Mol Gen Genomics (2006) 275: 297–309
DOI 10.1007/s00438-005-0081-9

ORIGINAL PAPER

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Catfish CC chemokines: genomic clustering, duplications, and expression after bacterial infection with *Edwardsiella ictaluri*

Received: 26 August 2005 / Accepted: 12 November 2005 / Published online: 8 December 2005
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Abstract Chemokines are a family of structurally related chemotactic cytokines that regulate the migration of leukocytes, under both physiological and inflammatory conditions. CC chemokines represent the largest subfamily of chemokines with 28 genes in mammals. Sequence conservation of chemokines between teleost fish and higher vertebrates is low and duplication and divergence may have occurred at a significantly faster rate than in other genes. One feature of CC chemokine genes known to be conserved is genomic clustering. CC chemokines are highly clustered within the genomes of human, mouse, and chicken. To exploit knowledge from comparative genome analysis between catfish and higher vertebrates, here we mapped to bacterial artificial chromosome (BAC) clones 26 previously identified catfish (*Ictalurus* sp.) chemokine cDNAs. Through a combination of hybridization and fluorescent fingerprinting, 18 fingerprinted contigs were assembled from BACs containing catfish CC chemokine genes. The catfish CC chemokine genes were found to be not only highly clustered in the catfish genome, but also extensively duplicated at various levels. Comparisons of the syntenic relationships of CC chemokines may help to explain the modes of duplication and divergence that resulted in the present repertoire of vertebrate CC chemokines. Here we have also analyzed the expression of the transcripts of the 26 catfish CC chemokines in head kidney and spleen in response to bacterial infection of *Edwardsiella ictaluri*, an economically devastating catfish pathogen. Such information should pinpoint research efforts on the CC chemokines most likely involved in inflammatory responses.

Keywords Chemokine · Fish · Catfish · Gene · Comparative · Duplication

Communicated by S. Hohmann

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Introduction

Chemokines are a superfamily of chemotactic cytokines in mammals and a crucial part of the innate immune response of higher vertebrates. They play roles in immunosurveillance under homeostasis as well as stimulating the recruitment, activation, and adhesion of cells to sites of infection or injury (Neville et al.1997; Moser and Loetscher 2001; Laing and Secombes 2004a). Recent research has found that some chemokine genes have important roles during normal development and growth (e.g. David et al. 2002; Molyneaux et al. 2003; Baoprasertkul et al. 2005). Chemokines 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. CC chemokines constitute the largest subfamily of chemokines with 28 CC chemokines identified from mammalian species (Bacon et al. 2003). 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 (Nomiyama et al. 2001; Wang et al. 2005). There are correlations between genomic archi-

tecture and the inducibility of their expression, with inflammatory CC chemokines constituting the large clusters, and a few homeostatic CC chemokines distributed among several chromosomes. Additionally, orthologies across species are relatively high between the non-clustered CC chemokines, but low when comparing the clustered CC chemokines of several species (Wang et al. 2005; Peatman et al. 2005).

Establishing orthology between fish and mammalian CC chemokines has been problematic. Sequence conservation of chemokines is low and duplication and divergence may have occurred at a significantly faster rate than in other genes. Concrete orthologues cannot be identified for the majority of CC chemokine transcripts found from catfish or trout based on either sequence identities or phylogenetics (Laing and Secombes 2004b; He et al. 2004; Peatman et al. 2005). Even gene organization (exon/intron) has been found to differ between evident orthologous chemokines in human, chicken, and catfish (Wang et al. 2005; Bao et al. 2006a). Genomic location of CC chemokines is important, therefore, in attempting to trace the origins of CC chemokines in teleosts and higher vertebrates. Comparisons of syntenic relationships of CC chemokines may help to explain the modes of duplication and divergence that resulted in the present repertoire of vertebrate CC chemokines.

Progress on identifying immune molecules in teleost fish has not traditionally come from the genome-enabled model species (*Danio rerio, Takifugu rubripes*). Rather it has been generated more slowly in several aquaculture species (catfish, salmonids, carps, flounders) where disease problems are a serious economic issue. The lack of even a draft genomic sequence in catfish makes cross-species comparisons of genomic neighborhoods much more difficult. We have used, therefore, a novel approach of overgo and cDNA hybridizations and bacterial artificial chromosome (BAC) fingerprinting and clustering to determine the architecture of the catfish CC chemokines without a draft genome sequence. Here we report the genomic architecture of previously sequenced catfish CC chemokine genes as well as their expression patterns after bacterial infection. Comparisons of CC chemokine arrangements and duplication between catfish, chickens, and humans reveal rapid multiplication of some chemokine genes.

Materials and methods

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 (Cai et al. 1998; Bao et al. 2005, 2006b; Xu et al. 2005). Each set of filters contained a 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 overgo probes of catfish CC chemokines were used to identify BAC clones with inserts likely containing chemokine genes. These positive BACs were then manually re-arrayed onto nylon filters and screened individually using labeled cDNA probes.

In order to rapidly generate a large number of gene-specific probes without extensive gel purification of probe fragments, the strategy of overgo hybridization was adopted (Han et al. 2000). With overgo hybridization, rather than isolating DNA segments for making probes, a short segment of a gene can be initially selected as the probe based on its unique presence in the genome. Most often, this uniqueness is determined by BLAST searches of the GenBank with the overgo probe sequence. In most cases, a probe of 40 bp long provides both very high specificity and easy handling during hybridization. Thus, primers of 24 bases long are selected within a gene sequence to be complementary to each other, but staggered with 8-bases pairing, leaving 16-base 5′-overhangs on both sides. Once the two overgo primers are mixed and annealed, they form a suitable substrate for DNA polymerase to fill in to the end of the overgo primers to produce a probe of 40 bp. Overgo primers were designed based on the coding sequence of the 26 chemokine cDNAs (Table 1). The overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/) with modifications (Bao et al. 2005, 2006b; Xu et al. 2005). Briefly, overgo primers of 24 bases were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, TX). Initially twenty-six overgos were pooled together. Overgos were labeled with 32P-dATP and 32P-dCTP (Amersham, Piscataway, NJ) according to Ross et al. (1999) at room temperature for 1 h in 40 µl reaction containing the following: 0.4 µl bovine serum albumin, 8 µl overgo labeling buffer (250 mM Tris, pH 8.0, 25 mM $MgCl_2$, 0.36% 2-mercaptoethanol, 1 mM dTTP, 1 mM dGTP, 1 M Hepes–NaOH, pH 6.6), 2 µl overgo primer mix, 1.5 µl 32P-dATP, 1.5 µl 32P -dCTP, 10 U Klenow polymerase (Invitrogen), and water to bring the volume to 40 µl. 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 hybridization 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 from the channel catfish BAC library. Approximately 200 positive BAC clones were identified through the hybridization of overgos for the 26 catfish CC chemokines. 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

Table 1 Overgo probes for BAC hybridization

Gene	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

duplicate on the membrane and allowed to dry. The membranes were placed in a dish containing 3M Whatman paper saturated with 10% SDS for 3 min, transferred to a second tray containing 0.5N NaOH, 1.5 M NaCl for 8 min without 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 cross-link function (120,000 $\mu J/cm^2$ for 25 s).

Probes based on catfish CC chemokine cDNAs were prepared from previously cloned plasmids. Probes were prepared using the random primer labeling method (Sambrook et al. 1989) 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 solution, 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.

Fluorescent fingerprinting and BAC contig construction

Positive BAC clones were fingerprinted and assembled, where possible, using the protocol described by Luo et al. (2003) with modifications. Briefly, BAC DNA was simultaneously digested with four 6-base pair (bp) recognizing restriction endonucleases (*Eco*RI, *Bam*HI, *Xba*I, *Xho*I) generating 3′ recessed ends and one 4-bp recognizing restriction endonuclease (*Hae*III) generating a blunt end. Each of the four recessed 3′ ends of restriction fragments was filled in using DNA polymerase with different fluorescent dyes using the SNaPshot kit (Applied Biosystems). Such labeling reactions allowed labeling of four sets of restriction fragments, providing a high level of confidence for contig assembly. Restriction fragments ranging from 50 to 500 bp were sized by an ABI PRISM 3130 XL automated sequencer producing *.fsa files. Genoprofiler (You and Lou 2003) converts *.fsa files to *.sizes files which can be utilized by FPC (Soderlund et al. 1997, 2000) for contig assembly. A 0.2–0.4 bp tolerance range was used in FPC, keeping the probability of coincidence (Sulston score, Sulston et al. 1988; 1989) low to avoid false assembly. A P value of 10^{-10} was used for contig assembly. The results of BAC fingerprinting from FPC are image files of each BAC contig. Data from contig assembly were used with previous hybridization data to obtain the patterns of CC chemokine genes. Fingerprinting does not allow the user

to discern the order of genes within each contig; therefore, the order of genes was arbitrarily assigned. Each fingerprint contig or singleton should represent a different genomic region based on its restriction pattern. In order to assess the reliability of the BAC contigs, we conducted two types of analyses. First, overgo hybridization and cDNA hybridization data were carefully analyzed to match the contigs assembled from fingerprinting; second, cutoff *P* values were varied, using a range of *P* values (10^{-10}, 10^{-8}, 10^{-6}, and 10^{-2}), in order to see how that would affect the contig assembly. Only by increasing the *P* value for assembly to 10^{-2}, an unacceptably low standard for assembly, are any of the contigs or singletons merged together. In cases where the combination of contig assembly and hybridization suggested the presence of multiple gene copies, the letters from A to F were assigned to differentiate between distinct genomic copies of the catfish CC chemokines.

Fish rearing, bacterial challenge and sampling

Channel catfish 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. Briefly, catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from a natural outbreak in Alabama (outbreak number ALG-02-414) and inoculated into brain heart infusion (BHI) medium and incubated in a shaker incubator at 28°C overnight. The bacterial concentration was determined using colony forming unit (CFU) per milliliter 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/l. A sham challenge was conducted the same way as the challenge except that no bacteria were added. After 2 h of immersion exposure, 15 fish were randomly taken and placed into a rectangular trough containing pond water with constant water flow throughout. Replicates of troughs were used to provide one trough for each sampling time point. For the control fish, 15 fish were incubated in a separate rectangular tank with the same fish density as the challenge tanks. However, due to facility limitations, control samples were only collected after the sham challenge. An additional trough was used to incubate the sham-challenged control fish till the conclusion of the challenge experiments 6 days after challenge. While clinical signs of enteric septicemia of catfish (ESC) were highly evident from challenged fish starting the third day after challenge including signs of hemorrhage, the sham-challenged control fish showed no clinical signs of the disease. Mortality of challenged fish started after 78 h after challenge and reached approximately 70% while the survival rate of the sham-challenged fish was 100% 6 days after sham challenge.

After challenge, head kidney and spleen samples were collected at 4 h, 24 h, and 3 days. At each time point, ten fish were sacrificed for sampling. The fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg/l before tissues were collected. Tissues were collected and snap frozen with liquid nitrogen, and kept in a −80°C ultra-low freezer until preparation of RNA. Because the tissues of the experimental fish were quite small, pooling of samples was necessary to provide sufficient tissue samples for use in validation of various immune-related genes. Samples of each tissue from ten fish were pooled at the stage of tissue samples to reduce the number of RNA extractions and costs. Tissue samples were carefully collected such that weight variation was minimal. 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.

RNA isolation and RT-PCR

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 −70°C freezer until used as template for reverse transcriptase PCR (RT-PCR). Total RNA was used for reverse transcriptase-PCR (RT-PCR) reactions. The RT-PCR reaction was conducted using two-step RT-PCR. RT was conducted by using M-MuLV reverse transcriptase (New England Biolabs). RT reactions were conducted in 40 µl containing 4 µg DNase I-treated RNA, 4 µl (40 µM) oligo dT primers, 8 µl (2.5 mM each) dNTPs, 1 µl RNase inhibitor, 1× RT reaction buffer, and 200 U of RT. Detailed procedures followed the instructions of the manufacturer. After RT reaction, 1 µl of the RT products was used as templates for PCR using JumpStart Taq polymerase (Sigma, St. Louis, MO). The reactions also included the primers of *β*-actin (Table 2), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: denaturation at 94°C for 3 min followed by 32 cycles of 94°C for 30 s, 56°C for 15 s, 72°C for 40 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 1.0% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., CA). Relative expression level was inspected visually and assessed by using the GelExpert program of the Gel Documentation System. 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.

Table 2 Primers for PCR and RT-PCR

Gene	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

Phylogenetic analyses and comparative genomics

Phylogenetic trees were drawn from ClustalW (Thompson et al. 1994) 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.

Comparisons of genomic organization and architecture of the CC chemokines among catfish, chicken, and humans were made with the aid of BLAST searches, phylogenetic sequence comparisons, and searches against the Ensembl genome browsers for human and chicken.

Results

Mapping catfish CC chemokine genes to BACs

We previously identified a total of 26 CC chemokines in catfish through the analysis of ESTs (He et al. 2004; Peatman et al. 2005) named SCYA101-SCYA126. In order to map these chemokine genes to BACs, overgo probes were designed based on the cDNA sequences and used to screen high-density BAC filters. Initially, pooled overgo probes for the 26 CC chemokines were used in the first screening that resulted in the identification of a pool of potential BACs positive to CC chemokine probes. The positive pool of BAC clones was picked from the arrayed BAC library and re-arrayed to nylon membranes for confirmation using individual cDNA probes. cDNA probes for each CC chemokine were used to screen the positive BACs.

As shown in Table 3, use of 26 cDNA probes in separate hybridizations resulted in 232 cumulative positive BAC hits for the catfish chemokine genes. The hybridization pattern, however, indicated that many of the chemokine probes had positive results on the same BAC clones. Considering these overlaps, only 92 distinct BAC clones were represented in the positive set. This pattern of distinct cDNA probes hybridizing to the same BAC clones strongly suggested the presence of clusters of catfish CC chemokine genes in the genome context.

Genomic clustering and duplication of catfish CC chemokine genes

Given the likelihood of genomic clustering of CC chemokine genes within the channel catfish genome, we utilized our pool of positive BAC clones (Table 3) for analysis using fluorescent fingerprinting to determine genomic copy numbers and cluster membership. By comparing cDNA hybridization results with fingerprint information, putative copy numbers were determined. For instance, SCYA102 cDNA hybridized to ten BAC clones (Table 3), and these ten clones fall into two

Table 3 Mapping of catfish CC chemokine genes to BACs through cDNA hybridization

Genes	Positive BAC clones
SCYA101	105_D15, 006_I13, 028_G4, 025_A20, 067_J3, 026_K13, 050_J5, 051_D5, 115_I22, 167_G22, 090_M4, 007_C11, 003_N13
SCYA102	104_A3, 088_M10, 164_N20, 044_A24, 069_N2,108_I9, 126_K10, 117_D24, 149_M19, 011_N1, 125_O17
SCYA103	039_K13
SCYA104	152_F2, 122_C9, 147_M12, 062_A9, 141_G12, 015_J14, 069_A16, 066_B19, 082_A13, 091_H12, 159_B7
SCYA105	50_J5
SCYA106	029_L5, 080_O10, 143_I8, 097_I13, 161_K1, 103_L4, 189_G23, 129_N10, 098_H1
SCYA107	006_I13, 067_J3, 050_J5, 090_M4, 007_C11, 003_N13, 184_M14, 071_C6
SCYA108	042_A8, 052_C23, 149_I8
SCYA109	029_L5, 080_O10, 143_I8, 097_I13, 161_K1, 103_L4, 189_G23, 129_N10, 098_H1
SCYA110	030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 119_E18, 179_H22
SCYA111	030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 121_I22, 129_P14, 119_E18
SCYA112	105_D15, 006_I13, 067_J3, 026_K13, 167_G22, 090_M4, 007_C11
SCYA113	037_D15, 052_F9
SCYA114	090_M4, 164_N20, 044_A24, 069_N2, 126_K10, 117_D24, 009_P8
SCYA115	105_D15, 099_C4, 179_H22, 006_I13, 028_G4, 025_A20, 067_J3, 026_K13, 050_J5, 051_D5, 115_I22, 167_G22, 090_M4, 007_C11, 003_N13
SCYA116	006_I13, 067_J3, 050_J5, 003_N13, 184_M14
SCYA117	105_D15, 030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 045_L17, 105_B8, 144_H14, 107_K11, 102_J7, 153_I24, 154_F9, 163_F4, 072_K10, 125_D4, 073_P7, 121_I22, 065_H1, 061_G20, 129_P14, 059_H18, 119_E18, 062_I3, 167_E6, 045_09
SCYA118	067_J3, 090_M4, 003_N13, 184_M14
SCYA119	104_A3, 088_M10, 164_N20, 044_A24, 069_N2, 108_I9, 126_K10, 117_D24, 149_M19, 009_P8, 049_P12
SCYA120	152_F2, 122_C9
SCYA121	105_D15, 030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 107_K11, 102_J7, 153_I24, 154_F9, 163_F4, 072_K10, 125_D4, 073_P7, 121_I22, 065_H1, 061_G20, 129_P14, 059_H18
SCYA122	030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 102_J7, 129_P14, 119_E18, 062_I3
SCYA123	042_A8, 152_F2, 122_C9, 029_N24, 147_M12, 052_C23, 149_I8, 143_P9, 139_D5
SCYA124	152_F2
SCYA125	031_N17, 042_B8, 188_D18, 192_L24, 163_G22, 158_L9
SCYA126	099_C4, 142_A8, 045_L17, 105_B8, 144_H14, 102_J7, 121_I22, 129_P14, 119_E18, 062_I3 047_K12

A total of 92 unique BACs are represented in a cumulative total of 232 positive clones

fingerprint contigs and a singleton (Table 4), suggesting the presence of three separate copies from different genomic loci.

The fingerprinted contigs and singletons (those BAC clones that did not assemble with others) are listed in Table 4. A total of 18 contigs were constructed after BAC fingerprinting, and an example of the contigs is shown in Fig. 1. Eight BAC clones for which we had hybridization data were not assembled into contigs and are listed as singletons at the bottom of the table. A pattern of gene duplication and clustering was immediately obvious from the merged data from fingerprinting and hybridization. Only five CC chemokines, SCYA103, SCYA105, SCYA108, SCYA113, and SCYA124 were present in a single copy. Five CC chemokines have at least two copies in the catfish genome-SCYA110, SCYA111, SCYA116, SCYA118, and SCYA125. Three genomic copies were found for eight of the catfish CC chemokines including SCYA102, SCYA104, SCYA106, SCYA109, SCYA119, SCYA120, SCYA122, and SCYA126. Four copies were found for five of the catfish CC chemokines including SCYA101, SCYA112, SCYA114, SCYA115, and SCYA126. Two CC chemokines, SCYA121 and SCYA123, had five genomic copies. Lastly, six distinct genomic copies were found for SCYA117 (Table 4).

Eighteen of the fingerprinted BAC contigs or singletons contained more than one catfish CC chemokine gene, illustrating extensive and repetitive genomic clustering. Clusters of genes ranged in size from containing eight genes (Contig 7) to containing only two (numerous contigs). Membership within the different contigs was often highly similar or identical, suggesting that segmental gene duplication was likely responsible for the genesis of many of these clusters. For example, there are three contigs containing SCYA106 and SCYA109, each in a different contig. Likewise, contigs 11 and 12 share identical members with the exception of SCYA115. Several of the catfish CC chemokines, such as SCYA117 and SCYA121, are present in both the smaller contigs (i.e. Contig 10) and the larger clusters (i.e. Contigs 9,11), indicating possible genomic rearrangements.

Genomic architecture and phylogenetic analysis

Genomic sequencing allowed us to previously obtain the encoding genes for 23 of the 26 catfish CC chemokine cDNAs (Bao et al. 2006a). When the deduced amino acid sequences of the coding regions of the 23 genes and the three cDNAs were subjected to phylogenetic analysis, and sequence similarity compared with genomic

Table 4 Contigs and singletons produced by fluorescent fingerprinting of catfish BAC clones

Contigs	Chemokines together based on fingerprinting and/or cDNA hybridization	BAC clones in each contig/singleton
1	125A	163_G22, 055_F15, 135_J15, 045_I14, 003_B23, 015_L09, 013_M08, 190_O24, 186_O14, 103_I22, 163_G22, 136_B14, 080_J22, 139_A20, 082_G20, 176_O19
2	103	039_K13, 183_K11, 151_B13, 064_C5, 068_K21, 127_B21, 182_I19, I82_K19, 120_O3, 071_O4, 110_J16, 176_K21
3	113	037_D15, 052_F9, 146_N23, 093_C8, 025_C1
4	102A-114C-119A	104_A3, 088_M10, 009_P8
5	102B-114A,B[a]-119B-107A	164_N20, 044_A24, 069_N2,108_I9, 126_K10, 117_D24, 149_M19, 011_N1, 071_C6
6	107B-101A-112A-115A-116A-118A	006_I13, 028_G4, 067_J3, 003_N13, 184_M14
7	107C-101B-112B-115B-116B-118B-114D-105	050_J5, 090_M4
8	101C-112C-115C	025_A20, 026_K13, 051_D5, 115_I22, 167_G22, 007_C11
9	101D-112D-115D-117a-121a	105_D15, 154_F9, 073_P7, 065_H1
10	117B-121B	153_I24, 072_K10, 125_D4, 061_G20, 059_H18, 167_E6, 045_09
11	117C-121C-110A-111A-122A-126A	030_D8, 003_P23, 041_O13, 142_A8, 121_I22, 129_P14, 102_J7, 163_F4
12	117D-121D-110B-111B-122B-126B-115E	099_C4, 149_D11, 045_L17, 105_B8, 144_H14, 119_E18, 179_H22
13	126C	047_K12, 108_F9, 103_G10
14	106A-109A	097_I13, 161_K1, 129_N10
15	106B-109B	029_L5, 080_O10, 143_I8, 103_L4
16	106C-109C	189_G23, 182_C23, 050_E23
17	108–123A	042_A8, 052_C23, 149_I8, 029_N24, 143_P9, 139_C13
18	104A-123B	147_M12, 062_A9, 141_G12, 015_J14, 069_A16, 066_B19, 082_A13, 091_H12, 159_B7
Singletons		
1	104B-120A,B[a]-123C	122_C9
2	104C-120C-123D-124	152_F2
3	123E	139_D5
4	117E-121E	107_K11
5	117F-122C-126D	062_I3
6	119C	049_P12
7	125B	158_L9
8	102C	125_O17

BAC contigs were constructed using fluorescent fingerprinting with a cutoff P value of 10^{-10}. BAC clones containing CC chemokine genes were initially selected for fingerprinting by pooled overgo probes, and, in most cases, also confirmed by using individual cDNA probes. Assignment of letters A–F to chemokine genes was arbitrary to differentiate between distinct copies of chemokines in different genomic regions

[a]indicates two distinct copies as determined by direct sequencing

location, several interesting patterns emerged (Fig. 2). In several instances there was a high correlation between sequence similarity and genomic architecture. For instance, SCYA123, SCYA108, SCYA124, SCYA120A, and SCYA120B are located together in the genome, and they also share a branch of the phylogenetic tree as analyzed by sequence similarities. A very strong clade containing SCYA111, SCYA121, SCYA117, and SCYA122 is present on the tree, and the four CC chemokines also are found together in Contigs 11–12. Two additional and similar correlations between tree position and genomic architecture can be seen for SCYA106-SCYA109 and for SCYA116-SCYA118-SCYA102-SCYA101-SCYA107-SCYA114A-SCYA114B (Fig. 2). Such correlations provide additional support for the theory that tandem and/or segmental gene duplications were involved in the evolution of the catfish CC chemokine genes.

Expression analysis of catfish CC chemokine genes

We previously reported the expression of 12 catfish CC chemokines (SCYA115-SCYA126) after challenge with

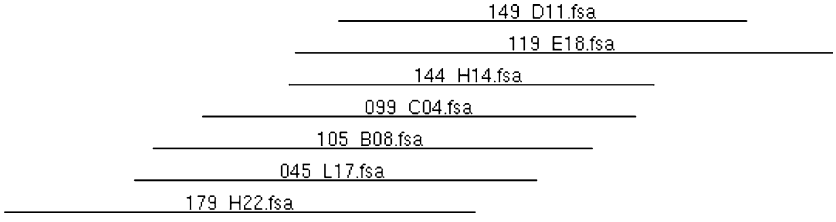

Fig. 1 Example of fingerprinted BAC contigs–contig 12 containing 117D-121D-110B-111B-122B-126B-115E. Identifiers on each *line* are BAC clone names. Note that the contigs do not allow the determination of CC chemokine gene arrangement order

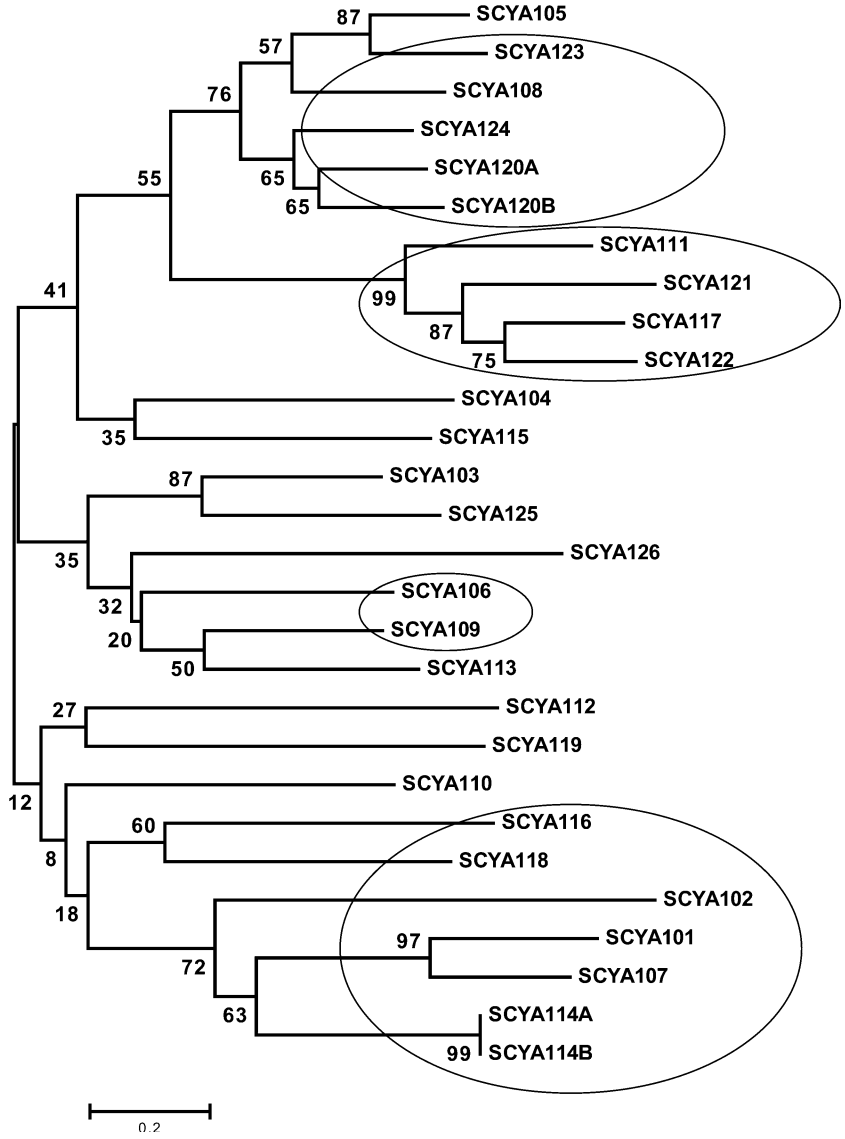

Fig. 2 The phylogenetic tree was drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA (3.0) package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. *Circles* indicate chemokines sharing both sequence similarity and genomic architecture as described in the text. GenBank accession numbers of the sequences used are: DQ173276 (SCYA101), DQ173277 (SCYA102), DQ173278 (SCYA103), DQ173279 (SCYA104), AY555502 (SCYA105), DQ173280 (SCYA106), DQ173281 (SCYA107), DQ173282 (SCYA108), DQ173283 (SCYA109), DQ173284 (SCYA110), DQ173285 (SCYA111), DQ173286 (SCYA112), DQ173287 (SCYA113), DQ173288 (SCYA114A), DQ182570 (SCYA114B), DQ173289 (SCYA115), DQ173290 (SCYA116), DQ173291 (SCYA117), DQ173292 (SCYA118), DQ173293 (SCYA119), DQ173294 (SCYA120A), DQ182569 (SCYA120B), DQ173295 (SCYA121), DQ173296 (SCYA122), CB937548 (SCYA123), DQ173297 (SCYA124), BM028237 (SCYA125), and DQ173298 (SCYA126)

Edwardsiella ictaluri (Peatman et al. 2005). To determine expression patterns of all known catfish CC chemokines, here we conducted expression analysis of the remaining 14 known catfish CC chemokines (SCYA101-SCYA114) using RT-PCR in the head kidney and spleen tissues from both healthy fish and fish challenged with the bacterial pathogen *E. ictaluri*. In order to be able to compare information on expression of all 26 known catfish CC chemokines, we present here the novel expression data combined with previously published expression data on SCYA115-126. As shown in Fig. 3, and summarized in Tables 5 and 6, four main expression patterns were observed. The majority (16) of the 26 CC chemokines were, on the whole, constitutively expressed with no effect observed after bacterial infection (Fig. 3). These included SCYA102, SCYA104, SCYA107, SCYA110, SCYA111, SCYA119, SCYA120, SCYA122, SCYA123, SCYA124, SCYA126, SCYA101, SCYA106, SCYA108, SCYA114, and SCYA103. Of these, SCYA101, SCYA106, SCYA108, and SCYA114 may be

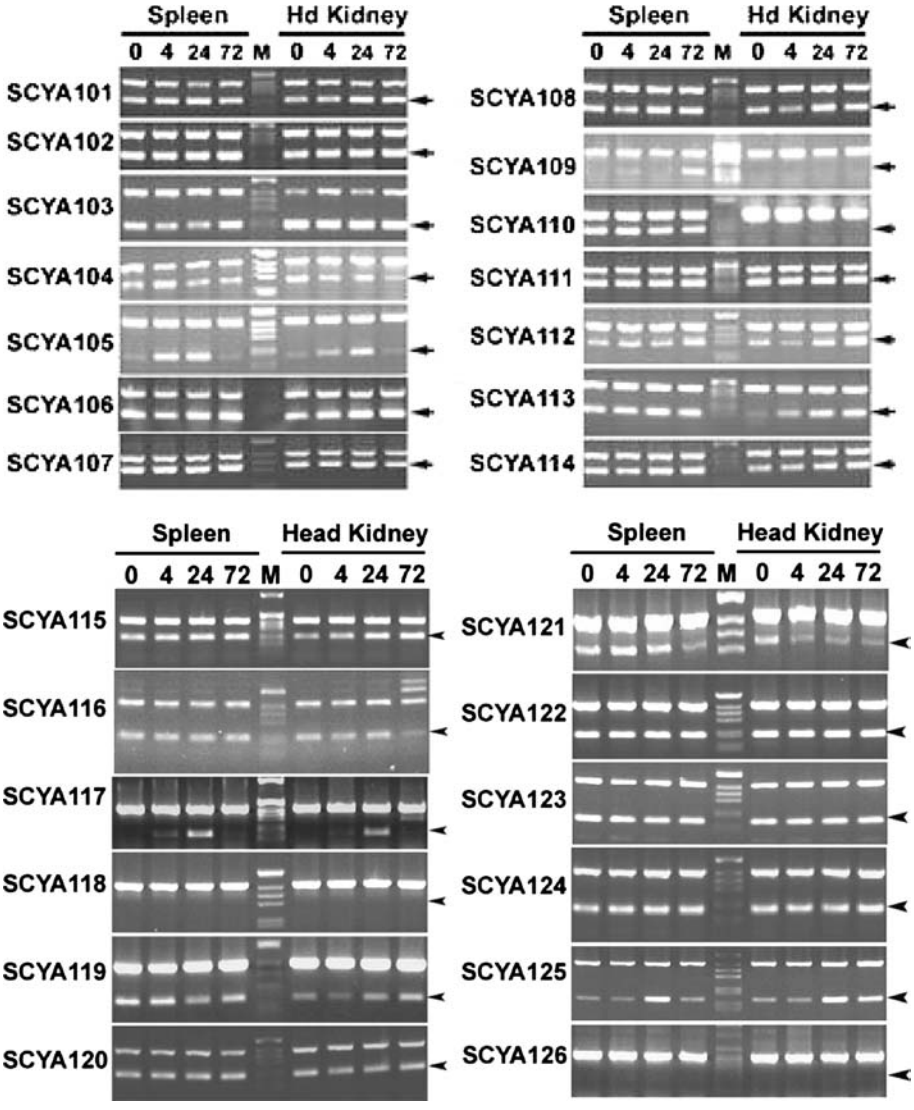

Fig. 3 Expression analysis of the 26 catfish CC chemokines using RT-PCR. RT-PCR reactions were conducted as described in Materials and methods. RT-PCR products were analyzed by agarose gel electrophoresis. Two tissues, spleen and head kidney (*Hd* kidney), were used in the study, as indicated at the *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. The RT-PCR product of the internal control, beta-actin, was not indicated, but in all cases, it was the upper band on the gel. 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

slightly upregulated, and SCYA103 may be slightly downregulated, but the extent of up- or downregulation was minor, and for the purpose of discussion here, we categorize them into the constitutively expressed group. In cases of SCYA114 and SCYA120, paralogous copies were identified. The RT-PCR primers should amplify both gene copies.

Seven of the 26 CC chemokines were upregulated upon bacterial infection (Fig. 3 and Table 5). These included SCYA105, SCYA109, SCYA112, SCYA113, SCYA115, SCYA117, and SCYA125. Of these upregulated CC chemokines, the most interesting were SCYA105, SCYA109, and SCYA117, which were expressed at very low levels before infection, but their expression was dramatically induced after challenge (Fig. 3, Peatman et al. 2005).

SCYA116 and SCYA121 were downregulated upon bacterial infection (Fig. 3 and Table 6). The downregulation was more evident with SCYA121, which showed a significant reduction of RT-PCR products in both the head kidney and spleen, but the response was more rapid in head kidney than in spleen (Fig. 3). SCYA116 expression was lower 3 days after infection; extra bands were detected using RT-PCR, possibly from unspliced products. With SCYA118 and SCYA126, no expression was detected at any time point of the analysis. In order to confirm the lack of expression for SCYA118 and SCYA126, PCR amplification was repeated with extended cycles, but no products were observed.

Differences were observed in the time points and tissues involving upregulation and downregulation. For instance, SCYA105, SCYA117 and SCYA125 were rapidly and highly induced after bacterial infection in both the spleen and the head kidney tissues, whereas SCYA109 was only induced in spleen, but no expression was detected from head kidney. In contrast, SCYA115 was moderately upregulated only in the head kidney, but not in the spleen. SCYA121 expression was downregulated in both spleen and head kidney tissues, but more rapidly in head kidney (Fig. 3).

Table 5 Upregulated CC chemokines

	Spleen			Head kidney		
	4 h	24 h	72 h	4 h	24 h	72 h
SCYA105	+ +	+ + +	NC	+ +	+ + +	NC
SCYA117	+ +	+ + +	+	+ +	+ + +	+
SCYA109	+	+	+ + +	0	0	0
SCYA112	NC	NC	+ +	NC	NC	+ +
SCYA113	NC	NC	NC	+	+ +	+ + +
SCYA115	NC	NC	NC	NC	+	+ +
SCYA125	NC	+ +	+	NC	+ +	+

NC denotes no change in expression; 0 indicated no expression detected; "+" indicates slightly up, "+ +" indicates intermediately up; and "+ + +" indicates greatly up. All comparisons of expression levels are within each individual gene and not among the other genes

Table 6 Downregulated CC chemokines

	Spleen			Head kidney		
	4 h	24 h	72 h	4 h	24 h	72 h
SCYA116	NC	NC	NC	NC	NC	–[a]
SCYA121	NC	NC	–	–	–	–

All comparisons of expression levels are within each individual gene and not among the other genes
[a]indicated the presence of additional PCR bands for SCYA116

Discussion

In this study, all 26 previously identified catfish CC chemokine cDNAs were mapped to BAC clones, setting the foundation for comparative genome analysis in the genomic regions containing chemokine genes. Through a combination of cDNA probe hybridizations and fluorescent fingerprinting, 18 fingerprinted contigs were assembled from BACs containing catfish CC chemokine genes. The catfish CC chemokine genes were found to be not only extensively clustered in the catfish genome, but also highly duplicated at various levels. As many as six copies of a single catfish chemokine were found from separate genomic regions. Although a draft genome is not available for catfish, our approach allowed us to study the local genomic architecture of the catfish CC chemokines in order to better understand the origins and orthologies of these important immune molecules. With genome-enablement still years away in many economically important species, our methods may serve as an important model for researchers working with other similar species who want to harness genome information on a limited budget. Here we have also analyzed the expression of the transcripts of the 26 catfish CC chemokines in head kidney and spleen in response to bacterial infection of *E. ictaluri*, an economically devastating catfish pathogen. Such analysis will allow us to concentrate research efforts on the CC chemokines most likely involved in inflammatory responses.

The clustering of CC chemokine genes on chromosomes was previously revealed in human, mouse, and chicken (Nomiyama et al. 2001; Wang et al. 2005). In humans, the largest group of CC chemokine genes is located on chromosome 17, and several clusters of CC chemokines genes are also found on chromosomes 7, 9, and 16. Chicken has a large cluster of CC chemokines on chromosome 19, with member genes orthologous to CC chemokines on human chromosome 17. A segment of mouse chromosome 11 additionally corresponds to human chromosome 17. In the case of both chicken and mouse, however, syntenies are only partially conserved with humans. For example, chicken has three genes on chromosome 19 corresponding to a single gene, CCL13, on human chromosome 17. Likewise, mouse has two genes CCL9 and CCL6 on chromosome 11 that lack orthologues in human despite conservation of the genomic neighborhood. This phenomenon, coupled with the high sequence similarity between the non-orthologous CC chemokines of a given species, is highly suggestive of a pattern of species-specific gene duplications and changes after species divergence. An expectation of distinct expansions of the CC chemokine family within each species means that identification of orthologues by phylogenetic analysis will be largely unsuccessful. Identification of a smaller ancestral set of CC chemokines and comparisons of genomic organization and architecture across species, therefore, may be more realistic aims for those describing novel sets of chemokines in lower vertebrates.

Duplication of CC chemokines within the human genome, before largely unanalyzed or ignored, has become an important matter for research only lately. The discovery that CC chemokine receptor 5 (CCR5) is an entry point for infection of cells by HIV-1 (Alkhatib et al. 1996), and that CCL3 and CCL4, by binding to CCR5, limit infection by HIV-1 (Nibbs et al. 1999), increased interest in understanding the chemokine repertoire and their functions. More recently, researchers have focused on CCL3 and CCL4 duplications and their correlation in disease severity (Townson et al. 2002). CCL3L1 and CCL4L1 have been discovered in segmental duplications on chromosome 17 (Modi 2004). Of greatest note has been a recent study strongly correlating copy number of a segmental duplication encompassing the gene encoding CCL3L1 with HIV/AIDS susceptibility. Possession of a copy number of CCL3L1 lower than the population average markedly enhances susceptibility (Gonzalez et al. 2005; reviewed by Julg and Goebel 2005). Additional correlations have been made between copy numbers or chemokine loci and other diseases such as tuberculosis (Jamieson et al. 2004). Chemokine architecture and duplication, therefore, is an important matter for investigation in studies of the innate immune components of lower vertebrates. Comparison of the major CC chemokine clusters across vertebrate species may reveal important patterns of divergence or conservation and help to pinpoint similar

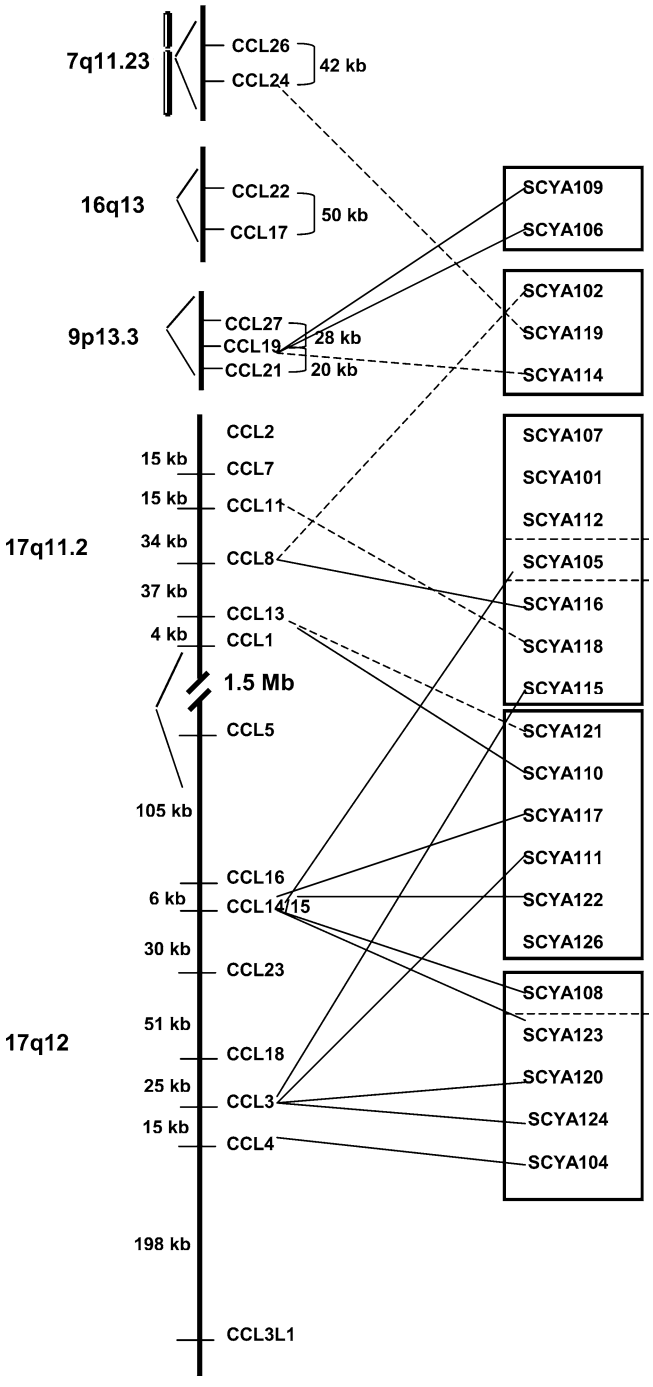

Fig. 4 A comparison of genomic contigs containing catfish CC chemokines with clusters of human CC chemokines on several human chromosomal segments. Chromosomal segment names for human are given on the *left*. Distances between the human CC chemokines are noted in kilo-bases (kb) or mega-bases (Mb). Orientation of the catfish contigs is unknown, and was arranged based on BLASTX identities as described in the text. *Dashed lines* between the catfish and human CC chemokines indicate especially low BLASTX identity. *Boxes* indicate catfish genomic contigs. *Dashed lines* within the catfish contigs surround SCYA105 and SCYA108, whose genomic contigs were merged with those shown to avoid showing duplicate copies in the figure. Note that the order and orientation of individual catfish CC chemokine genes were not determined and their relative positions shown in the contig (*box*) were arbitrary

disease quantitative trait loci (QTL) in agricultural species.

Sequence and phylogenetic analyses are currently not capable of establishing orthologies between the majority of mammalian CC chemokines and fish CC chemokines (He et al. 2004; Laing and Secombes 2004b; Peatman et al. 2005), probably because of the pattern of duplication and divergence described above. Nonetheless, we attempted to match the genomic segments containing catfish CC chemokine genes we obtained through fingerprinting with the largest of the human chromosome cluster of CC chemokines (Fig. 4). It must be noted that we are still missing the larger genomic context of these chemokine-containing contigs. Since only BAC clones positive for CC probes were included in the fingerprinting, we lack the surrounding genomic regions. A physical map of the catfish genome, currently under construction, when linked with available linkage maps, will tell us whether these contigs are distinct contigs on the same chromosome or on entirely different chromosomes. Based on our current knowledge, therefore, we aligned the larger of the catfish clusters with the human chromosomal segments using their top BLASTX identities. Some of the genomic segments of catfish appeared loosely conserved, in that all chemokine gene members shared highest identities with CC chemokines on the same chromosomal stretch in humans (i.e. SCYA108, SCYA123, SCYA120, SCYA124, and SCYA104). Other segments, such as the one containing SCYA107, SCYA101, SCYA112, SCYA105, SCYA116, SCYA118 and SCYA115, showed no discernible pattern of conservation. From this contig, SCYA101 and SCYA107 appear to be fish-specific CC chemokines (He et al. 2004) while SCYA112 shares clear orthology with human CCL20 which is localized on chromosome 2. Other chemokines from this contig share highest identities with CC chemokines on human chromosome 17 (Fig. 4). One notable feature of this "comparative map" was that many of the catfish CC chemokines share highest identity with CCL3 or CCL14. These CC chemokines may represent part of the ancestral repertoire before species-specific duplications and divergence. The human–mouse orthologies, for the most part, were strong and thus the comparison with the mouse was similar to that with the human. In chickens, however, in spite of suggested orthologies (Wang et al. 2005), the statistical support was not strong for the mammal–chicken orthologies regarding the CC chemokine genes.

Sequence similarities between the catfish CC chemokines correlated strongly with genomic architecture (Table 4, Fig. 2) strongly suggesting tandem and segmental gene duplications as the evolutionary mechanism responsible for the diversity of these molecules presently in catfish. The 26 CC chemokine cDNAs with the additional genomic copies revealed by fingerprinting leaves us with a tentative total of at least 75 genes. However, due to the relatively small pool of catfish ESTs in the GenBank, it is not possible yet to provide solid EST evidence for these genes. Using the 26 genes as

queries against catfish ESTs allowed the identification of 186 catfish CC chemokine-related ESTs. Cluster analysis using relatively stringent overlapping (90 bp) allowed us to identify 18 additional CC chemokines with protein sequences different from the queries. Thus, at the molecular level, it appeared that EST evidence as available now supports the presence of additional genes in channel catfish, consistent with our conclusions made from the BAC-based contig analysis. It is noteworthy that BAC-based physical analysis is accurate in answering whether sequences similar to CC chemokine genes physically exist in the catfish genome. Whether such sequences are transcribed requires further analysis using transcriptome approaches. Future work, including BAC sequencing and FISH, will help resolve questions related to the ontogeny of this large catfish gene family.

Most of the inducible/inflammatory human CC chemokines are highly clustered on chromosome 17, while the constitutive/homeostatic CC chemokines are on other chromosomes (Moser et al. 2004). Despite the extensive clustering of the catfish CC chemokines, a similar correlation between inducible expression and genomic architecture was not observed after infection with *E. ictaluri* in head kidney and spleen tissues. Patterns of expression of genes within the same genomic clusters often differed (i.e., SCYA117, SCYA121, SCYA122, SCYA126). It is possible that the highly duplicated nature of the catfish CC chemokine genes has allowed division of roles that may be manifested in spatial, temporal, or functional differences. With the inducible CC chemokines, such as SCYA105, SCYA109, SCYA112, SCYA113, SCYA115, SCYA117, and SCYA125, it is likely that they are involved in the attraction of leukocyte populations to the site of infection. Further functional studies are needed to pinpoint the catfish CC chemokines integral to successful innate immune responses against bacterial and viral pathogens, particularly with regard to their function in directing the leukocyte traffic after infection.

Acknowledgements This project was supported by a grant from USDA NRI Animal Genome Basic Genome Reagents and Tools Program (USDA/NRICGP 2003-35205-12827), in part by E-Institute of Shanghai Municipal Education Commission, Project number E03009, and in part by a Specific Cooperative Agreement with USDA ARS Aquatic Animal Health Laboratory under the Contract Number 58-6420-5-030. We thank Renee Beam, Karen Veverica, Esau Arana, and Randell Goodman for their excellence in the production and maintenance of fish used in this study and their assistance during challenge experiments.

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