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Complete Structure, Genomic Organization, and Expression of Channel Catfish (*Ictalurus punctatus*, Rafinesque 1818) Matrix Metalloproteinase-9 Gene

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In this study, the channel catfish (CC) matrix metalloproteinase-9 (MMP-9) gene was cloned, sequenced, and characterized at both the cDNA and the genomic DNA levels. The complete sequence of the CC MMP-9 cDNA consisted of 2,551 nucleotides, including one open reading frame and 5′- and 3′-end untranslated regions. The open reading frame potentially encoded a 686-amino-acid peptide with a calculated molecular mass (without glycosylation) of approximately 77.4 kDa, which included a signal peptide and potentially heavy *O*-glycosylation sites. CC MMP-9 did not have the tripeptide Arg-Gly-Asp motif. The degree of conservation of the CC MMP-9 amino acid sequence to human and mouse counterparts was 55%, while to those of other fish species was 67–74%. The full-length CC MMP-9 genomic DNA comprised 5,663 nucleotides, much shorter than human or mouse counterparts. The exon-intron structure followed the splice acceptor/donor consensus rule, and the sequence contained 13 exons. The MMP-9 transcript was constitutively expressed in restrictive CC tissues. This result should provide fundamental information for further exploration of the role of MMP-9 in fish pathophysiology.

Key words: matrix metalloproteinase-9 (MMP-9); channel catfish (CC); *Ictalurus punctatus*

The extracellular matrix network that has functions from supporting cell shape to coordinating the extracellular signaling system, is tightly regulated by a number of proteolytic enzymes, particularly the family of matrix metalloproteinases (MMPs).[1,2] MMPs are a large protein family, of at least 25 members, which share many common features in domain structures and functions, but differ in enzymatic specificities.[3–6] MMPs play many important roles in both physiological and pathological processes. In physiological conditions, MMPs cleave the extracellular matrix during normal tissue remodeling, such as morphogenesis, wound healing, angiogenesis, and leukocyte migration.[3,7,8] On the other hand, MMPs have been implicated in the pathogenesis of many diseases and invasive processes, such as asthma, lung fibrosis, liver cirrhosis, and tumor metastasis.[3,7,9,10] We are interested in MMP-9, because many recent studies have demonstrated that MMP-9 is induced during early infection by various microbial agents,[11–16] suggesting that MMP-9 plays a role in the pathogenesis of microbial infections.

MMP-9, also known as type IV collagenase, is a neutral zinc-dependent endopeptidase that has substrate specificity for gelatins and type IV intact collagens in the extracellular matrix.[3,5] It is predominantly found in neutrophils assessed by immunohistochemical staining in humans.[17] Its gene expression is regulated by various cytokines and growth factors.[17] In addition, MMP-9 is synthesized and secreted in an inactive proenzyme that is activated by a proteolytic cleavage.[3,17,18]

In teleost fish, the cDNA of MMP-9 gene has been recently cloned in zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), Japanese flounder (*Paralicthys olivaceus*), and medaka fish (*Oryzias latiles*),[19–23] but its genomic organization has not been characterized. Only limited information on the biological function of MMP-9 in fish is available. In addition, because of the diversity of fish species, it is important to understand the evolution and functions of the fish MMP-9 system. Hopefully, a model can be established for MMP-9 inhibitor screening and/or study of the roles of MMP-9 in pathogenesis.

Channel catfish (*Ictalurus punctatus*) production is the most important aquacultural industry in the southeastern United States, generating more than 450 million dollars annually.[24] In the course of studying the pathogenesis of enteric septicemia of catfish by *Edwardsiella ictaluri*, we observed that the CC MMP-9 expressed sequence tag, identified by subtraction suppression hybridization, was induced and up-regulated at the early stage of infection in catfish ovary cell culture (unpub-

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Abbreviations: CC, channel catfish; MMP-9, matrix metalloproteinase-9; RACE, rapid amplification of cDNA ends; UTR, untranslated region

lished data). This preliminary observation prompted us to speculate that MMP-9 plays a critical role at the early stage of *Edwardsiella ictaluri* infection. In order to provide a framework to answer this question, we undertook to isolate and characterize CC MMP-9 cDNA and genomic DNA. The MMP-9 transcript was constitutively expressed in restrictive tissues.

Materials and Methods

Animal. Channel catfish (NWAC 103 strain, weighed 20–25 gm) were used in this study. The fish were raised according to the methods and protocol established by ARS Aquatic Animal Health Research Unit.[25] Animal use in the experiments was approved by the Institutional Animal Care and Use Committee, Aquatic Animal Health Research Unit, MidSouth Area, Agricultural Research Service, United States Department of Agriculture.

Tissue samples. Fish were collected and euthanized by immersion in tricaine methane sulfonate (MS-222).[26] Gills, skin, spleens, livers, intestine, and head kidneys were aseptically excised.

RNA isolation. Total RNA from tissues was isolated using a Tri reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Total RNA was dissolved in DEPC-treated water. The quality and quantity of total RNA were determined with an Agilent Bioanalyzer using RNA 1200 chips (Agilent Technologies, Santa Clara, CA). Both 16S and 28S RNA were clearly identified.

Generation of rapid amplification of cDNA ends (RACE). After total RNA isolation, cDNA was synthesized and amplified using a GeneRacer kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. For 5′-RACE, 5 µg of total RNA were treated with calf intestinal phosphatase to remove the 5′-phosphate, treated with tobacco acid pyrophosphatase to eliminate the 5′-end cap structure, and then ligated to a GeneRacer RNA Oligo, 5′-CGACUGGAGCACGA-GGACACUGACAUGGACUGAAGGAGUAGAAA-3′. The treated RNA was then reverse transcribed into cDNA using Superscript™ reverse transcriptase III (Invitrogen) and random primers. For 3′-RACE, 5 µg of total RNA were reverse transcribed into cDNA using Superscript™ reverse transcriptase III (Invitrogen) and poly(dT) primers provided in the GeneRacer kit. Both 5′-RACE and 3′-RACE cDNAs were amplified by PCR. The primers for PCR amplification are listed in Table 1. Amplification was carried out on an ABI GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) as follows: initial activation of DNA polymerase at 94 °C for 5 min, followed by 5 cycles at 94 °C (20 s) and at 72 °C (1 min); 5 cycles at 94 °C (20 s) and at 70 °C (1 min); and 40 cycles at 94 °C (20 s), at 60 °C (1 min), and at 72 °C (1 min). After amplification, the mixture was incubated at 72 °C for 10 min. The PCR products were purified by agarose gel electrophoresis and cloned into a pCR4-TOPO TA cloning vector (Invitrogen). The ligated plasmid was transformed into TOP10™ *E. coli* by a heat-shock method. After enrichment for 60 min at 35 °C in SOC medium, the cells were streaked on LB plates containing 50 µg/ml of ampicillin and incubated at 35 °C overnight. Colonies were randomly picked and cultured in WU medium for sequencing.

Isolation and construction of genomic DNA libraries. Genomic DNA of CC head kidney was isolated using a DNeasy Tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Genomic DNA

Table 1. Synthetic Oligonucleotides Used in This Study

Oligonucleotide	Direction	Sequence	Tm (°C)
For sequencing			
GeneRacer 5′Primer (Invitrogen)	Forward	5′-CGACTGGAGCACGAGGACACTGA-3′	74
GeneRacer 3′Primer (Invitrogen)	Reverse	5′-GCTGTCAACGATACGCTACGTAACG-3′	78
Adaptor Primer 1 (Clontech)		5′-GTAATACGACTCACTATAGGGC-3′	59
MMP9605-F	Forward	5′-CTCCAGGTGAAGGGGTGCAGGGAGAT-3′	74
MMP9699-F	Forward	5′-TGCAGAGGGGGCATTGTGTCATTTCC-3′	74
MMP9192-F	Forward	5′-TGAGGGGGAGCCGTGTCAGTTTCCTT-3′	74
MMP9250-F	Forward	5′-ACGAGTGAAGGCCGCAGTGATGGAAA-3′	74
MMP9068-F	Forward	5′-ATGGACCCAAAACAGGCCCAGATCCT-3′	72
MMP9106-F	Forward	5′-GTGGATGCTCACGATGTGTTCCTCTAT-3′	71
MMP9185-F	Forward	5′-GGCGGCAGGTTGATAGAGTTGGATATG-3′	73
MMP9634-R	Reverse	5′-GAGCATCTCCCTGCACCCCTTCACCT-3′	74
MMP9275-R	Reverse	5′-TTTCCATCACTGCGGCCTTCACTCGT-3′	74
MMP9093-R	Reverse	5′-AGGATCTGGGCCTGTTTTGGGTCCAT-3′	72
MMP9027-R	Reverse	5′-CTTAAAGTGCACGTCCCAAGAACCATA-3′	70
MMP9362-R	Reverse	5′-GCTTGCCAGTCTCTTTCAGACCCAGTT-3′	73
For RT-PCR			
β-Actin-F	Forward	5′-CACCATTGGCAATGAGAGGTT-3′	66
β-Actin-R	Reverse	5′-TGAAGGATGGTTGGAAGAGGG-3′	67
MMP9605-F	Forward	5′-CTCCAGGTGAAGGGGTGCAGGGAGAT-3′	74
MMP9275-R	Reverse	5′-TTTCCATCACTGCGGCCTTCACTCGT-3′	74

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gaaaagacagagagtaagaccagccagaagaagaagacgaaagaagactcaacttcagaga    61

                                                M   R   I   S   I    5
ctttgacagactttcagaaatcagtggctgaaagctctcagc atg aga ata agt atc   118

 L   A   F   M   V   L   G   T   C   T   L   S   A   W   S ↑ H   21
ttg gca ttt atg gtt ctt ggg acg tgc act tta agt gca tgg agc cac  166

 P   I   K   T   I   F   V   N   F   P   G   D   V   I   K   N   37
cca att aaa acc atc ttc gtg aac ttc ccc ggg gat gta atc aaa aac  214

 T   I   D   L   E   L   A   E   S   Y   L   K   R   F   G   Y   53
acg ata gac tta gag ctg gct gaa agt tac ctg aag cgc ttt ggt tat  262

 M   E   I   L   D   K   S   G   R   Q   G   T   V   S   T   S   69
atg gag att ctg gat aag agt gga cga cag gga aca gtg tct act tcc  310

 K   A   L   R   R   L   Q   N   Q   L   G   L   K   E   T   G   85
aaa gct ctc agg agg ctg cag aat caa ctg ggt ctg aaa gag act ggc  358

 K   L   D   Q   P   T   I   D   A   I   K   T   P   R   C   G   101
aag ctg gat caa ccg aca att gat gcc ata aag aca cca cgc tgc ggt  406

 V   P   D   V   R   N   Y   Q   T   F   D   G   D   L   K   W   117
gtg cca gac gtc cgc aac tac cag aca ttt gat gga gat ctg aaa tgg  454

 D   H   N   D   V   T   Y   R   I   L   N   Y   S   P   D   L   133
gat cac aat gac gtt aca tac agg att ctg aac tac tca cca gac tta  502

 D   A   S   V   I   D   D   A   F   A   R   A   F   K   V   W   149
gat gcc tct gtt att gat gat gcc ttt gcg aga gcc ttc aag gtg tgg  550

 R   D   V   T   P   L   T   F   T   R   L   Y   N   G   I   A   165
aga gat gtc aca ccc cta act ttc aca cgt ctc tac aat ggc att gct  598

 D   I   M   I   L   F   G   K   R   D   H   G   D   P   Y   P   181
gac atc atg atc ttg ttt gga aaa agg gat cat ggc gat ccc tac cca  646

 F   D   G   K   D   G   L   L   A   H   A   Y   P   P   G   E   197
ttt gat ggg aaa gat ggc ctg ctg gca cat gct tat cct cca ggt gaa  694

 G   V   Q   G   D   A   H   F   D   D   D   E   Y   W   T   L   213
ggg gtg cag gga gat gct cac ttt gat gat gat gaa tac tgg acc ctt  742
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Fig. 1. *Continued.*

libraries were constructed using a Universal Genome-Walker kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The MMP-9 genomic DNA was amplified by PCR, sequenced, and assembled. The primers are listed in Table 1.

DNA sequencing. DNA sequencing reactions in both strands were carried out at the USDA ARS MidSouth Genomics Laboratory with an ABI 3100 Genetic Analyzer (Applied Biosystems). Big Dye termination version 3.1 chemistry was used. Sequencing chromatograms were edited for quality and trimmed to remove vector sequences using the Phred[27,28] and Lucy[29] computer programs. More than 10 clones were sequenced, and the same results were obtained. The CC MMP-9 amino acid sequence was deduced from the nucleic acid sequence using the Transeq program,[30] and was aligned with other MMP-9 amino acid sequences using ClustalW version 1.83 program.[31] Phylogenetic analysis was conducted using the MEGA version 3.1 program[32] based on the ClustalW alignment result.

RT-PCR. RT-PCR assays for MMP-9 gene expression in CC tissues were carried out by a two-step procedure

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 G   N   G   P   A   I   K   T   Y   F   G   N   A   E   G   A   229
ggc aat gga cca gca atc aag acc tac ttt ggc aat gca gag ggg gca  790

 L   C   H   F   P   F   R   F   E   G   K   S   Y   S   T   C   245
ttg tgt cat ttc cct ttc cgg ttt gaa ggg aag tca tat tcc act tgc  838

 T   T   E   G   R   E   D   G   L   P   W   C   A   T   T   A   261
acc act gaa ggt cgt gaa gat ggt ctg cca tgg tgt gcc acg act gct  886

 D   Y   G   K   D   G   K   Y   G   F   C   P   S   E   L   L   277
gac tat ggc aaa gat ggg aaa tat ggt ttc tgt cct agc gag ctt ctg  934

 Y   T   F   D   G   N   G   D   G   Q   A   C   V   F   P   F   293
tac aca ttt gat ggg aat gga gat ggt caa gca tgt gtc ttt cca ttt  982

 V   F   E   R   K   T   Y   T   S   C   T   T   E   G   R   D   309
gtg ttt gag cgg aaa aca tac acc agt tgc acc act gaa gga cgt gat  1030

 D   G   Y   R   W   C   A   T   T   A   N   F   D   Q   D   K   325
gat ggg tat cgc tgg tgc gct act aca gcc aat ttt gac cag gac aaa  1078

 K   Y   G   F   C   P   N   R   D   T   A   V   I   G   G   N   341
aaa tat ggg ttc tgt cct aac cga gat act gct gtg att ggt gga aac  1126

 S   E   G   E   P   C   Q   F   P   F   S   F   L   G   K   T   357
tct gag ggg gag ccg tgt cag ttt cct ttc agc ttc ctg ggg aaa acc  1174

 Y   T   S   C   T   S   E   G   R   S   D   G   K   L   W   C   373
tat act tcc tgc acg agt gaa ggc cgt agt gat gga aaa ctg tgg tgc  1222

 A   T   T   S   N   Y   D   K   D   S   K   W   G   F   C   P   389
gcc aca act agc aac tat gac aag gac agt aaa tgg gga ttt tgt cca  1270

 D   K   G   Y   S   L   F   L   V   A   A   H   E   F   G   H   405
gat aaa gga tac agt ctg ttc ctg gtg gca gcc cac gaa ttt gga cat  1318

 A   L   G   L   D   H   S   N   I   Q   D   A   L   M   Y   P   421
gct ctt ggt ctg gac cat tct aac att cag gat gct ctg atg tac ccc  1366

 M   Y   K   Y   I   A   D   F   S   L   H   Q   D   D   I   E   437
atg tac aaa tat ata gcg gac ttc tct ctg cat cag gac gac att gag  1414

 G   I   Q   Y   L   Y   G   P   K   T   G   P   D   P   T   P   453
ggc atc cag tat ctc tat gga ccc aaa aca ggc cca gac cct act cca  1462

 P   K   P   S   T   T   T   T   S   P   V   S   T   L   K   P   469
ccc aag cca tca acc act aca act tcc cca gtt tct act ctc aaa ccc  1510
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Fig. 1. *Continued.*

routinely used in our laboratory.[33–35] First, total RNA from various tissues was reverse transcribed into cDNA by Superscript reverse transcriptase III (Invitrogen Corp.) in the presence of random hexamers. Reverse transcriptase in the reaction mixture was not included in order to ensure that no transcription was from genomic DNA. For PCR, 50 µl reactions contained, in final concentrations, 1X PCR buffer (Takara Mirus Bio, Madison, WI), 3.0 mM $MgCl_2$ (Applied Biosystems), 200 µM dNTP (Takara Mirus Bio), 0.3 µM each of gene-specific primers (listed in Table 1), 1.25 U *ExTaq* HS DNA polymerase (Takara Mirus Bio) and cDNA templates. Amplification was performed initially at 94 °C for 2 min, followed by 25 cycles of 94 °C for 15 s, 62 °C for 1 min, and 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. The reaction mixture without cDNA template served as a negative control. The amplified PCR fragments were

```
 T   K   K   T   P   K   T   T   P   S   T   T   S   T   T   T   485
act aaa aaa aca cca aag act act cca tct aca act tcc acc aca act  1558

 P   S   V   Y   T   P   V   D   P   S   V   D   P   C   K   V   501
cca tct gtg tat acg cct gtg gat ccc tca gta gat cct tgc aag gtg  1606

 D   K   F   D   V   I   T   E   I   Q   G   E   P   H   F   F   517
gat aaa ttt gac gta atc aca gag atc caa gga gag cct cat ttc ttc  1654

 K   D   G   Y   Y   W   K   S   S   N   R   G   N   E   E   R   533
aaa gat ggg tac tac tgg aag tcc tca aat cgt ggt aac gag gaa cgg  1702

 K   G   P   F   I   V   S   E   R   W   P   A   L   P   A   E   549
aaa ggc cct ttc ata gtc tct gag aga tgg cct gcc ctg cca gct gag  1750

 L   D   T   A   F   E   D   P   L   T   N   K   M   Y   F   F   565
ctc gac act gct ttt gag gac cct ctt act aat aag atg tac ttc ttt  1798

 A   G   N   Q   F   W   V   F   T   G   Q   D   V   L   G   P   581
gca ggc aat cag ttc tgg gtg ttc act gga cag gat gta ttg gga ccg  1846

 R   R   I   E   K   L   G   L   P   V   S   L   K   K   V   E   597
cgc aga atc gag aaa ctt ggc ttg ccc gtc agc ttg aag aag gtt gaa  1894

 G   T   L   Q   R   G   K   G   K   V   L   L   F   S   G   E   613
gga aca ttg cag agg gga aaa ggc aag gtg ctg ctc ttt agt ggg gaa  1942

 D   Y   W   R   L   D   L   K   T   Q   Q   I   D   K   G   Y   629
gat tac tgg agg ctg gat ctg aag act cag cag atc gat aaa gga tat  1990

 P   R   H   I   D   V   T   F   G   G   V   P   V   D   A   H   645
cct cgc cac att gac gtg acc ttt ggt ggg gtt cca gtg gat gct cac  2038

 D   V   F   L   Y   K   G   N   Y   Y   F   C   R   D   I   Y   661
gat gtg ttc ctc tat aag gga aac tat tac ttc tgt cga gac atc tac  2086

 Y   W   R   M   T   S   R   R   Q   V   D   R   V   G   Y   V   677
tac tgg aga atg acc agc agg cgg cag gtt gat aga gtt gga tat gtc  2134

 Y   E   L   L   N   C   P   N   Y   *                           686
tat gaa ctc ctg aat tgt ccc aac tac tga atctgactttatcagattgaagtg  2188
ttataaggaaagcgcatctaactgaaatgcaaatcccagatatgtgttttaaaaaagaaagtat  2252
agtataattgaaagagtagaaactctgtaataatgtttttgtgttatattaagaaatatttgcc  2316
tgcagcaaataataaaattaataagggtaaagtagtaaggttacatttggtaaagtatggtaaa  2380
atatatttgtcctctctgttctacttctttttcatatagcctaaaattgcacaagtgaatgttt  2444
ttcacaattgtctaatttattataaaatcagtagcattttgtcatatgaataattaaataaat  2508
ttttattttaagtctcaaaaaaaaaaaaaaaaaaaaaaaaaaaa                     2551
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Fig. 1. Nucleotide Sequence (Lowercase) and Deduced Amino Acid Sequence (Uppercase in the One-Letter Amino Acid Code) of Channel Catfish Matrix Metalloproteinase-9.

The mRNA instability motifs (attta), polyadenylation signal sequence (aataaa), and polyadenylation tail are indicated by boldface and italics, underline, and italics, respectively. A signal peptide is illustrated in italics, and a potential cleavage site is indicated by ↑. The asterisk denotes the stop codon.

analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. Images were documented with a KODAK Gel Logic 440 Imaging System and processed with an Adobe Photoshop program (v. 7.0.1).

Accession no. The CC MMP-9 cDNA and genomic DNA sequences were deposited in the GenBank database under accession nos. DQ516066 and EF486306, respectively.

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                                                  ←----------------------Matrix

Japanese medaka      MRCSAFAWCLLLGISIQSSWSVPLK-SVFVTFPGDVIKNMSDTDLAESYLRKFGYMDTFQ 59
Fugu rubripes        MRSCTLVLLVVLGVTLQDGWSLPIK-SVFVDFPGDIIKNMTDIELADNYLKKFGYIDIQR 59
Bastard halibut      MRCCALAVCLVL-VIVQDGWSLPLR-SISVTFPGDILKNVTDTDLAETYLKRFGYLDKMH 58
Rainbow trout        MR--RVLALFVLGMCMLSGWCVPLKKSVSVTFPGDVLKNMTDTELANSYLQRFGYMDVQH 58
Zebrafish            MR-LGVLAFLVLGTCSLRAWCLPLK-SVFVTFPGDVIKNMTNTQLADEYLKRYGYVDVLQ 58
Common carp          MR-LGVVVVLVLGTCSLRAWSAPLK-SVFVTFPGDVIKNMTDNQLAEEYLKRYGYIDVLQ 58
Channel catfish      MR-ISILAFMVLGTCTLSAWSHPIK-TIFVNFPGDVIKNTIDLELAESYLKRFGYMEILD 58
                     **   .   .:*      .*. *:: :: * ****::**  : :**: **:::**::

                     Metalloprotease Domain---------------→    ←-------

Japanese medaka      RSGFQSLASTSKALKRMQRQLGLDETGQLDMQTLEAMKQPRCGVPDVANYKTFDGDLKWD 119
Fugu rubripes        RSGLDSVIST-KALKKMQKQLGLKETGELDKTTLEAMKQPRCGVPDVANYATFEGDLKWD 118
Bastard halibut      RSGFQSMVSTAKALKMMQRQMGLKETGKLDKSTLEAMKQPRCGVPDVANYQTFEGDLKWD 118
Rainbow trout        RSGFQSMASTSKALMRMQRQMGLEETGTLDKSTVAAMKAPRCGVPDVRSYQTFQGDLKWD 118
Zebrafish            RSGLQAVISNAKALKKLQRQLGLEETGLLDQPTVDAMKQPRCGVPDIRNYKTFDGDLKWD 118
Common carp          KSGLQAVVSTSKALMKLQQQLGLEETGSLDQPTIDAMKQPRCGVPDIRNYQTFEGDLKWD 118
Channel catfish      KSGRQGTVSTSKALRRLQNQLGLKETGKLDQPTIDAIKTPRCGVPDVRNYQTFDGDLKWD 118
                     :** :.  *. ***  :*.*:**.*** **  *: *:* *******: .* **:******

                     ------------------------Matrixin------------------------

Japanese medaka      HGDVTYRILNYSPDLDSSVTDDAFARAFKVWSDVTPLTFTRLFDGTADIMISFGKKDHGD 179
Fugu rubripes        HNDVTYRVLNYSPDMDSFVIDDAFVRAFRVWSDVTPLTFTRLFDGIADIMISFGKTDHGD 178
Bastard halibut      HNDVTYRTLNYSPDMESSLIDDAFARAFKVWSDVTPLTFTRLYEGTADIMISFGKADHGD 178
Rainbow trout        HHDITYRILNYSPDMGASLIDDAFARAFKVWSDVTPLTFTRLFDGIADIMVSFGKADHGD 178
Zebrafish            HTDVTYRILNYSPDMEASLIDDAFARAFKVWSDVTPLTFTRLFDGIADIMISFGKLDHGD 178
Common carp          HTDVTYRILNYSPDMEAPLIDDAFARAFKVWSDVTPLTFTRLYDGTADIMISFGRENHGD 178
Channel catfish      HNDVTYRILNYSPDLDASVIDDAFARAFKVWRDVTPLTFTRLYNGIADIMILFGKRDHGD 178
                     * *:*** ******: : : ****.***:** **********::* ****: **: :***
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Fig. 2. *Continued.*

Results and Discussion

Cloning and characterization of CC MMP-9 cDNA

In our previous study, we partially identified the CC MMP-9 gene by subtractive suppression hybridization after *Edwardsiella ictaluri* infection of the CC ovary cell line (Yeh and Klesius, unpublished). We further cloned and characterized this gene by the RACE method[36] in this study. The complete sequence of the CC MMP-9 cDNA gene consisted of 2,551 nucleotides. Analysis of the nucleotide sequence revealed one open reading frame and 5′- and 3′-end untranslated regions (UTR) (Fig. 1). The 5′-end UTR had 103 bases, shorter than the corresponding region of rainbow trout, which has 147 nucleotides,[20] but longer than 5′-UTR of zebrafish, which has 96 nucleotides.[19] The 3′-end UTR had 398 bases in length, longer than the corresponding region of mouse,[37] but shorter than rainbow trout or zebrafish.[19,20] As seen in mammalian and other fish mRNAs, the CC MMP-9 mRNA instability motifs (attta) (Fig. 1,

```
                    ------------------------------------→        ←Fibronectin

Japanese medaka     LYPFDGKDGLLAHAYPPGEGIQGDAHFDDDEFWTLGKGPVVKTYYGNADGAMCHFPFVFG 239
Fugu rubripes       FYPFDGKDGLLAHAYPPGEGVQGDAHFDDDEFWTLGKGPVVKTLYGNAEGAMCHFPFRFQ 238
Bastard halibut     PYPFDGRNGLLAHAYPPGEGVQGDAHFDDDEHWTLGNGPAVKTLYGNADGAMCHFPFTFE 238
Rainbow trout       GYPFDGKDGLLAHAFPPGEGIQGDAHFDDDENWTLGKGAAVKTSFGNAEGALCHFPFSFG 238
Zebrafish           PYPFDGKDGLLAHAYPPGEGTQGDAHFDDDEYWTLGSGPAIQTRYGNAEGAMCHFPFLFE 238
Common carp         PYPFDGKDGLLVHAYPPGEGIQGDAHFDDDEYWTLGSGPAIQTRYGNAEGAMCHFPFLFE 238
Channel catfish     PYPFDGKDGLLAHAYPPGEGVQGDAHFDDDEYWTLGNGPAIKTYFGNAEGALCHFPFRFE 238
                     *****::***.**:***** ********** ****.*..::* :***:**:***** *

                    Type II Domain 1-----------→        ←Fibronectin

Japanese medaka     GKTYTSCTSEGRADKLPWCSTTDDYDRDGKYGFCPSELLYTIGGNSDGAKCVFPFVFLGD 299
Fugu rubripes       NKPYKHCTSEGRLDDLPWCATTADYDKDKKYGFCPSELLYTFGGNANGEKCVFPFTFLGM 298
Bastard halibut     GKSYTSCTTDGRTDNLPWCATTADYSRDGKYGFCPSELLYTVGGNADGAKCVFPFVFLEK 298
Rainbow trout       GKQYSTCTTEGRSDNLPWCATTADYGRDKKFGFCPSELLYTFDGNSNGKACVFPFVFLGE 298
Zebrafish           GTSYSTCTTEGRTDGLPWCSTTADYDKDKKFGFCPSELLFTFDGNSNEAPCVFPFVFDGK 298
Common carp         GTSYSSCTTDGRTDGIPWCATTADYDKDKIFGFCPSELLFTFDGNSNEAPCVFPFVFLGV 298
Channel catfish     GKSYSTCTTEGREDGLPWCATTADYGKDGKYGFCPSELLYTFDGNGDGQACVFPFVFERK 298
                    .. *. **::** * :***:** **.:*  :********:*..**.:   *****.*

                    Type II Domain 2---------→        ←Fibronectin

Japanese medaka     EYDSCTTEGRRDGYRWCATTSNYDQDKKYGFCPNTDTTTIGGNAEGEPCHFPFEFLGKEY 359
Fugu rubripes       EYDSCTSEGRSDGFRWCATTKSFDEDKKYGFCPGRDTAVIGGNSDGELCHFPFIFQDKEY 358
Bastard halibut     EYDSCTKEGRSDGYRWCATTANFDQDQKYGFCPSRDTAVFGGNSEGEPCHFPFVFLGKEY 358
Rainbow trout       TYEGCTTEGRSDGYRWCSTTENFDKDKKFGFCPNRDTAVIGGNSEGEPCHFPFVFLGNKY 358
Zebrafish           KYDSCTTEGRNDGYRWCSTTANFDTDKKYGFCPNRDTAVIGGNSEGEPCHFPFTFLGNTY 358
Common carp         KYDSCTTDGRTDGYRWCATTANFDTDKKYGFCPNRDTAVIGGNSEGEPCQFPFIFLDKTY 358
Channel catfish     TYTSCTTEGRDDGYRWCATTANFDQDKKYGFCPNRDTAVIGGNSEGEPCQFPFSFLGKTY 358
                     * .**.:** **:***:** .:* *:*:****. **:.:***::** *:*** * .: *
```

Fig. 2. *Continued.*

bold and italics), which regulate mRNA degradation[38] were located within the 3′-end UTR. In addition, a polyadenylation signal sequence (aataaa) (Fig. 1, underlined) was located 24 bases upstream of the polyadenylation tail of 27 nucleotides (Fig. 1, italics). The open reading frame potentially encoded a 686-amino-acid peptide with a calculated molecular mass (without glycosylation) of approximately 77.4 kDa. In addition, as analyzed by the SignalIP 3.0 program,[39] the CC MMP-9 included a signal peptide with a cleavage site at positions 20 and 21 (AWS-HP). CC MMP-9, analyzed by the NetOGlyc 3.1 program,[40] had potentially heavy *O*-glycosylation sites between positions 447 and 490.

Alignment and phylogenetic analysis of CC MMP-9 amino acid sequence with other fish MMP-9 amino acid sequences

To determine the similarity of the predicted CC MMP-9 with known MMP-9 sequences deposited in

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                    Type II Domain 3-------→

Japanese_medaka     DSCTSEGRGDGKLWCGTTASYDDDKKWGFCPDQGYSLFLVAAHEFGHALGLDHSNIRDAL 419
Fugu_rubripes       DSCTSEGRGDGKLWCSTTANYGQDKKWGLCPDRGYSLFLVAAHEFGHALGLEHSTIKEAL 418
Bastard_halibut     DSCTSEGREDGKLWCSTTDNYDEDAKWGFCDDEGYSLFLVAAHEFGHALGLDHSNIREAL 418
Rainbow_trout       DSCTSEGRGDGRLWCATTSNFDTDTKWGFCQDRGYSLFLVAAHEFGHALGLDHSNIRNAL 418
Zebrafish           SSCTSEGRNDGKLWCGTTSNYDTDKKWGFCPDRGYSLFLVAAHEFGHALGLDHSNIKDAL 418
Common_carp         TSCTSEGRGDGKLWCATTSNYDTDKKWGFCPDKGYSLFLVAAHEFGHALGLDHSKIKDAL 418
Channel_catfish     TSCTSEGRSDGKLWCATTSNYDKDSKWGFCPDKGYSLFLVAAHEFGHALGLDHSNIQDAL 418
                     ******* **:***.** .:. * ***:* *.******************:**.*::**

Japanese medaka     MYPMYSYVEDFSLHEDDIEGIQYLYGSKTDPKPTVPQPKPPTTTATPDPDVTDSTDEPEP 479
Fugu rubripes       MYPMYSYVENFSLHKDDIEGIQYLYGSNTGPKLTPNPPIYPTT----DPIVTDSTDRP-- 472
Bastard halibut     MYPMYTYVEDFSLHKDDIEGIQYLYGRGTGPDPTPPQPTSTTT-------TPNPTEEP-- 469
Rainbow trout       MYPMYSYVEDFSLHKDDVEGIHYLYGSKTGPDPIPTIPSPGPD-----PKPDTTTTKS-- 471
Zebrafish           MYPMYKYVEGFPLHRDDIDGIQYLYGPRTGPEPTAPQPRTTTSSPVVPTKPSPSDKTT-- 476
Common carp         MYPMYKYIEDFSLNQDDIEGIQYLYGPKTGPNPTPPKPKTTTSSPVVPTKPTPSEKTT-- 476
Channel catfish     MYPMYKYIADFSLHQDDIEGIQYLYGPKTGPDPTPPKPSTTTTSPVSTLKPTKKTPKT-- 476
                    *****.*: .*.*:.**::**:****  *.*.     *   .              .
                                                  ←--------------------------------
Japanese medaka     TNRPATTTL-------NPLDPTKDACKVTKFDTITVIGGDLYFFKDGHFWRKSRKGDAAL 532
Fugu rubripes       ---TETTTP-------LPVDPSQDACTVTIFDSITAINGELHFFKNGIYWRMPSKSNAGV 522
Bastard halibut     ----EPTTP-------QPVDPTRDACKLTKFDTITMIENELHFFENGNYWKMPSRGDGGL 518
Rainbow trout       ---TTTTTT-------HPVDPSQSPCQINKFDTITEIDGDLHFFKDGQYWRMSSKTDGGL 521
Zebrafish           ---TASTT--------TQVVPSDDACQIKEFDAITEIQKELHFFKDGRYWKIS--GNGER 523
Common carp         ---TVSTT--------THVGPSQDACEIKEFDAITEIQKELHFFKDRHYWKIS--SNGER 523
Channel catfish     ---TPSTTSTTTPSVYTPVDPSVDPCKVDKFDVITEIQGEPHFFKDGYYWKSSNRGNEER 533
                         .**          : *: ..* :  ** ** *  : :**::  :*: .   :
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Fig. 2. *Continued.*

GenBank, the ClustalW program[31] was used to align the MMP-9 amino acid sequences (Fig. 2). Several common and distinctive features were observed in CC MMP-9. First, like their mammalian counterparts, CC MMP-9 consisted of many structural domains, including a signal sequence, a matrix metalloprotease domain, matrixin, three tandem fibronectin type II domains, and hemopexin-like repeats,[5] but the total length of CC MMP-9 was shorter than its human or mouse counterparts. Second, a zinc binding motif, $Ala^{400}His^{401}Glu^{402}Phe^{403}Gly^{404}$-$His^{405}Ala^{406}Leu^{407}Gly^{408}Leu^{409}Asp^{410}His^{411}$, and "Met turn" structure[5,8] were conserved throughout evolution. Also, another zinc catalytic site, $Pro^{98}Arg^{99}Cys^{100}$-$Gly^{101}Val^{102}Pro^{103}Asp^{104}$, in the matrix metalloproteinase domain which is indispensable for maintaining MMP-9 in an inactive state[8,41] was conserved among the human and fish species examined. It is surprising that CC MMP-9 did not have the tripeptide Arg-Gly-Asp motif, which is central in mediating cell attachment.[42] We then examined MMP-9 amino acid sequences from other species deposited in GenBank. We found that five out of seven fish species examined had the tripeptide

```
                    ----------------------------------------Hemopexin-like

Japanese medaka     ESPMLISERWPALPAVIDSAFEDILTKKLYFFSGTKFWVYTGKNVLGPRSIEKLGLPDSV 592

Fugu rubripes       KGPHAISEKWPQLPAVIDTAFEDTVTKKIYFFSGTKFWVYTGQNVMGPRSIEKLGLPPSI 582

Bastard halibut     KGPFSLSERWPALPAVIDSAFEDLLTKNMYFFSGNRFWVYTKEGVLGPRSIEKLGLPTSI 578

Rainbow trout       EGPFSMSKRWPAVPVVVDTAFEDLATKKIYFFSGTRFWVYTGQSVLGPRSIEKLGLSSTV 581

Zebrafish           KGPFMISAKWPALPAVINSAFEDHLTKKIYFFSERQFWVYSGNDVLGPRKIEKLGLPSDL 583

Common carp         KGPFFISEKWPALPATINSAFEEPLTKKIYFFADKQFWVYTGKEVLGPRKIEKLGLPSDL 583

Channel catfish     KGPFIVSERWPALPAELDTAFEDPLTNKMYFFAGNQFWVFTGQDVLGPRRIEKLGLPVSL 593

                    :.*  :* :** :*. :::***:  *:::***:  :***:: : *:*** ******.  :

                    Repeats-------------------------------------------------

Japanese medaka     QKVEGALQKGKDKVLLFSGENFWRLDVKTQKIDKGYPKYIDVVFGGVPIDAHDVFLYEGR 652

Fugu rubripes       QKVEGALQRGKDKVLLFSGDSYWRLNIKDQKMDKGYPKYTDVAFGGVPYKAHDVFQYKGN 642

Bastard halibut     QKVEGALQRGKGKVLLFTEESFWKFDLKSQKMDKGYPKSTDYVFGGVPNDAHDVFQYKGH 638

Rainbow trout       EKVEGALQRGKGKVLLFNGENYWRLDVEGQKIDTGYPRFTDLVFGGVPLDSHDVFQFKGN 641

Zebrafish           DKVEGSMQRGKGKVLLFNGENFWRLDVKAQLIDRGYPRFTDAAFGGVPIDSHDVFLYKGF 643

Common carp         DRVEGTVQRGKGKVLLFNGVNFWKLDVKAQMIDSGYPRSTDSVFGGVPIDSHDVFLHKGF 643

Channel catfish     KKVEGTLQRGKGKVLLFSGEDYWRLDLKTQQIDKGYPRHIDVTFGGVPVDAHDVFLYKGN 653

                    .:***::*:**.*****.  .:*::::: * :* ***:  * .***** .:**** .:*

                    ----------------------------->

Japanese medaka     TFFCRDQFYWRMNSRRQVDRVGYVKYDLLKCTNPSSRY 690

Fugu rubripes       SYFCQDRSYWRMNSRRQVDRVGNVKNDILKCVESY--- 677

Bastard halibut     MYFCRDSFYWRMNSRRQVDRVGYVKYDLLKCSDSY--- 673

Rainbow trout       SYFCRDSFYWRMNSNRQVDRVGYVKYDLLNCSGS---- 675

Zebrafish           FYFCRESFYWRMNAKRQVDRVGYVKYDLLKCSDIHSL- 680

Common carp         FYFCRESFYWRMNAKRQVDRVGYVRHDLLKC------- 674

Channel catfish     YYFCRDIYYWRMTSXRQVDRVGYV-YELLNCPNY---- 686

                     :**::  ****.: ******* *  ::*:*
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Fig. 2. Multiple Alignment of the Predicted Channel Catfish Matrix Metalloproteinase-9 with Those from Other Fish Species Deposited in Public Domains.

ClustalW (v.1.83) software[31] was used for sequence alignment *via* the European Bioinformatics Institute website. Gaps were introduced in the sequences, indicated by hyphens (-). Structural domains of matrix metalloproteinase-9 are indicated above the sequences. Japanese medaka (*Oryzias latipes*), BAA85770;[22] fugu rubripes (*Takifugu rubripes*), BAE06266; bastard halibut (*Paralichthys olivaceus*), BAB68366;[21] rainbow trout (*Oncorhynchus mykiss*), CAC85923;[20] zebrafish (*Danio rerio*), AAH53292;[19] common carp (*Cyprinus carpio*), BAB39390. Identical amino acids among all sequences are indicated by asterisks, and similarities are indicated by dots or colons.

Arg-Gly-Asp sequence in their MMP-9 (Fig. 2), while only human and dog among mammals possessed the sequence, indicating that it might not be critical to MMP-9 function. In addition, pair-wise comparison of amino acid sequences showed that MMP-9 was highly conserved among fish species (67%–74%), but not in mammalian MMP-9 (*e.g.*, 55% with human MMP-9) (Table 2).

To determine the evolutionary relatedness of MMP-9 among animals, phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.1 program[32] based on ClustalW alignment. As shown

Table 2. Channel Catfish Matrix Metalloproteinase-9 Protein Identity with Other Species[a]

Species	Percent identity	Accession no.
Zebrafish	73	Q7T317
Common carp	74	Q98TC6
Japanese medaka	70	Q9PVM5
Japanese flounder	71	Q90YB3
Rainbow trout	71	Q8QFQ6
Japanese pufferfish	67	Q4JF83
Mouse	55	NP_038627
Human	55	P14780

[a]Percent identity was calculated by WU-Blast2 *via* www.ebi.ac.uk.

in Fig. 3, MMP-9 from various fish formed a broadly supported clade, but was distinguishable from mammalian MMP-9. This indicates the diversity of fish MMP-9. In a recent study, we found a high degree of diversity in fish hemoglobin-β.[33] In addition, when MMP-2 was included in one phylogenetic analysis, we observed two distinguishable MMP-2 and MMP-9 groups, and CC MMP-9 fell within the MMP-9 group. These results indicate that the identified CC sequence was orthologous to MMP-9.

CC MMP-9 genomic organization

To elucidate the genomic organization of CC MMP-9, a Universal GenomeWalker kit was used for genomic DNA library construction. The full-length of the CC MMP-9 genomic sequence consisted of 5,663 nucleotides, much shorter than those of human (7,700 nucleotides[43]) and mouse (8,190 nucleotides[37]) (Fig. 4 and Table 3). Analysis by comparison of the genomic sequence with the cDNA sequence showed that, except at the intron acceptor #9 position, the exon-intron structure followed the splice acceptor (AG)/donor (GT) consensus rule.[44] Further analysis revealed that the CC MMP-9 genomic sequence contained 13 exons, sizes ranging from 104 nucleotides (exon 12) to 239 nucleotides (exon 2). Except for exons 9 and 13, the sizes of the MMP-9 exons were conserved among CC, human, and mouse (Table 3). On the other hand, the sizes of the CC MMP-9 introns varied from 92 nucleotides to 288 nucleotides, and were not conserved among CC, human, and mouse. In addition, a TATA box at the 263–266 position and a tetradecanoyl-phorbol 13-aceate response element at 296–302 were also observed in the 5′-end regulatory region. Unlike the human MMP-9 genome,[43] CC MMP-9 did not have the alternating C and A sequence at the 5′-end regulatory region.

Expression of MMP-9 in tissues of normal channel catfish

To determine the expression profile of the MMP-9 transcript in various CC tissues, total RNA from head kidney, spleen, liver, intestine, skin, and gill was isolated, followed by duplex RT-PCR using the primers listed in Table 1 and analysis by 2% agarose gel electrophoresis. The amplified MMP-9 and β-actin products had 529 and 210 nucleotides, respectively (Fig. 5). As seen in Fig. 5, the CC MMP-9 transcript was detected in spleen and gill of fish examined (n = 4), albeit at various levels, in agreement with other reports on rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), and Japanese flounder (*Paralicthys oliva-*

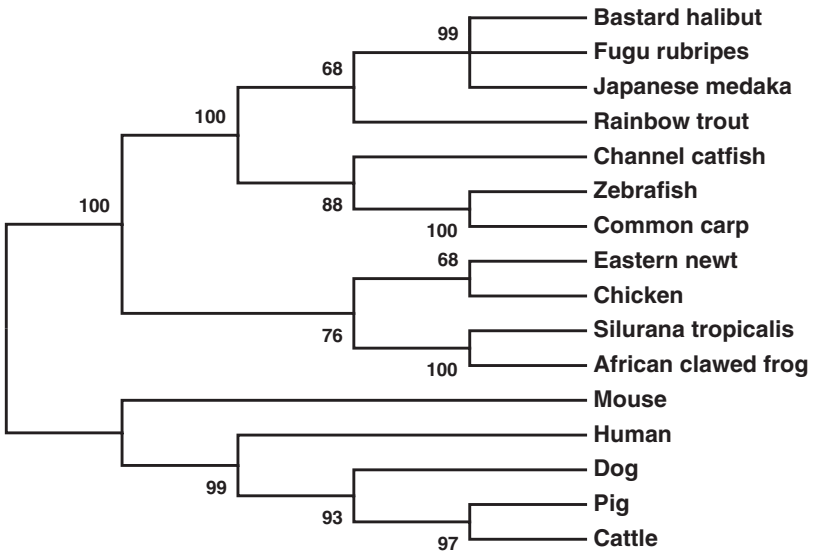

Fig. 3. Molecular Phylogenetic Relatedness of Matrix Metalloproteinase-9 Amino Acids.

In addition to the sequences from Fig. 2, the following amino acid sequences from GenBank were included in phylogenetic analysis by the neighbor-joining method (bootstrap replication 1,000 with its value > 50%) in MEGA version 3.1 phylogenetic analysis software:[32] eastern newt (*Notophthalmus viridescens*), AAX14805;[45] chicken (*Gallus gallus*), NP_989998;[4] silurana tropicalis (*Xenopus tropicalis*), AAH76927;[46] African clawed frog (*Xenopus laevis*), AAH57745;[46] mouse (*Mus musculus*), AAX90605; human (*Homo sapiens*), AAH06093;[46] dog (*Canis familiaris*), NP_001003219;[47] pig (*Sus scrofa*), NP_001033093; cattle (*Bos taurus*), NP_777169.[48] The numbers on the tree are bootstrapping values.

Table 3. Exon and Intron Junction and Sizes of Exons and Introns of Channel Catfish, Human, and Mouse MMP-9

Exon no.	Sequence				Exon sizes (bp)			Intron sizes (bp)		
					CC	Human	Mouse	CC	Human	Mouse
1	AGCTCT*CAG*C	*ATG***AGAATAAG**	**TGGCTGAA**	*GT*GAGTATT	135	138	141	263	870	437
2	TTGTTTC*CAG*	**AGTTACCTGAA**	**ACATATAG**	*GT*AACTATT	239	233	230	288	420	218
3	TTTTCTG*TAG*	**GATTCTGAACT**	**AAAAAGGG**	*GT*TAGTTTT	149	149	149	271	270	277
4	TATATTG*CAG*	**ATCATGGAGAT**	**TGGACCAG**	*GT*AAGAGTC	129	129	129	257	220	109
5	TTTCCTT*CAG*	**CAATCAAGACC**	**TAGCGAGC**	*GT*ATGTTCT	174	174	174	93	180	180
6	TTCTTCA*CAG*	**TTCTGTACACA**	**TAACCGAG**	*GT*GAGAGCC	174	174	174	172	325	350
7	GGGCCAA*CAG*	**ATACTGCTGTG**	**AGATAAAG**	*GT*AATTTTC	174	177	177	124	115	105
8	CTTGTCA*CAG*	**GATACAGTCTG**	**TCTCTATG**	*GT*ATGCACA	156	156	156	142	700	1155
9	CCTTATTAT*G*	**GACCCAAAACA**	**AAAGATGG**	*GT*AAGAACT	229	280	334	273	113	101
10	TTCCCAT*CAG*	**GTACTACTGGA**	**CTTTGCAG**	*GT*AACGCAA	140	140	140	92	240	269
11	TCTATTT*CAG*	**GCAATCAGTTC**	**TACTGGAG**	*GT*GAATATA	151	151	151	136	96	104
12	ATTTTCTT*AG*	**GCTGGATCTGA**	**CTATAAGG**	*GT*AATTTGC	104	104	104	164	1800	1150
13	TTCATTT*CAG*	**GAAACTATTAC**	**ACTAC***TGA*	A*T*CTGACTT	107	119	134			

Human and mouse data are from Huhtala *et al.*[43] and Masure *et al.*,[37] respectively.

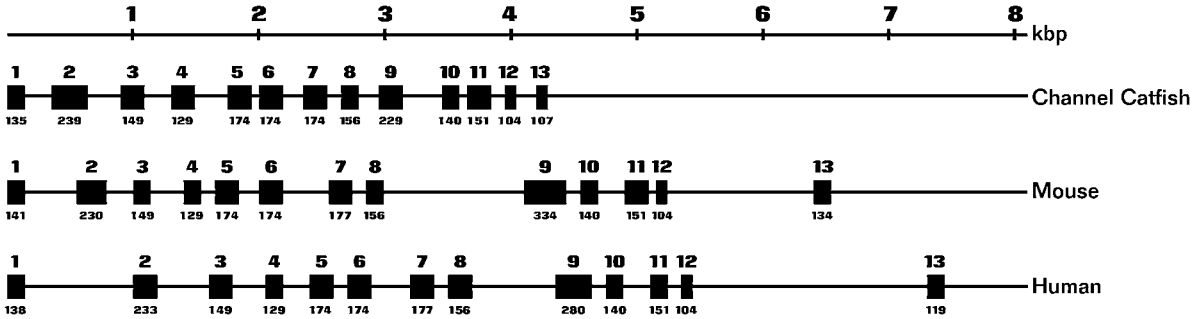

Fig. 4. Comparison of Matrix Metalloproteinase-9 Gene Organization of Channel Catfish, Human[43] and Mouse.[37]
Exons shown in filled boxes were joined by introns denoted by lines. The number and size of exons of each species are indicated above and below the boxes, respectively. The sizes of the introns for each species are listed in Table 3.

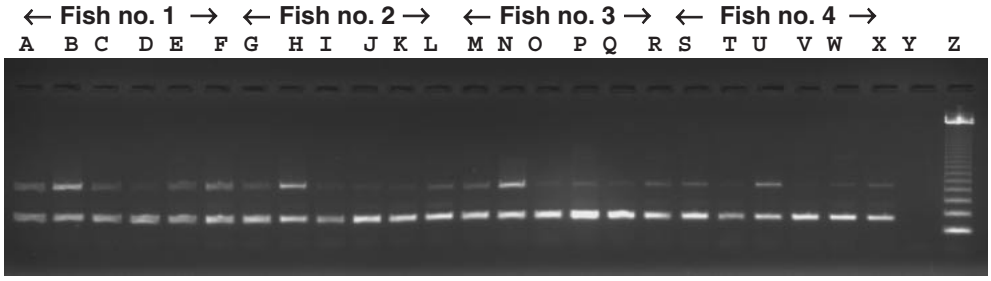

Fig. 5. Expression of Matrix Metalloproteinase-9 Transcript in Different Channel Catfish Tissues (n = 4).
Spleen (lanes A, G, M, and S), head kidney (lanes B, H, N, and T), liver (lanes C, I, O, and U), intestine (lanes D, J, P, and V), skin (lanes E, K, Q, and W), and gill (lanes F, L, R, and X). The sizes of matrix metalloproteinase-9 and β-actin products were 529 and 210 nucleotides, respectively. Lane Y, no cDNA added as a negative control; lane Z, 123 bp molecular weight standards (Invitrogen).

ceus).[20,21,23] In addition, the transcript was detected in head kidney from three fish. These results suggest that teleost MMP-9 is constitutively expressed in restrictive tissues. Its role in the teleost innate immune responses is under investigation.

In conclusion, the CC MMP-9 gene *via* the mRNA as well as genomic DNA was cloned and characterized. The transcript was detected constitutively in restrictive CC tissues. Further experiments to determine the roles of CC MMP-9 in *Edwardsiella ictaluri* pathogenesis in channel catfish are underway.

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