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Characterization of 23 CC chemokine genes and analysis of their expression in channel catfish (*Ictalurus punctatus*)

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Abstract

Chemokines are a large family of chemotactic cytokines playing crucial roles in the innate immune response. CC chemokines constitute the largest subfamily of chemokines, with 28 CC chemokines identified from mammalian species. However, the status of CC chemokines in teleosts is yet to be determined. We previously identified 26 catfish CC chemokine cDNAs from catfish. In this study, we isolated and sequenced 23 channel catfish CC chemokine genes amounting to a total of over 56 kb of genomic sequences. Genomic organization of the 23 CC chemokine genes was determined by comparing the generated genomic sequences with the previously identified cDNA sequences. Microsatellites were identified from 16 catfish CC chemokine genes allowing them to be utilized for genome mapping. Structural analysis indicated conservation of genomic organization of CC chemokine genes, which may facilitate the establishment of orthologies. Expression of all known catfish CC chemokine transcripts was assessed in nine important tissues. Of the 26 catfish CC chemokine genes, 14 were universally expressed, six were widely expressed in many tissues, while six were highly tissue-specific.

Keywords: Chemokine; Cytokine; Innate immunity; Fish; Catfish; Genome; Gene

1. Introduction

Interest in comparisons of the innate immune mechanisms of a wide spectrum of vertebrate species has grown considerably over the last decade [1–3]. Progress in sequencing the genomes (*Takifugu rubripes*, *Danio rerio, Tetraodon nigroviridis*) and expressed sequence tags (*Oncorhynchus mykiss, Ictalurus* sp.) of several fish species has been especially important for gaining a better understanding of the evolution of the molecular components working against pathogen invasion. Fish are the earliest class of vertebrates with active components of both the innate and adaptive systems of

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immunity. Additionally, their tremendous diversity and ability to adapt to a wide range of environmental challenges makes them an ideal group for study of early innate immune components [4].

Chemokines are a large family of chemotactic cytokines in mammals and a crucial part of the innate immune response of higher vertebrates. There they play roles in immunosurveillance under homeostasis as well as stimulating the recruitment, activation and adhesion of cells to sites of infection or injury [5–7]. They are structurally related small peptides, with the majority containing four conserved cysteine residues. Based on the arrangement of these conserved cysteine residues [8], chemokines were divided into four subfamilies—CXC (α), CC (β), C, and CX3C. CC chemokines constitute the largest subfamily of chemokines with 28 CC chemokines identified from mammalian species [9]. The largest number of CC chemokines found in a single species is 24 from humans, missing orthologues to the murine CCL6, CCL9/CCL10 and CCL12. The majority of human, murine and chicken CC chemokine genes are organized in gene clusters within their genomes. The largest clusters are found on human chromosome 17, mouse chromosome 11 and chicken chromosome 19 [10,11]. CC chemokines can be loosely characterized by their genomic location, with inflammatory CC chemokines constituting the large clusters, and a few homeostatic CC chemokines distributed individually among several chromosomes. Additionally, orthologies among species are relatively high between the non-clustered CC chemokines, but low when comparing the clustered CC chemokines of several species [11,12].

With the exception of reports of a small number of genomic CC chemokine sequences from banded dogfish, Japanese flounder and rainbow trout [13–15], research on fish CC chemokines has been limited to sequencing of cDNAs. Most studies have been restricted in scope to several sequences. Only studies in trout [16] and, in our lab, catfish [12,17], have attempted to survey the complete diversity of CC chemokines within a teleost species. Catfish has emerged lately as a model species for the study of the teleost immunity, with efforts being made to understand both adaptive and innate system components [18–22]. Our identification of 26 catfish CC chemokine cDNAs provided a foundation from which to conduct genomic sequencing and comparative analysis across species. The lack of even a draft genomic sequence in catfish made this task considerably more difficult than that faced by those with an assembled genome, because a large amount of sequencing is required as a first step. In this study, we used overgo and cDNA hybridizations to a catfish BAC library to isolate and sequence catfish CC chemokine genes. Here, we report their genomic structure, sequences and characteristics in relation to known CC chemokine genes in other vertebrate species. Despite the rapid identification of CC chemokines through EST analysis in catfish [12,17], their expression patterns have never been analyzed. Here, we also report the expression patterns of the 26 genes and their associated transcripts in various tissues.

2. Materials and methods

2.1. BAC library screening and BAC isolation

High-density filters of channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA), and screened using overgo hybridization probes [23–25]. Each set of filters contained 10 × genome coverage of the channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library.php?id = 103). The catfish BAC library was screened using a two-step procedure. First, pooled overgos representing catfish CC chemokines were used to identify BAC clones likely containing CC chemokine inserts. These positive BACs were manually re-arrayed onto nylon filters and screened individually using labeled cDNA probes.

Overgo primers were designed based on the coding sequence of 25 CC chemokine cDNAs from catfish. A suitable, non-redundant overgo could not be designed for one CC chemokine, SCYA120. It was isolated later by cDNA probing (Table 1). The overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/) with modifications. Briefly, overgos were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, Texas). A total of 25 overgos were pooled together, initially. Overgos were labeled with ^{32}P-dATP and ^{32}P-dCTP (Amersham, Piscataway, NJ) in overgo labeling buffer [26] at room temperature for 1 h. After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95 °C for 10 min and added to the hybridization tubes. Hybridization was performed at 54 °C for 18 h in a hybridization

Table 1
Overgo probes for BAC hybridizations

Genes	Upper primer (5′–3′)	Lower primer (5′–3′)
SCYA101	GCGTTGCTATTTCGCTGGCAAATC	CACACAGTCTCTCTCTGATTTGCC
SCYA102	GTGCTGCTTGCACTTTTTGGATGC	CAGGTGCAGTAGTGATGCATCCAA
SCYA103	GTCCTCTGTTTTCTCCTGCTTCTG	TTGGGTACATGCATGCCAGAAGCA
SCYA104	CCTGTCTTCAGTCCTTCACAATGG	CCGTTTGCATTCTGTGCCATTGTG
SCYA105	ACAAACGTCGTGTGTGTGCAAACC	ACCCACTCATCCTTGGGGTTTGCA
SCYA106	AACAGCGGCATCTGATATTGGCAC	CACACGTCCTGTTTCTGTGCCAAT
SCYA107	AGGCTTCCACCAAAGAAATCACCG	AATCCTGTGATGGGCACGGTGATT
SCYA108	GTAAACACCAGTGTGGAAACGCTG	AGAGGAAAGACCTGAGCAGCGTTT
SCYA109	CAACCGTAATGGCAAGAGCAAAGG	GGTCTTTCACTGAGCTCCTTTGCT
SCYA110	GAAACAGCACTGTGTGGATCCAAC	GTTGACCCAAACAGCTGTTGGATC
SCYA111	GCTCATGTTGTTCCTCCTACTTCC	GGGGGAATTTTCCCATGGAAGTAG
SCYA112	CCTCCACAAATGTGTGAACACCTC	GACATAGCCACGTGAAGAGGTGTT
SCYA113	CAAAGCCTGGTGGAATCCTACTAC	TCTCTGGAGTCTGAACGTAGTAGG
SCYA114	CCATCTGGACTGTAACAGATGCAG	CAGGGGCTCACTTTTTCTGCATCT
SCYA115	TTCACTGAAGGGATGCGTTTCACG	AGACGTTTTTGGTGCCCGTGAAAC
SCYA116	CATGGCCTTTTTGGACCACAGAGG	ATTCCCTGGTGGCATGCCTCTGTT
SCYA117	TCTACTCAGACGCTCAGCCTTTTG	TCAGGATGTGCAGGAGCAAAAGGC
SCYA118	TCCTAAGCAAGTCCGTGTGACAAG	CCAGTAGCTCACAATGCTTGTCAC
SCYA119	CTGCTCTATCCACTCTTCTTCTGC	AGAGGCAGAACACCATGCAGAAGA
SCYA120	cDNA probe	cDNA probe
SCYA121	AGATGAATCGTGTGGTTTTGGTCC	ATCAGGAAGAAGCCCAGGACCAAA
SCYA122	CAGCAAGGCTTCATTGTTACGACG	GGTTAGGGAACTTAGGCGTCGTAA
SCYA123	AACGTAGTGTGTGTGCAAACCCCA	TGCACCCACTTATCCTTGGGGTTT
SCYA124	CTCGACCTAACCTCAAACGTGTGT	TGGCCAGAGGATTTAAACACACGT
SCYA125	TTGACTCAGAGAGACCTCACCTTG	ACTGAATCGCATGGCTCAAGGTGA
SCYA126	CTCGTGCTGCTTATTCGTGGAAAG	TTGGTGCGCACAATCTCTTTCCAC

solution (50 ml of 1% BSA, 1 MM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). Filters were washed and exposed to X-ray film at −80 °C for 2 days.

Positive clones were identified according to the clone distribution instructions from CHORI, and picked out from the channel catfish BAC library. Approximately 200 positive BAC clones were identified through the hybridization of overgos for the 25 catfish CCLs. These 200 BAC clones were picked, cultured in 2 × YT media overnight, and manually arrayed on Immobilon nylon membranes (Millipore, Bedford, MA). Briefly, 4 μl of each overnight culture well was spotted in duplicate on the membrane and allowed to dry. The membranes were placed in a dish containing 3 M Whatman paper saturated with 10% SDS for 3 min, transferred to a second tray containing 0.5 N NaOH, 1.5 M NaCl for 8 min with no agitation, before being transferred to another dish containing 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA and immersed for 3 min with agitation. This second wash was repeated in a new dish with fresh solution. The membrane was air-dried at room temperature, and DNA was fixed to the membrane by UV cross-linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto-crosslink function.

Probes based on catfish CC chemokine cDNAs were prepared from previously cloned plasmids. Probes were prepared using the random primer labeling method [27] with a labeling kit from Roche Diagnostics (Indianapolis, IN). The membranes were pre-hybridized in 50% formamide, 5 × SSC, 0.1% SDS (w/v), 5 × Denhardt's and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA for 2 h. Hybridization was conducted for at least 16 h at 42 °C in 50% formamide, 5 × SSC, 0.1% SDS (w/v) and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA with probes added. 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 45 °C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography. Positive BAC clones were identified for each catfish CC

chemokine and BAC DNA was isolated with the Qiagen R.E.A.L Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

2.2. Confirmation of CC chemokine inserts by PCR

In order to confirm that BAC clones contained correct CC chemokine inserts, they were validated by PCR using isolated BAC DNA. Primers (Table 2) based on the catfish CC chemokine cDNA sequences were used. PCR reactions were carried out in a total volume of 10 μl containing 1 μl 10 × PCR buffer, 0.6 μl $MgCl_2$, 0.4 μl each of the reverse transcriptase (RT)–PCR primer, 1 μl each of dNTP at 2.5 mM and 1 unit of JumpStart Taq polymerase, all purchased from Sigma (St. Louis, MO). The samples were pre-denatured at 94 °C for 5 min, and then amplified for 32 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s. Upon completion of PCR, the reaction was incubated at 72 °C for an additional 5 min. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel and photographed with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

2.3. Genomic DNA sequencing

Positive BAC clones for each catfish CCL gene are shown in Table 3, were sequenced directly using the primer-walking method [4,24]. Fourteen picomoles of each primer were used for sequencing reactions. Before sequencing, a DyeEx 2.0 Spin Kit (Qiagen) was used to purify the isolated BAC DNA. BAC sequencing was performed in a 10 μl reaction using the BigDye Terminator v3.0 Ready Reaction kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions. Sequencing reaction products were analyzed on an ABI PRISM 3130XL automated sequencer (Applied Biosystems).

2.4. Tissue collection, RNA isolation and RT–PCR

For expression studies, samples from gill, head kidney, intestine, liver, ovary, skin, spleen, stomach

Table 2
Primers for PCR and RT–PCR

Genes	Upper primer (5′–3′)	Lower primer (5′–3′)
SCYA101	TGTGTGCTGTAAGGAGGTTTCC	TTCTGTGGCACGATTGTGGTCG
SCYA102	CTGCACCTGGTAACTACCGTCG	GTTTCTTTGGGATCCAGCGTGC
SCYA103	TGCATGTACCCAAGTTTGGCAC	TTCATCAGTTCTTGCACCCAGG
SCYA104	TCTCTCCTGCTGGTTCTGCTGG	TAATTTGTCGCCGGAGTCTTGG
SCYA105	AGATACCAGACACAACCGATCC	GCTGATCAGTTGTTTGCTTGCT
SCYA106	GTCTCTTGGAGAGCAAGCACTG	CATCAGCTCTCTGACCCAGTCG
SCYA107	CAGCCAGAAGATCCGAAGCCTC	TGGAAGTGGAGCCGGTTGTCTG
SCYA108	TGCAAACGAACCAGAACCATGC	TCGGTTGAGGTTGGATCACGTC
SCYA109	ACCAGCGACACTTTCGTTCCAC	GCTCTTGCCATTACGGTTGTCC
SCYA110	ATGAGGAACCTGACGGCTCTGC	AGCTGTTGGATCCACACAGTGC
SCYA111	AGACGCTACCTATCAAGCGCTC	CAGTTGCGTGAAAGCTGCAGTG
SCYA112	TCGCTGGATGCTGGCTTCTGTG	TGACCTTGTTATGAGGTTGCTG
SCYA113	TCCACAAAGCCTGGTGGAATCC	AGTTGTTCTTTGTCGCACGAGG
SCYA114	ATGAGGAGCCTGGCTGCCATAG	GATGCAGGGAGGCAGTGGTTGG
SCYA115	TGGTGCTGCTGAGTGCAGTCAC	ACCCAGGCGTCAGTGGGTTTGG
SCYA116	ACTCCACTTCTCAGCTGCCCTG	CAAGGTGAGGACGGGTCCAAGC
SCYA117	TCCTGCACATCCTGAGGATTGC	TCTCAGTAGCCGGGACTTCACG
SCYA118	CACCACTGCAGTGTTCTCCAGC	TCTCCTTTGGAGCATCTGGTGC
SCYA119	TGGTGTTCTGCCTCTGTGCCAG	TGTTCTGTGGAATGGTCACCTC
SCYA120	CTGCTGGTTCTGCTGGGTCTCG	TGCGGTCTGCACACGCCTTACG
SCYA121	TCTGCATCCATCTGCTGAGAAC	GTGCGTACGTGTTGCGTCTCAG
SCYA122	TGAGCTTCACACACCTGCTGAG	AGCCTTGCTGTTCACACTGTGC
SCYA123	TCCTTCACAGCGGCTCAGAGTG	TGGGGTTTGCACACACACTACG
SCYA124	GCCTTCAGTCCTTCACAACAGC	TGACATCAGGGTCTGCACACAC
SCYA125	CTTCAGCCTGGCACAAGGTTCG	CTAGCGCAAATGAGCCGACCTC
SCYA126	TTCTACAGCGCCACTGAGTCGA	AGTTAGGTCTCAGAAACGTTGC
Actin	AGAGAGAAATTGTCCGTGACATC	CTCCGATCCAGACAGAGTATTTG

Table 3
Primers used for sequencing the BAC clones containing the catfish CC chemokines

Gene	BAC	Primer sequence (5′–3′)
SCYA101	025_A20 003_N13	GGAAACGACTGCAACATGC; CTAGGATAATAAGCAGGCCATGC TACAACGACTCCGTGTTACAACCG; TAAGGGAGACTGGGGACGGTTGTA AATCCTGTTATGGGCACGTTG
SCYA102	125_O17 069_N02	CTGCTTGCACTTTTTGGATGC; CCGTCTTCCTGTTGCTTGC CGTGTTGAGAGGATAGACAGTACA; GTGCTGCTTGCACTTTTTGGATGC GATGCATAGTAGTGTGCTTCACC
SCYA103	039_K13	GGATGTTGCATCATCCATCTGTC; GTCCTCTGTTTTCTCCTGCTTCTG AGCGATTGAGCAACATTCTCTATTC
SCYA104	147_M12	GTGCAGCTACAGGCGTATG; TCTCTCCTGCTGGTTCTGCTGG
SCYA106	029_L5	TGTTCTTCCAAGCAGTCTAGGG; CATCAGCTCTCTGACCCAGTCG GTACTTTCAGCTTCCGAA; CTGCATGGCAAACCTAGACTG CAGTCTAGGTTTGCCATGCAG
SCYA107	006_I13	AGCCAGAAGATCCGAAGCC; CAGTAGCAAGTGCGTCAGAC AATCCTGTGATGGGCACGGTGATT; TGGAAGTGGAGCCGGTTGTCTG CTTGTGTATGTGGGTTATGGGTTC
SCYA108	149_I8 052_C23	CACCAGTGTGGAAACGCTG; TGCTAGACTGCTTCAGTCAAGAG GTACACTGGTTTGTAACATGACTGG; ATCTGCAGGGGAAAAATCATTACTG GTGTAAGTTTTCAACCCCTTGTTTC
SCYA109	143_I8	GCTGTCACAGAAAATCCAGGC; ACCAGCGACACTTTCGTTCCAC GAAGAAAATGGAAAGACCCCTTGAG; CAACCGTAATGGCAAGAGCAAAGG
SCYA110	105_B8	GACATTCTCACCTCTGAGTCTCA; GTAAACAAAAACGAGTGCTCGC GAAGCAGCACCACTGTACC; GTCCGGAGTGAGAGTTCGAG CTGCCCCATAAAGGCCATTG
SCYA111	041_O13 45_L17	GACAGACCCACGTTGTGC; GAGTATTGGAACAGCTAGGCG CAGCAGCTGATGACAGAACC; TCTTTAGGACGGAGGAGTTTACG AGACGCTACCTATCAAGCGCTC; GGGGGAATTTTCCCATGGAAGTAG
SCYA112	006_I13	AACTCTGCTCCGCGAAAC; TTCCTGACAAAGCGTAACCAG TGTAAAGCGACAATGCGTCTG; GACATAGCCACGTGAAGAGGTGTT
SCYA113	037_D15	CAGTGGTTTCAGCGTCTCAG; GGATTACAAGAGAGCAGTCACG
	052_F9	AGCACATAACAGATTGCCACTG; CAAAGCCTGGTGGAATCCTACTAC TCTCTGGAGTCTGAACGTAGTAGG; AGTTGTTCTTTGTCGCACGAGG
SCYA114	069_N02	GGCTCTGACAATAGTGCAGATG; GCGGCTCTGACAATAGTGC CTGCTGGTTCTGCTGGGTCTCG
SCYA115	179_H22	CAAGTACTCATCTTCTTTAATCTGTCCAT; AAATCTGCTCGAAACCCATCATAG GGACATCAAGTCAGGAAACCAG; ACAGGGGTAGCAGTGGTG TTCACTGAAGGGATGCGTTTCACG; CAATATCGCTGTGGTATGTAGCTG
SCYA116	090_M4	CTCACAGCATTCACGGGAC; CACTGGAAAGGCTTCAGCAC
	003_N13	ACTTGTGATGCCAAAGTACGG; CATGGCCTTTTTGGACCACAGAGG
SCYA117	059_H18	TATCGGTTCATTATAAGCCAAATCGAG; GAGACCTGCTGAGAACATGAAG GAATAGACACTGCATTAAGCAACAG; GCAATCCTCAGGATGTGCAG
SCYA118	003_N13	GAACACTGCAGTGGTGCAG; CTTTCACAATACACATTTAGCCATACAAG GTGGCCTAGATCTGTAAGGTCAG; CCAGTAGCTCACAATGCTTGTCAC
SCYA119	069_N2	CTGCTCTATCCACTCTTCTTCTGC;TGGTGTTCTGCCTCTGTGCCAG TTGTAGGTCTGGTTGGCCTAG
SCYA120	122_C9	GGCTCTGACAATAGTGCAGATG;CTGCTGGTTCTGCTGGGTCTCG GCGGCTCTGACAATAGTGC

Table 3 (*continued*)

Gene	BAC	Primer sequence (5′–3′)
SCYA121	153_I24	ATCAATAACATCCACGACCATCATC;CGATCTGCATCCATCTGCTG GCTATCCAAACATCTGCTGGAG;AGTGTTACTACTCATGAGTTCCTCAC
SCYA122	102_J7	TAGCGTCTGAGTAGAGCGC;CAGTGTTACGACGCCTAAGTTC
	45_L17	TAGCGTCTGAGTAGAGCGC;AGGTCTGCATTTCTTTCCCATC GTATTAAACTATAGTCTGCACCTATAGAC
SCYA124	152_F2	GTTGGTTAAAACTGGATATACAAGTATAC;GAAACAGCTCGTTCACAGGG
SCYA126	047_K12	CAGGGCTGTTTTGCTGGAC;GTCGTTGTGAGCATTCTGGAG CTCCAGAATGCTCACAACGAC

and trunk kidney were collected from ten fish. Samples of each tissue from ten fish were pooled. The pooled tissues were rapidly frozen with liquid nitrogen. In order to obtain samples representing the average of the ten fish, the pooled tissue samples were ground with a mortar/pestle to fine powders and were thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation [28]. RNA was isolated following the guanidium thiocyanate method [29] 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 RT–PCR. The RT–PCR reaction was conducted using a two-step approach with M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA) with the primers listed in Table 2. Detailed procedures followed the manufacturer's instructions. Briefly, 1 μg of total RNA was used in each first-strand reaction. PCR reactions were carried out as described above with two modifications. The primers of β-actin (Table 2) were added to serve as an internal control. The RT reaction product was used for PCR for 32 cycles. RT–PCR reactions were conducted for one gene at a time, and the images of agarose gels were compiled together into a single figure by using Adobe Photoshop and, therefore, expression levels should not, and cannot be compared from gene to gene, but only among tissues of the same gene.

2.5. Sequence and phylogenetic analyses

Genomic sequences were analyzed using a number of different software programs. In order to relate the genomic sequences to existing cDNA sequences in catfish, BLAST searches (Blastn) were conducted using each sequence as a query against all *Ictalurus* sp. sequences in the GenBank database. Highly related cDNAs were aligned with the genomic sequence using NCBI's Spidey program to predict the number of exons and introns, intron sizes and the genomic open-reading frame (ORF). The coding sequences were translated using a web-based translation tool (http://arbl.cvmbs.colostate.edu/molkit/translate/) into their deduced amino acid sequences. Multiple alignments were carried out using ClustalW [30]. For the identification of microsatellites, genomic sequences were scanned for microsattelite repeats for future marker development using the FastPCR program (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm).

Phylogenetic trees were drawn from ClustalW generated multiple sequence alignments of amino acid sequences using the neighbor-joining method [31] within the molecular evolutionary genetics analysis (MEGA 3.0) package [32]. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor joining trees was evaluated by 1000 bootstrapping replications.

3. Results

3.1. Identification of the channel catfish CC chemokine genes

Despite the recent identification of a large number of CC chemokine cDNAs from channel catfish through the analysis of expressed sequence tags (ESTs), their encoding genes have never been identified. In order to identify their encoding genes, overgo probes were designed based on the cDNA sequences and hybridized to high-density BAC

filters. Due to the large number of CC chemokine genes and their corresponding BACs, we first identified and confirmed at least one BAC for sequencing analysis. The CC chemokine-positive clones used for direct sequencing are shown in Table 3.

3.2. Sequencing of 23 catfish CC chemokine genes

Positive BAC clones for each catfish CCL cDNA, as confirmed by hybridizations using the cDNA probes, were sequenced directly using the primer-walking method. Positive BACs were obtained for all 26 CC chemokine cDNAs previously identified [12,17], but three, SCYA105, SCYA123 and SCYA125, could not be sequenced from the positive BACs due to technical difficulties. The remaining 23 CC chemokines were successfully sequenced from the coding exons into both the upstream and downstream regions. Their sequences have been submitted to GenBank with accession numbers listed in Table 4. The characteristics including the total sequenced length, number of exons, their ORFs, number of deduced amino acids and length of the upstream region sequenced are summarized in Table 4. Most of the channel catfish CC chemokine genes are highly compact, with the majority of introns smaller than 500 base pairs (bp). Typical intron splice motifs were observed at the 5′ (GT) and 3′ (AG) ends of each intron. The number of exons varied among the catfish CC chemokines. Approximately half (11 out of 23) of the CC chemokine genes have three exons and two introns. Ten catfish CC chemokines have a molecular organization containing four exons and three introns. Catfish SCYA112 contained five exons and four introns, a pattern found only in human CCL25. SCYA126 contains an interesting pattern of two exons and one intron (Table 4).

Efforts were made to sequence the upstream regions of the catfish CC chemokine genes. In a few cases, it was not possible to obtain additional

Table 4
Characteristics of the genomic sequences of 23 CC chemokines from channel catfish (*Ictalurus punctatus*)

Genes	Accession numbers	Total length sequenced (bp)	ORF (bp)	Number of amino acid	Upstream sequenced (bp)	Exons	Orthology
SCYA101	DQ173276	2491	345	114	790	4	
SCYA102	DQ173277	3495	303	100	919	4	
SCYA103	DQ173278	2524	261	86	1165	4	CCL21/25
SCYA104	DQ173279	1040	288	95	10	3	CCL22
SCYA106	DQ173280	2734	333	110	869	4	CCL19
SCYA107	DQ173281	2558	291	96	834	4	
SCYA108	DQ173282	2093	306	101	791	3	
SCYA109	DQ173283	1995	375	124	1108	3	CCL19
SCYA110	DQ173284	2143	300	99	1310	3	
SCYA111	DQ173285	2804	237	78	1221	3	
SCYA112	DQ173286	2636	324	107	612	5	CCL20
SCYA113	DQ173287	2340	324	107	327	4	CCL19
SCYA114	DQ173288	3053	336	111	287	4	
SCYA115	DQ173289	3525	300	99	845	3	
SCYA116	DQ173290	1885	324	107	822	4	
SCYA117	DQ173291	1905	291	96	280	4	
SCYA118	DQ173292	3229	567	188	968	3	
SCYA119	DQ173293	1922	357	118	106	4	
SCYA120	DQ173294	1388	276	91	84	3	
SCYA121	DQ173295	2362	246	81	938	3	
SCYA122	DQ173296	2795	243	80	830	3	
SCYA124	DQ173297	2751	288	95	1150	3	
SCYA126	DQ173298	2814	279	92	1324	2	CCL27/28
Total		56,482 bp			17,590		

Open reading frames were determined by alignment of the genomic sequences with existing cDNA sequences. Exons and introns were determined using the Spidey program. Genomic sequences with their associated coding sequences are available at the GenBank accession numbers provided. Orthologies were determined previously by phylogenetic analysis [12].

upstream sequences, possibly because of duplication of sequences within the target BAC clone. All sequenced catfish CC chemokines contained the pattern of four conserved cysteine residues with two adjacent cysteines at the N-terminus characteristic of CC chemokines.

3.3. Identification of microsatellite repeats within the catfish CC chemokine genes

Microsatellites are useful for genome mapping because of their high levels of sequence polymorphism [33]. Identification of microsatellites associated with genes provides the potential for mapping them onto chromosomes. Informatic mining of the channel catfish CC chemokine gene sequences revealed that the majority of catfish CC chemokine genes sequenced contained one or more microsatellite repeats (Table 5). These will be of importance in placing these chemotactic cytokines onto the catfish linkage map. Since their BAC locations are now known, these genes can also be easily localized to a BAC-based physical map for catfish which is currently under construction. Mapping to both the physical and linkage map will allow integration of the two maps.

3.4. Tissue expression analysis of catfish CC chemokine genes

Large numbers of catfish chemokine genes have been identified, but tissue expression analysis has not been conducted. In order to assess the patterns of expression of the catfish CC chemokine genes, RT–PCR was used to study the expression of mRNA of the catfish CC chemokines in various tissues (Fig. 1). The expression of the CC chemokine transcripts fell into three broad categories, universally expressed, widely expressed and tissue-specific.

Of the catfish CC chemokines, 14 exhibited a pattern of universal expression in all tissues tested. These included SCYA101, SCYA102, SCYA103, SCYA106, SCYA108, SCYA110, SCYA111, SCYA113, SCYA114, SCYA115, SCYA120, SCYA123, SCYA124 and SCYA125. Of these, several chemokines had relatively low expression in ovarian tissue including SCYA102, SCYA111, SCYA113, SCYA114 and SCYA125.

Six catfish CC chemokines were widely expressed with mRNA being detected in the majority of tissues, although not necessarily at the same levels across the tissues, but were not expressed in at least one tissue tested (Fig. 1). Two of these six chemokines, SCYA112 and SCYA116, were not expressed in the liver; three of the six chemokines, SCYA104, SCYA119 and SCYA122, were not

Table 5
Identification of microsatellites associated with the catfish CC chemokine genes

Genes	Repeat motif	Repeat location*	Structural location
SCYA102	$(taa)_{18}$	1789	3rd Intron
	$(ac)_{14}$	3377	Downstream
	$(ctt)_{11}$	3444	Downstream
SCYA106	$(gt)_{18}$	966	1st Intron
SCYA108	$(aaat)_{6}$	538	Upstream
	$(at)_{15}$	1387	2nd Intron
SCYA110	$(ac)_{24}$	810	Upstream
	$(ata)_{19}$	1484	1st Intron
SCYA111	$(ta)_{35}$	1285	1st Intron
SCYA112	$(ta)_{32}$	27	Upstream
	$(att)_{20}$	1025	1st Intron
SCYA113	$(ca)_{10}$	302	Upstream
	$(ttc)_{12}$	486	1st Intron
SCYA114	$(ca)_{8}$	2348	Downstream
SCYA115	$(ca)_{9}$	149	Upstream
	$(ga)_{11}$	1770	2nd Intron
	$(ga)_{11}$	1798	2nd Intron
	$(gt)_{17}$	2159	2nd Intron
SCYA116	$(at)_{40}$	1699	3′ UTR
	$(at)_{21}$	1781	3rd Intron
SCYA117	$(ac)_{19}$	226	5′ UTR
	$(ca)_{10}$	428	2nd Intron
SCYA118	$(ata)_{7}$	2154	2nd Intron
	$(taa)_{7}$	2177	2nd Intron
	$(tta)_{7}$	2275	2nd Intron
	$(ttg)_{7}$	2344	2nd Intron
SCYA120	$(cat)_{14}$	29	3′ UTR
SCYA122	$(att)_{16}$	1317	1st Intron
SCYA124	$(tca)_{9}$	1118	3′ UTR
	$(at)_{40}$	1291	1st Intron
SCYA126	$(ata)_{9}$	2398	Downstream
	$(gt)_{18}$	2769	Downstream

Multiple microsatellites within a single sequence are separated by "---". Repeat location refers to the first base within the nucleotide sequence with the accession numbers as listed in Table 4. Upstream and downstream refer to the microsatellite being located on the genomic sequence 5′ or 3′, respectively, of available cDNA sequences from catfish.

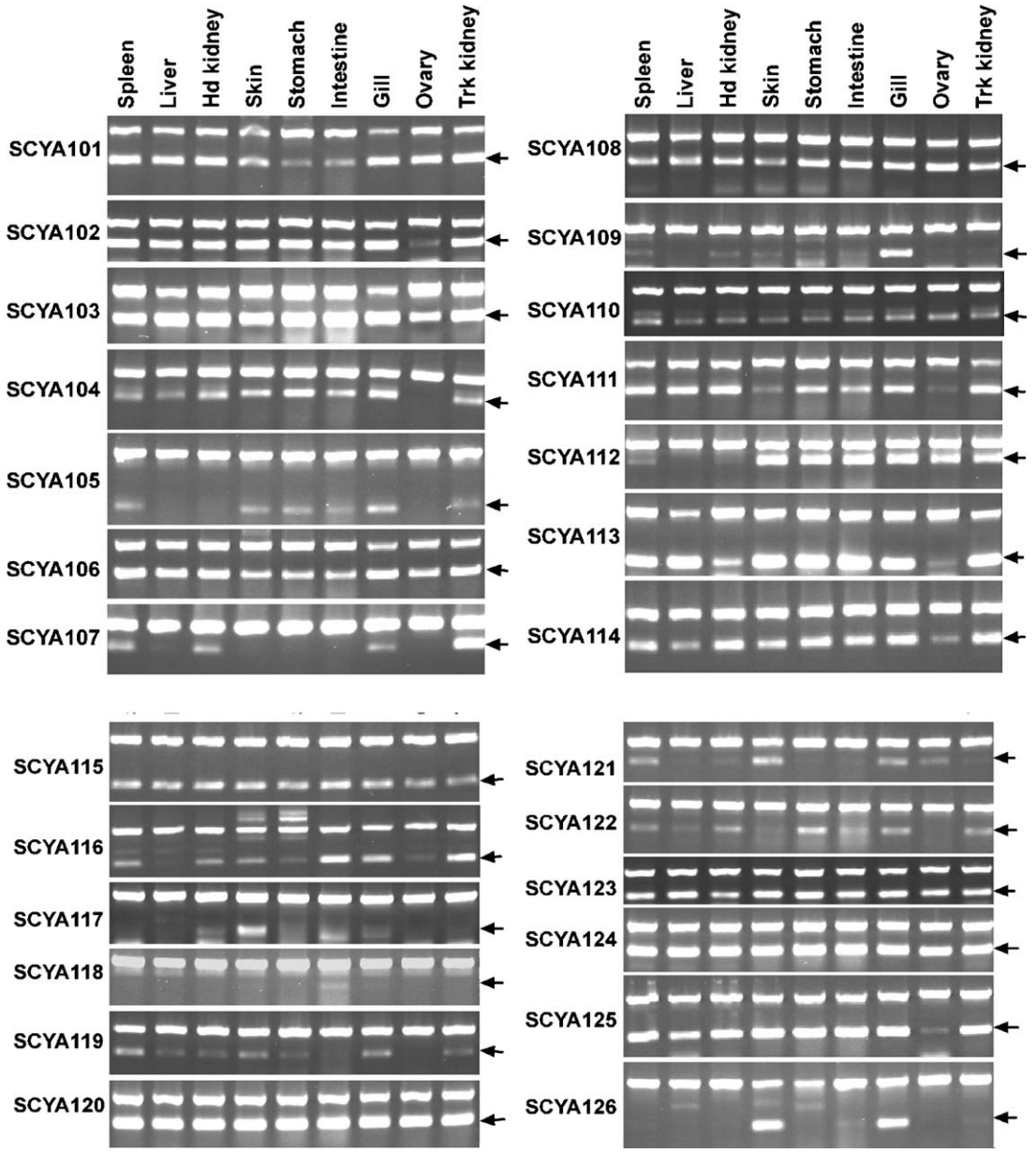
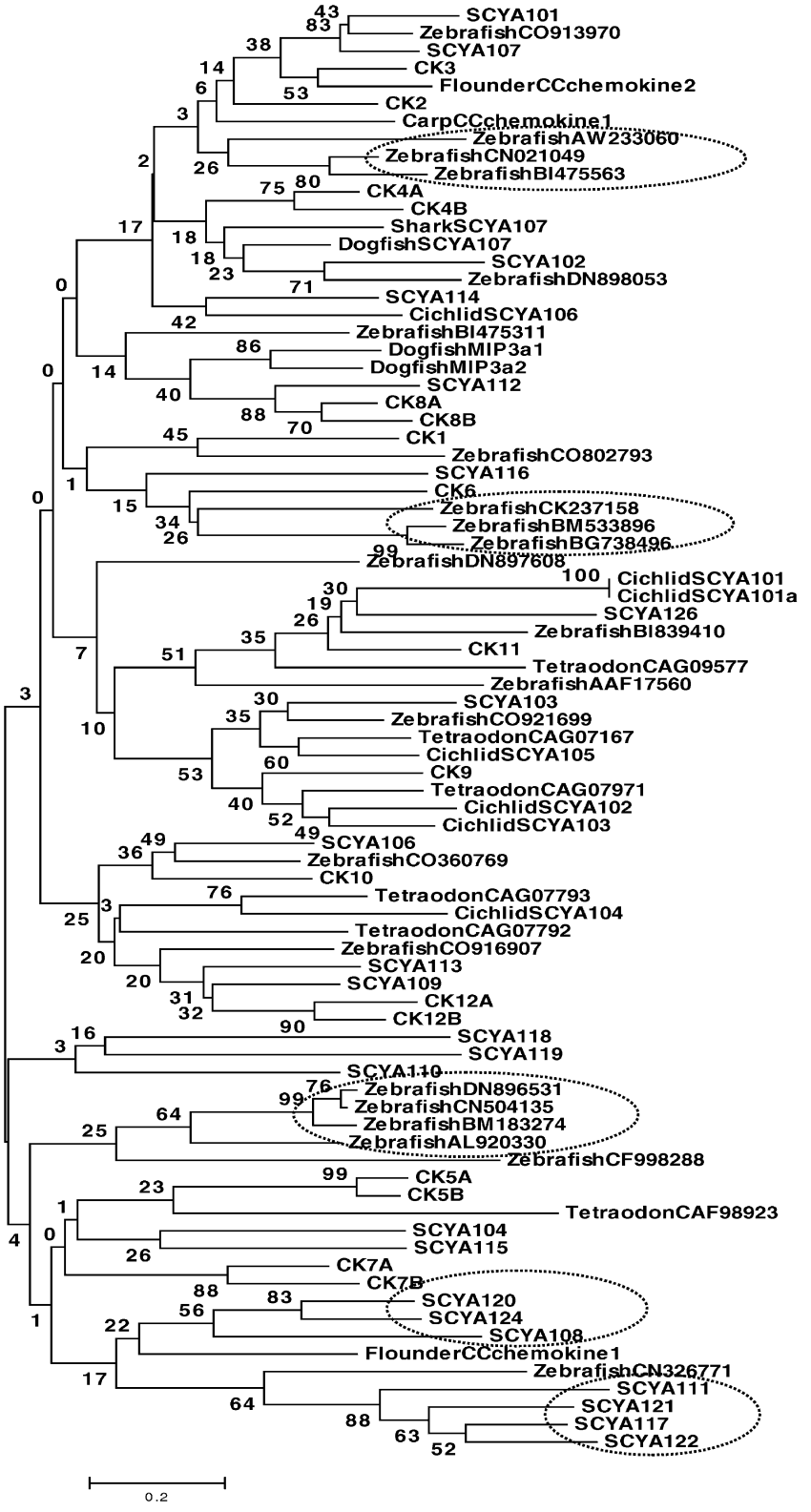


Fig. 1. Expression analysis of the 26 catfish CC chemokines using RT–PCR. RT–PCR reactions were conducted as described in the Section Materials and methods, and their products were analyzed by agarose gel electrophoresis. Nine tissues were used in the study, as specified on the top of the figure, with abbreviations for head kidney (hd kidney) and trunk kidney (trk kidney). The names of the catfish CC chemokines were indicated on the left margins of each panel of the gels. Arrows indicate the expected positions of the catfish CC chemokine RT–PCR products. In each RT–PCR reaction, beta-actin gene was used as an internal control. In all cases, the upper band was from beta-actin.

expressed in ovarian tissue, and SCYA105 was not expressed in the liver or in the ovarian tissue.

Six of the catfish CC chemokines were expressed in a tissue-specific manner, as shown in Fig. 1. SCYA118 mRNA was only detected in the intestine. SCYA109 was expressed strongly in the gill, not detected in the liver and present weakly in the remaining tissues tested. SCYA107 mRNA was detected strongly in spleen, head kidney, gill and trunk kidney, and weakly in liver, but its expression


SCYA101
ZebrafishCO913970
SCYA107
CK3
FlounderCCchemokine2
CK2
CarpCCchemokine1
ZebrafishAW233060
ZebrafishCN021049
ZebrafishBI475563
CK4A
CK4B
SharkSCYA107
DogfishSCYA107
SCYA102
ZebrafishDN898053
SCYA114
CichlidSCYA106
ZebrafishBI475311
DogfishMIP3a1
DogfishMIP3a2
SCYA112
CK8A
CK8B
CK1
ZebrafishCO802793
SCYA116
CK6
ZebrafishCK237158
ZebrafishBM533896
ZebrafishBG738496
ZebrafishDN897608
CichlidSCYA101
CichlidSCYA101a
SCYA126
ZebrafishBI839410
CK11
TetraodonCAG09577
ZebrafishAAF17560
SCYA103
ZebrafishCO921699
TetraodonCAG07167
CichlidSCYA105
CK9
TetraodonCAG07971
CichlidSCYA102
CichlidSCYA103
SCYA106
ZebrafishCO360769
CK10
TetraodonCAG07793
CichlidSCYA104
TetraodonCAG07792
ZebrafishCO916907
SCYA113
SCYA109
CK12A
CK12B
SCYA118
SCYA119
SCYA110
ZebrafishDN896531
ZebrafishCN504135
ZebrafishBM183274
ZebrafishAL920330
ZebrafishCF998288
CK5A
CK5B
TetraodonCAF98923
SCYA104
SCYA115
CK7A
CK7B
SCYA120
SCYA124
SCYA108
FlounderCCchemokine1
ZebrafishCN326771
SCYA111
SCYA121
SCYA117
SCYA122
0.2

was not detected in other tissues. SCYA121 mRNA was detected strongly in spleen, skin and gill, moderately in ovary and weakly in the remaining tissues. SCYA117 mRNA was expressed strongly in the skin, weakly in head kidney, gill and intestine, but was not expressed in other tissues. SCYA126 mRNA was expressed strongly only in the skin and gill and weakly in the intestine.

4. Discussion

In the present study, we have sequenced 23 channel catfish CC chemokine genes. This represents the first and largest set of fish chemokine genes that has been characterized at the genomic level. Over 56,000 bp of genomic sequences were sequenced. Genomic organization of the 23 CC chemokine genes was determined by comparing the generated genomic sequences with previously identified cDNA sequences. Microsatellites were identified from 16 catfish CC chemokine genes allowing them to be utilized for genome mapping. Expression of all known catfish CC chemokine transcripts was assessed in nine important tissues.

We previously conducted extensive phylogenetic analysis of known vertebrate CC chemokines and concluded that orthologies could not be established between many of the teleost CC chemokines and the mammalian CC repertoire [12]. As shown in Table 4, only seven channel catfish CC chemokines had identifiable orthologies with the mammalian CC chemokines. Similar observations were made in analysis of trout and chicken CC chemokines [11,16]. The lack of orthologies among vertebrate CC chemokines suggests the involvement of several evolutionary mechanisms. First, there may have been extensive fish-specific gene duplications of chemokine genes. The identification of 26 CC chemokines from catfish, as compared to 24 from humans, from a limited resource of ESTs support this notion, and indicates that a larger number of catfish CC chemokines genes may exist. Second, phylogenetic analysis of the current research suggests extensive gene duplications within fish species, with sequences of a single species clustering together exclusive of the sequences of even closely related species (Fig. 2). Third, the evolutionary pace of chemokine genes may have been significantly faster in teleosts than in mammals. Our previous analysis [12] indicated that sequence similarities among the CC chemokines of mammalian species were greater than among the CC chemokines of teleost fish species. The highly divergent sequences would not support a reliable phylogenetic analysis for the establishment of orthologies.

Fish biologists should seek alternative strategies in the analysis of chemokine identities in teleosts. While establishing orthologies between many fish and mammalian chemokines may not be possible, phylogenetic analysis of fish CC chemokines may provide important information. Given the large numbers of CC chemokine genes likely to be identified from teleost species in the near future and given the tremendous diversity of fish species, phylogenetics may eventually provide us a detailed history of chemokine evolution in lower vertebrates. Functional analysis, conducted in several important fish species, will allow additional inferences as to the roles of this diverse family of molecules in teleost immune responses.

Here, we analyzed a large set of teleost CC chemokines, particularly those from channel catfish, rainbow trout, zebrafish and *Tetraodon nigroviridis*. As shown in Fig. 2, there appeared to be many clades with reasonable bootstrapping support. As more and more fish chemokine sequences become available, more meaningful and comprehensive

Fig. 2. Phylogenetic tree drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA (3.0) package. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. GenBank accession numbers are included in the names on the tree for unpublished fish CC chemokines. SCYA101-SCYA126 refer to the catfish CC chemokines with GenBank accession numbers listed in Table 4. The accession numbers for the trout (CK-) CC chemokines are AF093802(CK1), AF418561(CK2), AJ315149(CK3), CA371157(CK4A), CA352593(CK4B), CA383670(CK5A), CA374135(CK5B), CA355962(CK6), CA355962(CK7A) and CA346976(CK7B), CB494647(CK8A), CA353159(CK8B), CA378686(CK9), CA361535(CK10), BX072681(CK11), CA358073(CK12A) and CA346383(CK12B). Other fish CC chemokines included carpCCchemokine1(AB010469), flounderCCchemokine1 (AU090535) and flounderCCchemokine 2(AB117523), cichlidSCYA101(AY178962), cichlidSCYA101a(AY178964), cichlidSCYA102(AY178963), cichlidSCYA103(AY178965), cichlidSCYA104(AY178966), cichlidSCYA105(AY178967), cichlidSCYA106(AY178968), sharkSCYA107(AY178970), dogfishSCYA107(AB174767), dogfishMIP3a1(AB174768) and dogfishMIP3a2(AB174766). Circles indicate highly related sequences from the same species.

phylogenetic analysis may become feasible. Phylogenetic analysis suggested the possibility of rapid gene multiplication within species of fish, as highly clustered CC chemokines within each fish species fell into clades. This was especially true for zebrafish and channel catfish where a larger number of CC chemokines are available for analysis. In rainbow trout, gene duplication was notable, with pairs of its CC chemokines being placed together in strongly supported clades [16].

Analysis of genomic structure and organization may provide additional tools for comparing the CC chemokine families of vertebrate species. One of the objectives of this work was to reveal the genome structures and organizations of the catfish chemokine encoding genes, allowing comparisons to other fish chemokine genes as they become available. An interesting finding of this work was the varied exon structures of the catfish CC chemokines. Of the 24 human CC chemokines, roughly half have an organization of three exons and two introns. This was also the case in channel catfish. Catfish appear to possess ten CC chemokines with an organization of four exons and three introns.

An analysis of exon structure and size in the catfish CC chemokines with orthologies to the human set revealed only partial conservation. SCYA106, SCYA109 and SCYA113 are all related to human CCL19, which is encoded by four exons. However, while SCYA106 and SCYA113 also have four exons, SCYA109 is encoded by only three. SCYA103 and related human CC chemokine, CCL21, both possess a four exon structure, while SCYA104 and its orthologue CCL22 are both encoded by three exons. SCYA112, an orthologue of human CCL20 is encoded by five exons rather than the four found in humans. Likewise, SCYA126 differs from its orthologues in that it is encoded by only two exons.

Although CC chemokine sequences and genomic structures are not known from many fish species, limited analysis suggested that genomic organization may have been more conserved among related fish CC chemokines. For instance, channel catfish SCYA101 and SCYA107 are both highly related to a zebrafish CC chemokine (accession number CO913970), and all three chemokines had four exons. Similarly, channel catfish SCYA102 is related to the dogfish CC chemokine SCYA107 [13] and a zebrafish chemokine (accession number DN898053), and all three fish chemokines had four exons.

Analysis of the genomic structures of related CC chemokines from multiple fish species may reveal structural patterns specific to the fish lineage or conserved across the evolutionary spectrum. Unfortunately, at this time, there are extremely few cases where the genomic sequences of orthologous CC chemokines from multiple fish species are available for comparison with the human set. This dearth of information also makes it difficult to determine whether exon sizes are conserved between orthologous chemokines. Many CC chemokine genes within both the catfish and human sets are encoded by exons of similar sizes, and it is difficult, therefore, to judge whether sizes are the result of evolutionarily conservation between orthologues of the two species. Three CC chemokine genomic sequences are available from dogfish [13], and a comparison of these sequences with their catfish orthologues is illustrative of the points made above. Two orthologues of human CCL20 and SCYA112 were sequenced from dogfish, *Trsc*-MIP-3α1 and *Trsc*-MIP-3α2. A comparison across the three species reveals that, while catfish SCYA112 encodes an additional small exon (Exon 2) not seen in the other species, all the sequences share two highly conserved exons. Catfish exons three (118 bp) and four (78 bp) equate to exons two and three in *Trsc*-MIP-3α1 (112 bp, 78 bp), *Trsc*-MIP-3α2 (103 bp, 78 bp), and Human CCL20 (115 bp, 78 bp). Here, the availability of related sequences from additional fish species allowed the detection of patterns of structural conservation that were otherwise not evident. The third dogfish CC chemokine, *Trsc*-SCYA107, as mentioned above, is related to catfish SCYA102, but neither gene has a clear orthologue among the human CC chemokines. The sizes of the second and third exons may be conserved between dogfish (121 bp, 78 bp) and catfish (127 bp, 68 bp), but additional orthologous sequences are needed from other species before an accurate judgment can be made. As more genomic structures become available from other fish species, it may become possible to use genomic organization as a complementary approach to phylogenetic analysis for the establishment of orthologies.

SCYA126 has only one intron, unlike all other reported CC chemokines. EST analysis suggested the presence of both the spliced and unspliced transcripts (BM027974 and BM029630) in the skin cDNA library [34]. We previously reported the incomplete cDNA sequence for SCYA126 missing the first 25 amino acids. Through genomic sequence

analysis, the complete cDNA sequence is now available. The unspliced transcript (BM029630) is complete at the 5' but retains the 89 bp intron found in the genomic sequence. This unspliced transcript was likely detected during RT–PCR analysis of SCYA126 (see Fig. 1).

RT–PCR analysis indicated that the majority of the chemokine genes were expressed in all tested tissues, suggesting possible homeostatic functions in immunosurveillance more than the better known functions of chemotactic attraction of leukocytes in immune-related tissues. However, six catfish CC chemokines appeared to exhibit tissue-specific expression. In particular, SCYA118 was detected only from the intestine, and SCYA109 was strongly expressed only in the gill, while SCYA107, SCYA121, SCYA117 and SCYA126 were expressed highly in immune-related tissues only, suggesting their specific roles in inflammatory responses. Functional analysis of these chemokines is required to confirm these speculations. In our previous report [12], expression of SCYA118 and SCYA126 was not detected in the infected tissues of head kidney or spleen. Through tissue expression studies here, it is clear that these CC chemokines were highly tissue specific, and they were only expressed in intestine, and skin and gill, respectively.

Making sense of the divergence, duplication and diversity of CC chemokine gene families in fish is by no means a straightforward task. We are still attempting to assemble a puzzle that is missing many of its pieces. However, the catfish CC chemokine gene family described here should aid in our eventual understanding of the origins of vertebrate innate immunity, and the evolutionary mechanisms that produced the CC chemokine repertoire present in mammalian species. Specifically, the genomic sequences should allow cross-species comparisons for the identification of conserved gene sequences, particularly the regulatory elements involved in infection or LPS-induced gene expression.

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