

Constitutive expression of three novel catfish CXC chemokines: homeostatic chemokines in teleost fish

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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 remedial 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.

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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 (Murphy et al., 2000). The major subfamilies are the CC and CXC chemokines.

CXC chemokines were initially identified as potent mediators of neutrophil chemotaxis (Walz et al., 1987; Yoshimura et al., 1987), but are now known to function also in chemotaxis of monocytes and lymphocytes (Koch et al., 1992; Strieter et al., 1995). Altogether 16 CXC chemokines have been identified from mammals, although a smaller number was identified from any given species. Thus, 15 were identi-

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fied 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 (Laing and Secombes, 2004a). 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 (Strieter et al., 1995). In fish, however, the ELR motif was not found in several CXCL8-like chemokines (Najakshin et al., 1999; Lee et al., 2001; Laing et al., 2002b; Inoue et al., 2003a, 2003b; Chen et al., 2005).

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 (Oppenheim et al., 2000). 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 (Laing et al., 2002a). Two CXC chemokines from carp, CXCa and CXCb, have been functionally characterized, but lack orthology to known mammalian CXC chemokines (Huisling et al., 2003b; Savan et al., 2003). Recently, a CXCL10-like chemokine was identified from channel catfish and blue catfish (Baoprasertkul et al., 2004). A CXCL12-like expressed sequence tag (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 (David et al., 2002). A CXCL14 homologue has also been identified from zebrafish (Long et al., 2000) 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 (Huisling et al., 2004). 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 (stromal cell-derived factor-1 (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 (Baoprasertkul et al., 2004; Chen et al., 2005).

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 (Ju et al., 2000; Cao et al., 2001; Karsi et al., 2002; Kocabas et al., 2002). The putative CXC chemokine clones were subjected to complete sequencing analysis. Plasmid DNA was prepared by the alkaline lysis method (Sambrook et al., 1989) using the Qia-gen 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™ 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 (Altschul et al., 1990; Gish and States, 1993; Serapion et al., 2004) to identify gene similarities. Further analysis of open reading frame, sequence similarities, and multiple alignments were conducted using ClustalW 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 ClustalX (Thompson et al., 1997). Percentage of amino acid identities were recorded after all multiple alignments. Phylogenetic tree was drawn by the neighbor joining method (Saitou and Nei, 1987) 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. Chal-

lenge experiments were conducted as previously described (Dunham et al., 1993) with modifications (Baoprasertkul et al., 2004; Chen et al., 2005). Briefly, the catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of enteric septicemia of catfish (ESC) bacteria, *Edwardsiella 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 millilitre 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 3×10^7 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 l⁻¹, 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 (Chomczynski and Sacchi, 1987) 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 2× 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 thermoprofiles: 45 °C for 15 min for one cycle (reverse transcription reaction), the samples were pre-denatured at 94 °C for 2 min, then the samples were amplified for 40 cycles with

Table 1
Primer sequences for three catfish CXC chemokines

Gene (Accession no.)	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	ACAGTCTGTGGTGGAGTAC
	RT-PCR lower	ACATAGTGCTTCTTTGGACCAC
	cDNA probe upper	GACAGTCTGTGGTGGAGTCACTTCAACC
	cDNA probe lower	GTTGCAAGTCTAACACTTGG
	Overgo A	CAAATGCAGATGCACCAGGAAAGG
	Overgo B	GTATCGTATCTTGGGGCCTTTCT
β-Actin	RT-PCR upper	AGAGAGAAATTGTCCGTGACATC
	RT-PCR lower	CTCCGATCCAGACAGAGTATTTG

94 °C for 15 s, 45 °C for 30 s, and 68 °C 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 Corporation, San Mateo, CA).

2.6. BAC library screening

High density filters (10× 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 (Peatman et al., in press). Overgo primers (Table 1) were designed using an overgo maker program. The primers were purchased from Sigma Genosys (Woodlands, TX), then labeled with ³²P-dATP and ³²P-dCTP (Amersham, Piscataway, NJ) in 10 mg/ml bovine serum albumin, overgo labeling reaction 1× buffer (Ross et al., 1999) 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 95 °C for 10 min and added into hybridization tubes that had been under pre-hybridization for 2 h. The filters were hybridized at 50 °C for 18 h in 50 ml hybridization solution (1% BSA, 1 mM 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 -80 °C 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 2× 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 *EcoRI* and *HindIII* 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 Stratilinker 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, 5× SSC (Sambrook et al., 1989), 0.1% SDS (w/v), 5× 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 (Sambrook et al., 1989) with a labeling kit from Roche Diagnostics (Indianapolis, IN). The nylon membranes were washed first in 500 ml of 2× SSC for 10 min, followed by three washes in 0.2× 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 *HindIII* and *PstI* 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 (Fig. 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.

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

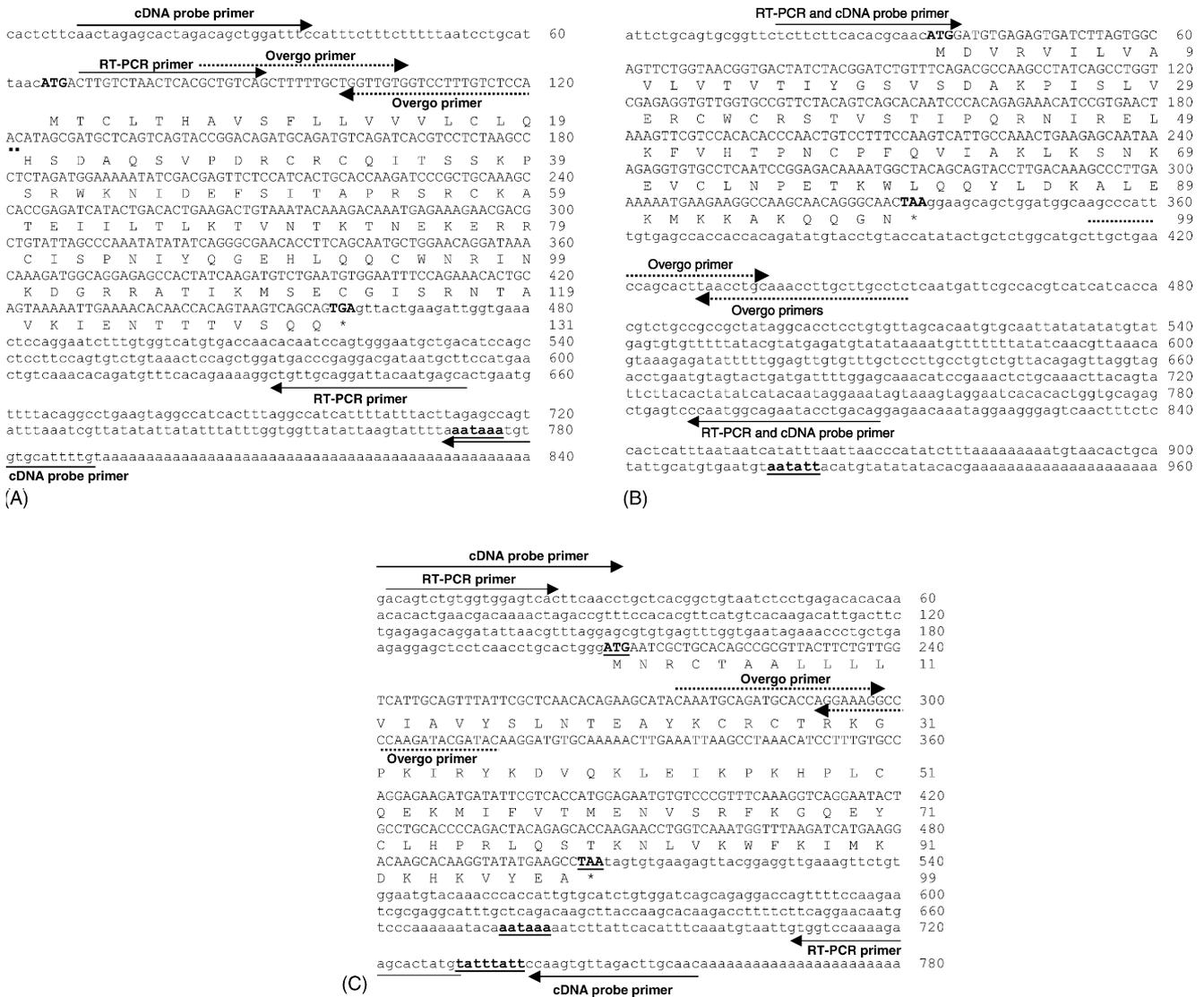


Fig. 1. cDNA and deduced amino acid sequences of the catfish CXCL2-like (A), CXCL12 (B), and CXCL14 (C) chemokine. The nucleotide sequences are on the upper lines and the amino acid sequences are in the lower lines. The translation start codon ATG and the termination codon TAA or TGA is bold. The putative 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.

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 (Fig. 1B). Similar to chicken CXCL12 (Read et al., 2005), 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 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

Table 3
Pairwise 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

Table 4
Pairwise 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

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 TATT-TATT exists 20-bp upstream of the poly (A)⁺ tail (Fig. 1C). The channel catfish CXCL14 shares high amino acid sequence similarity with both carp and zebrafish CXCL14 (Table 4).

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 (Fig. 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.

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).

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 Fig. 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

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

Gene	Location in 384-well 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

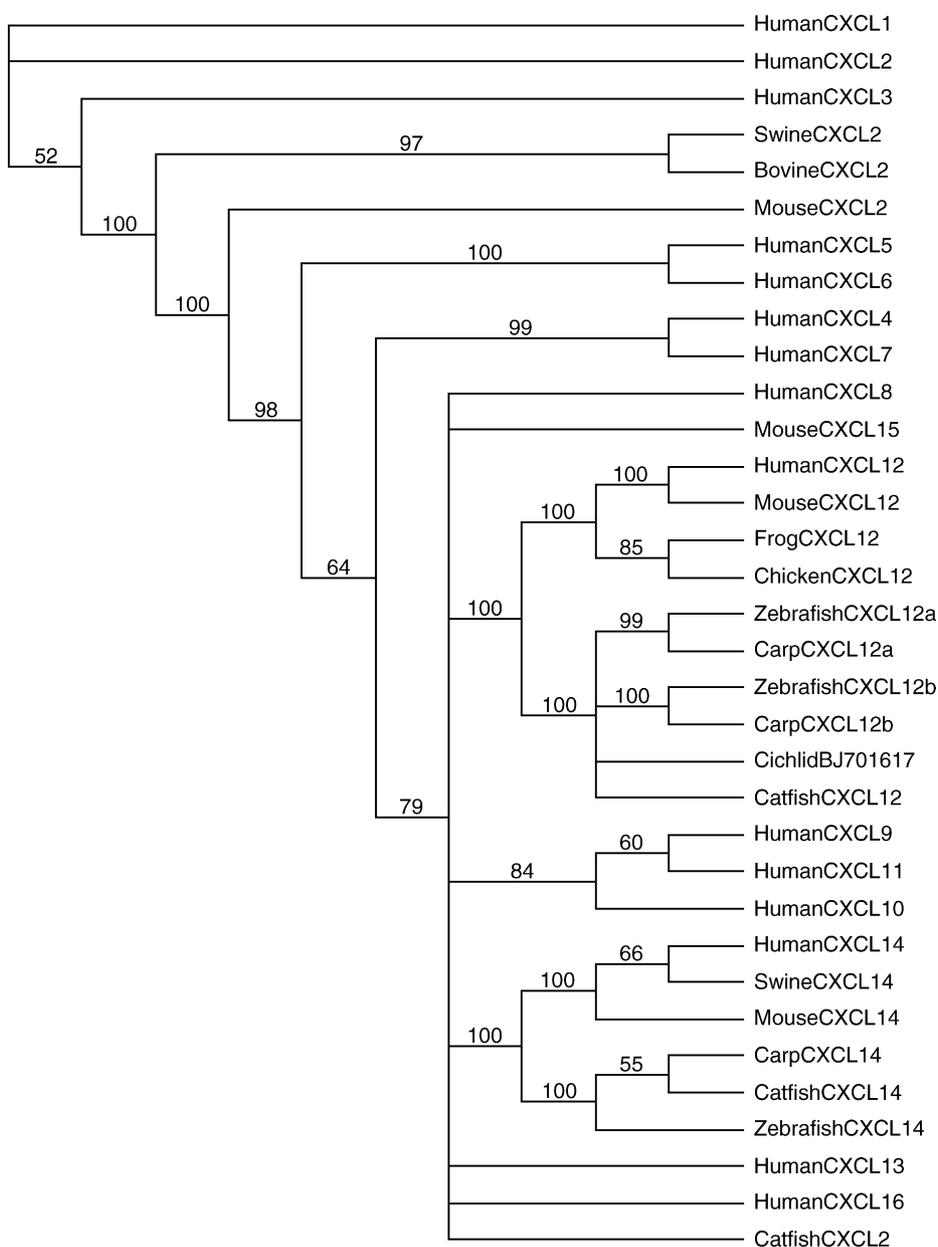


Fig. 2. Phylogenetic analysis of the three newly identified catfish CXC chemokines. The phylogenetic tree was constructed with ClustalX and PAUP using the neighbor joining method. The numbers on the branches represent the confidence level of 10,000 bootstrap replications. The sequences and their accession 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), and catfish CXCL2-like (AY836754).

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 *Hind*III (Fig. 4) or *Eco*RI (not shown), confirming the presence of a single copy of CXCL12 gene in the catfish genome.

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*RI (Fig. 5A) or *Hind*III (Fig. 5B). With *Eco*RI, only one band was positive for the CXCL14 probe, whereas with *Hind*III, three bands were positive for the CXCL14 probe. The identical restriction fragment profiles confirm

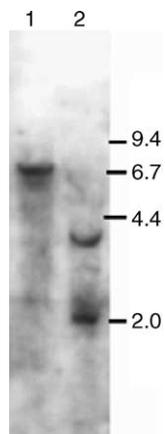


Fig. 3. Genomic Southern blot analysis of the catfish CXCL2-like chemokine. Genomic DNA was digested with *HindIII* (lane 1) or *PstI* (lane 2), and electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized with CXCL2-like cDNA probes. Molecular weight standard (kb) is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

the presence of a single copy of CXCL14 in the catfish genome.

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 very high, higher, in fact, than expression levels of the β -actin gene. Interest-

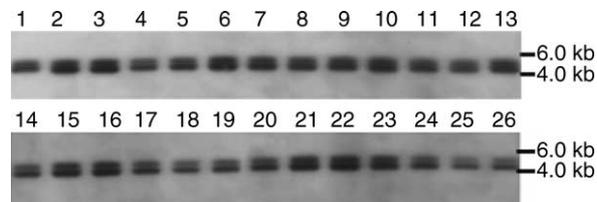


Fig. 4. Southern blot analysis of the catfish CXCL12 locus using BAC clones. BAC DNAs were digested with *HindIII*, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized with CXCL12 cDNA probes. Lanes 1–26 contained BAC DNA from 26 different BAC clones (see Table 5).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

ingly, equal levels of CXCL2-like chemokine were expressed in all tissues, regardless of whether the tissues were immune-related (Fig. 6A).

Catfish CXCL12 was also expressed in all 11 tested tissues (Fig. 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 (Long et al., 2000; Huising et al., 2004), CXCL14 was expressed highly in the brain (Fig. 6C). Strong expression was also noted in ovary, gill, and head kidney.

CXCL12 plays an important role in early development, specifically primordial cell migration in zebrafish and colonization of gonad in mouse (David et al., 2002; Ara et al.,

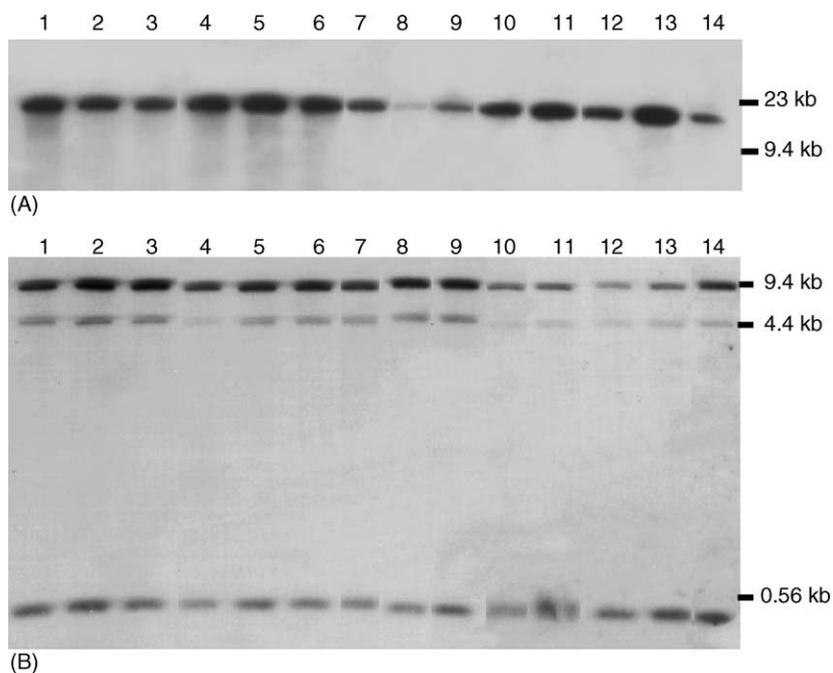


Fig. 5. Southern blot analysis of the catfish CXCL14 locus using BAC clones. BAC DNAs were digested with *EcoRI* (A) or *HindIII* (B), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized with the catfish CXCL14 cDNA probes. Lanes 1–14 contained BAC DNA from 14 different BAC clones (see Table 5). Molecular weight standard (kb) is indicated on the right margin.

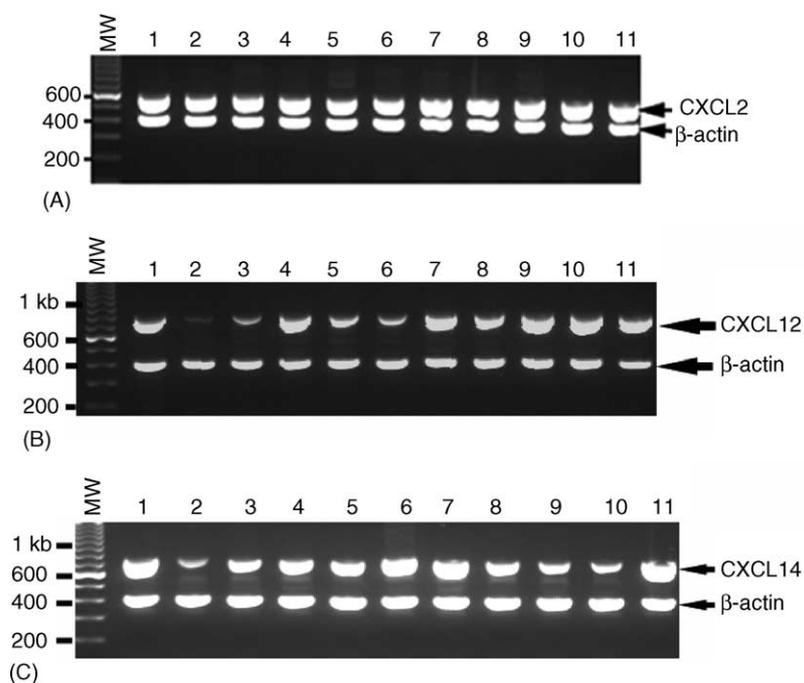


Fig. 6. RT-PCR analysis of gene expression of CXCL2-like (A), CXCL12 (B), and CXCL14 (C) in various tissues. RT-PCR reactions were conducted as detailed in the text. MW, molecular weight; 1, ovary; 2, skin; 3, muscle; 4, intestine; 5, stomach; 6, brain; 7, gill; 8, liver; 9, spleen; 10, trunk kidney; 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.

2003; Knaut et al., 2003; Molyneaux et al., 2003; Raz, 2003; Li et al., 2004). 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 h after fertilization and expressed constitutively at high levels thereafter (Fig. 7).

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 *E. ictaluri*, the causative agent of enteric septicemia of catfish. Analysis using RT-PCR indicated that the expression

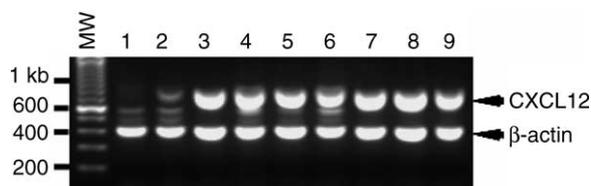


Fig. 7. RT-PCR analysis of CXCL12 gene expression during early development. RT-PCR analysis was conducted as described in Section 2. (MW) molecular weight standard; (1) 8 h after fertilization; (2) 24 h after fertilization; (3) 48 h after fertilization; (4) 4 days after fertilization (hatching); (5) 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.

of all three CXC chemokines was not affected by the bacterial infection (Fig. 8A–C). 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–C).

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 (He et al., 2004; Laing and Secombes, 2004b) indicate that more CXC chemokines may be identified after further genome sequencing and analysis.

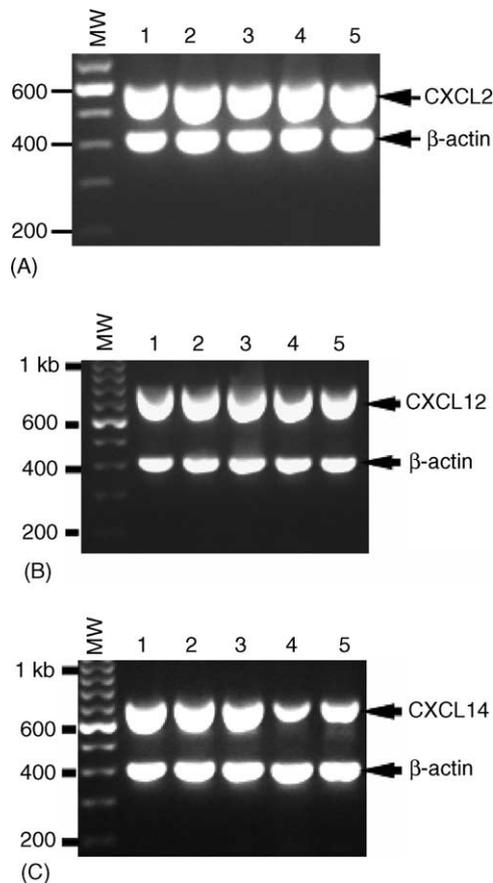


Fig. 8. RT-PCR analysis of CXC chemokine gene expression after bacterial challenge with *Edwardsiella ictaluri*. RT-PCR analysis was conducted as described in Section 2. Samples of head kidney were collected and expression of CXCL2-like (A), CXCL12 (B), and CXCL14 (C) was analyzed at various time points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

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 (Wolpe et al., 1989; Biedermann et al., 2000; Liu et al., 2000). However, several reports of constitutive expression of CXCL2 have been made (Saavedra et al., 1999; Luan et al., 2001; Matzer et al., 2001; Savard et al., 2002). Saavedra et al. (1999) 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. (2004) 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 (Huisling et al., 2004) and in zebrafish (Doitsidou et al., 2002) 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 (McGrath et al., 1999; Sleeman et al., 2000; Lazarini et al., 2003; Klein and Rubin, 2004). The former chemokine also plays important roles in reproductive organ development (Ara et al., 2003; Molyneaux et al., 2003; Raz, 2003). In addition, Kurth et al. (2001) found constitutive expression of CXCL14 in skin and intestine suggesting the chemokine functions homeostatically in macrophage development by recruiting precursors to fibroblasts. Huisling et al. (2003a) 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 *E. 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 (Baoprasertkul et al., 2004; Chen et al., 2005). 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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