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In silico identification and expression analysis of 12 novel CC chemokines in catfish

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Abstract Chemokines, a superfamily of chemotactic cytokines involved in recruitment, activation, and adhesion of a variety of leukocyte types to inflammatory foci, are a crucial component of the immune system of Sarcopterygiian vertebrates. Although all mammalian chemokines are believed to have been found, the status of these molecules in Actinopterygii was unknown until recently. The identification of chemokines in fish species has been complicated by low sequence conservation and confusion over expected numbers. Earlier discoveries of single fish chemokines coupled with rapidly expanding genetic resources in these species have recently provided a foundation for large-scale in silico discoveries of these important immune regulators. We report here the identification and expression analysis of 12 new CC chemokine sequences from catfish. When added to our previous report of 14 catfish CC chemokines, the number of CC chemokines in catfish now stands at 26, two more than known from humans. Establishing orthologous relationships among the majority of catfish CC chemokines, a newly available set of chicken CC chemokines, and their mammalian counterparts remain difficult, suggesting high levels of duplication and divergence within individual species.

Keywords Chemokine · Cytokine · Innate immunity · Fish · Catfish

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Introduction

With the advent of the genomics era, great efforts have been made in mammalian species to identify and characterize the primary gene components of innate immunity including chemokines, cytokines, and complement factors. In the last 5 years, the resources developed from the mammalian species have expedited similar efforts in the fish research community.

Fish have an intermediate phylogenetic position between species possessing only innate defenses (i.e., *Drosophila*) and species depending heavily on adaptive immunity (i.e., human, mouse). Much can be learned, therefore, about the origin, function, and regulation of immunity through immunological studies in fish. Furthermore, the tremendous diversity of fish, the largest group of extant vertebrates with more than 23,000 species, allows the study of immune adaptations over broad temporal, spatial, and life history differences.

Chemokines, chemotactic cytokines, are crucial mediators of inflammation and important components of innate immunity. They comprise a superfamily of molecules involved in stimulating the recruitment, activation, and adhesion of cells to sites of infection or injury (Neville et al. 1997; see Laing and Secombes 2004a for review). They are structurally related small peptides, with the majority containing four conserved cysteine residues. Based on the arrangement of these conserved cysteine residues (Murphy et al. 2000), 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).

CC chemokines constitute the largest subfamily of chemokines with 28 CC chemokines identified from mammalian species (Bacon et al. 2003). They mediate chemotaxis of mononuclear cells in contrast to neutrophils for CXC chemokines. 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.

Based on their functions and structural characteristics, the mammalian CC subfamily has been traditionally divided into several subgroups including the monocyte chemotactic proteins (MCPs) and the macrophage inflammatory proteins (MIPs) (Mantovani et al. 2003; Laing and Secombes 2004a). Recently, chemokines have been grouped more broadly into three functional subfamilies: *inflammatory*, *homeostatic*, and *dual function* (Moser et al. 2004). Despite some continued changes, this system of CC chemokine classification should prove useful in the initial assessment of chemokine diversity and conservation in nonmammalian species.

The identification of CC chemokines in nonmammalian species has been slow, due in part to the rapid sequence divergence rate of CC chemokines. In addition, most CC chemokines are small in size with fewer than 100 amino acids. As a result, molecular cloning based on hybridization or polymerase chain reaction (PCR) using heterologous probes or primers designed from sequences of different species is not very effective. Similarly, low sequence conservation hinders sequence analysis using bioinformatic approaches at the nucleotide level. Nevertheless, small, important discoveries of fish CC chemokines in rainbow trout, carp, Japanese flounder, and dogfish have been made (Dixon et al. 1998; Liu et al. 2002; Fujiki et al. 1999; Kono et al. 2003; Khattiya et al. 2004; Inoue et al. 2005). The recent adoption of genomic approaches to search EST collections has resulted in more rapid discovery of fish chemokines. Seven new chemokines from two cichlid fish, *Paralabidochromis chilotes* and *Melanochromis auratus*, and catshark *Scyliorhinus canicula* were reported (Kuroda et al. 2003), followed by sequencing and analysis of 14 CC chemokines from catfish (He et al. 2004) and 15 trout CC chemokines (Laing and Secombes 2004b). The in silico identification of these last two large sets of CC chemokines, in particular, has challenged previous notions about chemokine diversity in fish. The higher amino acid similarities between chemokines of the same species, and to a lesser extent, between chemokines of closely related species, make these two sets of fish chemokines invaluable as "probes" for the in silico identification of further chemotactic cytokines in fish. The rapid expansion of catfish ESTs (Ju et al. 2000; Cao et al. 2001; Karsi et al. 2002; Kocabas et al. 2002) allows such a genomic approach to be applied for the identification of additional catfish CC chemokines.

Here, we have identified and conducted sequence analysis on 12 novel CC chemokines in catfish. As part of our efforts to understand the genetic components of the catfish immune system in the context of disease (Baoprasertkul et al. 2004, 2005; Chen et al. 2005), we also analyzed expression of the 12 newly identified CC chemokines after bacterial challenge with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC). ESC is a fast-acting bacterial pathogen responsible for large economic losses to the commercial catfish industry. The rapidly expanding repertoire of fish chemokines, CC and CXC, has important implications for both our understanding of comparative immunology and our investigation of the immune system of fish.

Materials and methods

In silico identification of putative catfish chemokines

The previously published sets of 14 CC chemokines from channel catfish and 18 CC chemokines from trout along with the known 28 mammalian CC chemokines were used as queries against NCBI's "estothers" database. tBLASTn (Altschul et al. 1990) searches were used to identify homologous EST sequences with a cutoff p value of 0.01. All catfish sequences were selected from this pool and transferred to Vector NTI's (Invitrogen, Carlsbad, CA) Contig Express program. Multiple copies of the same putative catfish CC chemokine sequence identified by multiple BLAST searches were eliminated. Remaining sequences were clustered in the Contig Express program along with the nucleotide sequences of the published catfish chemokines. Those sequences that clustered with the published catfish chemokines were excluded from analysis, whereas representative clones from new contigs and singletons were selected for further analysis. BLASTX searches were run on this set of putative novel catfish CC chemokine sequences to search for proper open reading frames, sequencing errors, and the distinct CC motif. After identification of proper open reading frames, the nucleotide sequences were translated to amino acids using a web-based translation tool (http://www.arbl.cvmbs.colostate.edu/molkit/translate/). The 12 CC chemokines identified here from channel catfish were named in order of discovery using the four-letter genus and species heading and the "SCYA" designation. The "100" series was continued so as not to suggest wrongly orthology with mammalian chemokines (He et al. 2004). For example, the first novel catfish CC chemokine described here is catfish SCYA115.

Sequence analysis of 26 catfish CC chemokines

The 14 previously published catfish CC chemokines (He et al. 2004) were combined with the 12 newly identified catfish CC chemokines for simultaneous analysis. The relevant sequences were retrieved from GenBank for multiple sequence alignments using ClustalW (Thompson et al. 1994). Percentage of amino acid identities was recorded after all multiple alignments using the DNASTAR software package (Lasergene, Madison, WI; Serapion et al. 2004).

Fish 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 (Dunham et al. 1993) with modifications (Baoprasertkul

et al. 2004; Chen et al. 2005). Briefly, catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. Head kidney and spleen were collected from challenged fish. Samples were collected from ten fish at each point including control (before challenge), and 4, 24, and 72 h after challenge. Samples of each tissue from ten fish were pooled. In order to obtain samples representing the average of the ten 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 (Chomczynski and Sacchi 1987) 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).

RT-PCR analysis of expression after bacterial infection

Sequences of the RT-PCR primers and expected product sizes are shown in Table 1. RT-PCR reactions were conducted using the 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 of total RNA (~200 ng), 2 μl (100 ng) each of the upper and lower primer, 2 μl of 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°C for 15 min for one cycle (reverse transcription reaction), the samples were predenatured at 94°C for 2 min, then the samples were amplified for 29 to 32 cycles (depending on initial trials) at 94°C for 15 s, 53°C for 30 s, and 72°C for 30 s. Upon the completion of PCR, the reaction was incubated at 72°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.

Phylogenetic analysis

Phylogenetic trees were drawn from ClustalW generated multiple sequence alignments of amino acid sequences using the neighbor-joining method (Saitou and Nei 1987) within the Molecular Evolutionary Genetics Analysis [MEGA (3.0)] package (Kumar et al. 2004). Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1,000 bootstrapping replications. Trees were also constructed using the minimum evolution method in MEGA (3.0) to verify overall topology. Trials of bootstrapping indicated that bootstrap values were similar using the two methods. Numerous trees were constructed using different groups of available chemokine sequences. After many rounds of initial analysis, the approach of Wang et al. (2005) for phylogenetic analysis of chicken CC chemokines using two trees was ultimately adopted here. Amino acid sequences of catfish, trout, chicken, and human/mouse CC chemokines were used in both trees.

Results

Identification and sequence analysis of CC chemokines from catfish

A total of 12 novel CC chemokines were identified here from channel catfish, and they are herein referred to as SCYA115-126. These chemokines were initially identified by BLAST searches, followed by sequence analysis for the conserved sequence characteristics of CC chemokines. BLASTX searches identified the chemokines sharing highest similarity with the catfish CC chemokines from both mammalian and fish species. However, many of the catfish CC sequences had overlapping hits because of the

Table 1 Primer sequences used in this study

Chemokine	RT-PCR upper	RT-PCR lower	Product size
SCYA115	tggtgctgctgagtgcagtcac	acccaggcgtcagtgggtttgg	208
SCYA116	actccacttctcagctgccctg	caaggtgaggacgggtccaagc	222
SCYA117	tcctgcacatcctgaggattgc	tctcagtagccgggacttcacg	167
SCYA118	caccactgcagtgttctccagc	tctcctttggagcatctggtgc	287
SCYA119	tggtgttctgcctctgtgccag	tgttctgtggaatggtcacctc	258
SCYA120	ctgctggttctgctgggtctcg	tgcggtctgcacacgccttacg	205
SCYA121	tctgcatccatctgctgagaac	gtgcgtacgtgttgcgtctcag	315
SCYA122	tgagcttcacacacctgctgag	agccttgctgttcacactgtgc	206
SCYA123	tccttcacagcggctcagagtg	tggggtttgcacacacactacg	172
SCYA124	gccttcagtccttcacaacagc	tgacatcagggtctgcacacac	186
SCYA125	cttcagcctggcacaaggttcg	ctagcgcaaatgagccgacctc	171
SCYA126	ttctacagcgccactgagtcga	agttaggtctcagaaacgttgc	199
β-actin	agagagaaattgtccgtgacatc	ctccgatccagacagagtatttg	396

high sequence similarity between some of the queries (see below) and because mammalian CC chemokines can also be highly similar. For example, human CCL8 and CCL11 share 70.1% amino acid identities. Establishing orthologous relationships based solely on BLAST identities, therefore, is impossible at this point, resulting only in a tangled web of shared similarity between multiple chemokines.

Multiple sequence analysis of the 26 catfish CC chemokines, the 12 newly identified CC chemokines plus the 14 previously published CC chemokines, using ClustalW (Fig. 1) revealed that all sequences contain the four conserved cysteine residues that are characteristic of CC chemokines. Other residues show high, if not universal, conservation in the catfish CC chemokine family (Fig. 1).

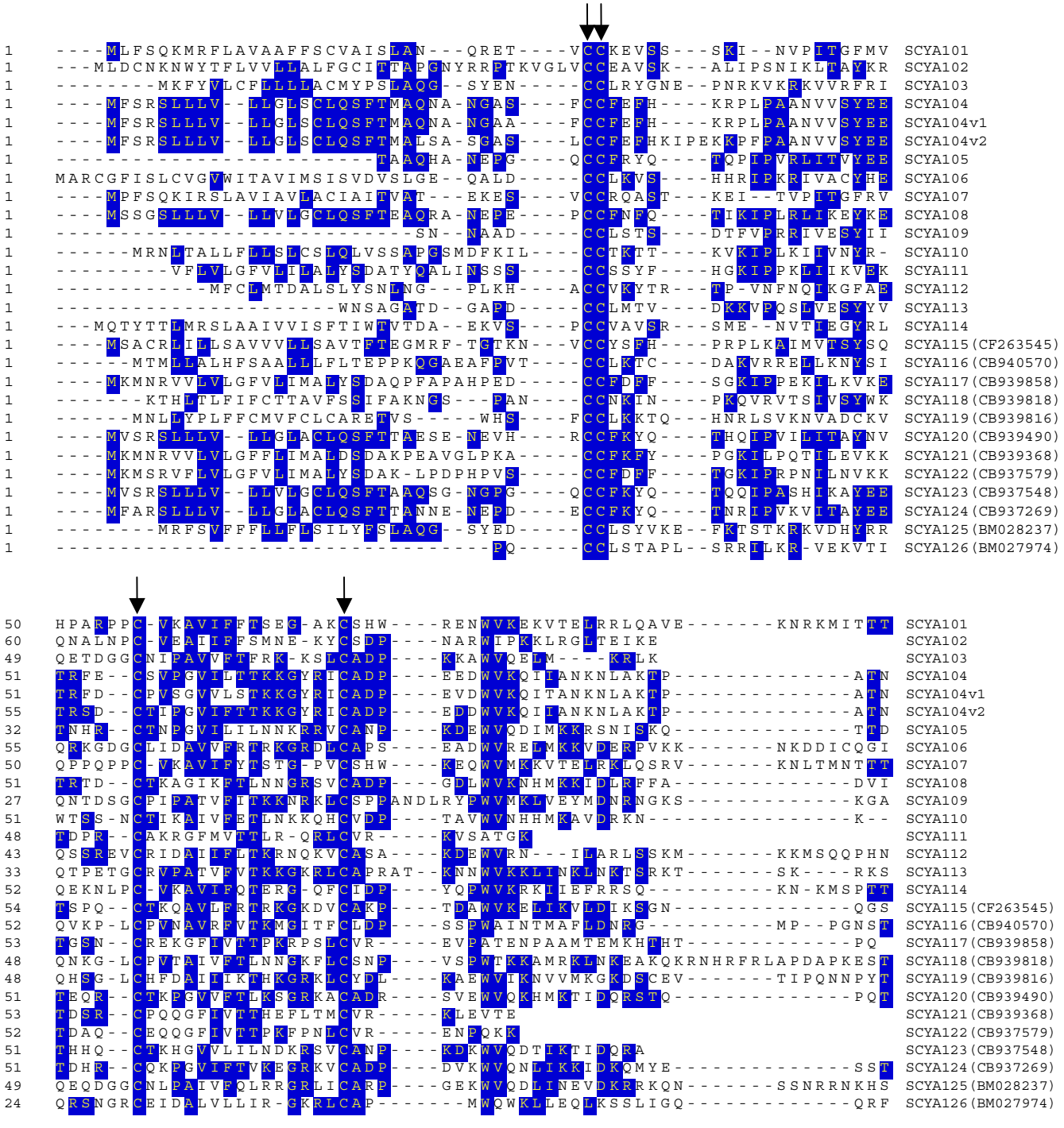


Fig. 1 Partial multiple sequence alignment of all known catfish CC chemokines in which the characteristic four conserved cysteine residues are noted with *arrows*. Catfish CC chemokines identified in this study are SCYA115-126. Nucleotide accession numbers are included for the new CC chemokines. Two variants of SCYA104 (described in He et al. 2004) are included for purposes of comparison

Table 2 Amino acid identities (%) shared between all known catfish CC chemokines

102	25.0																														
103	19.8	19.8																													
104	15.8	14.7	18.6																												
104v1	11.6	14.7	17.4	**91.6**																											
104v2	15.2	13.1	27.9	**87.4**	**83.2**																										
105	10.1	15.2	26.6	35.4	32.9	34.2																									
106	16.4	17.0	33.7	18.9	20.0	18.2	20.3																								
107	**59.6**	22.0	22.1	14.7	9.5	11.1	15.2	12.7																							
108	16.8	15.0	25.6	43.2	43.2	44.4	46.8	20.8	11.9																						
109	7.6	11.4	26.6	21.5	22.8	20.3	19.0	36.7	15.2	13.9																					
110	13.8	20.2	23.3	23.4	24.5	28.7	22.8	23.4	11.7	24.5	26.6																				
111	11.8	6.6	18.4	19.7	17.1	14.5	17.1	6.6	18.4	22.4	13.2	14.5																			
112	15.0	13.0	24.4	14.7	13.7	20.2	26.6	16.8	17.8	16.8	21.5	27.7	6.6																		
113	15.3	14.1	23.5	25.9	25.9	24.7	19.0	30.6	22.4	17.6	41.8	20.0	14.5	20.0																	
114	25.2	19.0	15.1	11.6	11.6	12.1	8.9	14.5	32.4	11.9	15.2	16.0	9.2	17.8	18.8																
115	20.2	10.1	25.6	26.3	28.4	30.3	21.5	27.3	15.2	23.2	22.8	21.3	15.8	17.2	25.9	13.1															
116	10.3	10.0	23.3	16.8	17.9	15.2	13.9	14.0	6.5	17.8	27.8	26.6	11.8	15.0	22.4	20.6	17.2														
117	14.6	8.3	15.1	14.7	12.6	19.8	16.5	11.5	6.3	22.9	16.5	14.9	**53.9**	13.5	15.3	8.3	13.5	10.4													
118	14.0	14.0	23.3	14.7	16.8	19.2	20.3	18.2	13.4	18.8	25.3	25.5	10.5	19.6	27.1	16.2	17.2	22.4	10.4												
119	10.5	14.0	18.6	18.9	15.8	10.1	10.1	15.5	11.9	7.9	20.3	19.1	11.8	19.6	11.8	13.5	10.1	15.0	6.3	14.4											
120	15.5	13.4	25.6	41.1	40.0	42.3	45.6	22.7	12.4	51.5	24.1	30.9	25.0	15.5	24.7	12.4	19.6	16.5	16.7	15.5	19.6										
121	16.0	6.2	6.2	18.5	17.3	19.8	15.2	12.3	13.6	24.7	12.7	17.3	48.7	13.6	11.1	4.9	16.0	18.5	**60.5**	14.8	14.8	21.0									
122	11.3	13.8	8.8	17.5	15.0	22.5	16.5	11.3	13.8	27.5	13.9	22.5	**51.3**	15.0	12.5	7.5	12.5	13.8	**66.3**	16.3	6.3	17.5	**58.8**								
123	12.2	16.7	25.6	44.4	44.4	44.4	**57.0**	21.1	16.7	**58.9**	19.0	20.0	22.4	17.8	15.3	10.0	25.6	22.2	18.9	22.2	16.7	**60.0**	18.5	23.8							
124	15.8	17.9	29.1	46.3	43.2	45.3	46.8	22.1	15.8	**51.6**	22.8	26.6	27.6	18.9	21.2	13.7	24.2	20.0	18.9	16.8	18.9	**64.2**	24.7	22.5	**57.8**						
125	14.2	17.0	**52.3**	20.0	16.8	19.2	21.5	23.6	12.3	22.8	30.4	25.5	17.1	19.8	30.6	17.9	18.2	17.0	11.5	17.9	19.8	20.6	14.8	15.0	25.6	26.3					
126	7.5	10.4	25.4	16.4	17.9	13.4	19.4	23.9	6.0	10.4	32.8	9.0	7.5	22.4	31.3	14.9	16.4	7.5	4.5	9.0	20.9	6.0	3.0	4.5	11.9	9.0	22.4				
SCYA	101	102	103	104	104v1	104v2	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125				

Values greater than 50% are in bold

A comparison of amino acid identities of the 26 putative catfish CC chemokines revealed that the majority of catfish CC chemokines had low levels of similarity with one another with a few exceptions (Table 2). SCYA104v1 and SCYA104v2, described previously by He et al. (2004), share very high identity with SCYA104 (>80% amino acid identity), possibly as the result of allelic variation or recent gene duplication. The other catfish CC chemokines share markedly lower identities, but several had similarities above 50%, most notably among SCYA 111/117/121/122, among SCYA 105/108/120/123/124, and between SCYA 101/107 and SCYA 103/125. These could represent possible CC chemokine subgroups within channel catfish. Although orthologues are difficult to establish for these subgroups (see below), functional subgroups sharing high identities are common in the mammalian species (i.e., the MCP and MIP groups). Altogether, 13 of the 26 catfish CC chemokines share higher than 50% amino acid identities with at least one other member of the family. Likewise, Laing and Secombes (2004b) found that 12 of 18 trout chemokines they investigated shared higher than 50% amino acid identity with at least one other member of the trout CC chemokine family.

Phylogenetic analysis of catfish CC chemokines

The relationships of the 26 catfish CC chemokines were investigated further in the context of a group of species stretching across a broad phylogenetic spectrum. The larger groups of systematically identified, analyzed, and published CC chemokines from trout (Laing and Secombes

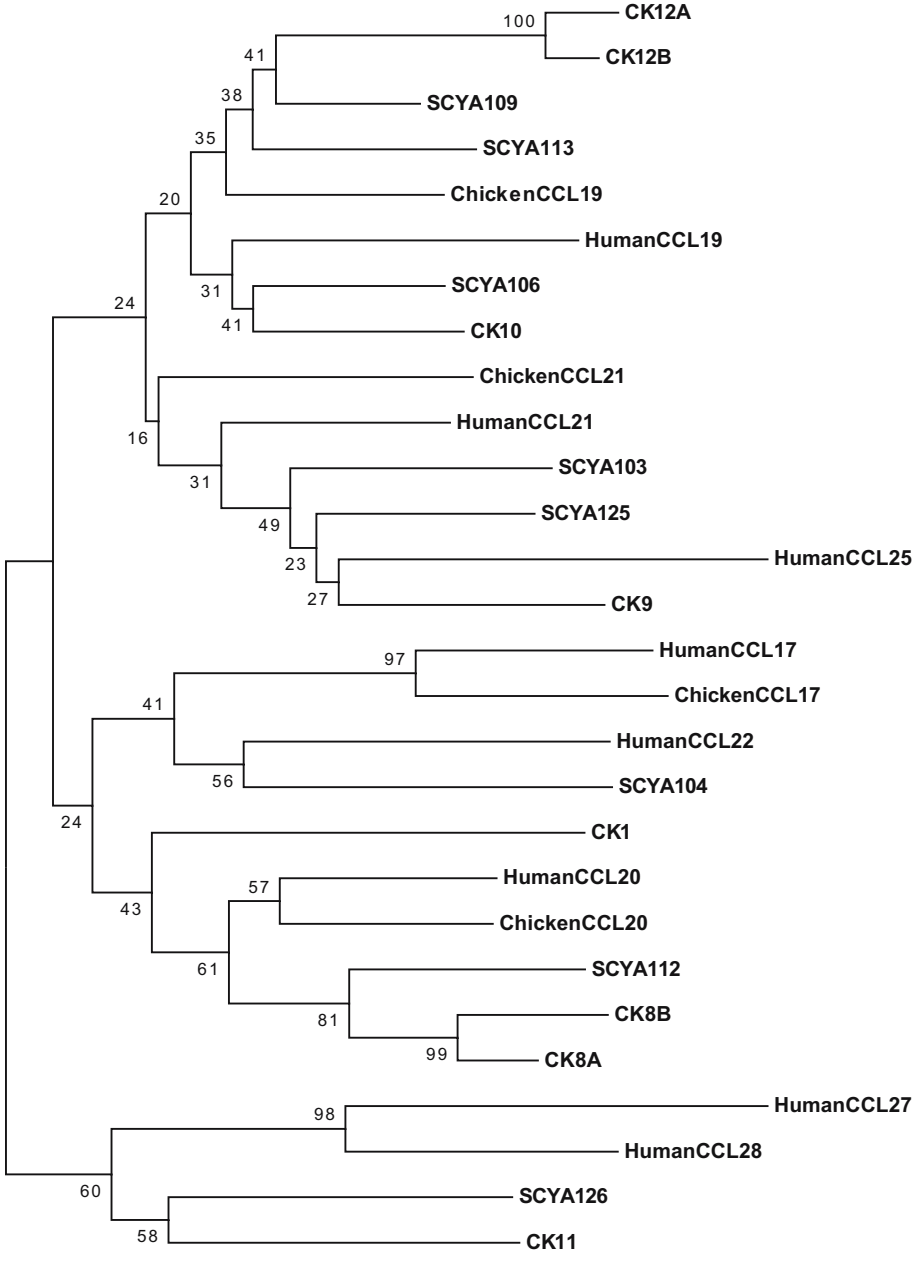


Fig. 2 Phylogenetic trees were drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA (3.0) package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor-joining tree was evaluated by 1,000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. The accession numbers for the representative mammalian CC chemokines are Q92583 (CCL17), Q99731(CCL19), P78556(CCL20), O00585 (CCL21), O00626(CCL22), O15444(CCL25), Q9Y4X3 (CCL27), and Q9NRJ3(CCL28). The accession numbers for chicken CC chemokines are BI067703(CCL17), BX929857 (CCL19), AY037861(CCL20), and CR522995(CCL21). The accession numbers for the catfish (SCYA−) CC chemokines are: AY555500(SCYA103), AY555501(SCYA104), AY555503(SCYA106), AY555506(SCYA109), AY555509(SCYA112), AY555510(SCYA113), BM028237(SCYA125), and BM027974(SCYA126). The accession numbers for the trout (CK−) CC chemokines are AF093802(CK1), CB494647 (CK8A), CA353159(CK8B), CA378686(CK9), CA361535 (CK10), BX072681(CK11), CA358073(CK12A), and CA346383(CK12B)

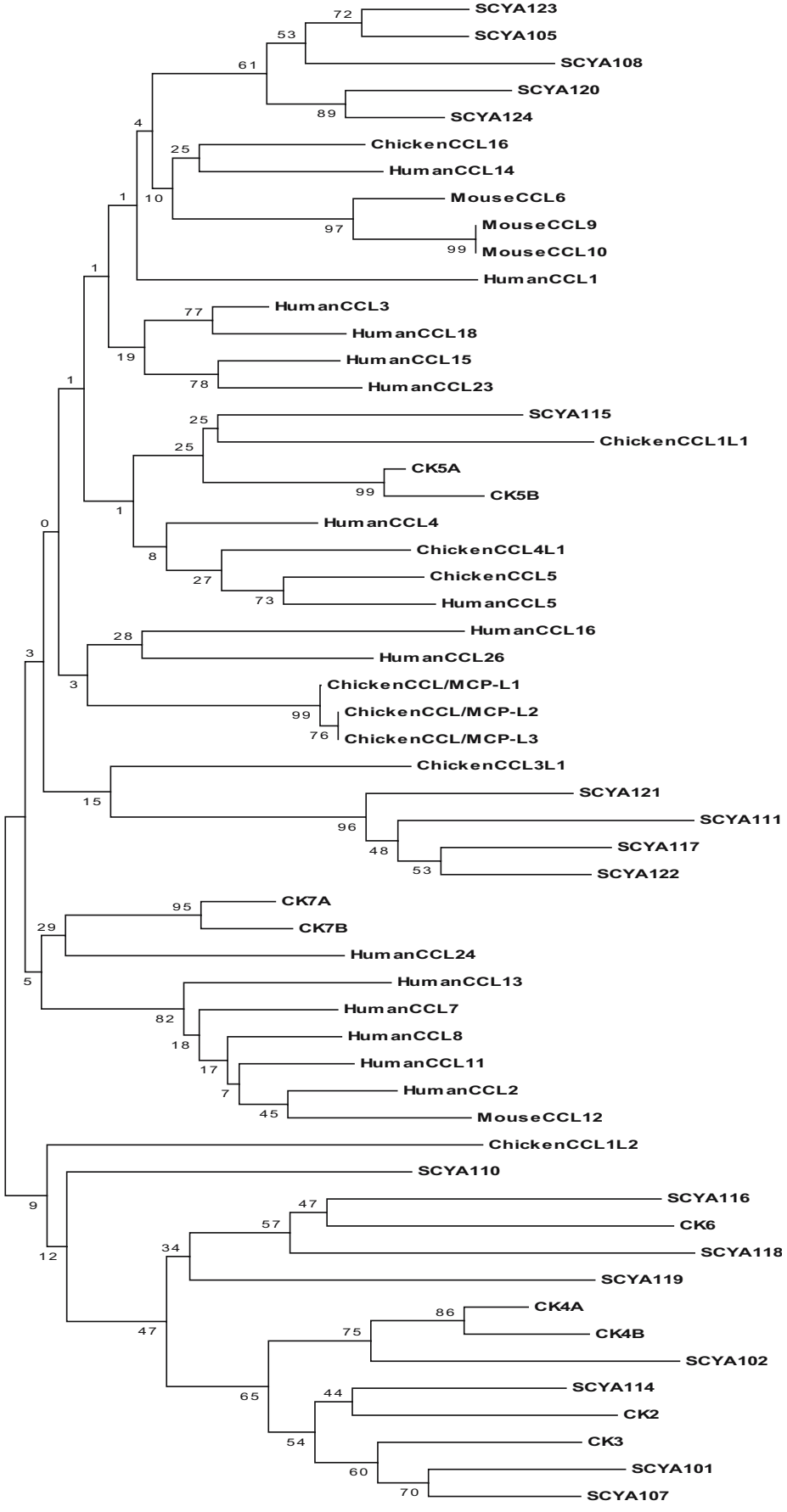
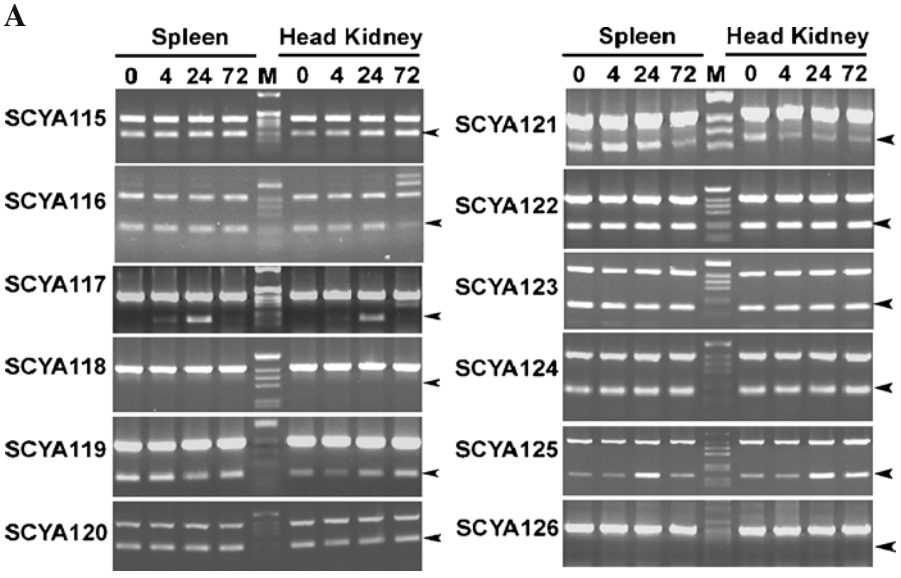


Fig. 3 Phylogenetic trees were drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA (3.0) package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1,000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. The accession numbers for the representative mammalian CC chemokines are P22362(CCL1), P13500(CCL2), P10147(CCL3), P13236(CCL4), P13501(CCL5), P27784(CCL6), P80098(CCL7), P80075(CCL8), P51670(CCL9), P51670(CCL10), P51671 (CCL11), Q62401(CCL12), Q99616(CCL13), Q16627 (CCL14), Q16663(CCL15), O15467(CCL16), P55774 (CCL18), P55773(CCL23), O00175(CCL24), and Q9Y258 (CCL26). The accession numbers for chicken CC chemokines are BX935885(CCL1L1), L34552(CCL1L2), CF258095 (CCL3L1), AY037860(CCL/ MCP-L1), CK610423(CCL/ MCP-L2), CK610627(CCL/ MCP-L3), AY037859(CCL5), AJ243034(CCL4L1), and Y18692(CCL16). The accession numbers for the catfish (SCYA−) CC chemokines are AY555498 (SCYA101), AY555499 (SCYA102), AY555502 (SCYA105), AY555504 (SCYA107), AY555505 (SCYA108), AY555507 (SCYA110), AY555508 (SCYA111), AY555511 (SCYA114), CF263545 (SCYA115), CB940570 (SCYA116), CB939858 (SCYA117), CB939818 (SCYA118), CB939816 (SCYA119), CB939490 (SCYA120), CB939368 (SCYA121), CB937579 (SCYA122), CB937548 (SCYA123), and CB937269 (SCYA124). The accession numbers for the trout (CK−) CC chemokines are AF418561 (CK2), AJ315149(CK3), CA371157(CK4A), CA352593 (CK4B), CA383670(CK5A), CA374135(CK5B), CA355962 (CK6), CA355962(CK7A),and CA346976(CK7B)

A



B

	Spleen			Head kidney		
	4h	24h	72h	4h	24h	72h
Upregulated						
SCYA115	NC	NC	NC	NC	+	++
SCYA117	++	++++	+	++	++++	+
SCYA125	NC	++	+	NC	++	+
Downregulated						
SCYA116	NC	NC	NC	NC	NC	-*
SCYA121	NC	NC	-	-	-	-
Constitutively expressed						
SCYA119	NC	NC	NC	NC	NC	NC
SCYA120	NC	NC	NC	NC	NC	NC
SCYA122	NC	NC	NC	NC	NC	NC
SCYA123	NC	NC	NC	NC	NC	NC
SCYA124	NC	NC	NC	NC	NC	NC
Not detected						
SCYA118	0	0	0	0	0	0
SCYA126	0	0	0	0	0	0

Fig. 4 Expression analysis of the 12 novel catfish CC chemokines using RT-PCR (**a**). RT-PCR reactions were conducted as described in the Materials and methods. RT-PCR products were analyzed by agarose gel electrophoresis. Two tissues, spleen and head kidney, were used in the study, and they are indicated at the top of the figure. The names of the catfish CC chemokines were indicated on the left margins of each panel of the gels. Samples from healthy fish (0) and infected fish at 4 h (4), 24 h (24), and 72 h (72) were used. Molecular marker (M) was 1-kb ladder purchased from Invitrogen. *Arrows* indicate the expected positions of the catfish CC chemokine RT-PCR products. Note that RT-PCR reactions were conducted for one gene at a time, and the images of agarose gels were compiled together into a single figure and, therefore, expression levels can only be analyzed separately for each gene. Note also that 32 PCR cycles were used for SCYA119 and SCYA121, whereas 29 cycles were used for the remaining chemokines. The main results of the RT-PCR reactions were summarized in **b** and grouped into four categories: up-regulated, down-regulated, constitutively expressed, and no expression detected. *NC* no change, + slightly up-regulated, ++ moderately up-regulated, ++++ highly up-regulated, − down-regulated, 0 no expression detected. The asterisk (*) indicated the presence of additional PCR bands for SCYA116

2004b), chicken (Wang et al. 2005), and mammalian species (human and mouse) were used to build phylogenetic trees. All sequences were evaluated together using both the neighbor-joining and minimum evolution methods. The homeostatic or dual function mammalian CC chemokines and a subset of catfish, trout, and chicken CC chemokine sequences that consistently grouped with them were separately aligned and a phylogenetic tree constructed (Fig. 2). The remaining set of catfish, trout, and chicken CC chemokines were more closely related to the inflammatory mammalian CC chemokines but formed only weak clades with them. These sequences were also separately aligned and a tree constructed (Fig. 3). The two-tree approach for CC chemokine analysis was also used by Wang et al. (2005) (see Discussion) and resulted in trees with higher overall bootstrap values. The overall topology of both trees was confirmed using the minimum evolution method (not shown). Bootstrap values given by the two methods were highly similar.

In Fig. 2, several catfish CC chemokines are consistently grouped into clades with mammalian CC chemokines. SCYA126 is grouped with human CCL27 and CCL28 sequences in a clade supported by moderate bootstrapping values. Human CCL20 groups with catfish SCYA112 as well as three trout chemokine sequences and a chicken orthologue. This relationship is also supported by moderate

bootstrapping values. SCYA104 was consistently grouped with significant bootstrapping support with human CCL22 in a clade without chicken or trout membership. SCYA103 and SCYA125, along with trout CK9, were placed repeatedly in clades with human CCL25 albeit with lower bootstrapping values. The top of the tree in Fig. 2 holds three catfish CC chemokines, SCYA109, SCYA113, and SCYA 106, that consistently grouped with human CCL19 in all trees and methods evaluated, but whose exact positions within the clade are not well supported.

The phylogenetic tree in Fig. 3 contains the inflammatory mammalian CC chemokines and those sequences from catfish, trout, and chicken that, through evaluation of new and previously published trees, were determined to be related to them. This tree was characterized by tight species-specific clades regardless of attempts to use different methods or sequences from additional species. The overall topology of the tree did not differ significantly between application of the neighbor-joining method (shown) and the minimum evolution method (not shown). Consistent clades of catfish SCYA123/105/108/120/124 and SCYA 121/111/117/122 formed in all trees evaluated with no clear orthologues in trout, chicken, or human/mouse. SCYA115 was grouped repeatedly with CK5A and CK5B, and chicken CCL1L1, and shares low levels of similarity with human CCL4 and CCL5. Another large group of catfish CC chemokines, these with apparent trout orthologues, is found at the bottom of the tree. SCYA102, SCYA114, SCYA101, and SCYA107 were grouped together in a fairly strong clade with four trout CC chemokines but appear only distantly related to any known chicken or mammalian CC chemokines. SCYA116, SCYA118, and SCYA119 were consistently grouped with trout CK6, but their relation to the mammalian CC chemokines is also still unclear.

Expression analysis

CC chemokine expression was analyzed by RT-PCR in head kidney and spleen tissues from both healthy fish and fish challenged with the bacterial pathogen *E. ictaluri*. As summarized in Fig. 4, four main expression patterns were observed. The first group of catfish CC chemokines, SCY A115, SCYA117, and SCYA125, were up-regulated upon bacterial infection; the second group of catfish CC chemokines, SCYA116 and SCYA121, were down-regulated upon bacterial infection; the third group of catfish CC chemokines, SCYA119, 120, 122, 123, and 124, were constitutively expressed; and finally, with SCYA118 and SCYA126, no expression was detected at any point of the analysis. In order to confirm the lack of expression for SC YA118 and SCYA126, PCR amplification was repeated with extended cycles, but no products were observed.

Minor differences were observed in the points and tissues in which up-regulation and down-regulation occurred. For instance, SCYA117 and SCYA125 were rapidly and highly induced after bacterial infection in both the spleen and the head kidney tissues, whereas SCYA115 was moderately up-regulated only in the head kidney. Similarly, SC YA121 expression was down-regulated in both spleen and head kidney tissues, but more rapidly in the latter tissue (Fig. 4). For SCYA116, it appeared that the mature RNA product decreased at 72 h postinfection in head kidney, but bands with higher molecular weights were amplified, likely representing unspliced transcripts.

Discussion

A foundation of small chemokine discoveries in fish combined with rapidly expanding genetic resources and bioinformatics tools has allowed us to probe the diversity of chemokine genes in catfish. Here we have identified and conducted sequence and expression analysis on 12 novel putative CC chemokines. Coupled with our prior sequencing report, we report a catfish CC chemokine family with at least 26 representatives, two more than are currently known from humans. The identification of a large repertoire of CC chemokines from catfish furthers our understanding of the immune system of Actinopterygiian vertebrates and provides novel perspectives in comparative immunology. The importance of chemokines as regulators of inflammation and host defense has long been understood. Chemokine ligands and their receptors are increasingly targets of therapeutic approaches in a vast array of disease studies (i.e., Nagar and Gorelick 2004; Lomas-Neira et al. 2005; Eugenin et al. 2005).

The identification of chemokine genes in species with an intermediate phylogenetic position between fish and mammals (birds and reptiles) has been eagerly anticipated by many (including the authors) for their potential in drawing more concrete connections between chemokines in Actinopterygii and Sarcopterygii. The recent work of Wang et al. (2005) in identifying chemokines in the chicken genome provides a new piece of the puzzle. They identified 13 CC chemokine ligands from chicken. Although they leave open the possibility of further small discoveries (i.e., they identified CCR9 but not its ligand CCL25), they conclude that a substantial number of chemokine genes were present before the divergence between aves and mammals, and that more duplications of chemokines occurred in mammals than in aves after the divergence.

Other findings from the chicken and trout studies of CC chemokines provide perspective for the results presented here. The majority of human CC chemokine ligands not present in a large cluster on chromosome 17 possess likely orthologues in catfish, trout, and chicken. In sharp contrast, those human CC chemokines on chromosome 17 cannot be clearly grouped with CC chemokines from the other species. Although there are exceptions to this observation (i.e., human CCL5), it is borne out by the two phylogenetic trees (Figs. 2 and 3) presented here and in numerous other studies (Wang et al. 2005; Inoue et al. 2005; Laing and Secombes 2004b; MacKenzie et al. 2004). The presence of a large group of highly similar human CC chemokines in close genomic proximity that cluster more tightly with each other than with the sequences of other species is suggestive of tandem gene duplications after species divergence.

Chicken also has a cluster of CC chemokine genes on its chromosome 19, which demonstrate partially conserved synteny with human chromosome 17. Duplication has also likely occurred on the chicken chromosome 19 (i.e., chicken CCL/MCP group in Fig. 3) but to a far lesser extent than in humans. Catfish also appears to possess groups of highly similar species-specific CC chemokines suggestive of gene duplications after species divergence (Fig. 3). Two of these groups account for 9 of the 26 CC chemokines identified in catfish. Although the genomic location of these genes is not yet known, we are currently in the process of mapping these catfish chemokines to chromosomal segments using overgo hybridizations (Peatman et al. 2004).

We have also investigated CC chemokine diversity in other teleost species with rich genetic resources (not presented). The number of zebrafish (*Danio rerio*) putative CC chemokines identified (18) corresponded more closely with catfish than the seven putative CC chemokines we were able to identify from the genome of the spotted green pufferfish, *Tetraodon nigroviridis*. Trees including zebrafish and pufferfish sequences (not shown) demonstrated that zebrafish also possesses clusters of highly similar species-specific chemokines. The inclusion of additional unpublished sequences from other fish species did not change the overall topology of the phylogenetic trees presented.

High levels of duplication and divergence coupled with the short amino acid length of most CC chemokines make the naming of newly identified sequences difficult. Identities gleaned from BLAST results are inconclusive and often misleading. BLASTX searches often generated overlapping hits because of the high sequence similarity between some of the queries and among subject chemokine sequences within the GenBank databases. Establishing orthologous relationships based solely on BLAST identities, therefore, is impossible. Wang et al. (2005) relied properly on phylogenetic trees and syntenies for naming the chicken CC chemokines, but they admit that the information upon which seven of the sequences were named was inadequate. We followed the example of Wang et al. (2005) in separating our sequences between the mammalian inflammatory (chromosome 17) and homeostatic/dual function (other chromosomes) CC chemokines. When catfish and trout sequences were added, the lack of clear orthology between many of the chicken and mammalian CC chemokines resulted in their being consistently placed in clades lacking the mammalian sequence for which they were named (Fig. 3). Given these realities, we believe that using nomenclatures distinct from the mammalian system (i.e., SCYA101-126) is presently the best approach in catfish and other nonmammalian species in order to avoid confusion.

Expression analysis is one tool available with which to characterize novel putative CC chemokines. We examined the expression patterns in head kidney and spleen of the 12 catfish CC chemokines identified here after infection with *E. ictaluri*, a fast-acting bacterial fish pathogen. Three of the CC chemokines were up-regulated, two were down-regulated, five exhibited constitutive expression, and expression of two of the chemokines was not detected in these samples. No strong correlations were observed between position on the phylogenetic trees and expression patterns as seen by Laing and Secombes (2004b) with stimulation by tumor necrosis factor (TNFα). We speculate that the catfish CC chemokines sharing high levels of sequence identity may have been derived from recent gene duplications and thus may reside in the same or similar genomic patterns as those on human chromosome 17, but further studies, particularly physical mapping and functional analysis of the catfish CC chemokines, are required to provide firm answers.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bacon K, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Matsushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, Van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zoon K (2003) Chemokine/chemokine receptor nomenclature. Cytokine 21:48–49

Baoprasertkul P, Peatman E, Chen L, He C, Kucuktas H, Li P, Simmons M, Liu Z (2004) Sequence analysis and expression of a CXC chemokine in resistant and susceptible catfish after infection of *Edwardsiella ictaluri*. Dev Comp Immunol 28:769–780

Baoprasertkul P, He C, Peatman E, Zhang S, Li P, Liu Z (2005) Constitutive expression of three novel catfish CXC chemokines; homeostatic chemokines in teleost fish. Mol Immunol 42:1355–1366

Cao D, Kocabas A, Ju Z, Karsi A, Li P, Patterson A, Liu Z (2001) Transcriptome of channel catfish (*Ictalurus punctatus*): initial analysis of genes and expression profiles of the head kidney. Anim Genet 32:169–188

Chen L, He C, Baoprasertkul P, Xu P, Li P, Serapion J, Waldbieser G, Wolters W, Liu Z (2005) Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with *Edwardsiella ictaluri*. Dev Comp Immunol 29:135–142

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159

Dixon B, Shum B, Adams EJ, Magor KE, Hedrick RP, Muir DG, Parham P (1998) CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunol Rev 166:341–348

Dunham RA, Brady Y, Vinitnantharat S (1993) Response to challenge with *Edwardsiella ictaluri* by channel catfish, *Ictalurus punctatus*, selected for resistance to *E. ictaluri*. J Appl Aquac 3:211–222

Eugenin EA, Dyer G, Calderon TM, Berman JW (2005) HIV-1 tat protein induces a migratory phenotype in human fetal microglia by a CCL2 (MCP-1)-dependent mechanism: possible role in NeuroAIDS. Glia 49:501–510

Fujiki K, Shin DH, Nakao M, Yano T (1999) Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. Immunogenetics 49:909–914

He C, Peatman E, Baoprasertkul P, Kucuktas H, Liu Z (2004) Multiple CC chemokines in channel catfish and blue catfish as revealed by analysis of expressed sequence tags. Immunogenetics 56:379–387

Inoue Y, Saito T, Endo M, Haruta C, Nakai T, Moritomo T, Nakanishi T (2005) Molecular cloning and preliminary expression analysis of banded dogfish (*Triakis scyllia*) CC chemokine cDNAs by use of suppression subtractive hybridization. Immunogenetics 56: 722–734

Ju Z, Karsi A, Kocabas A, Patterson A, Li P, Cao D, Dunham R, Liu Z (2000) Transcriptome analysis of channel catfish (*Ictalurus punctatus*): genes and expression profile from the brain. Gene 261:373–382

Karsi A, Cao D, Li P, Patterson A, Kocabas A, Feng J, Ju Z, Mickett KD, Liu Z (2002) Transcriptome analysis of channel catfish (*Ictalurus punctatus*): initial analysis of gene expression and microsatellite-containing cDNAs in the skin. Gene 285:157–168

Khattiya R, Ohira T, Hirono I, Aoki T (2004) Identification of a novel Japanese flounder (*Paralichthys olivaceus*) CC chemokine gene and an analysis of its function. Immunogenetics 55:763–769

Kocabas A, Li P, Cao D, Karsi A, He C, Patterson A, Ju Z, Dunham R, Liu ZJ (2002) Expression profile of the channel catfish spleen: analysis of genes involved in immune functions. Mar Biotechnol 4:526–536

Kono T, Kusada R, Kawahara E, Sakai M (2003) The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder *Paralichthys olivaceus*. Vaccine 21:446–457

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Kuroda N, Uinuk-ool TS, Sato A, Samonte IE, Figueroa F, Mayer WE, Klein J (2003) Identification of chemokines and a chemokine receptor in cichlid fish, shark, and lamprey. Immunogenetics 54:884–895

Laing KJ, Secombes CJ (2004a) Chemokines. Dev Comp Immunol 28:443–460

Laing KJ, Secombes CJ (2004b) Trout CC chemokines: comparison of their sequences and expression patterns. Mol Immunol 41: 793–808

Liu L, Fujiki K, Dixon B, Sundick RS (2002) Cloning of a novel rainbow trout (*Oncorhynchus mykiss*) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDNA fragments containing Au-rich elements. Cytokine 17:71–81

Lomas-Neira JL, Chung CS, Wesche DE, Perl M, Ayala A (2005) In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 versus KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury. J Leukoc Biol, in press

Mackenzie S, Liarte C, Iliev D, Planas JV, Tort L, Goetz FW (2004) Characterization of a highly inducible novel CC chemokine from differentiated rainbow trout (*Oncorhynchus mykiss*) macrophages. Immunogenetics 56:611–615

Mantovani A, Locati M, Sozzani S (2003) CC chemokines. In: Thomson AW, Lotze MT (eds) The cytokine handbook. Elsevier, London, pp 1083–1097

Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. Trends Immunol 25:75–85

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52:145–176

Nagar AB, Gorelick FS (2004) Acute pancreatitis. Curr Opin Gastroenterol 20:439–443

Neville LF, Mathiak G, Bagasra O (1997) The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. Cytokine Growth Factor Rev 8:207–219

Peatman EJ, Wei X, Feng J, Liu L, Kucuktas H, Li P, He C, Rouse D, Wallace R, Dunham R, Liu Z (2004) Development of expressed sequence tags from Eastern oyster (*Crassostrea virginica*): lessons learned from previous efforts. Mar Biotechnol 6:S491–S496

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Serapion J, Kucuktas H, Feng J, Liu Z (2004) Bioinformatic mining of type I microsatellites from expressed sequence tags of channel catfish (*Ictalurus punctatus*). Mar Biotechnol 6:364–377

Thompson JD, Higgins DG, Gibson TJ (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Wang J, Adelson DL, Yilmaz A, Sze SH, Jin Y, Zhu JJ (2005) Genomic organization, annotation, and ligand-receptor inferences of chicken chemokines and chemokine receptor genes based on comparative genomics. BMC Genomics 6:45