 103_A15 was used for sequencing. A genomic sequence of 3967 bp was obtained and the sequence was deposited to GenBank with accession number GU122922. NCBI's Spidey program was used to analyze the genomic structure by aligning the genomic DNA sequence and

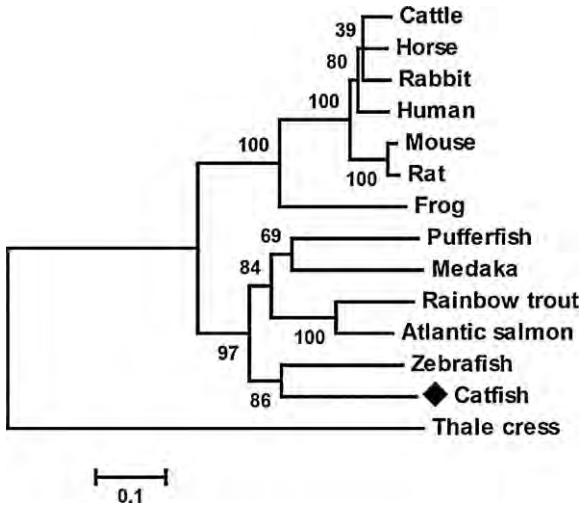

Fig. 3. Phylogenetic analysis of the catfish MMP-13 gene with related MMP-13 gene sequences. The phylogenetic tree was drawn by using the minimum evolution method in MEGA 4.0 package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The catfish MMP-13 gene is marked with a black diamond. Accession numbers of utilized sequences are as follows: rat (P23097), mouse (P33435), horse (O18927), cattle (O77656), rabbit (O62806), human (P45452), frog (Q10835), Atlantic salmon (ACI34278.1), rainbow trout (NP_001117671.1), *medaka* (NP_001098185.1), pufferfish (CAF90586.1), zebrafish (AAQ07962.1), and thale cress (NP_182030.1).

Table 1
Pairwise similarities of selected MMP-13 proteins.

Medaka	Rainbow trout	Zebrafish	Salmon	Human	
56	61	61	62	49	Catfish
	63	55	65	47	*Medaka*
		57	82	50	Rainbow trout
			60	51	Zebrafish
				49	Salmon

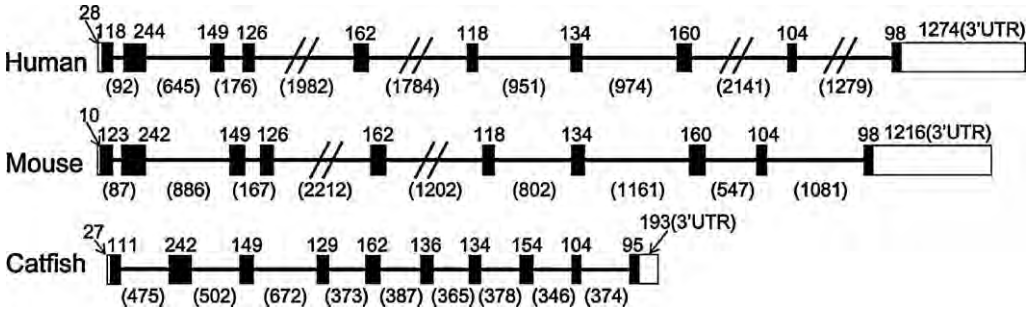

Fig. 4. Schematic diagram of vertebrate MMP-13 gene structures. Exons are represented by boxes. Solid boxes indicate the coding region while open boxes indicate untranslated regions. The length of exons is indicated by numbers in base pairs on the top of boxes. The number in each parentheses below gene structure indicates the length of intron. Double slashes indicate non-proportional representation of the introns as specified.

cDNA sequence. The data reveals that MMP-13 gene consists of 10 exons and 9 introns (Fig. 4).

3.4. Determination of genomic copy numbers of MMP-13 gene in channel catfish

A single copy of MMP-13 gene was found to exist in the channel catfish genome by Southern blot analysis. As shown in Fig. 5, a single band was observed with restriction enzyme digestion using *Hin*d III, suggesting a single copy of MMP-13. Digestion using *Pst* I and *Eco* RI generated two bands each (Fig. 5). An internal *Pst* I site was identified in the MMP-13 gene. Two *Eco* RI sites were identified in the MMP-13 genome sequence covered by probe, suggesting three bands are generated. Considering the position in the MMP-13 genome sequences, one of these bands is only 106 bp in the genomic DNA, which was too small to be observed in the Southern blot membrane. These results suggest the presence of a single copy of the MMP-13 gene in the catfish genome.

3.5. Analysis of MMP-13 gene expression in various tissues

In order to gain insight into the tissue distribution of MMP-13 gene, RT-PCR was conducted with RNA isolated from various healthy tissues from channel catfish. As shown in Fig. 6, MMP-13 is expressed in all tissues tested, including stomach, heart, blood, brain, head kidney, trunk kidney, liver, intestine, gill, ovary, skin, spleen and muscle. Tissue preferences appeared in the analysis. The strongest expression of MMP-13 was observed in the head kidney and trunk kidney. Moderate levels of MMP-13 expression were observed in blood, gill, skin, and spleen. Lower levels of MMP-13 expression were observed in stomach, brain, liver, intestine, muscle, heart, and ovary (Fig. 6).

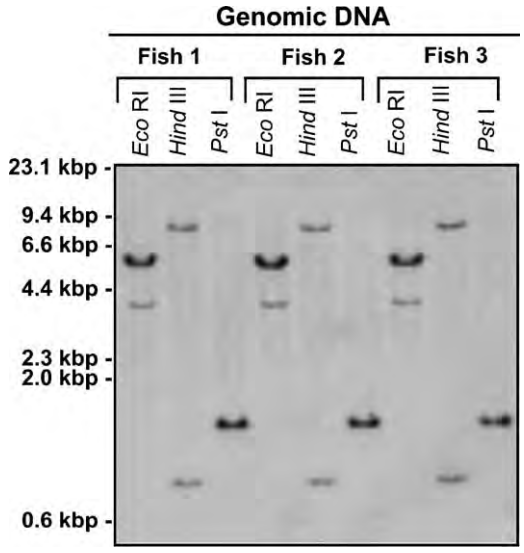

Fig. 5. Southern blot analysis of the catfish MMP-13 gene using genomic DNA of three catfish individuals. Procedures are detailed in Section 2. Kb markers are indicated on the left margin.

3.6. MMP-13 gene expression after bacterial challenge

Quantitative real-time RT-PCR was conducted to provide an accurate assessment of MMP-13 expression in channel catfish after bacterial infection in the catfish liver, spleen, skin and intestine. As indicated in Fig. 7, the expression of MMP-13 is significantly ($p < 0.05$) up-regulated in several tissues and at several time points as compared to control tissue expression. In the catfish liver, MMP-13 was slightly increased at 4 and 24 h post-infection, which decreased by day 3, but had significantly spiked by day 7. In the spleen, MMP-13 expression was significantly up-regulated at 4 h post-challenge, but decreased through 3 days. A significant spike in expression was observed at 7 days post-challenge in spleen. In the skin tissue, a slight increase in expression was seen at 4 h post-challenge, which decreased through day 3 of the challenge. A significant spike in expression was observed in the skin at day 7. A marked increase in MMP-13 expression in intestine was observed at 7 days post-challenge (Fig. 7).

3.7. MMP-13 gene expression during embryo development

In order to determine the expression of MMP-13 mRNA during normal channel catfish embryogenesis, embryos were collected at different time points and analyzed via quantitative real-time RT-PCR. Basal levels appeared to be maintained through the first 24 h post-fertilization. MMP-13 gene expression slightly increased in the first day after fertilization. However, beginning at 48 h post-fertilization, MMP-13 was highly up-regulated. Expression of MMP-13 maintained at an elevated level at 72 and 96 hpf, although they are less than that observed at 48 hpf. MMP-13 was also highly up-regulated in newly hatched fish (Fig. 8).

Fig. 6. RT-PCR analysis of catfish MMP-13 gene expression in various healthy channel catfish tissues. RT-PCR products were analyzed on an agarose gel. The positions of the RT-PCR amplified band of MMP-13 and β-actin are indicated on the left margin. The tissues are as following: 1, stomach; 2, heart; 3, blood; 4, brain; 5, head kidney; 6, trunk kidney; 7, liver; 8, intestine; 9, gill; 10, ovary; 11, skin; 12, spleen; 13, muscle.

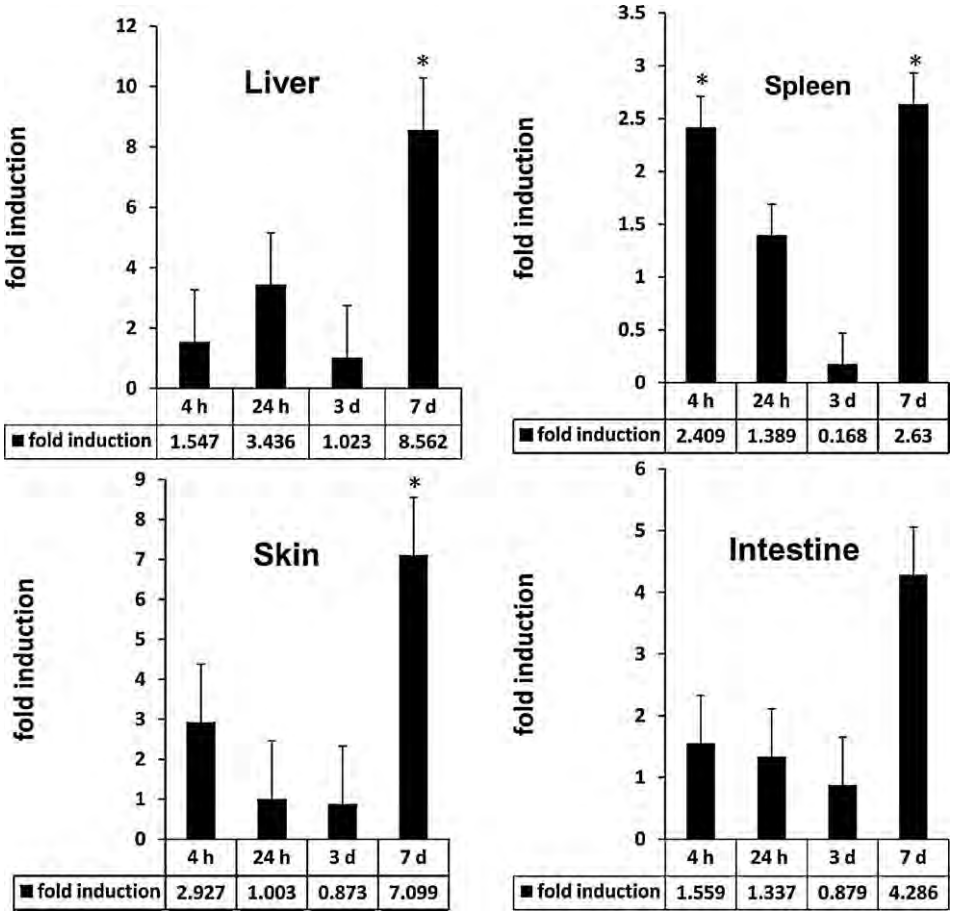

Fig. 7. Quantitative real-time RT-PCR analysis of expression of channel catfish MMP-13 gene after *E. ictaluri* infection in liver, spleen, skin and intestine, respectively. The RNA samples were collected at 4 h, 24 h, 3 and 7 days post-treatments. Relative expression was measured as fold change over control samples taken at the same time point as normalized to change in expression in the 18S rRNA control. Asterisks indicate statistical significance at the level of $p < 0.05$.

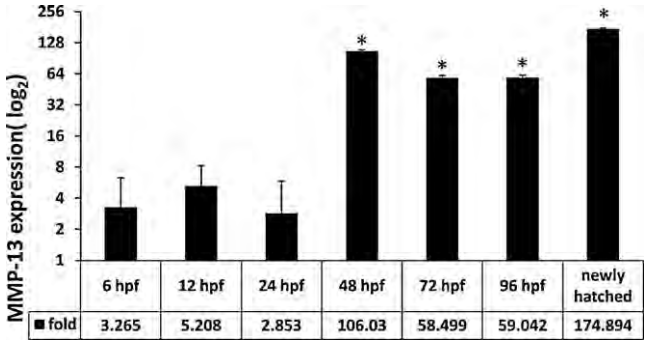

Fig. 8. Expression of MMP-13 gene during channel catfish embryogenesis. Embryos were collected at eight different time points: 2 h post-fertilization (hpf), 6, 12, 24, 48, 72, 96 hpf, and newly hatched. Relative expression was measured as fold change compare to the sample taken at the 2 hpf as normalized to change in expression in the 18S rRNA control. The *Y*-axis is displayed logarithmically based on 2.

4. Discussion

MMPs are key modulators of many biological processes, including regulation of matrix degradation, cell cycle, apoptosis, and embryogenesis. However, little is known of the structure and function of MMPs in teleost fish species. Here, the complete cDNA and genomic DNA of catfish MMP-13 were sequenced and analyzed. The expression of MMP-13 was measured after bacterial infection and during embryogenesis.

Catfish MMP-13 consists of a signal peptide, a peptidoglycan binding domain, a ZnMc domain, and four sequential hemopexin-like domains, as determined by the SMART program (Fig. 1). Peptidoglycan is the main component of the bacterial cell wall of both Gram-positive and Gram-negative bacteria. Peptidoglycan binding domains (PGBD) are found at the N- or C-terminus of a variety of enzymes involved in bacterial cell wall degradation [45]. The majority of vertebrate matrix metalloproteinases contain a PGBD at the N-terminus, but little is known regarding the functional capabilities of these domains in MMPs. A previous study demonstrated that peptidoglycan of *Staphylococcus aureus* could induce enhanced protein levels of MMP-9 in human blood [46], but the authors did not examine binding relationships. More recent research has revealed that, upon infection, murine MMP-12 is mobilized to macrophage phagolysosomes where it adheres to bacterial cell walls and disrupts cellular membranes resulting in bacterial death [47]. Although no investigation into the mode of MMP-12 adherence to the bacterial cell wall was conducted, the study represented the first report of direct antimicrobial activity by a matrix metalloproteinase. Further work is needed to clarify whether teleost MMPs, including catfish MMP-13, possess antimicrobial capabilities to adhere to invading pathogenic bacteria and resolve infection by direct killing.

As shown in Fig. 2, the catfish MMP-13 signal peptide is not well conserved when compared to those of other fish species. However, the zinc-binding motif HEXXHXXGXXH in the catalytic domain is highly conserved, so is the "cysteine switch" motif PRCGXPD, known to coordinate with the zinc atom in the catalytic domain. MMP-13 is synthesized in a latent form, and this Cys–Zn^{+} interaction keeps proMMP-13 inactive until dissociation and exposure of the active site [48].

Compared to mammalian counterparts, MMP-13 gene structure, especially exon numbers and sizes are highly similar, suggesting high conservation through evolution (Fig. 4). Phylogenetic analysis shows that the MMP-13 genes from fish species fall into one clade, separate from frog and other mammalian sequences (Fig. 3).

Many MMP family members have been reported to have a role in inflammation and innate immunity [2]. MMPs not only take part in repairing epithelium and participate in the removal of bacterial pathogens, but also directly modulate the activities of chemokines and cytokines. Recently, several reports have expanded our knowledge of the diverse immune roles of MMPs. MMP-12 (macrophage elastase), as discussed above, has been shown to directly kill bacteria within murine macrophages. Collagenases, such as MMP-1 (collagenase-1), have been reported to be involved in innate immunity [23]. MMP-1 knockout beetles were more susceptible to the entomopathogenic fungus *Beauveria bassiana*. Elevated expression of MMP-1 stimulated immune-competent hemocytes. In teleost fish, Castillo-Briceno et al. [49] have recently shown that proteolytic fragments derived from collagen and produced by the action of host MMPs, including MMP-13, during infection, are sensed by fish phagocytes. Their report represents a first indication of the mechanisms by which teleost fish respond to infection-mediated tissue injury. MMP-13 has been reported as an immune-related gene in Japanese flounder based on up-regulation of expression after infection of *E. tarda* [35]. Similarly, our study indicates that catfish MMP-13 likely participates in immune processes given significant increases in expression in several tissues over the course of *E. ictaluri* infection. As shown in Fig. 7, the induced expression of catfish MMP-13 was significantly up-regulated in spleen at 4 h after infection, and increased expression levels were detected as well in liver, suggesting potential roles for MMP-13 in innate immune responses. While outside of the scope of the present report, we speculate that catfish MMP-13 may carry out diverse roles over the course of infection. It may recognize and bind peptidoglycan through its PGBD and degrade the bacterial cell wall. Catfish MMP-13 likely helps to generate proteolytic fragments of collagen released by pathogen-mediated damage to the host [50], activating phagocytes and potentiating the broader innate immune response [51]. At 7 days after bacterial infection, the expression of catfish MMP-13 was induced in skin as well as in intestine. Late or lagging expression of MMP-13 may indicate the start of remodelling processes that help to repair widespread tissue damage caused by *E. ictaluri*. A recent report indicated that MMP-13 in mouse aids in skin wound healing through involvement in keratinocyte migration, angiogenesis and contraction [52]. MMP-13 has been shown to be involved in intestinal remodelling during metamorphosis in frog [53] and intestinal ulceration in human [54]. Taken together, our expression data suggests the potential involvement of catfish MMP-13 in a wide range of immune-relevant processes from pathogen elimination to wound healing. The universal, constitutive expression of MMP-13 in all examined healthy tissues indicates that stores of MMP-13 are available to rapidly respond to infection or tissue damage throughout the host in addition to carrying out putative homeostatic processes. Further studies are required to conclusively demonstrate and clarify the pleiotropic roles of catfish MMP-13, and other MMP family members, in immune responses.

In addition to MMP-13 involvement in immune functions, MMP-13 also appears to be important during the embryonic development of catfish. The expression of MMP-13 varied during normal channel catfish embryonic development. The MMP-13 mRNA levels were relatively low in the first day after fertilization. According to the stages of normal development of brown bullhead catfish, *Ictalurus nebulosus* [55] and Northcutt's modification to channel catfish development [56], this period consists of stages of blastulation, and gastrulation, during which the main events are cell cleavage and movement. Low expression of catfish MMP-13 during these stages suggests that it may play a minor role in early embryonic development. Catfish MMP-13 expression sharply increased from 24 to 48 h post-fertilization, a time during which a number of morphogenetic movements occur after gastrulation, including neurulation, and formation of tail bud, heart, and somites [56]. High levels of catfish MMP-13 indicate this gene may be involved in primary organogenesis. Channel catfish MMP-13 embryonic data corroborate a similar expression pattern to that observed in zebrafish [16].

In conclusion, the expression of catfish MMP-13 is modulated by bacterial infection, and displays a conserved pattern of expression during embryogenesis, suggesting its importance in both immune and developmental processes. Further studies are needed to fully elucidate functional roles for catfish MMPs in a host of homeostatic and immune settings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.01.001.

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