CHARACTERIZATION OF INNATE IMMUNE GENES OF CATFISH: CXC

CHEMOKINES AND TOLL-LIKE RECEPTORS

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CHARACTERIZATION OF INNATE IMMUNE GENES OF CATFISH: CXC CHEMOKINES AND TOLL-LIKE RECEPTORS

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CHARACTERIZATION OF INNATE IMMUNE GENES OF CATFISH: CXC CHEMOKINES AND TOLL-LIKE RECEPTORS

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Puttharat Baoprasertkul, daugther of Yingsak Baoprasertkul and Wannee Baoprasertkul, was born September 3, 1969, in Nakorn Srithammarat, Thailand. She graduated from Kasetsart University in Bangkok, Thailand with a Bachelor of Science degree majoring in Fisheries in 1994. After working as a fisheries biologist at the Department of Fisheries, Bangkok, Thailand for two years, she attended the University of Tasmania, Australia in 1996 and obtained a Master degree of Science majoring in Fish Genetics in 1998. She entered Graduate School at the Department of Fisheries and Allied Aquacultures, Auburn University to pursue a Doctor of Philosophy degree in August 2001.

DISSERTATION ABSTRACT

CHARACTERIZATION OF INNATE IMMUNE GENES OF CATFISH: CXC CHEMOKINES AND TOLL-LIKE RECEPTORS

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Innate immunity is known as the first line of defense against pathogenic invasions. Fish, as lower vertebrates, are believed to maintain their healthy status mostly by relying on their innate immunity. Of the many types of genes involved in innate immunity, chemokines and toll-like receptors (TLRs) are known to play critical roles during early stages of microbial infections. Chemokines represent a superfamily of small molecular cytokines involved in recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci, while TLRs have been elucidated to serve as receptors for specific pathogen-associated molecular patterns (PAMPs).

In order to better understand catfish immune responses, I have characterized five genes involved in the innate immunity including CXCL2, CXCL10, CXCL12, CXCL14

and TLR2. While the chemokine genes shared high levels of sequence identities and structural features with their counterparts from other species, the catfish (*Ictalurus sp*.) TLR2 is an intronless gene, dissimilar to those from fugu (*Takifugu rubripes*), flounder (*Paralichthys olivaceus)*, and human (*Homo sapiens*), but similar to that of zebrafish (*Danio rerio*). Analysis of expression of these genes provided functional hints. While CXCL10 was highly inducible upon an infection with Gram-negative *Edwardsiella ictaluri*, causative agent of enteric septicemia of catfish (ESC), CXCL2, CXCL12, and CXCL14 were constitutively expressed, suggesting their homeostatic functions in addition to serving as chemokines. The catfish TLR2 was also respond to Gram-negative ESC bacteria, although a major role of TLR2 response is for Gram-positive bacterium.

This work also mapped the chemokines and the TLR2 to BAC clones, setting the foundation for mapping them to the catfish physical map. Various copy numbers seemed to exist in the catfish genome. CXCL2, CXCL12, and CXCL14 exist as a single copy gene in the catfish genome; TLR2 appeared to have two genomic copies, whereas CXCL10 appeared to have a multi-gene family with at least four copies arranged in a tandem fashion. While this research for the first time identified the chemokine and TLR genes in catfish, functional studies are required to answer many of the interesting questions raised by this work.

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I. INTRODUCTION

Immunity, the state of protection from disease, has both non-specific and specific components. Immunologists have long been interested in the components of the innate (non-specific) immune system and their interactions with the better-characterized adaptive immune system. Innate or nonspecific immunity refers to the basic resistance to disease that a species possesses. Innate defenses have the potential for quick, powerful, non-specific responses to a wide range of pathogens. An effective innate immune response to an acute disease is often the difference between life and death for the host organism. Innate immunity comprises defense barriers at various levels including physical barriers, physiologic barriers, cellular barriers, and molecular barriers.

The aqueous environment is critical for fish to survive, as well as facilitating transmission of many water-borne pathogens. For fish that are in constant and intimate contact with their aquatic environment, physical barriers are extremely important. The scales, skin, and surface of mucous membranes are all crucial barriers against microbial and parasitic invasions. The fish mucous layer contains antimicrobial substances that can effectively stop an infection at its earliest stages.

The physiological barriers of innate immunity include pH, temperature, oxygen, and various soluble factors. If the pathogens are able to breach the physical barrier, they next face the soluble factors such as anti-bacterial peptides, lysozyme, complement factors and acute-phase proteins [1,2].

At the cellular level, the host is capable of ingestion of extracellular macromolecules and particles through endocytosis and phagocytosis. At the same time, the cellular component of innate immunity is stimulated upon recognition of pathogen-associated molecular patterns (PAMPs) [3,4] including lipopolysaccharide (LPS), lipoproteins, flagellin, and host-derived cytokines. The invasion of pathogenic microorganisms often causes tissue damage that induces a series of complex responses collectively known as the inflammatory response. Key players in the inflammatory response include cytokines, a group of low molecular weight proteins which function as messengers of the immune system in cell-to-cell communication.

With the advent of the genomics era, great efforts were made in mammalian species to identify and characterize the primary gene components of innate immunity including chemokines, cytokines, complement factors, and toll-like receptors. In the last five years, the resources developed from the mammalian species have expedited similar efforts in the fish research community.

Fish represent a transition point on the evolutionary spectrum between species possessing only innate defenses and species depending heavily on adaptive immunity. Much can be learned, therefore, about the origins, functions and regulation of immunity through the study of fish. Furthermore, the tremendous diversity of fish, the largest group of extant vertebrates with over 23,000 species, allows the study of immune

adaptations over broad temporal, spatial, and life history differences. Non-specific immunity is particularly important to cultured fish [1]. Intensive aquaculture often results in acute disease outbreaks. For example, enteric septicemia of catfish (ESC) is characterized by heavy mortalities only four days after infection. This short time-frame means that a specific response (antibody production) cannot be effectively mounted and the fish is left to rely on innate resistance in the initial exposure to the pathogen. Correlations between survival from disease and expression of innate immune-related genes, therefore, provide an exciting basis for research in fish immunology.

Inflammation, the attempt to localize cellular injury caused by an infectious agent, is an important part of inducible innate immunity and can be seen within 1-2 days of infection. Chemokines, as mediators of inflammation, are crucial components of innate immunity.

Chemokines are a large superfamily of chemotactic cytokines that stimulate the recruitment, activation and adhesion of cells to sites of infection or injury [5]. They are structurally-related small peptides, with the majority containing four conserved cysteine residues. Based on the arrangement of these conserved cysteine residues [6,7], chemokines were divided into four subfamilies: CXC (α), CC (β), C, and CX3C. Corresponding to these subfamilies of chemokine proteins, their encoding genes were designated by SCY (for small inducible cytokines) followed by a letter A, B, C, or D (for CC, CXC, C, and CX3C, respectively). CXC and CC are the two major subfamilies. To date, 16 CXC chemokines, 28 CC chemokines, two C chemokines, and one CX3C chemokine have been identified from mammalian species [8].

Despite the availability of these mammalian chemokines for comparative immunology, low sequence conservation has hindered their identification in fish through PCR or hybridization-based techniques. Nevertheless, as the volume of sequencing in aquatic species has increased, *in silico* identification of chemokines has become practical in some species. The availability of a growing set of fish chemokines on public databases should have a cascading effect through the fish research community as identification of chemokines in one species facilitates their identification in closely related species and so on. Such an "information cascade" is necessary, if we are to conduct accurate phylogenetic analyses and establish reasonable orthologous relationships between fish chemokines and their mammalian counterparts.

Recently, by using comparative study from available draft genome in non-mammalian species, about 127 chemokines and 70 chemokine receptors in seven species, including zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*) have been identified and their evolutionary relationships were inferred [9]. The gradual identification of a small number of fish chemokines has provided a foundation from which we can now explore the total extent of chemokine diversity in lower vertebrates such as catfish.

The CXC subfamily, the second largest group of chemokines, has its first two conserved cysteine residues separated by one amino acid. The 16 CXC chemokines reported from mammals tend to be clustered together on chromosome locations. The CXC group can be divided into two subgroups based on the presence and absence of an ELR motif (glutamic acid (E), leucine (L) and arginine (R)). The ELR subgroup of CXC chemokines includes CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL15. These CXC chemokines specifically attract neutrophils that express receptors

CXCR1 and CXCR2. They are expressed in a wide range of cells in response to many stimulants, particularly pro-inflammatory cytokines such as IL-1 and TNF [5]. Their major role is to promote the adherence of neutrophils to endothelial cells and subsequent migration along a gradient of chemokines associated with matrix proteins and cell surfaces toward inflammatory sites. ELR-containing chemokines are all angiogenic and chemotactic for endothelial cells [10]. The non-ELR subgroup includes CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, and CXCL16. They attract lymphocytes and monocytes, with poor chemotactic ability for neutrophils [11]. Most of this subgroup are angiostatic and possess anti-angiogenic properties.

Only a few fish chemokines orthologous to the mammalian CXC chemokines have been identified. The first report of a fish CXC chemokine came from lamprey (*Lampetra fluviatilis*) [12]. This CXC chemokine was designated as LFCA-1 and showed the highest similarity to human IL-8 (CXCL8). Presently, six fish CXC chemokines with highest amino acid similarity to CXCL8 have been identified from flounder (*Paralichthys olivaceous*), rainbow trout (*Oncorhynchus mykiss*), silver chimaera (*Chimaera phantasma*), banded dogfish (*Triakis scyllia*) and channel catfish [13-18]. Unlike human CXCL8, all fish CXCL8 sequences are lacking an ELR-motif.

In addition to the fish CXC chemokines described above, a 100 amino acid chemokine was identified in rainbow trout as being related to the CXCL9, CXCL10 and CXCL11 subset, but with highest similarity to CXCL10 [19]. Two CXC chemokines from carp, CXCa and CXCb, have been functionally characterized, but lack orthology to known mammalian CXC chemokines [20,21].

A CXCL12-like EST sequence has been detected from zebrafish which is 44% identical to human CXCL12 at the amino acid level and appears to have a role in developmental processes such as the control of primordial cell migration [22]. Recently, Knaut et al. [23] discovered additional function of zebrafish CXCL12 for ganglion formation of sensory neurons. A CXCL14 homologue has also been identified from zebrafish [24] that is predicted to have a role in the development of the acoustico-lateralis system in non-haematopoietic cells of the central nervous system of fish. Recently, orthologues of CXCL12 and CXCL14 have been isolated from carp, of which CXCL12 was duplicated [25].

The other important innate immune component which is a focus in this study is toll-like receptors (TLRs) which act as germline-encoded receptor to recognize microbial pathogens. A series of germline-encoded receptors in the host innate system are known as pattern recognition receptors (PRRs) that can specifically recognize a highly conserved structures which are exclusively found on pathogens, referred to as pathogen-associated molecular patterns (PAMPs) [26]. Several of the best known examples of PAMPs are lipoproteins, lipopolysaccharide (LPS), dsRNA and flagellin. A successful, appropriate innate immune response can lead to rapid elimination of pathogens long before an antibody-based defense could potentially be mounted from clonal selection and expansion processes that may take several days to weeks. If a pathogenic invasion cannot be fully controlled by an innate immune response, several of its components are crucial stimulators of the later acquired immune response.

Toll gene was initially identified in *Drosophila* as an important receptor for embryonic dorsal-ventral development [27,28]. Subsequent work found this gene is also involved in defense system against a fungal infection [29].

The first toll-like receptor (TLR) in mammals, homologous to the *Drosophila* toll gene, was discovered and designated as TLR4 which functions as a receptor for lipopolysaccharide (LPS) [30,31]. TLRs are defined by the presence of a Toll/IL-1 receptor (TIR) domain in their cytoplasmic portion and by leucine-rich repeats (LRR) in their extracellular domain [32]. Each TLR recognizes and responds to different types of pathogens such as lipopeptides (TLR2), dsRNA (TLR3), LPS (TLR4), flagellin (TLR5), and nucleic acid and heme motifs (TLR7-9) [33]. At least 11 TLRs have been identified to-date from mammals.

The discovery of TLRs in teleost fish was reported in fugu (*Takifugu rubripes*) genome with a total of nine TLRs: TLR1, TLR2, TLR3, TLR5, TLR7, TLR8, TLR9, TLR21 and TLR22 [34]. The first seven fugu TLRs are structurally similar to those of human, while the last two are unique in fish species. The authors suggested that the fish lineage may have lost TLR4 while TLR21 and TLR22 were lost in mammals. Stafford et al. [35] later found TLR in goldfish macrophage with a constitutive expression. The up-regulation was detected in cultured macrophage after 3 and 6 hr post-stimulation with LPS, heat-killed *Aeromonas salmonicida*, and live *Mycobacterium chelonei*.

A large set of TLRs and their adapter proteins in zebrafish have been identified by two research groups at the same time [36,37]. Altogether, 19 TLRs, two interleukin receptors and four adapter genes from zebrafish genomic database have been identified, including TLR4 which does not exist in fugu genome. An additional two TLRs were

later discovered in Japanese flounder (*Paralichthys olivaceus)* by Hirono et al. [38]. JF-TLR2 and JF-TLR22 were identified from flounder cDNA clones, encoding 818 and 961 amino acid residues, respectively. Their expression was induced after peptidoglycan and polyI:C stimulation.

Several more TLRs from teleost fish have been reported since late 2004. The membrane and soluble form of TLR5 were found in rainbow trout that was up-regulated after flagellin induction, thus indicating the role of acute phase protein sensing during bacterial infection [39,40]. Although the soluble form of rainbow trout TLR5 (rtTLR5S) does not exist in humans, a recent study showed this protein bound flagellin and was able to function as an adjuvant amplifying human TLR5-mediated NF-kappaB activation [41]. Complete sequences as well as expression analysis of TLR3 and two signaling molecules, IL-1 receptor associated kinases 4 (IRAK-4) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), were recently reported in zebrafish [42]. TLR3 was also recently reported in rainbow trout, which was up-regulated after poly (I:C) stimulation, while either no change and down-regulation were found after *Yersinia ruckeri* infection [43].

For catfish, only partial sequences of TLR3 and TLR5 were identified, and their expression in channel catfish and back-cross hybrid (F1 male (blue x channel) x female channel) catfish was induced after *E. ictaluri* infection [44,45]. In addition, Peterson et al. [46] found TLR3 and TLR5 also play a role during embryogenesis in channel catfish and hybrid catfish (channel x blue).

Due to the importance of innate immune responses, specifically during the early period of infection, the study of chemokines and TLRs will provide more information on

fish immunology. Catfish, the predominant aquaculture species in the US, suffer heavy mortalities from ESC disease. The disease accounts for close to $30 million dollars in farm losses each year. Therefore, the understanding of catfish immune response in relation to resistance and susceptibility may help us to understand the fundamental mechanisms for the observed differences in resistance against ESC between channel catfish and blue catfish.

In this dissertation, I identified, cloned, and characterized five innate immune related genes from catfish including four CXC chemokines and one TLR: CXCL2 (MIP-2), CXCL10 (interferon-γ inducible protein-10 or IP-10), CXCL12 (SDF-1), CXCL14 (BRAK), and TLR2. In addition to analysis of gene structures and organization, genomic location and copy numbers, I have focused my energy to analyze the expression of the five genes in relation to tissues, and after infection with ESC. Particular attention was given to the differential expression between channel catfish and blue catfish to search for any clues that may be correlated with the observed difference in resistance against ESC between the two species. The dissertation is organized into three major chapters: one chapter on CXCL10 and its differential expression after infection with ESC, one chapter on characterization of the genes and their homeostatic expression of CXCL2, CXCL12, and CXCL14, and the third chapter on molecular characterization of TLR2. Because the major findings of these three chapters are published, I believe it is most productive to organize these chapters as separate and independent chapters, with this general introduction, followed by a section of overall conclusions.

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II. DIFFERENTIAL EXPRESSION PROFILES OF CHEMOKINE CXCL10 IN RESISTANT AND SUSCEPTIBLE CATFISH AFTER INFECTION OF *EDWARDSIELLA ICTALURI*

Abstract

Chemokines represent a superfamily of small molecular cytokines involved in recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci. We cloned and sequenced the cDNA, and analyzed expression of CXCL10 (IP-10) chemokine from channel catfish and blue catfish to study its potential roles in resistance to infectious diseases. Sequence analysis of PCR amplicons from a single F1 hybrid catfish indicated that channel catfish and blue catfish may have a multigene family of the IP-10 gene. Catfish IP-10 was expressed in a wide range of tissues including head kidney, spleen, liver, gill, skin, stomach, and intestine, but not in the muscle. Fish challenged with intracellular bacterium *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), showed dramatically elevated levels of IP-10 expression, as quantified with real time RT-PCR. Differential expression profiles were observed between resistant and susceptible channel catfish strains and blue catfish. Blue catfish were characterized by only modest induction in comparison to the drastic elevation of IP-10 in channel catfish. Taken together, our results suggest a role for chemokine IP-10 in the innate immune response of catfish to ESC.

1. Introduction

The roles of the innate and acquired immune systems vary according to an organism's position in the evolutionary spectrum. Invertebrates and early chordates possess only innate immunity while higher vertebrates depend heavily on acquired immunity [1]. However, immune recognition, signaling, and gene regulatory mechanisms are remarkably conserved [2]. Bony fish use both systems of immunity, making them an excellent model for the study of the evolutionary interactions between the two. As immunologists learn more about the response of bony fish to prominent diseases, they have discovered that the innate immune system is often the very important factor in resistance. Diseases characterized by rapid progression through the host organisms are affected more by the fast-acting innate components (cytokines, chemokines, and complement factors *etc*.) than by the delayed responses of acquired immunity. Thus, research into the innate components of immunity is critical for the comprehension and eventual prevention of fish diseases.

Several innate immune systems have been identified in bony fish including chemokines (CC, CXC, and CX3C chemokines and their receptors), cytokines (IL-1, interferons, TGF-β, TNF-α), acute phase proteins (SAA, SAP, CRP, a2M, and the complement component C3-C9, MASP, MBL, Bf), NK cell receptors, and molecules upstream and downstream of the Toll signaling pathways [1]. Of these innate immune molecules, chemokines serve as pioneer passengers at inflammatory sites. Upon localized physical or chemical insult, phagocytes (macrophages and neutrophils) are activated in response to microbial antigens or compounds released from damaged cells.

Among the mediators of inflammation released by the activated phagocytes are an array of chemokines and cytokines. These compounds in turn lead to increased vascular permeability, proliferation and recruitment of leukocytes, and the synthesis of acute phase proteins by the liver for fighting off the pathogens.

Chemokines represent a superfamily of small molecular cytokines involved in recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci [3]. Based on the arrangement of the first two cysteine residues of the amino acid sequences [4,5], chemokines were divided into four groups: CXC (α), CC (β), C (γ), and CX3C (δ) subfamilies. The CXC and CC are the two major families of chemokines. The CXC family includes IL-8, GRO/MGSA, MIP-2, ENA-78, NAP-2, MIG, GCP-2, PF4 and CXCL10 (interferon-γ inducible protein-10, IP-10). The biological effects of CXC family gene products can be considered as predominantly stimulatory towards neutrophils (PMNs). They are generally involved in promoting acute, PMN-driven inflammatory reactions [6].

IP-10 is a pleiotropic member of the CXC chemokines superfamily [3]. Up-regulation of IP-10 expression has been reported after bacterial infection [7] while down-regulation of its expression was reported after Mumps virus infection in human leydig cells [8]. It has been suggested that IP-10 had a role in CXCR3 mediated activation of eosinophils [9] and tissue regeneration in multiple models of liver and bile duct injury [10]. IP-10 cDNA has been cloned from a number of mammals including human, rat, mouse, and rhesus monkey. In fish, three sequences are highly similar to the IP-10 sequences---one from common carp [11] (accession number BAB88677), and two from rainbow trout [12] designated as VHSV-induced protein-7 (accession number

AAM18466) and VHSV-induced protein-8 (accession number AAM18467). The cDNA for the catfish IP-10 was not available and its role in early defenses after infection is not known. The objectives of this study were to characterize the IP-10 cDNA, analyze its expression before and after infection, and compare its expression in catfish with different genetic backgrounds and different resistance to the bacterial disease enteric septicemia of catfish (ESC). *Edwardsiella ictaluri* infection in channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) causes ESC. Under artificial challenge conditions, this disease progresses rapidly causing heavy mortalities starting four days after infection, when antibody-based defense is not yet in place [13]. This observation made many to believe that the innate immunity may play a crucial role for the survival of catfish from ESC disease. Under natural and artificial challenge conditions, channel catfish are generally susceptible to the infection of *E. ictaluri* while blue catfish are generally resistant [14]. Thus, the two closely-related catfish species offer an excellent research system for understanding early defense reactions after infection, especially the differential responses attributed to innate immunity. Channel catfish (*Ictalurus punctatus*) is a classical model within lower vertebrates for the study of comparative immunology [15, 16, 17]; and major progress has been made in understanding structure and organization of genes involved in both the innate and adaptive immunity [e.g.,18, 19, 20, 21, 22]. We report here molecular cloning, sequencing, and expression of IP-10 gene in catfish. The chemokine exhibits dramatic differences in expression profiles after infection in the resistant blue catfish and susceptible channel catfish suggesting a potential role through innate immunity in the early defenses of catfish.

2. Materials and Methods

2.1. Fish and challenge experiments

Three strains of catfish were used: Marion Select, Kansas Random of channel catfish (*Ictalurus punctatus*) and D&B strain of blue catfish (*I. furcatus*). Challenge experiments were conducted as previously described [23] with modifications. Briefly, the three strains of catfish were communally challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from natural outbreak in Alabama (outbreak number ALG-02-414) and inoculated into brain heart infusion (BHI) medium and incubated in a shaker incubator at 28°C overnight. The bacterial concentration was determined using colony forming unit (CFU) per ml by plating 10 µl of 10-fold serial dilutions onto BHI agar plates. At the time of challenge, the bacterial culture was added to the tank to a concentration of 3 x10^7 CFU/ml. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/ml. After 2 h of immersion exposure, 45 fish (15 fish each from the three strains) were randomly taken and placed into a rectangular trough containing pond water with constant water flow through. Eight troughs were used in the experiments including one trough for the controls for a total of 120 fish per strain. For the control fish, 15 fish of each strain were incubated in a separate rectangular tank with the same fish density as the challenge tanks. The only difference was that ESC bacteria were not added. After 2 h, these control fish were incubated in a separate trough at the same density as the challenged fish.

2.2. Tissue preparation and RNA isolation

Samples were collected from 10 fish of each strain before challenge as unchallenged control. After challenge, samples were collected at various times after infection: at 4 h, 24 h, 3 days, and 7 days. Samples were also collected from fish that were dying during a period between day 4 and day 7 after challenge. At each time point, 10 fish were sacrificed for sampling from each strain. The fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg per liter before tissues were collected. The following tissues were collected from 10 fish of the same strain at a given time point: head kidney, spleen, skin, gill, intestine, stomach, muscle, and liver; samples from each tissue type were combined. Tissues were kept in a –80 °C ultra-low freezer until preparation of RNA.

Pooled tissues from 10 fish were rapidly frozen with liquid nitrogen and were ground with a mortar and pestle. A fraction of the mixed tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [24] using the Trizol reagents kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Extracted RNA was stored in a -70°C freezer until used as template for real time reverse transcriptase PCR (real time RT-PCR).

2.3. Plasmid preparation and sequencing

Plasmid DNA was prepared by the alkaline lysis method [25] using the Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid

DNA (about 0.5-1.0 µg) were used in sequencing reactions. Chain termination sequencing [26] was performed using cycleSeq-farOUT™ polymerase (Display Systems Biotech, Vista, CA). The PCR profiles were: 95 °C for 30 s, 55 °C for 40 s, 72 °C for 45 s for 30 cycles. An initial 2 min denaturation at 96 °C and a 5 min extension at 72 °C were always used. Sequences were analyzed on an automatic LI-COR DNA Sequencer Long ReadIR 4200 or LI-COR DNA Analyzer Gene ReadIR 4200. BLAST searches [27, 28, 29] were conducted to determine gene identities. The EST analysis was conducted as we previously reported [30, 31].

2.4. PCR amplified segment of IP-10 gene

A blood sample from a single individual F1 hybrid catfish (channel catfish x blue catfish) was obtained for genomic DNA extraction. Genomic DNA (100 ng) was used to amplify the IP-10 gene segments. Specific primers (Table 1) were designed to amplify regions with single nucleotide polymorphisms (SNPs) and a 7-bp deletion to reveal sequence variation within IP-10 genes. The PCR profiles were: 94°C for 30 s, 45°C for 60 s, 72°C for 60 s for 40 cycles. An initial 3 min denaturation at 94°C and a 5 min extension at 72°C were always used. PCR products were cloned into pCR 2.1-TOPO cloning vector (Invitrogen). Clones were grown, isolated and sequenced following the above protocol (section 2.3.)

Table 1 Primers used in analysis of sequence variation and RT-PCR or real time RT-PCR

Primer name	Primer sequences (5' to 3')
IP-10 upper for sequence variation & RT-PCR	TCTGAATTTACTAAGAAATAC
IP-10 lower for sequence variation & RT-PCR	CTCATAAATTAAACAGT
IP-10 upper for real time RT-PCR	CCAGTGTAAGGAGGTGTT
IP-10 lower for real time RT-PCR	CTCTAATCCTGCCGTGATG
β–actin upper	AGAGAGAAATTGTCCGTGACATC
β–actin lower	CTCCGATCCAGACAGAGTATTTG

2.5. RT-PCR

Total RNA was used for reverse transcription-PCR (RT-PCR) reactions. The RT-PCR reaction was conducted using SuperScript^{TM} III One Step RT-PCR System (Invitrogen). The system contained a mixture of SuperScript^{TM} III reverse transcriptase and the Platinum Taq DNA polymerase in an optimized buffer. Detailed procedures followed the instructions of the manufacturer. Briefly, the following was added to a reaction of 50 µl: 25 µl 2X reaction mix, 1 µl total RNA (~100 ng), 1 µl (100 ng) each of the upper and lower primer (Table 1), 2 µl SuperScript III RT/Platinum Taq polymerase mix, and water to bring the reaction volume to 50 µl. The reaction also included the primers of β-actin (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 ºC for 15 min for one cycle (reverse transcription reaction), the samples were pre-denatured at 94 ºC for 2 min, then the samples were amplified for 40 cycles with 94 ºC for 15 s, 45 ºC for 30 s, 68 ºC for 1 min. Upon the completion of PCR, the reaction was incubated at 68 °C for an

additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5 % agarose gel and documented with a Gel Documentation System (Nucleotech Corp., CA).

2.6. Real time PCR using a Lightcycler

Total RNA was used for reverse transcription real time PCR (RT-real time PCR). Concentration and quality of total RNA was determined by spectrophotometry (optical density 260/280 ratio) and electrophoresis. Primers used in real time RT-PCR are shown in Table 1. β–actin was used as an internal control for real time RT-PCR. A standard curve was constructed by using various copy numbers of a plasmid containing IP-10 cDNA. Real time RT-PCR reactions of the standard curves were always included in all runs in order to relate quantitative data from run to run. One-step real time RT-PCR was carried out in a LightCycler (Roche Applied Science, Indianapolis, IN) using a Fast Start RNA Master SYBR Green I reagents kit (Roche Applied Science) following manufacturer's instructions with modifications. Briefly, all real time RT-PCR reactions were performed in a 10-μl total reaction volume (9 μl master mix and 1 μl RNA template). A five-step experiment protocol was run on the LightCycler: (i) reverse transcription, 20 min at 61 °C; (ii) denaturation, 30 s at 95 °C; (iii) amplification repeated 50 times, 1 s at 95 °C, 1 s at 55 °C, 13 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 per second; (v) cooling, 30 s at 40 °C. Concentration of cDNA in each sample was calculated from the standard curve. Each sample was normalized to the equivalent of the reference gene, β–actin. The ratio between IP-10 and β–actin was used for the purpose of comparisons.

3. Results

3.1. cDNA cloning and sequence analysis

cDNA clones for the channel catfish and blue catfish IP-10 genes were obtained by homology comparisons of EST sequences. During EST analysis of channel catfish, four clones were found to be homologues of IP-10; one from the brain EST clone IpBrn01096 (accession number BE212851) [32], one from spleen EST clone IpSpn01194 (accession number BM425159), and two from head kidney ESTs IpHdk01736 and IpHdk02546 (accession numbers BE468362 and BE469394). One blue catfish EST clone IfHdk01187 (accession number BQ097353) was identified to include the blue catfish IP-10 cDNA. All these clones harbored complete coding sequences of the IP-10 gene.

Sequence analysis indicated that the four cDNA clones of the channel catfish IP-10 gene represented two types of cDNAs: IpBrn01096 and IpHdk01736 had identical sequences, and IpHdk02546 and IpSpn01194 had identical sequences. The two types of the channel catfish and one type of blue catfish IP-10 cDNAs were completely sequenced and the sequences have been deposited to GenBank with accession numbers AY335949, AY335950, and AY335951.

The two types of channel catfish cDNAs were quite different; they had 31 single nucleotide polymorphic sites within 600 bp sequences, or about 5% divergence, and one 7-bp deletion/insertion 18 bp downstream of the termination codon (Figure 1). In addition, the two types of cDNA clearly had alternative polyadenylation sites. A single

base change within the poly $(A)^+$ signal sequences AATAAA into AAGAAA in IpBrn01096 led to polyadenylation at a site 75 bp downstream (Figure 1).

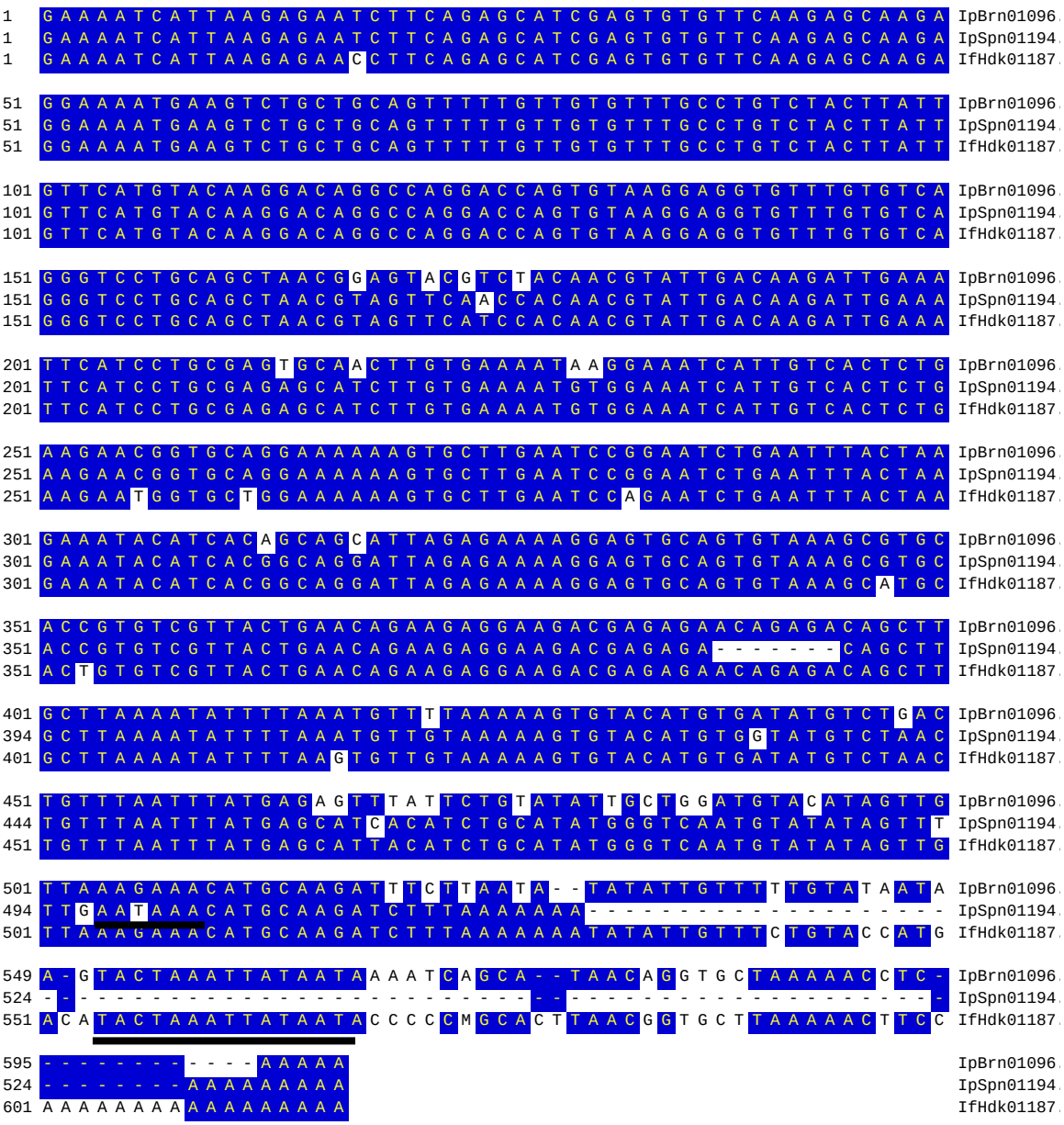

Figure 1. Sequence alignments of the two types of channel catfish CXCL10 (IP-10) cDNAs and the blue catfish IP-10 cDNA. Note the high levels of sequence variation between the two channel catfish sequences (IpBrn01096 and IpSpn01194). Potential polyadenylation signals were underlined. Alternative polyadenylation sites were used in the two channel catfish IP-10 cDNAs.

The polymorphic rate between the two types of cDNAs of the channel catfish IP-10 gene was much higher than the average polymorphic rate within genes of channel catfish [33]. Even between the genes of channel catfish and blue catfish, the average SNP rate among sequenced ESTs was found to be 1.32% (1.32 SNPs/100 bp) [33]. While a 5% sequence difference existed between the two channel catfish cDNAs, one of the two channel catfish cDNAs (IpSpn01194) was very similar to the blue catfish sequence with 2% sequence divergence. This may indicate that the two cDNAs from channel catfish actually represented paralogues of the IP-10 gene. This speculation is strengthened when the deduced amino acid sequences were analyzed (Figure 2). There was only one amino acid difference between the channel catfish IP-10 (encoded by IpSpn01194) and the blue catfish IP-10, but there were seven amino acid differences between the channel catfish IP-10 encoded by the two different types of transcripts (Figure 2).

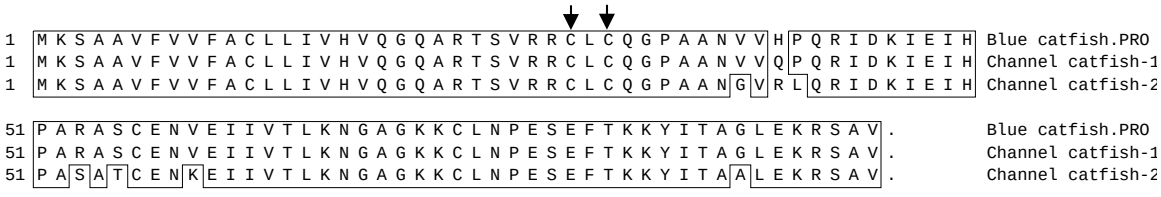

Figure 2. Aignments of the deduced amino acid sequences of the two types of the channel catfish IP-10 and the blue catfish IP-10. Arrows indicated the two cysteine residuals involved in the CXC motif.

Alignment of amino acid sequences of the IP-10 genes from catfish with those from common carp, rainbow trout, human, and rat indicated high levels of amino acid sequence conservation (Figure 3). The deduced amino acid sequences of catfish IP-10 was 51% similar to the carp sequences, 47% similar to the rainbow trout sequences, 38% similar to the rat sequences, and 33% similar to the human sequences.

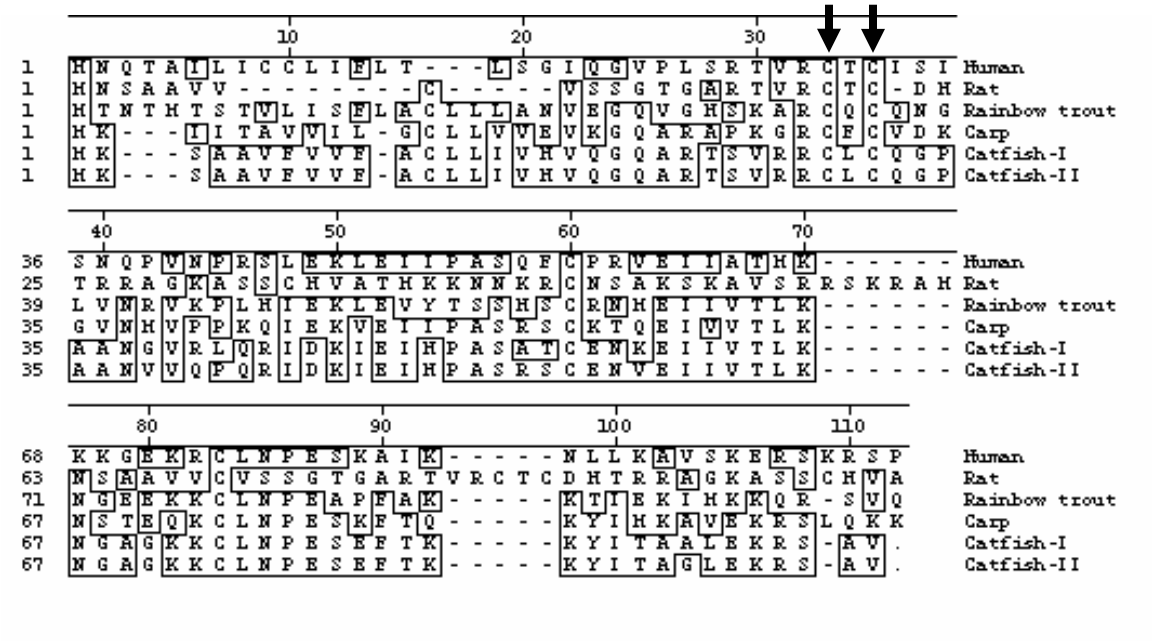

Figure 3. Similarity comparison of the deduced amino acid sequences of the IP-10 genes from human, rat, rainbow trout, carp, and catfish. Arrows indicated the two cysteine residuals involved in the CXC motif.

3.2. Channel catfish may have a multigene family of IP-10 genes

cDNA sequence analysis indicated that the two types of IP-10 transcripts within channel catfish are highly divergent. They could represent transcripts of two paralogues

of IP-10 genes from channel catfish, or could represent transcripts of alternative alleles in the population of the same gene since multiple individuals were used for the construction of the cDNA libraries. If the cDNAs represented transcripts of the same gene with alternative alleles, then only one type of PCR product should result from a single sperm or egg, considering that channel catfish and blue catfish are diploid organisms. In contrast, if the cDNAs represented transcripts from paralogous genes, then more than one type of PCR products should result. PCR primers were designed to amplify fragments spanning a region rich in SNPs and also including the 7-bp deletion/insertion (Table 1). Genomic DNA from a single F1 hybrid catfish (channel catfish x blue catfish) was amplified by PCR. The PCR products were cloned and sequenced. Among 32 clones sequenced, 14 types of cDNA sequences were found (Table 2). Obviously, in the F1 hybrid catfish, one allele each should be expressed from a given gene of channel catfish and blue catfish. This data indicated that a multigene family may exist in channel and blue catfish.

Table 2 Sequence variations as analyzed from PCR products of a single F1 hybrid catfish. Of the 32 sequenced clones, 14 types of sequences were found with base substitutions at various positions as indicated and a 7-bp deletion (del)

STS#	Sequence variation (positions start with the PCR Primer: TCTGAATTTACTAAGAAATAC where first T was position 1																			
	27	30	32	67	68	76	89	91	106	129	162	167	169	187	203 - 209	229	232	237	255	263
00043	G	A	G	C	T	T	G	C	G	T	A	C	A	G	7bp	T	G	G	A	A
00044	G	A	G	T	T	T	G	C	G	T	G	C	G	A	7bp	T	A	G	A	A
00045	G	A	G	T	T	T	G	C	G	C	G	C	G	A	7bp	A	A	G	A	G
00046	A	A	C	T	T	T	A	T	G	C	G	C	G	A	7bp	T	A	T	A	G
00047	G	A	G	T	T	T	G	C	G	T	A	C	A	A	7bp	T	G	G	A	A
00048	A	G	C	T	T	T	G	C	G	T	G	C	G	A	7bp	T	A	G	A	A
00049	G	A	G	T	T	T	G	C	G	C	G	C	G	A	del	T	A	G	G	A
00050	G	A	G	T	T	T	G	C	C	C	G	T	G	A	7bp	T	A	T	A	G
00051	G	A	G	T	T	T	G	C	C	C	G	C	G	A	7bp	T	A	G	A	G
00052	A	A	C	T	T	T	A	T	G	C	G	C	G	A	7bp	T	A	G	A	G
00053	G	A	G	T	T	A	G	C	G	T	G	C	G	A	7bp	T	A	G	A	G
00054	G	A	G	C	T	T	G	T	G	C	G	C	G	A	7bp	T	A	G	A	G
00055	G	A	G	T	T	T	G	C	G	T	G	C	G	A	7bp	T	A	G	A	G
00056	G	A	G	C	C	T	G	C	G	T	G	C	G	A	7bp	T	A	G	A	A

3.3. IP-10 is expressed in a wide range of tissues

Previous EST analysis indicated that IP-10 gene is expressed in several tissues since it was sequenced from the cDNA libraries made from the brain, head kidney, and spleen of the four libraries that we have sequenced [30, 34]. In order to analyze tissue expression of IP-10 in channel catfish, RT-PCR was conducted using total RNA from various tissues. As shown in Figure 4, except muscle tissue from which no RT-PCR products of IP-10 were detected, IP-10 was detected from all tested tissues including head kidney (anterior kidney), liver, spleen, intestine, stomach, skin, and gill. It appeared that

IP-10 was expressed at relatively low levels in all the healthy tissues tested because the RT-PCR products could not be detected at lower cycle numbers of PCR. It took 35 cycles to see a faint band from spleen, head kidney, and gill, but 40 cycles were required to visualize the band of other tissues. This is consistent with IP-10 being an inflammatory Th1 chemokine expressed at low levels under healthy conditions.

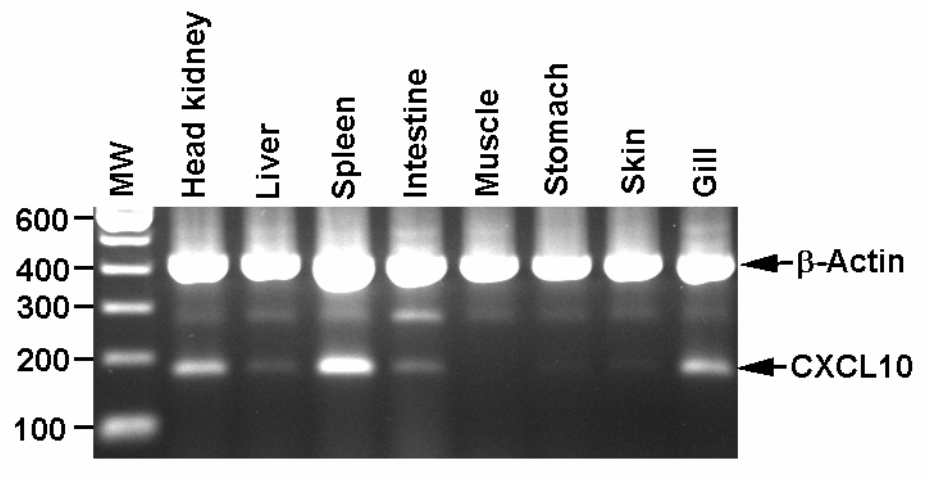

Figure 4. Expression of the channel catfish IP-10 in various tissues as analyzed by RT-PCR. MW, molecular weight standards with the sizes indicated on the left margin in base pairs (bp); tissues are indicated on the top of the gel. CXCL10 (IP-10) gene-specific RT-PCR products are indicated on the right margin, along with β–actin RT-PCR products as an internal control.

3.4. Drastic induction of IP-10 expression after infection of channel catfish with bacterial pathogen Edwardsiella ictaluri

The production of IP-10 has been found in several animal models of infection, especially infections where IFN-γ is known to play an important role in host defense [35, 36]. In humans, several recent studies reported elevated expression of IP-10 after bacterial infections both in vitro [7] and in vivo [37, 38], but little is known about the role of IP-10 in bacterial infections in fish. Here we determined the levels of IP-10 gene expression in channel catfish after *E. ictaluri* challenge using real time PCR. In both Marion Select and Kansas Random strains, IP-10 expression was dramatically induced in head kidney after challenge (Figure 5). As mentioned above, the background expression of IP-10 before challenge was low in both strains. Upon challenge, high levels of IP-10 expression were detected in Kansas Random strain as soon as 4 h after challenge. The expression reached very high levels (60 times of the level of controls) at 24 h after challenge. The overall expression was lower three days after challenge. At seven days after challenge, the expression returned to much lower levels, only slightly higher than the level prior to challenge. In Marion Select strain, the pattern was very similar to that of Kansas Random strain except that the high level of IP-10 expression was detected at 24 h after challenge, but not at the first sampling time of 4 h after challenge (Figure 5).

IP-10 expression was higher in moribund fish than in survivors. Mortalities started to occur four days after challenge in the experiments. In order to determine IP-10 expression in moribund fish, real-time observation was made in the first week after challenge. As the infected fish became moribund, they often went through a period of

"spinning swimming". Tissue samples were collected immediately after spinning swimming when they lost balance. These fish were regarded as dead fish. As shown in Figure 5, IP-10 expression in the moribund fish was high as compared to the survivors. However, the moribund fish were collected in a period of three days, day-4 to day-7 after challenge, whereas the surviving fish were collected at a given time point.

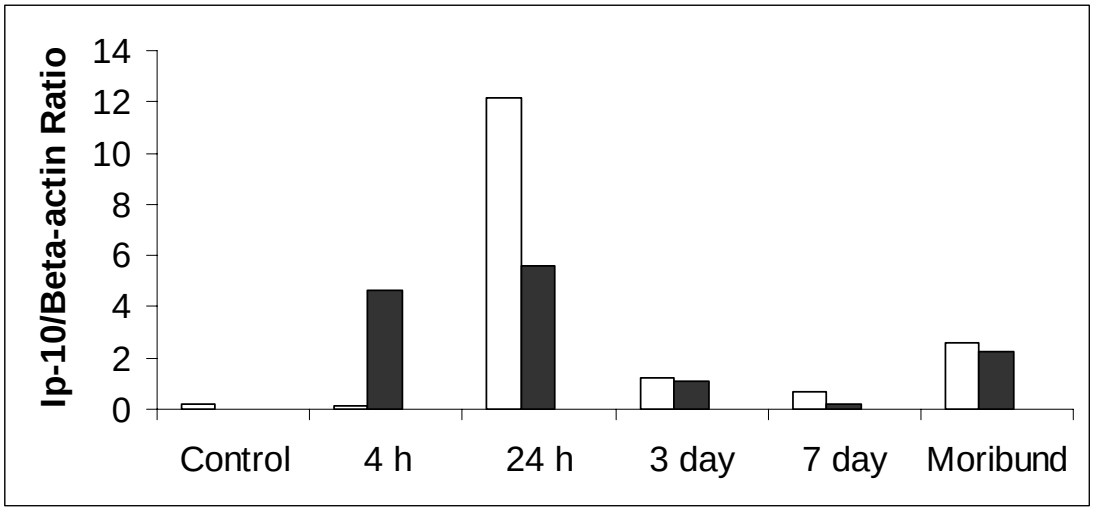

Figure 5. Changes of CXCL10 (IP-10) chemokine mRNA levels in head kidney of channel catfish as analyzed by real time RT-PCR. Data is expressed as the ratio of the IP-10 RNA and the β–actin RNA at various times: control, immediately before challenge; 4 h, 24 h, 3 day, and 7 day were time points at which the head kidney tissue samples were collected. Moribund fish were head kidney samples collected from moribund fish between day 4 to day 7 after challenge. Open bar, Marion Select strain; and solid bar, Kansas Random strain of channel catfish.

3.5. Differential expression profile of IP-10 in channel catfish and blue catfish after infection

In spite of the differences in resistance to *E. ictaluri* among various channel catfish strains, they are generally susceptible to the disease. In contrast, blue catfish (*I. furcatus*) are generally resistant to the disease. To determine if the IP-10 expression differed between channel and blue catfish after bacterial challenge, IP-10 levels were examined in head kidney and spleen at various times after challenge. As discussed above, IP-10 expression was dramatically induced at 24 h after challenge in channel catfish (Figures 6A and 6B). However, IP-10 expression was only modestly (about 3 fold) induced in blue catfish in both the head kidney (Figure 6A) and spleen tissues (Figure 6B).

A B

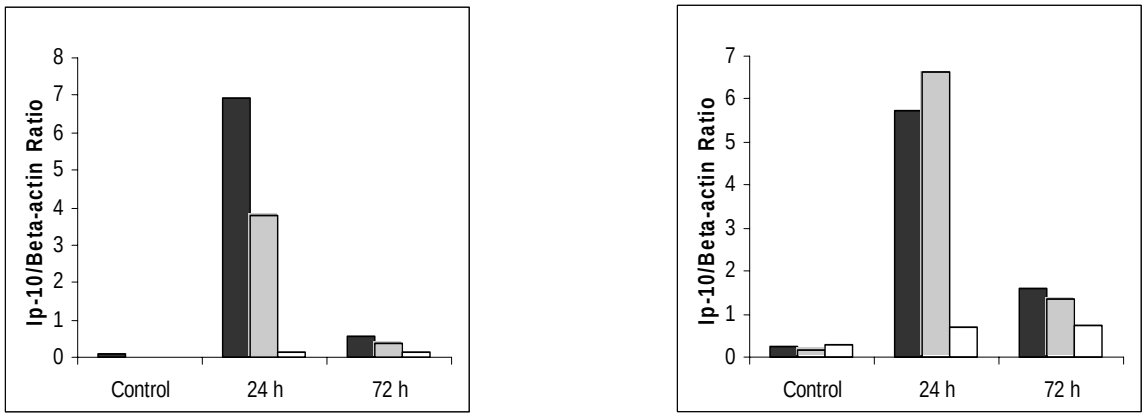

Figure 6. Changes of CXCL10 (IP-10) chemokine mRNA levels in head kidney (A) and spleen (B) of channel catfish and blue catfish as analyzed by real time RT-PCR. Data is expressed as the ratio of the IP-10 RNA and the β–actin RNA at various times: control, immediately before challenge; and 24 h and 72 h after challenge when the tissue samples

were collected. Solid bar, Marion Select strain of channel catfish (*Ictalurus punctatus*); sketched bar, Kansas Random strain of channel catfish; open bar, D&B strain of blue catfish (*I. furcatus*).

4. Discussion

Innate immunity is widely believed to play a very important role in resistance against major bacterial diseases in catfish. As one of the innate immune responses, chemokines are key components in the process of leukocyte recruitment in inflammatory sites. The interactions of various chemokines with their receptors on leukocytes allow activation and chemotaxis of neutrophils, eosinophils, lymphocytes, and monocytes for migration to the sites of evolving inflammation. We have identified and characterized the IP-10 (IP-10) chemokine from both channel catfish and blue catfish. The molecular cloning of the chemokine provides necessary molecular tools for characterization of its expression during infection. This work was the first in characterization of IP-10 expression during bacterial infection in a teleost fish species.

An EST approach was used to identify the IP-10 cDNAs. Clearly, the EST approach was effective not only in identification of the chemokine cDNAs, but also in providing detailed sequence information allowing identification of sequence variants. In this work, we have sequenced two types of IP-10 cDNAs with 5% sequence divergence. Because fifteen fish were used in making the cDNA libraries, one possibility of cDNA sequence divergence is that the cDNAs represent allelic variation among the individuals used for library construction. However, this level of sequence divergence was high as

compared to the observed average sequence divergence among channel catfish orthologous genes [33]. Alternatively, these cDNAs may actually represent transcripts of different genes (paralogues). Our results using PCR-sequencing indicated that channel catfish may have a multigene family, supporting the notion that the sequenced two types of IP-10 cDNAs were transcripts of paralogous genes.

Channel catfish strains show various levels of resistance to ESC caused by infection of *E. ictaluri*. In terms of resistance, Kansas Random is one of the most resistant strains while Marion Select is one of the most susceptible (Dr. Dunham, Auburn University, unpublished). The mechanisms of resistance are unknown at present. This work characterized IP-10 expression in these two strains. Although the overall pattern of IP-10 expression after bacterial challenge is very similar, the onset of elevation of IP-10 gene expression is more rapid in Kansas Random than in Marion Select. Unfortunately, no samples were collected between 4 h and 24 h after challenge so the exact time difference in IP-10 induction was not revealed. It is speculated that early onset of lymphocyte recruitment to the inflammatory sites may help clear the pathogenic bacteria. CXC chemokines can be divided into two classes based on the presence of the glutamate-leucine-arginine (ELR) motif preceding the CXC sequence. While the ELR-containing CXC chemokines like interleukin-8 and growth-related oncogene alpha (Groα) preferentially attract neutrophils [39, 40, 41, 42, 43], the non-ELR-containing IP-10 and monokine induced by IFN-γ (MIG) selectively attract T lymphocytes and NK cells [44, 45, 46, 47]. If confirmed, the early onset of IP-10 expression could be part of the mechanisms accounting for the greater resistance to ESC in Kansas Random involving Th1-type immune response. IP-10 specifically binds to CXCR3 [46, 48]. Recently, it has

been demonstrated that CXCR3 is preferentially expressed on Th1-type lymphocytes [49, 50]. A Th1-type immune response is associated with the release of Th1-type cytokines, such as IFN-γ and IL-2, and known to enhance cell-mediated immunity, which is important for host defense against intracellular pathogens [37]. It seems likely that IP-10 contributes to the selective recruitment of Th1 cells in sites of inflammation with high IFN-γ production.

A Th1 response is usually observed in infections with intracellular infectious agents such as mycobacteria, leishmania or viruses. *E. ictaluri* is an intracellular bacterium [51] and, therefore, a Th1 response was expected. While the early Th1 response may help the host to clear bacteria, the prolonged and highly elevated Th1 immune response may actually have pathogenic potential. The high level of sustained expression of IP-10 in moribund fish provided some indirect evidence to support this hypothesis.

Blue catfish is not a perfect host for *E. ictaluri*. Although blue catfish can be naturally infected by this pathogen, they are generally resistant to ESC. While it can not be excluded that antibody-based immunity plays a role in this resistance, innate resistance appears to play a greater role because most often, heavy mortalities occur only 4-5 days after infection when specific immune response is not yet active [14]. This work demonstrated that IP-10 is highly differentially expressed in channel and blue catfish. Upon infection, IP-10 expression was induced drastically (60-200 fold) in channel catfish within 24 h after challenge, while IP-10 expression was only modestly induced (about 3-fold) in blue catfish. This indicates that IP-10 and its related immune response could potentially be part of the mechanisms accounting for the general species resistance of

blue catfish to ESC. Further analysis and comparison of expression of genes involved in early immune response between channel and blue catfish is warranted in order to gain more complete understanding of the roles of IP-10 in the resistance of blue catfish against ESC.

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III. CONSTITUTIVE EXPRESSION OF THREE NOVEL CATFISH CXC CHEMOKINES: HOMEOSTATIC CHEMOKINES IN TELEOST FISH

Abstract

Chemokines are best known for their vital role in leukocyte chemotaxis as part of the larger inflammatory response. Expression analysis and functional characterization of chemokines in mammalian species have often overlooked the role of these proteins under homeostatic conditions. Recent investigations of chemokine diversity in teleost fish have also centered on the immune-related functions of chemotactic cytokines such as CXCL8 and CXCL10. While a disease-based approach to chemokines is essential to the development of remediative therapies for both human and animal infections, it may be a poor measure of the overall complexity of chemokine functions. As part of a larger effort to assess the conservation of chemokine diversity in teleost fish, we report here the identification of three novel, constitutively expressed CXC chemokines from channel catfish (*Ictalurus punctatus*). Phylogenetic analyses indicated that two of the three CXC chemokines were orthologues for mammalian CXCL12 and CXCL14, respectively. Whereas a clear orthology could not yet be established for the third CXC chemokine, it shared highest amino acid identity with mammalian CXCL2. All three CXC chemokines show expression in a wide range of tissues, and early expression during development was observed for CXCL12. The expression of this new set of catfish CXC chemokines was not induced during challenge by infection of *Edwardsiella ictaluri*, the causative agent of the fish pathogen enteric septicemia of catfish. In contrast to the gene duplication of CXCL12 in carp and zebrafish, Southern blot analysis indicated that all three catfish CXC chemokines exist as single copy genes in the catfish genome suggesting that gene duplication of CXC chemokines in specific teleost fish was a recent evolutionary event.

1. Introduction

Chemokines are traditionally viewed as a superfamily of chemotactic cytokines involved in the recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci. While this definition summarizes the predominant role of chemokines, as it is currently understood, it fails to capture a greater complexity of functions both immune and non-immune that some chemokines possess. Chemokines are classified into four groups—C, CC, CXC, and CX3C—based on the arrangement of conserved cysteine residues that determine their tertiary structure [1]. The major subfamilies are the CC and CXC chemokines.

CXC chemokines were initially identified as potent mediators of neutrophil chemotaxis [2,3], but are now known to function also in chemotaxis of monocytes and lymphocytes [4,5]. Altogether 16 CXC chemokines have been identified from mammals, although a smaller number was identified from any given species. Thus, 15 were identified from humans (lacking CXCL15) and 13 were identified from mouse (lacking CXCL7, CXCL8, and CXCL16).

CXC chemokines have been divided into two subgroups based on the presence or absence of the ELR motif (glutamic acid (E), leucine (L), arginine (R)). The ELR subgroup of CXC chemokines includes CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL15. These CXC chemokines specifically attract neutrophils that express CXCR1 and CXCR2. They are expressed in a wide range of cells in response to many stimulants, particularly pro-inflammatory cytokines such as IL-1 and

TNF [6]. Their major role is to promote the adherence of neutrophils to endothelial cells and subsequent migration along a gradient of chemokines associated with matrix proteins and cell surfaces toward inflammatory sites. ELR containing chemokines are all angiogenic and chemotactic for endothelial cells [5]. In fish, however, the ELR motif was not found in several CXCL8-like chemokines [7-12].

The non-ELR subgroup includes CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, and CXCL16. They attract lymphocytes and monocytes, with poor chemotactic ability for neutrophils [13]. Most of this subgroup are angiostatic and possess anti-angiogenic properties.

Only a few fish orthologous to the mammalian CXC chemokines have been identified. In addition to the CXCL8-like chemokines described above, a 100 amino acid chemokine was identified in rainbow trout as being related to the CXCL9, CXCL10 and CXCL11 subset, but with highest similarity to CXCL10 [14]. Two CXC chemokines from carp, CXCa and CXCb, have been functionally characterized, but lack orthology to known mammalian CXC chemokines [15,16]. Recently, a CXCL10-like chemokine was identified from channel catfish and blue catfish [17]. A CXCL12-like EST sequence has been detected from zebrafish which is 44% identical to human CXCL12 at the amino acid level and appears to have a role in developmental processes such as the control of primordial cell migration [18]. A CXCL14 homologue has also been identified from zebrafish [19] that is predicted to have a role in the development of the acoustico-lateralis system in non-haematopoietic cells of the central nervous system of fish. Recently, orthologues of CXCL12 and CXCL14 have been isolated from carp, of which CXCL12 was duplicated [20]. Here we report the identification and expression analysis of three

novel channel catfish CXC chemokines. Two of the chemokines are clearly orthologous to mammalian CXCL12 (SDF-1) and CXCL14 (BRAK), whereas the third chemokine shares low amino acid identity with mammalian CXCL2 (MIP-2). This third chemokine will be referred to here as CXCL2-like chemokine. Southern blot analysis indicated that a single copy existed in the catfish genome for all three CXC chemokines. All three catfish CXC chemokines are expressed in a wide range of tissues, including those not traditionally associated with the immune response. In head kidney, a rough equivalent of the mammalian bone marrow, expression of the three CXC chemokines after bacterial challenge was constitutive, demonstrating marked differences from the inducible expression profiles of previously characterized CXCL8 and CXCL10 in catfish [17,12].

2. Material and Methods

2.1. Identification of CXC chemokines and sequencing analysis

The three CXC chemokines were initially identified from BLAST analysis of expressed sequence tags [21-24]. The putative CXC chemokine clones were subjected to complete sequencing analysis. Plasmid DNA was prepared by the alkaline lysis method [25] using the Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid DNA (about 0.5-1.0 μg) were used in sequencing reactions. Chain termination sequencing was performed using cycleSeq-farOUT^{TM} polymerase (Display Systems Biotech, Vista, CA). The PCR profiles were: 95 °C for 30 s, 55 °C for 40 s, 72 °C for 45 s for 30 cycles. An initial 2 min denaturation at 96 °C and a 5 min extension at

72 °C were always used. Sequences were analyzed on an automatic LI-COR DNA Sequencer Long ReadIR 4200 or LI-COR DNA Analyzer Gene ReadIR 4200.

Sequences were initially analyzed by BLAST searches [26-28] to identify gene similarities. Further analysis of open reading frame, sequence similarities, and multiple alignments were conducted using Clustal W method within MegAlign program of DNAStar software package (Lasergene, Madison, WI).

2.2. Phylogenetic analysis

The relevant sequences were retrieved from GenBank for multiple sequence alignments using Clustal X [29]. Percentage of amino acid identities were recorded after all multiple alignments. Phylogenetic tree was drawn by the neighbor-joining method [30] in PAUP using amino acid sequence p-distances. The topological stability of the neighbor joining trees was evaluated by 10,000 bootstrapping replications.

2.3. Fish rearing and bacterial challenge

Channel catfish larvae were reared at the hatchery of the Auburn University Fish Genetics Research Unit. Challenge experiments were conducted as previously described [31] with modifications [17,12]. Briefly, the catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from a natural outbreak in Alabama (outbreak number ALG-02-414) and inoculated into brain heart infusion (BHI)

medium and incubated in a shaker incubator at 28°C overnight. The bacterial concentration was determined using colony forming unit (CFU) per ml by plating 10 µl of 10-fold serial dilutions onto BHI agar plates. At the time of challenge, the bacterial culture was added to the tank to a concentration of 3 x10^7 CFU/ml. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/ml. After 2 h of immersion exposure, 15 fish were randomly taken and placed into a rectangular trough containing pond water with constant water flow through. Replicates of troughs were used to provide one trough for each sampling time point in order to randomize sampling fish without any human bias at any time points. For the control fish, 15 fish were incubated in a separate rectangular tank with the same fish density as the challenge tanks. The only difference was that ESC bacteria were not added. After 2 h, these control fish were incubated in a separate trough at the same density as the challenged fish.

2.4. Tissue sampling and RNA extraction

Eleven tissues were collected from healthy channel catfish including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney. Head kidney was collected from challenged fish. Samples were collected from 10 fish at each time point including control (before challenge), 24 h, 3 days, and 7 days after challenge. Samples were also collected from dying fish during a period between day 4 and day 7 after challenge. The experimental fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg per liter before tissues were collected. Samples of each tissue from 10 fish were pooled. Tissues were quick frozen in liquid nitrogen and

kept in a –80 °C ultra-low freezer until preparation of RNA. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powders and thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [32] using the Trizol reagents kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Extracted RNA was stored in a -80°C freezer until used as template for reverse transcriptase PCR (RT-PCR).

2.5. RT-PCR

RT-PCR reactions were conducted using SuperScript™ III One Step RT-PCR System (Invitrogen). The system contained a mixture of SuperScript™ III reverse transcriptase and the Platinum *Taq* DNA polymerase in an optimized buffer. Detailed procedures followed the instructions of the manufacturer. Briefly, the following was added to a reaction of 50 µl: 25 µl 2X reaction mix, 1 µl total RNA (~100 ng), 1 µl (100 ng) each of the upper and lower primer (for sequences, see Table 1), 2 µl SuperScript III RT/Platinum *Taq* polymerase mix, and water to bring the reaction volume to 50 µl. The reaction also included the primers of β-actin (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 ºC for 15 min for one cycle (reverse transcription reaction), the samples were pre-denatured at 94 ºC for 2 min, then the samples were amplified for 40 cycles with 94 ºC for 15 s, 45 ºC for 30 s, 68 ºC for 1 min. Upon the completion of PCR, the reaction was

incubated at 68 °C for an additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5 % agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

Table 1 Primer sequences for three catfish CXC chemokines

Gene (Accession number)	Primer name	Primer sequences (5' to 3')
CXCL2-like (**AY836754**)	RT-PCR upper	CTTGTCTAACTCACGCTGTCAG
	RT-PCR lower	TGCTCATTGTAATCCTGCAACAG
	cDNA probe upper	AACTAGAGCACTAGACAGCTGGATTTC
	cDNA probe lower	CAAAATGCACACATTTATTT
	Overgo A	GCTGTCAGCTTTTTGCTGGTTGTG
	Overgo B	GTTGGAGACAAAGGACCACAACCA
CXCL12 (**AY836755**)	RT-PCR and cDNA probe upper	TCTTCTTCACACGCAACATGG
	RT-PCR and cDNA probe lower	CTGTCAGGTATTCTGCCATTGG
	Overgo A	TTGCTGAACCAGCACTTAACCTGC
	Overgo B	GAGGCAAGCAAGGTTTGCAGGTTA
CXCL14 (**AY836756**)	RT-PCR upper	ACAGTCTGTGGTGGAGTCAC
	RT-PCR lower	ACATAGTGCTTCTTTTGGACCAC
	cDNA probe upper	GACAGTCTGTGGTGGAGTCACTTCAACC
	cDNA probe lower	GTTGCAAGTCTAACACTTGG
	Overgo A	CAAATGCAGATGCACCAGGAAAGG
	Overgo B	GTATCGTATCTTGGGGCCTTTCCT
β-actin	RT-PCR upper	AGAGAGAAATTGTCCGTGACATC
	RT-PCR lower	CTCCGATCCAGACAGAGTATTTG

2.6. BAC library screening

High density filters (10X genome coverage) of a BAC library purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA), were screened using overgo hybridization probes [33]. Overgo primers (Table 1) were designed using an overgo maker program. The primers were purchased from Sigma Genosys (Woodlands, Texas), then labeled with ^{32}P-dATP and ^{32}P-dCTP (Amersham, Piscataway, NJ) in 10 mg/ml bovine serum albumin, overgo labeling reaction 1X buffer

[34] and double distilled water and incubated for 1 h at room temperature with Klenow polymerase (Invitrogen). Unincorporated nucleotides were removed using Sephadex G50 spin columns. Probes were denatured at 95ºC for 10 min and added into hybridization tubes that had been under pre-hybridization for 2 h. The filters were hybridized at 50ºC for 18 h in 50 ml hybridization solution (1%BSA, 1mM EDTA at pH 8.0, 7% SDS, 0.5 M sodium phosphate, pH 7.2). The filters were washed at room temperature and exposed to X-ray film at -80ºC for 24 h. Positive clones were identified according to the clone distribution pattern from CHORI. The clone locations were indicated by a number-a letter-a number system. The first number indicates the plate number, and a letter followed by a number after the dash indicates the location of the positive BAC within the 384-well plate. For instance, 38-A17 indicates the positive clone is located in plate 38, row A, column 17. Positive clones were picked for culture in 2X YT medium. After overnight culture, BAC DNA was isolated using the Perfectprep® BAC 96 BAC DNA isolation kit (Brinkmann instruments, Inc., Westbury, NY).

2.7. Southern blot hybridization

In order to characterize the CXC chemokine loci, Southern blot analysis was conducted using either genomic DNA or BAC DNA. In case of BAC-based Southern blot analysis, BAC DNA was first digested with restriction endonuclease *Eco*R I and *Hin*d III separately as specified for the specific CXC chemokine, and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 0.4 M NaOH overnight. DNA was

fixed to the membrane by UV cross linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto crosslink function. The membrane was washed in 0.5% SDS (w/v) at 65°C for 15 min and then pre-hybridized in 50% formamide, 5X SSC [25], 0.1% SDS (w/v), 5X Denhardt's and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA (100 μg/ml) overnight. Hybridization was conducted overnight at 42°C in the same solution with appropriate probes added. In all three cases, the cDNA amplified using primers listed in Table 1 was used as the probes. The probe was prepared using the random primer labeling method [25] with a labeling kit from Roche Diagnostics (Indianapolis, Indiana). The nylon membranes were washed first in 500 ml of 2X SSC for 10 min, followed by three washes in 0.2X SSC with SDS at 0.2% (w/v) at 65°C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography. The rationale for the BAC-based Southern analysis was that if the clones represented a single copy, the same hybridization pattern would be expected. Alternatively, if the clones were from different genomic copies (more than one gene), then variations in the hybridization pattern would be expected with the exception of the clones harboring the gene of interest at the end of its insert, which should be rare.

In the case of Southern blot using genomic DNA, the procedures were the same as described above except that genomic DNA was first digested with *Hin*d III and *Pst* I restriction endonucleases.

3. Results

3.1. Characteristics of three novel catfish CXC chemokines

The clones for the putative CXC chemokines were initially identified by BLAST analysis of ESTs. After their initial identification, their complete cDNAs were sequenced. The sequences of the three cDNAs have been deposited to GenBank with accession numbers **AY836754**, **AY836755**, and **AY836756**. BLAST similarity comparisons indicated that **AY836754** is similar to CXCL2; **AY836755** is similar to CXCL12; and **AY836756** is similar to CXCL14.

The channel catfish CXCL2-like cDNA was identified from a head kidney cDNA library. The appropriate EST clone, AUF_IpHdk_41_O14, was completely sequenced. Its full length cDNA (791 bp) encodes a protein of 131 amino acids. It contains a 64 nucleotide 5'-untranslated region (5'-UTR), and a 331 nucleotide 3'-UTR. A typical AATAAA polyadenylation signal sequence exists 14-bp upstream of the poly $(A)^+$ tail (Figure 1A). The channel catfish CXCL2-like chemokine is most similar to the mouse CXCL2, but shares only 17%-24% amino acid identities with various CXCL2 ligands from mammals (Table 2). Although CXCL2 belongs to the ELR-containing subgroup of CXC chemokine, there is no ELR motif in the channel catfish CXC chemokine, a similar situation to the CXCL8 chemokines found in various fish species in which ELR motifs have also not been found.

Table 2 Pairwise sequence similarities of CXCL2 chemokines from various species

	Catfish CXCL2-like	Swine CXCL2	Bovine CXCL2	Mouse CXCL2	Human CXCL2
Catfish CXCL2-like	100				
Swine CXCL2	17.8	100			
Bovine CXCL2	21.4	82.7	100		
Mouse CXCL2	24.0	61.0	61.2	100	
Human CXCL2	16.8	74.8	79.6	62.0	100

The channel catfish CXCL12 cDNA was identified from the head kidney cDNA library with an EST clone, AUF_IpHdk_42_L09. Its full length cDNA (939 bp) encodes a protein of 99 amino acids. It contains a 34 nucleotide 5'-UTR and a 605 nucleotide 3'-UTR. There is no typical poly $(A)^+$ signal sequence, but a AATATT sequence exists 17-bp upstream of poly $(A)^+$ tail (Figure 1B). Similar to chicken CXCL12 [35], two important evolutionary conserved residues for receptor activation: lysine (K) and proline (P) located in the KPVSLSYR motif (before CXC) are also found in the catfish CXCL12 sequence. The catfish CXCL12 shares highest amino acid sequence similarity with the zebrafish CXCL12a chemokine and appears to be more closely related to the CXCL12a variants of both zebrafish and carp (Table 3).

Table 3 Pair wise sequence similarities of CXCL12 chemokines from various species

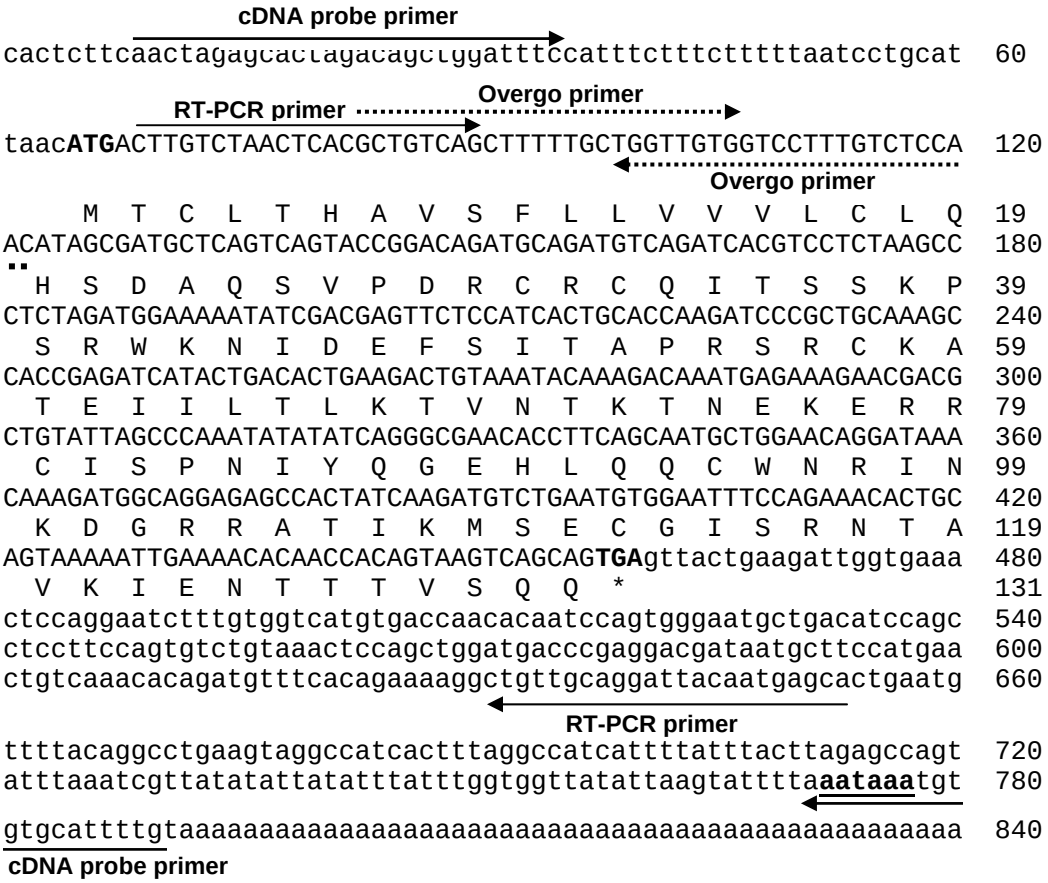
	Catfish CXCL12	Carp CXCL12a	Carp CXCL12b	Zebrafish CXCL12a	Zebrafish CXCL12b	Cichlid BJ701617	Chicken CXCL12	Mouse CXCL12	Human CXCL12
Catfish CXCL12	100								
Carp CXCL12a	70.7	100							
Carp CXCL12b	64.9	71.1	100						
Zebrafish CXCL12a	73.7	86.9	76.3	100					
Zebrafish CXCL12b	62.9	70.1	90.7	75.3	100				
Cichlid BJ701617	67.3	68.4	67.0	72.4	66.0	100			
Chicken CXCL12	39.4	38.4	40.2	40.4	34.0	35.7	100		
Mouse CXCL12	42.4	39.4	41.2	43.4	45.4	36.7	54.2	100	
Human CXCL12	44.6	41.3	41.3	44.6	46.7	38.0	70.7	90.2	100

The channel catfish CXCL14 cDNA was identified from the gill cDNA library with an EST clone, AUF_IpGil_01_G21. Its full length cDNA (757 bp) also encodes a protein of 99 amino acids. It contains a 5'-UTR of 206 nucleotides and a 3'-UTR of 251 nucleotides. There is no typical poly $(A)^+$ signal sequence in the cDNA, but a highly A/T-rich sequence TATTTATT exists 20-bp upstream of the poly $(A)^+$ tail (Figure 1C). The channel catfish CXCL14 shares high amino acid sequence similarity with both carp and zebrafish CXCL14 (Table 4).

Table 4 Pair wise sequence similarities of CXCL14 chemokines from various species

	Catfish CXCL14	Carp CXCL14	Zebrafish CXCL14	Swine CXCL14	Mouse CXCL14	Human CXCL14
Catfish CXCL14	100					
Carp CXCL14	97.3	100				
Zebrafish CXCL14	92.0	94.6	100			
Swine CXCL14	51.8	53.6	50.0	100		
Mouse CXCL14	58.9	60.7	57.1	79.5	100	
Human CXCL14	50.0	51.8	48.2	69.6	82.1	100

A.




```
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    M  T  C  L  T  H  A  V  S  F  L  L  V  V  V  L  C  L  Q  19
ACATAGCGATGCTCAGTCAGTACCGGACAGATGCAGATGTCAGATCACGTCCTCTAAGCC  180
  H  S  D  A  Q  S  V  P  D  R  C  R  C  Q  I  T  S  S  K  P  39
CTCTAGATGGAAAAATATCGACGAGTTCTCCATCACTGCACCAAGATCCCGCTGCAAAGC  240
  S  R  W  K  N  I  D  E  F  S  I  T  A  P  R  S  R  C  K  A  59
CACCGAGATCATACTGACACTGAAGACTGTAAATACAAAGACAAATGAGAAAGAACGACG  300
  T  E  I  I  L  T  L  K  T  V  N  T  K  T  N  E  K  E  R  R  79
CTGTATTAGCCCAAATATATATCAGGGCGAACACCTTCAGCAATGCTGGAACAGGATAAA  360
  C  I  S  P  N  I  Y  Q  G  E  H  L  Q  Q  C  W  N  R  I  N  99
CAAAGATGGCAGGAGAGCCACTATCAAGATGTCTGAATGTGGAATTTCCAGAAACACTGC  420
  K  D  G  R  R  A  T  I  K  M  S  E  C  G  I  S  R  N  T  A  119
AGTAAAAATTGAAAACACAACCACAGTAAGTCAGCAGTGAgttactgaagattggtgaaa  480
  V  K  I  E  N  T  T  T  V  S  Q  Q  *                       131
ctccaggaatctttgtggtcatgtgaccaacacaatccagtgggaatgctgacatccagc  540
ctccttccagtgtctgtaaactccagctggatgacccgaggacgataatgcttccatgaa  600
ctgtcaaacacagatgtttcacagaaaaggctgttgcaggattacaatgagcactgaatg  660
ttttacaggcctgaagtaggccatcactttaggccatcatttatttacttagagccagt  720
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gtgcattttgtaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  840
```

B.


RT-PCR and cDNA probe primer
attctgcagtgcggttctcttcttcacacgcaacATGGATGTGAGAGTGATCTTAGTGGC 60
M D V R V I L V A 9
AGTTCTGGTAACGGTGACTATCTACGGATCTGTTTCAGACGCCAAGCCTATCAGCCTGGT 120
V L V T V T I Y G S V S D A K P I S L V 29
CGAGAGGTGTTGGTGCCGTTCTACAGTCAGCACAATCCCACAGAGAAACATCCGTGAACT 180
E R C W C R S T V S T I P Q R N I R E L 49
AAAGTTCGTCCACACACCCAACTGTCCTTTCCAAGTCATTGCCAAACTGAAGAGCAATAA 240
K F V H T P N C P F Q V I A K L K S N K 69
AGAGGTGTGCCTCAATCCGGAGACAAAATGGCTACAGCAGTACCTTGACAAAGCCCTTGA 300
E V C L N P E T K W L Q Q Y L D K A L E 89
AAAAATGAAGAAGGCCAAGCAACAGGGCAACTAAggaagcagctggatggcaagcccatt 360
K M K K A K Q Q G N * 99
tgtgagccaccaccacagatatgtacctgtaccatatactgctctggcatgcttgctgaa 420
Overgo primer
ccagcacttaacctgcaaaccttgcttgcctctcaatgattcgccacgtcatcatcacca 480
Overgo primers
cgtctgccgccgctataggcacctcctgtgttagcacaatgtgcaattatatatatgtat 540
gagtgtgtttttatacgtatgagatgtatataaaatgtttttttatatcaacgttaaaca 600
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ctgagtcccaatggcagaatacctgacaggagaacaaataggaagggagtcaactttctc 840
RT-PCR and cDNA probe primer
cactcatttaataatcatatttaattaacccatatctttaaaaaaaaatgtaacactgca 900
tattgcatgtgaatgtaatattacatgtatatatacacgaaaaaaaaaaaaaaaaaaaaa 960

C.

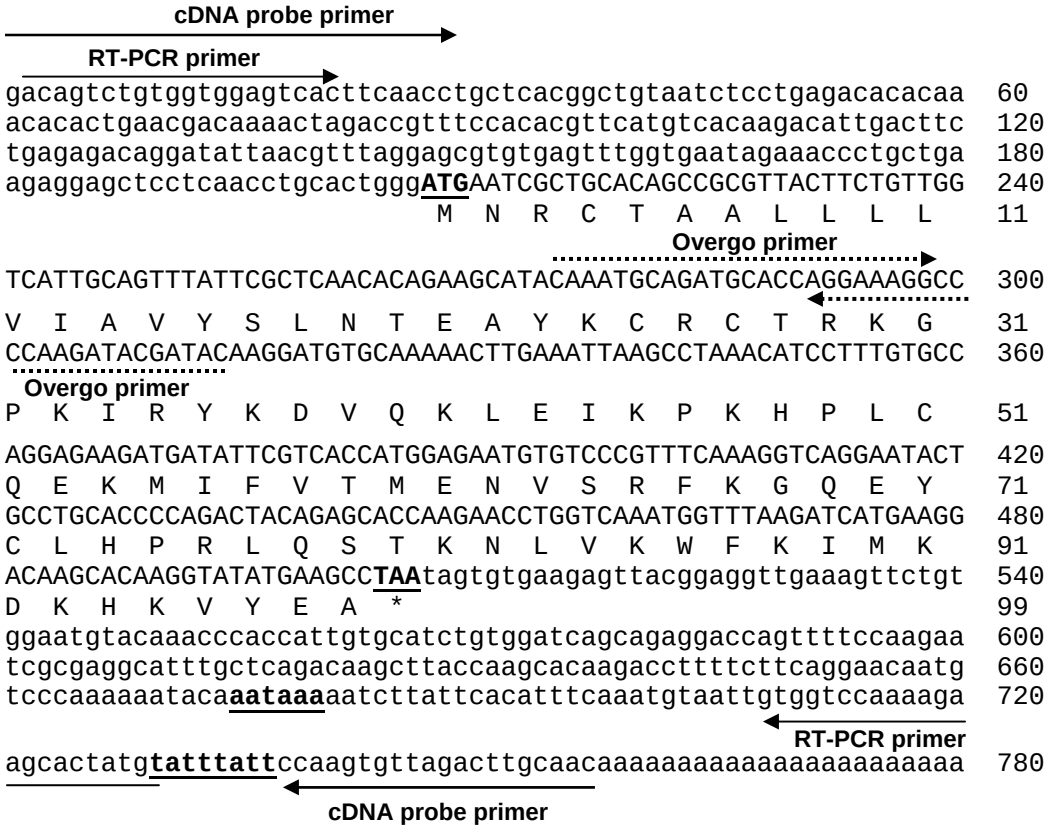


Figure 1. cDNA and deduced amino acid sequences of the catfish CXCL2-like (A), CXCL12 (B) and CXCL14 (C) chemokine. The nucleotide sequences are on the upper lines and the amino acid sequences are in the lower lines. The translation start codon ATG and the termination codon TAA or TGA is bold. The putative poly $(A)^+$ signal sequences AATAAA or AT-rich tracts are in bold font and underlined. The locations of the RT-PCR primers, cDNA probe primers, and overgo primers as well as their orientations are indicated by arrows as specified in the figure.

3.2. Phylogenetic analysis

Phylogenetic analysis using the neighbor joining method indicated that orthologies can be established for catfish CXCL12 and CXCL14. Catfish CXCL12 was placed into a clade containing CXCL12 from various organisms with very strong statistical support. The orthology of catfish CXCL14 could also be established, as it falls into a clade containing CXCL14 from various organisms (Figure 2). The lack of CXCL2-like sequences from species intermediate to fish and mammals meant that the catfish chemokine could not be included in a clade containing CXCL2; its orthology cannot be presently established.

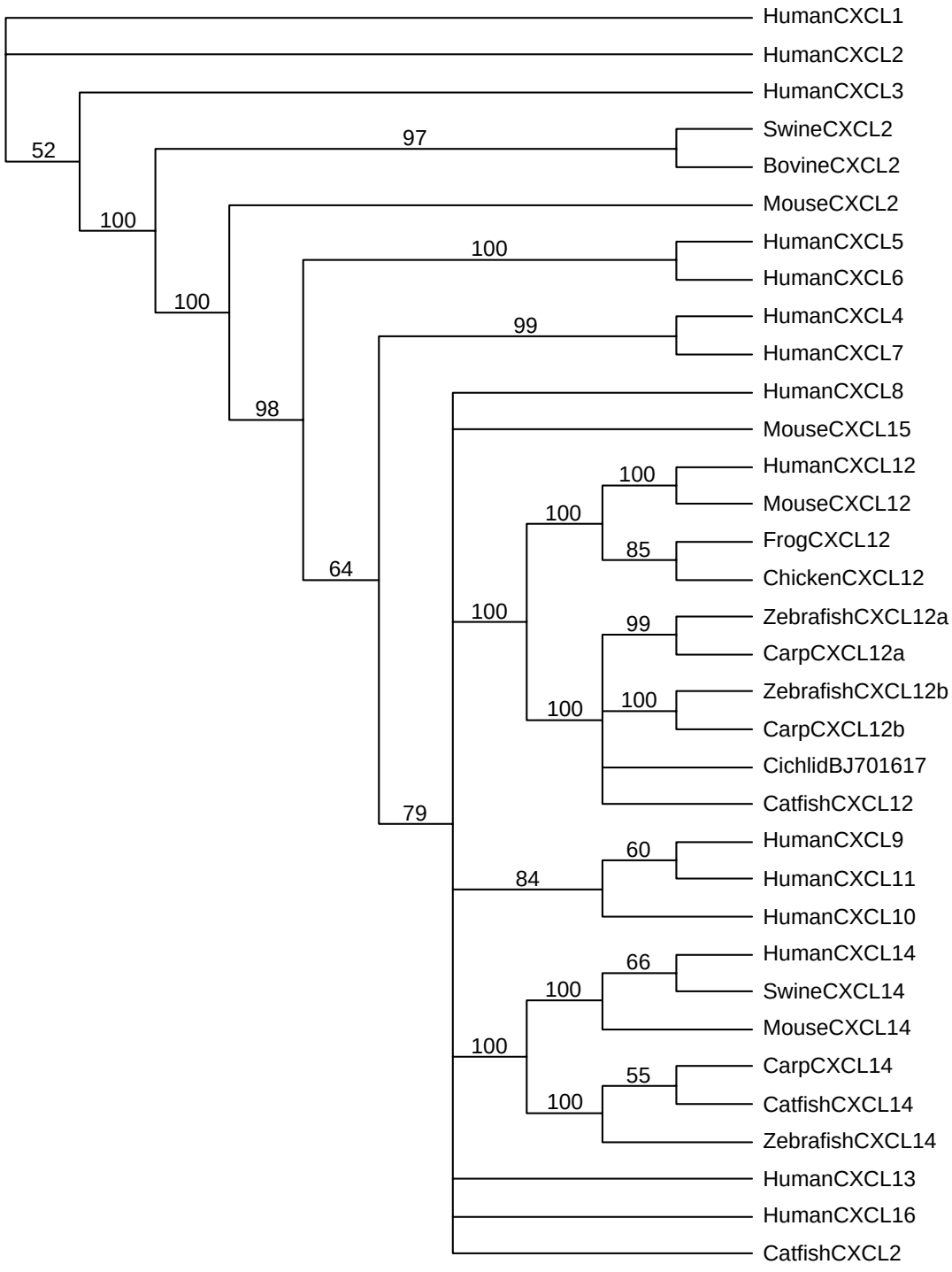


Figure 2. Phylogenetic analysis of the three newly identified catfish CXC chemokines. The phylogenetic tree was constructed with Clustal X and PAUP using the neighbor

joining method. The numbers on the branches represent the confidence level of 10,000 bootstrap replications. The sequences and their accession numbers used in the phylogenetic analysis are as the following: human CXCL1 (**P09341**), human CXCL2 (**NP_002080**), human CXCL3 (**NP_002081**), swine CXCL2 (**NP_001001861**), bovine CXCL2 (**NP_776724**), mouse CXCL2 (**NP_033166**), human CXCL5 (**NP_002985**), human CXCL6 (**NP_002984**), human CXCL4 (**NP_002610**), human CXCL7 (**NP_002695**), human CXCL8 (**NP_000575**), mouse CXCL15 (**NP_035469**), human CXCL12 (**NP_954637**), mouse CXCL12 (**AAH46827**), frog CXCL12 (**CAC82196**), chicken CXCL12 (**AAR91696**), zebrafish CXCL12a (**NP_840092**), carp CXCL12a (**AJ627274**), zebrafish CXCL12b (**NP_932334**), carp CXCL12b (**AJ536027**), catfish CXCL12 (**AY836755**), human CXCL9 (**NP_002407**), human CXCL11 (**NP_005400**), human CXCL10 (**P02778**), human CXCL14 (**NP_004878**), swine CXCL14 (**AAQ75577**), mouse CXCL14 (**Q9WUQ5**), carp CXCL14 (**AJ536028**), catfish CXCL14 (**AY836756**), zebrafish CXCL14 (**NP_571702**), human CXCL13 (**AAH12589**), human CXCL16 (**NP_071342**), catfish CXCL2-like (**AY836754**).

3.3. Assigning three CXC catfish chemokines to BAC clones

Comparative genome analysis requires anchorage of genes onto BACs. As part of our ongoing effort to map known genes to BACs, we conducted hybridization using overgo probes. As shown in Table 5, hybridization using a CXCL2-like overgo allowed identification of three CXCL2-like positive BACs. Similarly, 26 BAC clones were identified to contain CXCL12, and 14 BAC clones contain CXCL14 chemokine (Table 5).

Table 5 Location of BAC clones positive to three novel catfish CXC genes from high density BAC filters containing 10X genome coverage of the CHORI212 BAC library

Gene	Location in 384-wells plate
CXCL2-like	38-A17, 119-I6, 119-I17
CXCL12	19-P10, 24-M3, 31-O18, 34-F17, 37-E2, 38-D13, 52-M13, 59-J23, 68-F22, 81-L19, 90-M1, 96-H15, 99-D13, 111-G22, 130-I9, 135-F1, 136-C7, 139-M18, 142-G24, 158-J22, 160-D11, 170-N10, 184-K1, 185-P24, 189-J24, 191-N17
CXCL14	10-I2, 20-F3, 26-D1, 35-K6, 39-N17, 40-F10, 53-M9, 60-K1, 79-P24, 118-P19, 123-I24, 143-G19, 144-J3, 164-F2

3.4. Determination of genomic copy numbers

In order to determine the copy number of the chemokine genes in the channel catfish genome, two approaches were taken. Initially, genomic Southern blot analysis was conducted with CXCL2-like chemokine. As shown in Figure 3, however, the sizes of some of these chemokine genes may be quite large, resulting in multiple bands. In the absence of genomic sequences, it is difficult to make a conclusion on genomic copy numbers. Therefore, BAC-based Southern hybridization was conducted. If the gene is present in multiple copies in different genome locations, then clones positive to the gene probes should generate different restriction patterns with the gene probes, regardless of the number of bands. As shown in Fig. 4, all 26 CXCL12-positive BAC clones produced identical restriction patterns using *Hin*d III (Figure 4) or *Eco*R1 (not shown), confirming the presence of a single copy of CXCL12 gene in the catfish genome.

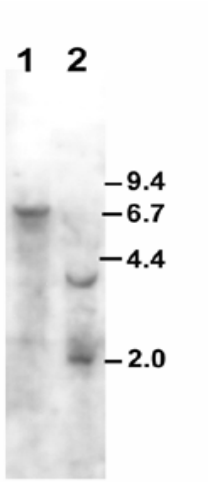


Figure 3. Genomic Southern blot analysis of the catfish CXCL2-like chemokine. Genomic DNA was digested with *Hin*d III (lane 1) or *Pst* I (lane 2), and electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized with CXCL2-like cDNA probes. Molecular weight standard (kb) is indicated on the right margin.

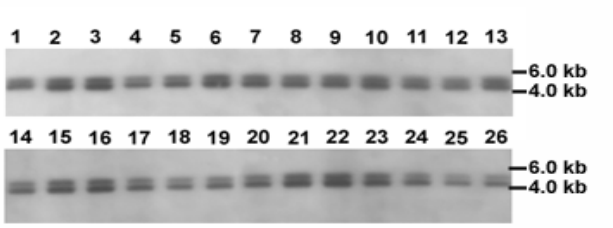
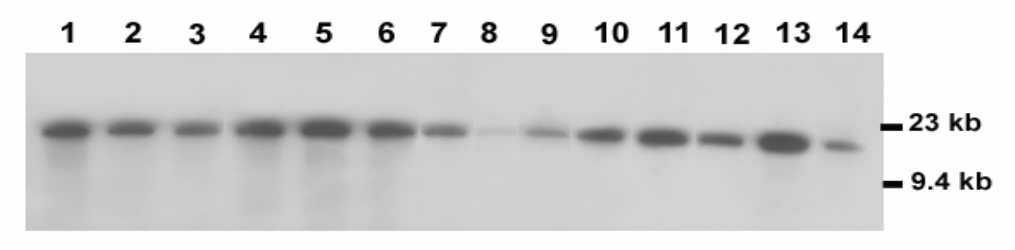


Figure 4. Southern blot analysis of the catfish CXCL12 locus using BAC clones. BAC DNAs were digested with *Hin*d III, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized with CXCL12 cDNA probes. Lanes 1-26 contained BAC DNA from 26 different BAC clones (see Table 5). Molecular weight standard (kb) is indicated on the right margin.

The channel catfish genome also contains a single copy of CXCL14. BAC-based Southern blot analysis produced identical restriction patterns with 14 BAC clones using *Eco*R1 (Figure 5A) or *Hin*d III (Figure 5B). With *Eco*R1, only one band was positive for the CXCL14 probe, whereas with *Hin*d III, three bands were positive for the CXCL14 probe. The identical restriction fragment profiles confirm the presence of a single copy of CXCL14 in the catfish genome.

A.



B.

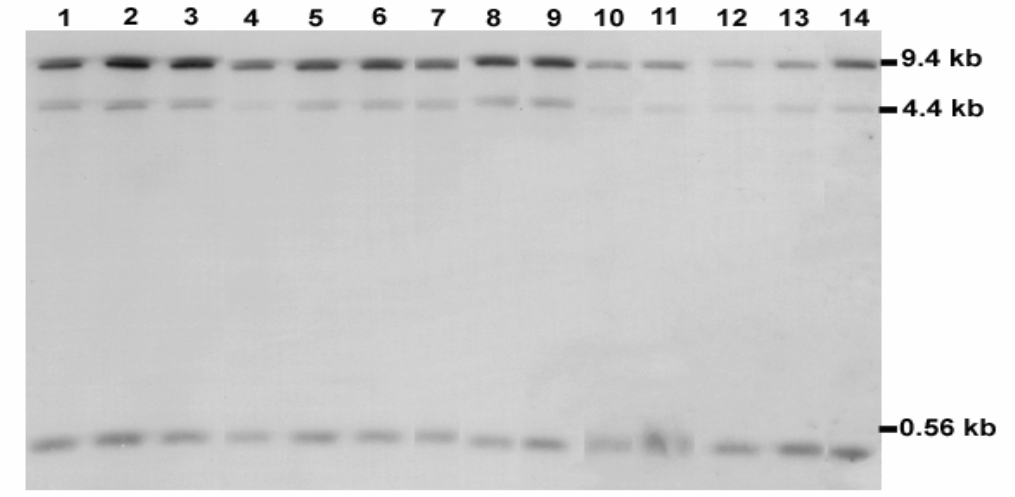


Figure 5. Southern blot analysis of the catfish CXCL14 locus using BAC clones. BAC DNAs were digested with *Eco*R1 (A) or *Hin*d III (B), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized with the catfish CXCL14

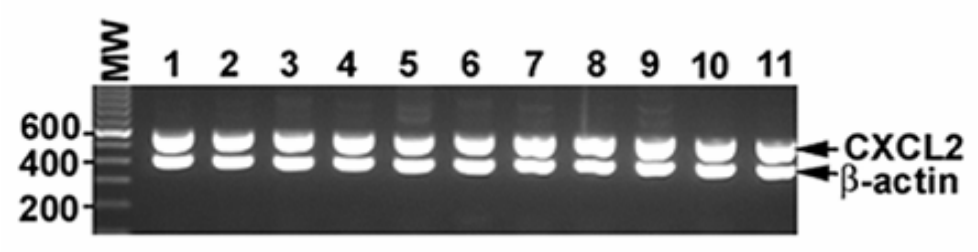
cDNA probes. Lanes 1-14 contained BAC DNA from 14 different BAC clones (see Table 5). Molecular weight standard (kb) is indicated on the right margin.

3.5. Expression of three CXC chemokines in normal catfish tissues and developmental expression of CXCL12

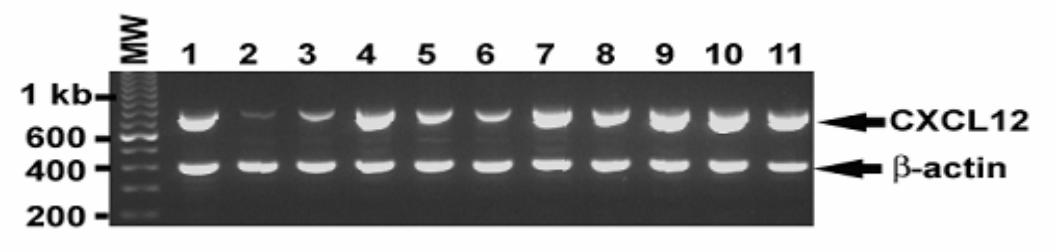
Constitutive expression of the CXCL2-like chemokine was observed in all tested tissues including ovary, skin, muscle, intestine, stomach, brain, gill, liver, spleen, trunk kidney, and head kidney. The expression level was much higher than expression levels of the β-actin gene. Interestingly, equal levels of CXCL2-like chemokine were expressed in all tissues, regardless of whether the tissues were immune-related (Figure 6A).

Catfish CXCL12 was also expressed in all 11 tested tissues (Figure 6B). Expression levels appeared to be lower in the skin, muscle, and brain. The overall expression was high, at a level comparable to the expression of β-actin. Very similarly, CXCL14 was expressed at high levels in various tissues. The notable exceptions were in the skin and trunk kidney where the expression was lower. In relation to its functions in the central nervous system as characterized in zebrafish and carp [19,20], CXCL14 was expressed highly in the brain (Figure 6C). Strong expression was also noted in ovary, gill, and head kidney.

A.



B.



C.

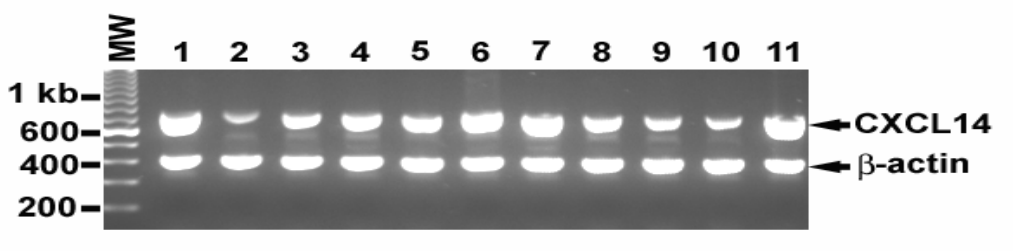


Figure 6. RT-PCR analysis of gene expression of CXCL2-like (A), CXCL12 (B), and CXCL14 (C) in various tissues. RT-PCR reactions were conducted as detailed in the text. MW, molecular weight; 1, ovary; 2, skin; 3, muscle; 4, intestine; 5, stomach; 6, brain; 7, gill; 8, liver; 9, spleen; 10, trunk kidney; 11, head kidney. The positions of RT-PCR products for each CXC chemokine and β-actin are indicated by the arrows on the right margins. Molecular weight standard is indicated on the left margins.

CXCL12 plays an important role in early development, specifically primordial cell migration in zebrafish and colonization of gonad in mouse [18,36-40]. Its expression during the time course of development was analyzed using RT-PCR. As shown in Fig. 7, CXCL12 was expressed quite early during development. Its mature mRNA was detected at 24 hours after fertilization and expressed constitutively at high levels thereafter (Figure 7).

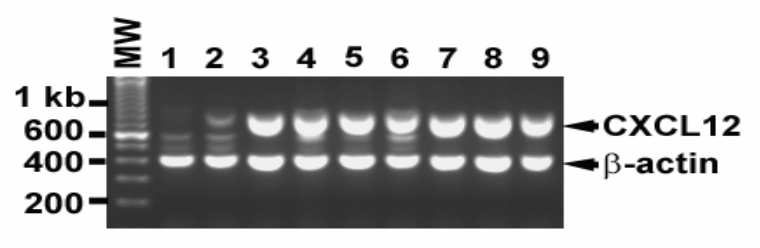


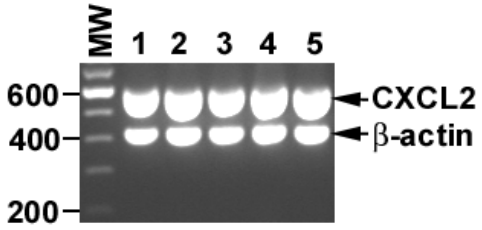
Figure 7. RT-PCR analysis of CXCL12 gene expression during early development. RT-PCR analysis was conducted as described in Materials and Methods. MW, molecular weight standard; 1, 8h after fertilization; 2, 24h after fertilization; 3, 48h after fertilization; 4, 4 days after fertilization (hatching); 5, 6 days after fertilization (first feeding); 6, 9 days after fertilization; 7, 13 days after fertilization; 8, 16 days after fertilization; 9, 20 days after fertilization. The positions of RT-PCR products for each CXC chemokine and β-actin are indicated by the arrows on the right margins. Molecular standards are indicated on the left margins.

3.6. Expression of three CXC chemokines in the head kidney tissue of infected fish

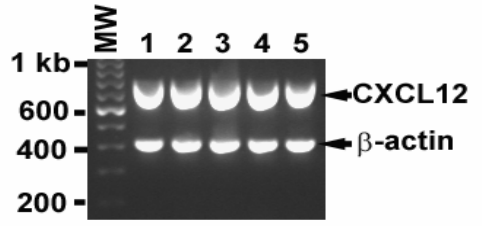
The expression of the three CXC chemokines was analyzed in the head kidney tissues of catfish after challenge with *Edwardsiella ictaluri*, the causative agent of enteric

septicemia of catfish (ESC). Analysis using RT-PCR indicated that the expression of all three CXC chemokines was not affected by the bacterial infection (Figure 8A, 8B, and 8C). High levels of expression were observed in control fish, and in fish challenged with the ESC bacteria at 24 h, 3 days, and 7 days after challenge. Similar expression was also observed from moribund fish (Fig. 8A, 8B, and 8C).

A.



B.



C.

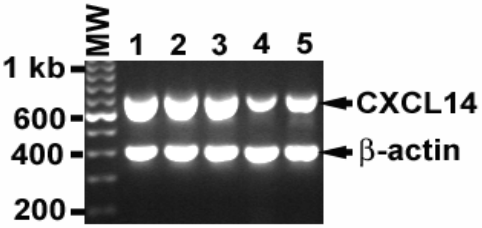


Figure 8. RT-PCR analysis of CXC chemokine gene expression after bacterial challenge with *Edwardsiella ictaluri*. RT-PCR analysis was conducted as described in Materials and Methods. Samples of head kidney were collected and expression of CXCL2-like (A), CXCL12 (B), and CXCL14 (C) was analyzed at various time points after bacterial challenge: 1, no challenge control; 2, 24h after challenge; 3, 3 days after challenge; 4, 7

days after challenge; and 5, moribund fish collected between day 4 and 7 after challenge. The positions of the RT-PCR products of CXCL2-like, CXCL12, and CXCL14, as well as that of β-actin are indicated by arrows on the right margins. Molecular weight standards are indicated on the left margins.

4. Discussion

We report here three new constitutively-expressed CXC chemokines from channel catfish. Phylogenetic analysis demonstrates strong orthologies between two of the catfish chemokines and mammalian CXCL12 and CXCL14. Although the orthology of the third CXC chemokine could not be established, it represents a novel CXC chemokine from teleost fish most similar to CXCL2. Expression of these three novel catfish chemokines differs from our previous work with CXCL8-like chemokine, and CXCL10-like chemokine in channel catfish. While dramatic upregulation was observed after bacterial infection for the CXCL8- and CXCL10-like chemokines, the catfish CXCL2-like, CXCL12, and CXCL14 were constitutively expressed.

Five CXC chemokines have now been identified from channel catfish. While this number is still significantly less than the number known in mammalian species, recent discoveries of large sets of CC chemokines in fish species [41,42] indicate that more CXC chemokines may be identified after further genome sequencing and analysis.

The identity of the catfish CXCL2-like chemokine remains to be resolved. It shares only 24% amino acid identity with mouse CXCL2 and even less with other mammalian species. While future sequencing efforts in intermediate species may provide

sequences that allow catfish CXCL2-like chemokine to cluster concretely with mammalian counterparts on a phylogenetic tree, presently we can only speculate as to orthology. An examination of the functional and expression characteristics of mammalian CXCL2, however, provides some interesting insights. CXCL2 is traditionally classified as an inducible chemokine, stimulated by bacterial cell wall components and induced by pro-inflammatory cytokines [43-45]. However, several reports of constitutive expression of CXCL2 have been made [46-49]. Savvedra et al. [46] noted fairly high constitutive expression of CXCL2, however, they were unable to detect a change of expression after *Candida albicans* infection. The authors suggested that CXCL2 may normally function in the regulation of polymorphonuclear neutrophils (PMNs). A recent study by Matzer et al. [50] found constitutive expression of CXCL2 in the bone marrow of mice even after infection with bacteria, *Yersinia enterocolitica*. CXCL2 is believed to be produced by a subset of bone marrow granulocytes in mice. Our observation, therefore, of high constitutive expression of catfish CXCL2-like chemokine during challenge in head kidney (functionally similar to bone marrow), may represent a conservation of homeostatic functions between teleost and mammalian species.

The genome duplication of CXC chemokines in certain fish species appears to be a recent evolutionary event. While two copies of CXCL12 were found in carp [20] and in zebrafish [51] only a single copy was identified here from channel catfish. Zebrafish and carp are closely related taxonomically, both species are part of the Cyprinid family. However, catfish as an order, Siluriformes, is tightly grouped on the tree of life with Cypriniformes, containing zebrafish and carp, as ostariophysan fishes. Considering the

small evolutionary distance separating these species, it was somewhat surprising to not find a second copy of catfish CXCL12. Catfish CXCL12 shares highest identity with the *a* forms of both zebrafish and carp CXCL12. Although the high levels of similarity of the *a* and *b* types of CXCL12 in these fish make it unlikely that another copy of catfish CXCL12 was missed in screening the catfish BAC library, the possibility remains.

Chemokines are best known for their vital role in leukocyte chemotaxis as part of the larger inflammatory response. Expression analysis and functional characterization of chemokines in mammalian species has often overlooked the role of these proteins under homeostatic conditions. Recent investigations of chemokine diversity in teleost fish have also centered on the immune-related functions of chemotactic cytokines such as CXCL8 and CXCL10. While a disease-based approach to chemokines is essential to the development of therapies for both human and animal infections, it may be a poor measure of the overall complexity of chemokine function. Both CXCL12 and CXCL14 are known to be expressed constitutively in the developing central nervous system [52-55]. The former chemokine also plays important roles in reproductive organ development [36,38,39]. In addition, Kurth et al. [56] found constitutive expression of CXCL14 in skin and intestine suggesting the chemokine functions homeostatically in macrophage development by recruiting precursors to fibroblasts. Huising et al. [57] stated that CXCL12 and CXCL14 functions not only can be related to immune defense, but also pleiotropic functions. A full understanding of chemokine complexity, though currently a good ways off, will allow us to assess properly each chemokines role within the organism, explain the apparent redundancies of function, and identify the most valuable targets for remediative disease therapies in humans and fish.

As part of a larger effort to assess the conservation of chemokine diversity in teleost fish, we report here the identification of three novel, constitutively-expressed CXC chemokines from channel catfish (*Ictalurus punctatus*). All three novel CXC chemokines were found to be non-inducible under bacterial challenge with *Edwardsiella ictaluri*, the causative agent of ESC disease. Their expression profiles stand in notable contrast with CXCL10 and CXCL8-like chemokines in catfish that were induced more than 3-60 times after challenge depending on the genetic background [17,12]. CXCL2-like, CXCL12, and CXCL14 catfish chemokines may lack significant roles in the inflammatory response but, instead, insert their functions during normal developmental processes. Their high constitutive expression in several non-immune tissues suggests the pleiotropic nature of their functions and their importance to homeostasis.

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IV. STRUCTURAL CHARACTERIZATION AND EXPRESSION ANALYSIS OF TOLL-LIKE RECEPTOR 2 GENE FROM CATFISH

Innate immunity has long been regarded as a host organism's initial line of defense against pathogen invasion. The roles of proinflammatory cytokines, chemokines, and complement factors as immune activation signals and the roles of NK cells, phagocytes, antimicrobial peptides, and the membrane attack complex as immune effectors have been well characterized for some time in mammalian species. The molecular components involved during initial contact between host and pathogen, however, were poorly understood until recently. To detect non-specific antigens, a fast-acting, non-clonal mechanism of pathogen recognition, differing significantly from that of adaptive immunity, was clearly required. The discovery of a family of receptors homologous to the *Drosophila* receptor Toll provided molecular evidence of such a mechanism in vertebrate species [1,2]. These Toll-like receptors (TLRs) were found to recognize specific structures on pathogens termed pathogen-associated molecular patterns (PAMPs) [3] and utilize conserved signaling pathways to activate pro-inflammatory cytokines and type-1 interferons [4,5]. TLRs are defined by the presence of a Toll/IL-1 receptor (TIR) domain in their cytoplasmic portion and by leucine-rich repeats (LRR) in their extracellular domain [6]. At least 11 TLRs have been identified to-date from mammals. Functional studies have revealed that these receptors recognize and respond to a wide range of exogenous as well as endogenous ligands. Roach et al. [7], in their study of the evolution of vertebrate TLRs, identified six major subfamilies, each recognizing a general class of PAMPs. Under this classification, individual TLRs recognize either lipopeptides (TLR2), dsRNA (TLR3), lipopolysaccharide (LPS, TLR4), flagellin (TLR5), nucleic acid and heme motifs (TLR7-9) [7]. The number of reported TLR ligands, however, continues to increase rapidly, frustrating attempts to characterize TLR

specificity. Additionally, reports of multiple TLRs acting together in pathogen recognition and signaling indicate that much work is still needed to complete our understanding of the TLR family [4,8].

TLR2 is best known as a receptor recognizing conserved components of Gram-positive bacteria such as lipoteichoic acid (LTA), peptidoglycans (PGN), and lipoproteins [9,10]. However, TLR2 interacts with a wide range of additional ligand types including zymosan, derived from yeast, glycosylphosphatidylinositols (GPIs) from protozoan parasites, LPS of Gram-negative bacterium *Porphyromonas gingivalis*, and the LPS of zoonotic pathogen *Leptospira interrogans* [11-14]. While TLR4 is traditionally considered the receptor for PAMPs of Gram-negative bacteria, recent studies have shown that TLR2 may function together with TLR4 [15] or independently in this role [16]. Additionally, TLR2 can form functional pairs with TLR1 or TLR6, capable of recognizing and specifically responding to a variety of PAMPs [17,18].

TLR2 structure [19,20] and functional patterns (above) have been investigated extensively in mammalian species. More recently in teleost fish, the structures and expression patterns of TLR2 have been reported in fugu (*Takifugu rubripes*), zebrafish (*Danio rerio*) and Japanese flounder (*Paralichthys olivaceus*) [21-24]. As part of our efforts to gain a better understanding of the innate immune response of channel catfish (*Ictalurus punctatus*), the primary aquaculture species in the US, here we characterized the complete cDNA of catfish TLR2, identified and sequenced the TLR2 gene from the catfish BAC library, determined TLR2 genomic structure, assessed catfish TLR2 conservation through sequence and phylogenetic analysis, examined the expression patterns of catfish TLR2 in various healthy tissues, and compared TLR2 expression after

infection with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), in susceptible (channel) and resistant (blue) catfish (*Ictalurus furcatus*).

A channel catfish partial cDNA sharing highest BLASTX identity with TLR2 sequences from fish and mammalian species was identified through analysis of catfish ESTs [25-27]. High-density filters of a channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Each set of filters contained 10X genome coverage of the channel catfish BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). As part of ongoing efforts to physically map important genes, an overgo probe was designed based on the partial TLR2 cDNA sequence and hybridized to the catfish BAC library. Sequences of the overgo primers are: Overgo A: GCAGCATGATACAGTTAGCGTTTC, and Overgo B: GTGAGGTGCTTCAACAGAAACGCT. Overgo hybridization was conducted as described [28-30]. Positive clones were identified according to the clone distribution instructions from CHORI, and one clone, BAC_153_N05, was picked out for sequencing analysis. The catfish TLR2 gene was sequenced by primer walking and its sequence has been deposited in GenBank with accession number DQ372072.

BLAST searches were conducted to determine gene identities and the full open reading frame of catfish TLR2, and the DNASTAR software package was used for sequencing analysis [31]. The MegAlign program of the DNASTAR package was used for TLR2 TIR domain sequence alignment using ClustalW. The receptor structure was characterized based on amino acid sequence using the simple modular architecture research tool (SMART) [32; http://smart.embl-heidelberg.de/] and TMHMM programs [33].

The full-length of amino acid sequences from known TLR2 genes was retrieved from GenBank for phylogenetic analysis. ClustalW was used for multiple alignments of amino acid sequences.

Challenge experiments of Marion select strain from channel (*Ictalurus punctatus*), and blue catfish (*I. furcatus*) were conducted at the hatchery of the Auburn University Fish Genetics Research Unit as previously described [34] with modifications [28,35]. Briefly, catfish were challenged in a rectangular tank by immersion exposure for two hours with freshly prepared cultured from single colony of ESC bacteria, *Edwardsiella ictaluri*, from natural outbreak in Alabama (the outbreak number ALG-02-414).

Eleven tissues were collected from healthy channel catfish including head kidney, spleen, intestine, stomach, skin, muscle, liver, trunk kidney, ovary, brain and gill. Head kidney and spleen were collected from challenged channel catfish and blue catfish. Samples were collected from 10 fish at each time point including control (0 hr), 4 hr, 24 hr, 72 hr after challenge, and moribund fish. Samples of each tissue from 10 fish were pooled. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powder and thoroughly mixed. A fraction of the tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [36] using the Trizol reagents kit from Invitrogen following manufacturer's instructions. Extracted RNA was stored in a -80°C freezer until used as template for reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR.

RT-PCR was used to study an expression of catfish TLR2 in various normal tissues. RT-PCR reactions were conducted by two step RT-PCR using M-MuLV reverse

transcriptase (New England Biolabs, Ipswich, MA). RT reactions were conducted in 40 µl containing 4 µg DNase I-treated RNA, 4 µl (40 uM) oligo dT primers, 8 µl (2.5 mM each) dNTPs, 1 µl RNase inhibitor, 1X RT reaction buffer, and 200 units of RT. Detailed procedures followed the instructions of the manufacturer. The sequences of primers for RT-PCR of TLR2 are: TLR2 upper primer: ACAGGCTTACGTCGCTGGAC, TLR2 lower primer: TCCAGAACGGTCAGGCTCAC. After RT reaction, 1 µl of the RT products was used as templates for PCR using JumpStart Taq polymerase (Sigma, St. Louis, MO). The reactions also included the gene specific primers and primers of β-actin serving as an internal control. The sequences of the RT-PCR primers for the beta-actin internal control are: beta-actin upper primer: AGAGAGAAATTGTCCGTGACATC, beta-actin lower primer: CTCCGATCCAGACAGAGTATTTG. The reactions, for both healthy and challenged tissues, were completed in a thermocycler with the following thermo-profiles: denaturation at 94ºC for 2 min followed by 38 cycles of 94ºC for 30 s, 57ºC for 30 s, 72ºC for 1 min. Upon the completion of PCR, the reaction was incubated at 72°C for an additional 10 min. The RT-PCR products were analyzed by electrophoresis on a 1.0 % agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

Quantitative real-time RT-PCR using a LightCycler (Roche) was carried out to characterize catfish TLR2 expression after ESC infection. Concentration and quality of total RNA from spleen and head kidney was determined by spectrophotometry (optical density 260/280 ratio) and electrophoresis. Primers for TLR2 and internal control used in quantitative real-time PCR were the same as RT-PCR. One-step quantitative real-time PCR was carried out in the LightCycler using a Fast Start RNA Master SYBR Green I

reagents kit (Roche) following manufacturer's instructions with modifications as previously described [34]. Relative expression ratios were obtained by normalizing the expression of the target gene, as determined by mean crossing point deviation, by that of a non-regulated reference gene, β-actin, using the Relative Expression Software Tool 384 v. 1 (REST) developed by Pfaffl et al. ([37]; http://www.wzw.tum.de/gene-quantification/). Each reaction was carried out in triplicate. Expression ratio results were tested for significance by a randomization test built into the software. All ratios are relative to expression of the gene in 0 hr (control) RNA samples.

Analysis of the domains of the catfish TLR2 gene using the SMART program revealed that it encodes the two motifs characteristic of TLR genes, several leucine rich repeats (LRRs) in the N-terminal region and a toll-interleukin-1 receptor (TIR) domain at the C-terminal end (Figure 1). Two microsatellites were detected in the upstream and downstream genomic regions, an $(AT)_9$ repeat at 632 bp and an $(AC)_{21}$ repeat at 3,606 bp.

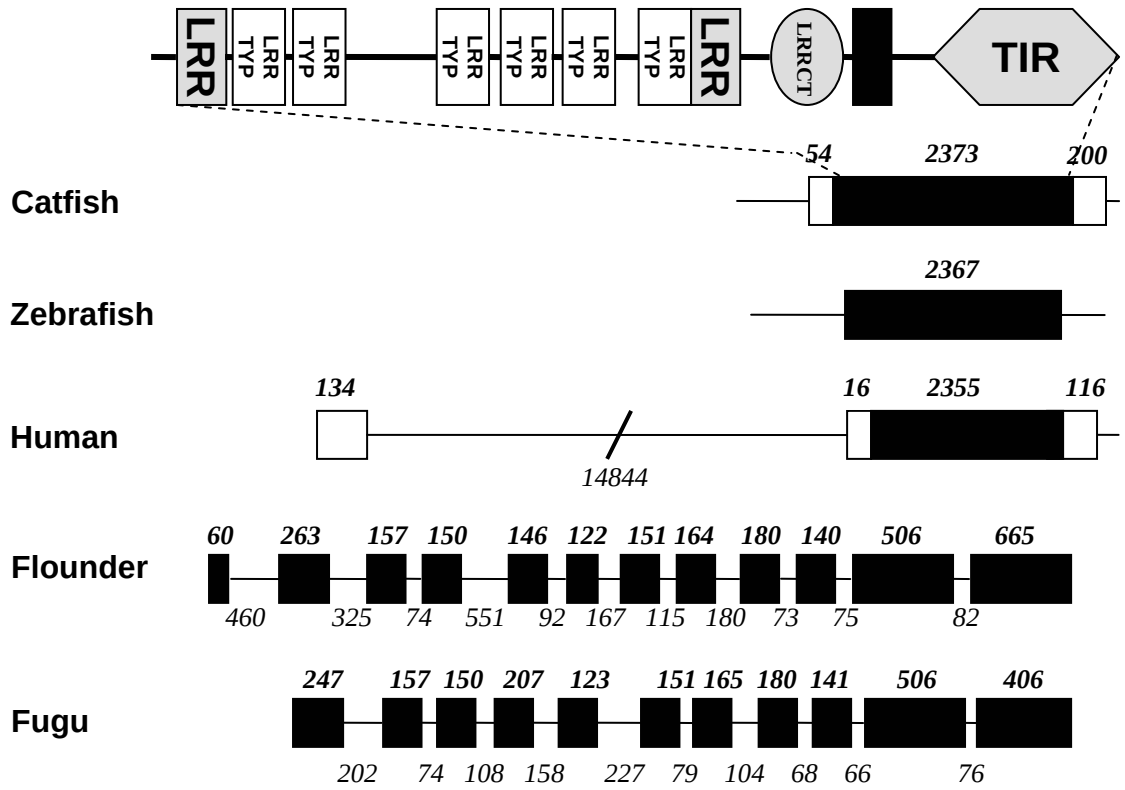

Figure 1. Schematic representation of catfish TLR2 domains, predicted by SMART and TMHMM programs. Domains include the leucine rich repeats (rectangles) and C-terminal leucine rich repeats (oval), followed by a putative transmembrane region (black rectangle), and a TIR domain (hexagon). A comparison of TLR2 genomic structure and organization from catfish, zebrafish, human, flounder and fugu is also presented. Exons are represented by boxes; solid boxes represent coding region of the gene; white boxes represent 5'-untranslated region (UTR) and 3'-UTR. Their sizes in base pairs are shown on the top of the boxes. Introns are represented by a line and the size is shown below the line.

A striking feature of the catfish TLR2 gene is that it does contain any introns and is therefore, an intronless gene. Structurally, catfish TLR2 consists of one exon with

an open reading frame of 2,373 bp, encoding a protein of 790 amino acid residues (Figure 1). Catfish TLR2 has a 5'-untranslated region (UTR) of 54 bp and a 3'-UTR of 200 bp, as determined by comparing the catfish cDNA and gene sequences. The gene structure of one coding exon in catfish TLR2 is shared by zebrafish and human TLR2 genes, but not by fugu or Japanese flounder TLR2 genes which are encoded by 11 and 12 exons, respectively [21,24]. Before the structure of the zebrafish TLR2 gene was available, it was hypothesized that a large number of introns were acquired in fish TLR2 genes after the divergence of vertebrates [21,24]. Our results, coupled with those from zebrafish [23], clearly suggest that in catfish and zebrafish, TLR2 genes are intronless. Both catfish and zebrafish belong to Ostariophysi and are more closely related each other phylogenetically than to fugu. This raises interesting questions about the timing of intron invasion and possible functional/evolutionary reasons behind the striking structural differences in TLR2 genes.

A ClustalW-generated multiple sequence alignment using all available TLR2 sequences from fish species as well as those of other vertebrates indicated high levels of amino acid sequence conservation within the TIR domain (Figure 2). We first performed sequence alignment without using the rainbow trout sequences (Accession number AJ628348 and AJ878915). Catfish TLR2 shared highest full length amino acid sequence similarity with zebrafish (60.2%), and the lowest similarity was found in mouse and human (41.1%) (Table 1). As expected, higher amino acid identities were found among the conserved TIR domain, ranging from 82.2% in zebrafish to 65.1% in flounder (Table 1). We then conducted sequence alignment with the rainbow trout sequences designated as TLR2 in the GenBank (AJ878915), but the similarities between the catfish and the

trout sequences were low. The overall similarities were approximately 20% with the entire amino acid sequences, and the similarities within the TIR domain were 43%. Further BLAST searches suggested that the trout sequence AJ878915 was most similar to flounder TLR3. As toll-like receptors share some level of similarities among them, especially within the TIR domains, the establishment of orthologies among all teleost TLR genes requires availability of additional TLR gene sequences from various species.

Figure 2. Alignment of the catfish (DQ372072) TLR2 TIR domain with those of zebrafish (NP_997977), Rainbow trout I (AJ628348), Rainbow trout II (AJ878915), fugu (AAW69370), Japanese flounder (BAD01044), chicken (BAB16113), cow (NP_776622), horse (AAR08196), mouse (NP_036035), pig (BAD91799), and human (NP_003255) TLR2 genes.

Table 1. Percentages of amino acid identities between catfish TLR2 and those from other species using ClustalW. Percentages were compared based on full-length and TIR domain amino acids sequences

Catfish	Full sequence (%)	TIR domains (%)
Zebrafish	60.2	82.2
Rainbow Trout I (AJ628348)	20.1	43.1
Rainbow Trout II (AJ878915)	19.6	43.8
Fugu	46.6	65.8
Flounder	47.7	65.1
Chicken	41.3	72.4
Pig	42.3	71
Cow	42.5	70.3
Horse	41.2	71
Mouse	41.1	70.3
Human	41.1	72.4

RT-PCR analysis using total RNA from various healthy tissues of channel catfish indicated that TLR2 is ubiquitously expressed albeit at different levels. High levels of TLR2 expression were detected in liver, brain, and gill while only low TLR2 expression was detected in skin and muscle (Figure 3). Similar ubiquitous patterns of TLR2 expression were found in various healthy tissues of fugu and flounder [21,24], whereas expression was not detected in several non-immunological zebrafish tissues such as testis and heart [22].

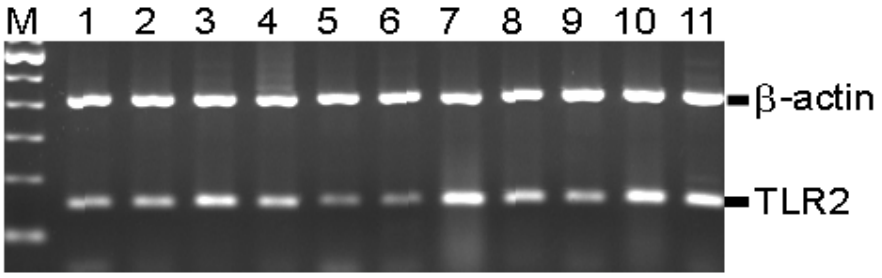

Figure 3. RT-PCR analysis of catfish TLR2 gene expression in various healthy tissues. RT-PCR products were analyzed on an agarose gel. M, 100 bp molecular weight; 1, head kidney; 2, spleen; 3, intestine; 4, stomach; 5, skin; 6, muscle; 7, liver; 8, trunk kidney; 9, ovary; 10, brain; 11, gill. The positions of the RT-PCR amplified bands of beta-actin and TLR2 are indicated on the right margin.

In order to assess potential roles of TLR2 during bacterial infection in catfish, quantitative real-time PCR was conducted using RNA isolated from the head kidney and spleen of blue and channel catfish at several time points after challenge with *Edwardsiella ictaluri*, the causative agent of ESC. Channel catfish are highly susceptible to ESC infection while blue catfish are more resistant [38]. TLR2 expression changes for both species in infected spleen samples were small and did not rise to a level of statistical significance (Figure 4). Expression for both species in spleen was down-regulated approximately two-fold at 4 hr, and upregulated two-fold at 24 hr relative to the 0 hr control. At 72 hr post infection, channel catfish TLR2 expression in spleen increased three-fold, while a two-fold down-regulation was observed in blue catfish. In infected head kidney samples, a consistent pattern of down-regulation was observed from both species over all time points. Expression changes in blue catfish, however, were larger

(up to eight-fold) and statistically significant, while smaller, insignificant changes were recorded from channel catfish. Catfish TLR2 appears to respond to ESC infection by down-regulation post infection, although further characterization is needed in the future. In contrast, a pattern of quick up-regulation of expression after ESC infection has been characteristic of many other genes involved in the catfish innate immune response including CC and CXC chemokines [28,35,36,39,40], antimicrobial peptides [29,41-43], and proinflammatory cytokines [44].

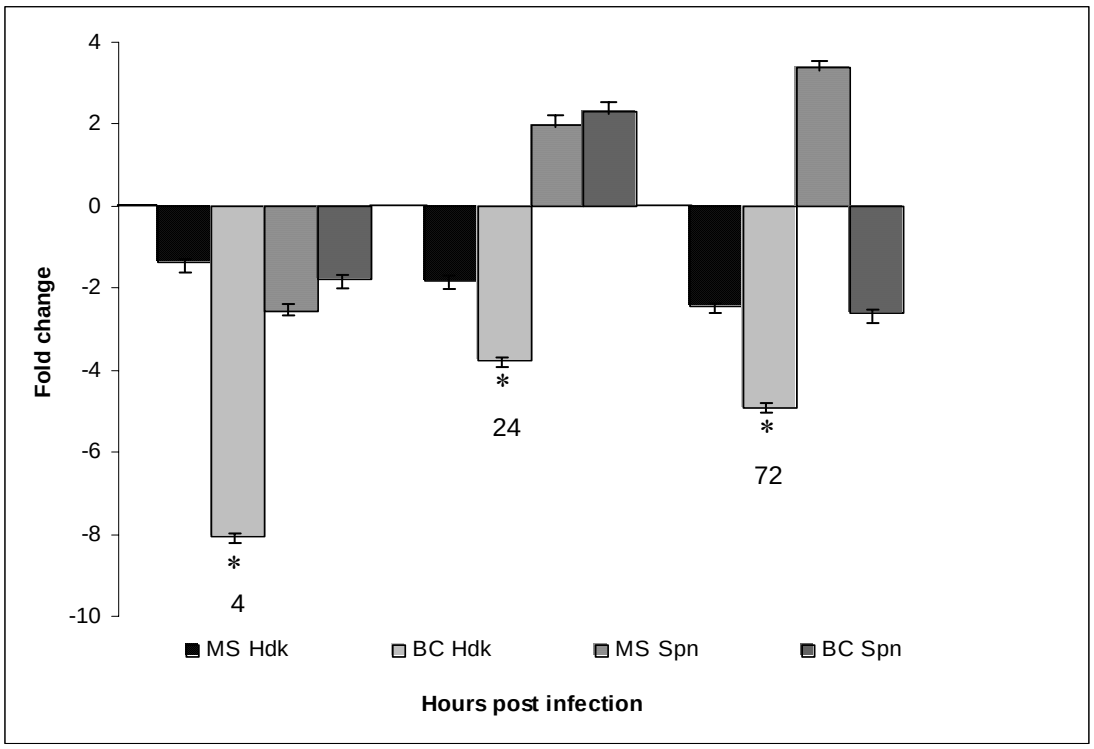

Figure 4. Real-time PCR analysis of catfish TLR2 gene expression in head kidney and spleen following infection with *Edwardsiella ictaluri* at different time points (4h, 24h and 72h). Fold change indicates the ratio of expression at the specified time after ESC exposure to that at 0 hour before ESC infection, as normalized with an internal reference gene of catfish beta-actin. Three independent Lightcyler reactions were run for each

sample and statistical analysis was conducted using the software REST. Error bars indicate standard error and an asterisk indicates statistical significance. MS and BC signify Marion select strain of channel catfish and blue catfish, respectively.

Induction of TLR2 expression has also been reported in Japanese flounder peripheral blood leukocytes (PBLs) at 1 hr and 3 hr after PGN and polyI:C stimulation, respectively [24]. Meijer et al. [23] reported that zebrafish TLR2 expression was induced in fish infected with *Mycobacterium marinum*. Both previous studies in fish utilized Gram-positive bacteria or their components as stimulants or infectious agents. However, our results with Gram-negative bacteria *E. ictaluri* combined with the observed gene induction after stimulation with dsRNA polyI:C [24] indicate that TLR2 may function more widely in fish than previously assumed. Similar studies of TLR3, classically considered a receptor for viral dsRNA, in catfish after infection with *E. ictaluri* revealed induced expression [45,46]. All these observations suggest that toll-like receptors may be involved in a more complex patterns of ligand binding and recognition than previously thought [7]. Further work is needed to determine the pathogen specificities of TLRs in fish species.

All vertebrate species studied to-date possess a functional, well-conserved TLR2 gene, indicating strong selective pressure for the gene's ability to recognize important classes of PAMPs. Conservation of microbial PAMPs has likely led to the maintenance of a highly conserved TLR family across vertebrate species. The interspecies conservation of the TLR multigene family differs significantly from other immune families of genes under less selective restraints [7,28,47].

Much work remains in assessing the potential of TLRs in immune research and their applications in mammalian and fish species. TLR2 research in mammals continues to uncover polymorphisms associated with infectious and inflammatory diseases [20,48,49]. Two microsatellites revealed by genomic sequencing of the TLR2 gene environ should allow mapping of the TLR2 gene and future QTL analysis in catfish. Additionally, understanding the function of fish TLRs and their pathogen specificities may lead to the development of better immunostimulants for use in commercial aquaculture [50]. The future identification and characterization of additional TLR family members and the components of their signaling pathways [51] in aquaculture species should further our knowledge of the teleost innate immune response.

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V. CONCLUSIONS

During the course of my studies, I have characterized genes and their expression for four CXC chemokines, CXCL2, CXCL10, CXCL12 and CXCL14, and toll-like receptor 2. All the genes were identified and the cDNAs were completely sequenced. All the genes were mapped to BACs, allowing their mapping to BAC-based physical maps as the physical mapping project is independently conducted in our laboratory. Expression analysis was conducted on all the five genes in relation to tissue distribution in healthy fish, as well as after infection with *E. ictaluri*.

All four catfish CXC chemokines reported in this dissertation have four conserved cysteine residues and the first two conserved cysteines exhibit a typical characteristic of CXC arrangements. However, differences were noticed. For instance, catfish CXCL2 lacks the ELR motif, a situation similar to teleost fish CXCL8, although both belong to the ELR subgroup. It is possible that the ELR motif is only conserved among mammals, but not among fish. The catfish chemokines exhibited a high level of amino acid identities to those from other vertebrates, and mostly close to those of zebrafish when present.

In spite of putative identities, orthologies can not yet be established for all the identified chemokines. Use of phylogenetic analysis was able to establish orthologies for the catfish CXCL12 and CXCL14, but the orthology can not be established for catfish CXCL2, possibly due to the lack of CXCL2-like sequences from species intermediate to fish and mammals. Therefore, more sequences are required from other species in a wide spectrum of evolutionary ladder in order to establish orthologies for all CXC chemokines. I believe that as more related sequences become available from various species, it will be possible to establish orthologies for more chemokines. However, it is also possible that the rapid sequence divergence, when coupled to the rapid gene duplication, may make establishment of orthologies very difficult by phylogenetic analysis. It may also require functional analysis to further assure the orthologous relations among various chemokines.

CXCL2, CXCL12 and CXCL14 existed as a single copy gene in the catfish genome. However, a multigene family of CXCL10 was detected in catfish. Using a single individual of F1 hybrid catfish revealed the presence of multiple haplotypes of CXCL10 cDNAs. Southern blot analysis indicated the presence of multiple copies of CXCL10 in a tandem arrangement (data not shown). Exact genomic arrangements and their expression requires further research.

All four catfish CXC chemokines were expressed in a wide range of tissues in healthy fish. They are expressed most highly in immune-related tissues such as head kidney and spleen. However, the catfish chemokines exhibited quite different expression profiles after ESC infection. While a strong induced expression of the catfish CXCL10 was observed after infection, CXCL2, CXCL12, and CXCL14 exhibited homeostatic

expression regardless of infection. This suggests that these chemokines may have additional functions other than serving as chemokines during inflammatory responses.

The catfish TLR2 gene is conserved in the LRR and TIR domains, highly similar to those from mammals and other fish species. It also shared a high level of amino acid sequences similarity within TIR domain with TLR2 from other teleost fish and mammals. However, its genome organization was quite different from that of the TLR2 genes from other species. The catfish TLR2 was an intronless gene, similar to the gene structure of zebrafish TLR2, but different from the TLR2 genes from fugu, flounder, and human, all of which has introns.

The catfish TLR2 was constitutively expressed in various tissues of healthy fish, and the expression was changed after bacterial challenge in spleen of catfish. This raises the very interesting question as to the roles of TLR2 in infections by Gram-negative bacteria. It has been known as a receptor for Gram-positive bacteria, but this research indicated its involvement also in Gram-negative bacterial infections. Further research is required to understand its functions.

Future studies should include functional studies. As most past studies have established the main functions of the CXC ligand molecules and TLR receptors, many genome researchers tend to neglect the functional studies. This research actually triggered a great need for functional studies. A few examples are listed here: First, the three CXC ligands CXCL2, CXCL12, and CXCL14 were constitutively expressed, with little changes with bacterial challenges. This suggested that they may have homeostatic functions other than just as messengers for inflammatory responses; second, multiple CXCL10 genes were detected in catfish. How are the multigenes regulated? Do they

have a partitioned function in terms of space, time, or both? Third, the changing of TLR2 expression by ESC infection suggested that the functions of fish TLR2 could be pleotropic and certainly should be studied. A complete understanding of their functional complexity will allow us to assess properly each chemokine and TLR role within the organism, explain the apparent redundancies of function seen in chemokine and TLR families, and identify the most valuable targets for remediative disease therapies in fish.