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Developmental and Comparative Immunology 29 (2005) 135–142
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with *Edwardsiella ictaluri*

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Received 18 August 2003; revised 16 February 2004; accepted 22 June 2004
Available online 12 August 2004

Abstract

Chemokines are important mediators for innate immunity involved in recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci. While almost all chemokines have been identified from mammals, only a handful of fish chemokines have been identified. Here we report molecular cloning, sequence analysis, and expression of a channel catfish gene resembling interleukin-8 (IL-8). The gene has two alternatively spliced transcripts encoding 114 and 111 amino acids, respectively. The gene has four exons and three introns, typical of the CXC chemokine gene organization. In spite of the structural conservation through evolution, the piscine IL-8 genes showed a much greater sequence divergence than their counterparts among mammals. RT-PCR indicated that both spliced forms were expressed. Expression of the IL-8 like gene was up-regulated 3–5-fold in channel catfish and blue catfish after infection with pathogenic bacteria *Edwardsiella ictaluri*.

Keywords: Interleukin-8; Fish; Cytokine; Catfish; Chemokine; Alternative splicing; Gene

1. Introduction

Chemotactic cytokines attract and activate specific types of leukocytes to the sites of inflammation or injury [1,2]. Based on the arrangement of the first two conserved cysteine residues [3,4], chemokines are divided into four distinct groups: CXC, CC, C, and CX_3C. In humans and mouse, nearly all of chemokines have been identified including 16 CXC, 28 CC, two C, and one CX_3C chemokines [2]. CXC chemokines can be further divided into two subgroups based on the presence of the glutamate–leucine–arginine (ELR) motif preceding the CXC sequence. While the ELR-containing CXC

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doi:10.1016/j.dci.2004.06.011

chemokines like interleukin-8 (IL-8) preferentially attract neutrophils [5–11], the non-ELR-containing chemokines selectively attract T lymphocytes and NK cells [12–15].

IL-8 is produced in response to stimulation by pro-inflammatory cytokines or bacterial lipopolysaccharides, and stimulates target cells by binding to CXCR1 or CXCR2 [16–18]. IL-8 has been cloned from mammals ([19], reviewed in Ref. [2]), chicken [20], and a number of fish including flounder [21], rainbow trout [22,23], lamprey [24], and banded dogfish [25], but has not been reported from channel catfish (*Ictalurus punctatus*). Channel catfish has served as a classical model for the study of comparative immunology [26–32]. Molecular cloning and characterization of its chemokines should help in elucidation of innate immunity in catfish. Here we report molecular cloning and sequence analysis of an IL-8 like gene from channel catfish, and its expression in relation to infection of catfish by *Edwardsiella ictaluri*, the causative agents of enteric septicemia of catfish (ESC).

2. Materials and methods

2.1. cDNA libraries and ESTs

As part of a different project, a cDNA library was constructed from mRNA isolated from the spleen of channel catfish as we previously reported [33]. A total of 1204 ESTs was sequenced from the spleen library. The IL-8 like cDNA clones were initially identified from these ESTs.

2.2. Plasmid preparation and sequencing analysis

For the sequencing of the IL-8 like cDNA clones, plasmid DNA was prepared by the alkaline lysis method using Qiagen's Spin Column Mini-plasmid kit. Three microliters of plasmid DNA (about 0.5–1.0 μg) were used in sequencing reactions. Chain termination sequencing was performed using thermosequenase kit (Amersham, Piscataway, NJ). The PCR profiles were: 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 45 s for 30 cycles. An initial 2 min denaturation at 96 °C and a 5 min extension at 72 °C were always used. Sequences were analyzed on an automatic LI-COR DNA Sequencer Long ReadIR 4200 or LI-COR DNA Analyzer Gene ReadIR 4200. BLAST searches [34] were conducted to determine gene identities. Matches were considered to be significant only when the probability (P) was less than 1×10^{-4} using BLASTX [35]. Sequences of IL-8 related clones were edited using the EDITSEQ program and aligned using the Clustal W method within the MEGALIGN program of the DNAStar package (Lasergene, WI). Complete cDNA sequences for each IL-8 like clone were obtained by sequence analysis using primer walking. Multiple sequence alignments were generated using CLUSTALX (version 1.83). Phylogenetic trees were constructed from CLUSTAL generated alignments using Neighbor-joining method, and displayed using the TREEVIEW program.

2.3. PCR amplification of the genomic segment and sequence analysis

Genomic DNA containing the channel catfish IL-8 like gene was amplified by PCR. Primers were designed from 5′ and 3′ untranslated regions of the cDNA sequences (5′-CACGATGAAGGCTGCAACTC-3′ and 5′-CATTGTGTAACGAACTGTGTG-3′, respectively). The gene was amplified from genomic DNA using 40 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min. The PCR product was purified using a Qiagen PCR purification kit and then subjected to direct DNA sequencing analysis using an ABI PRISM 3100 automatic sequencer at the core facility of Sequencing and Genomics Laboratory at Auburn University.

2.4. Fish and challenge experiments

Two strains of catfish were used: Marion Select of channel catfish and D&B strain of blue catfish. Challenge experiments were conducted as previously described [36]. Briefly, catfish were communally challenged by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri* (outbreak number ALG-02-414). At the time of challenge, the bacterial culture was added to the tank to a concentration of 3×10^7 CFU/ml. After 2 h of immersion exposure, the fish were incubated in rectangular troughs containing pond water with constant water flow through, and their head kidney

tissues were collected at the time of challenge (controls), 24 h, and 72 h after challenge. The experiment involving expression kinetics was conducted by mixing the tissue samples of 10 fish. The tissues were grounded to fine powder and mixed really well before a sample was taken to assess the average of the gene expression.

2.5. RT-PCR and real time RT-PCR

Total RNA was used in a 20-μl reverse transcriptase (RT) reaction containing the following: 5 μg RNA, 1× first-strand buffer (Life Technologies), 10 units of RNase Block Ribonuclease Inhibitor (Stratagene, CA), 500 ng of synthetic oligo $(dT)_{18}$ primer, 0.4 mM each of dATP, dCTP, dGTP, dTTP, and 400 units of the M-MLV reverse transcriptase (Life technologies, Inc., Bethesda, MD). The reaction was incubated at 37 °C for 1 h, then at 70 °C for 15 min to stop the reaction. After the first strand cDNA synthesis, 1/10 volume of the RT reaction (2 μl) was used for PCR amplification using the IL-8 like gene specific primers. The RT-PCR primers were designed from the coding regions of the channel catfish IL-8 like gene (IL-8 upper primer: 5′-CAC--CACGATGAAGGCTGCAACTC-3′; IL-8 lower primer: 5′-TGTCCTTGGTTTCCTTCTGG-3′). The reaction also included the primers of β-actin (upper primer: 5′-AGAGAGAAATTGTCCGTGACATC-3′, and lower primer: 5′-CTCCGATCCAGACAGAG-TATTTG-3′), serving as an internal control. After an initial incubation at 94 °C for 3 min, the RT-PCR was carried out at 94 °C for 40 s, 58 °C for 1 min, 72 °C for 1 min for 25 cycles. Upon the completion of PCR, the reaction was incubated at 72 °C for an additional 10 min. The RT-PCR products were electrophoresed on a 2.0% agarose gel (GIBCOBRL) and documented with a Gel Documentation System (Nucleotech Corp., CA).

Total RNA was used for reverse transcription real time PCR (real time RT-PCR). Concentration and quality of total RNA was determined by spectrophotometry (optical density 260/280 ratio) and electrophoresis. Primers for IL-8 used in real time RT-PCR were as follows: upper 5′-AGGCTG-CAACTCCTAC-3′ and lower, 5′-TTTGAACAG-GAGGCACT-3′. β-actin was used as an internal control for real time RT-PCR. The primers for β-actin real time RT-PCR was the same as for RT-PCR as described in Section 2.5 above. A standard curve was constructed by using various copy numbers of a plasmid containing IL-8 cDNA. Real time RT-PCR reactions of the standard curves were always included in all runs in order to relate quantitative data from run to run. One-step real time RT-PCR was carried out in a LightCycler (Roche Applied Science, Indianapolis, IN) using a Fast Start RNA Master SYBR Green I reagents kit (Roche Applied Science) following manufacturer's instructions with modifications. Briefly, all real time RT-PCR reactions were performed in a 10-μl total reaction volume (9 μl master mix and 1 μl RNA template). A five-step experiment protocol was run on the LightCycler: (i) reverse transcription, 20 min at 61 °C; (ii) denaturation, 30 s at 95 °C; (iii) amplification repeated 50 times, 1 s at 95 °C, 1 s at 55 °C, 13 s at 72 °C; (iv) melting curve analysis, 5 s at 95 °C, 15 s at 65 °C, then up to 95 °C at a rate of 0.1 °C/s; (v) cooling, 30 s at 40 °C. Concentration of cDNA in each sample was calculated from the standard curve. Each sample was normalized to the equivalent of the reference gene, β-actin. The ratio between IL-8 and β-actin was used for the purpose of comparisons.

3. Results and discussion

3.1. Cloning of the catfish IL-8 like cDNAs

Six clones were identified as IL-8 like transcripts through analysis of ESTs using a spleen cDNA library. These clones were then completely sequenced. As summarized in Table 1, the catfish IL-8 like cDNAs included two types of alternatively spliced transcripts; the longer transcript encodes a peptide of 114 amino acids, while the shorter transcript is 9 bp shorter leading to the loss of three amino acids (amino acids 23–25, Fig. 1). Two types of polyadenylated transcripts were observed. In the first type of transcripts (AY140806), poly A tail was added 257 bp downstream of the translation termination codon TGA; while in the second type (AY140803 and AY140804), poly A tail was added 555 bp downstream of the TGA termination codon.

Table 1
A summary of the alternative spliced and alternative polyadenylated transcripts of the catfish IL-8 like gene

Accession number	Characteristics	ORF (amino acids)
AY140806	Poly A at 257 bp downstream of TGA, a poly A signal AATAAA exists 14 bp upstream of poly A	114
AY140803	Poly A at 555 bp downstream of TGA, a poly A signal AATAAA also exists 14 bp upstream of poly A	114
AY140804	Alternative splicing leading to the deletion of three amino acids (23–25), poly A same as AY140803	111
AY140805/AY140802	Partial spliced products	–

3.2. The structure of the catfish IL-8 like gene and sequence analysis

Genomic DNA segment containing the channel catfish IL-8 like gene was amplified using PCR and sequenced. Sequence analysis confirmed alternative splicing and alternative polyadenylation as described above. Comparison of the genomic sequences with cDNA sequences indicated the presence of four exons and three introns, typical of the gene organization of CXC subfamily of chemokines (Fig. 2). Genomic organization of the IL-8 like gene was very similar to other known IL-8 genes with the same exon/intron arrangement. The splice sites have been also well conserved through evolution, especially for the first two introns (Fig. 2). The lengths of exon 1 and exon 2 remained almost identical among the IL-8 genes of human, chicken, rainbow trout, and channel catfish, but the splice sites for intron 3 were more variable.

In spite of the structural and organizational conservation through evolution, great levels of sequence variation existed among the piscine IL-8 genes (Fig. 3). Unlike in mammals where a high level of sequence conservation was found with over 70% identities among human, bovine, swine, and sheep IL-8 (not shown), the similarities among the few known fish IL-8 genes were relatively low (Fig. 3). Overall the fish IL-8 amino acid sequences shared about 30% identity with those of mammals. An interesting observation was that the sequence conservation among the IL-8 genes of fishes was also low [24]. Considering the similar evolutionary distances among the mammals and among the fishes analyzed [37], it appeared that the molecular evolution of fish IL-8 was much faster than that of the mammals. Although IL-8 was a member of the ELR-containing CXC chemokine, ELR motif was not present in any fish IL-8 genes characterized to date [2,21–25].

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aagcttcacgATGAAGGCTGCAACTCTCACAGTGCTGCCCTTGATCATCTTTGCACTGACTGCAATACTTTGTGAAGTTT
          M  K  A  A  T  L  T  V  L  P  L  I  I  F  A  L  T  A  I  L  C  E  V
CTGCAGGATGGGGAGAAGGAAAAGCAGAGCGTTGTTTCTGCCAAAAGAAAGCACTGGAAAAGGTAAGACCTGCACTTGTG
S  A  G  W  G  E  G  K  A  E  R  C  F  C  Q  K  K  A  L  E  K  V  R  P  A  L  V
AAGAAGTTTGAGGTATTCCCTCCAAGTGCCTCCTGTTCAAATACTGAGATCATATTATCTCTGAAGCAAGGAATGAAGGT
 K  K  F  E  V  F  P  P  S  A  S  C  S  N  T  E  I  I  L  S  L  K  Q  G  M  K  V
CTGCCTGGATCCAGAAGGAAACCAAGGACAGAAAGTCTTAACTGGACAGAAACTAAAGATAAAGTCCAAGGGAAGAAGGG
  C  L  D  P  E  G  N  Q  G  Q  K  V  L  T  G  Q  K  L  K  I  K  S  K  G  R  R
GAAAGAAGCAGAAGAAGATCAAACAGCAAAACTGAagtttacagctggagaattctacacatatatgattgctcaaccttt
G  K  K  Q  K  K  I  K  Q  Q  N  *
ttcgcattactgtttcatatgtgattagtcagtcaatataataatctcactgtgctctctgttgtttcaaatgcattact
ttccacagctaaatcacaggccatgaggtatatatgttaactgcatatatataaatgtagtaaaggtgattattgtatgt
gtatcttcttgctcgtatttactctgatttataataaatgattagctatgccaaacactcgcctgaagaaatcatgtatg
tatttgtgtgagcagatgaaacctattctgtctttttttctatcaagtaattataagaaattgagcaatatttttttaatta
cttctaatcaaattacctgacagtgtattaatagataacacacagttcgttacacaatgtataatgatattaatattatg
cacacagtgatactacaaaatgaacatttaacaatagatgttagattgattgactaccggatggattgattgaaagctga
accaaatcaaaataaaaccttaatacagttaaaaaaaaaaaa
```

Fig. 1. cDNA sequence of the catfish IL-8 like gene with deduced amino acids below the nucleotide sequences. The ATG start codon, the TGA termination codon, and the polyadenylation signal AATAAA are bold underlined. The deleted three amino acids in one of the alternative spliced transcripts are shaded.

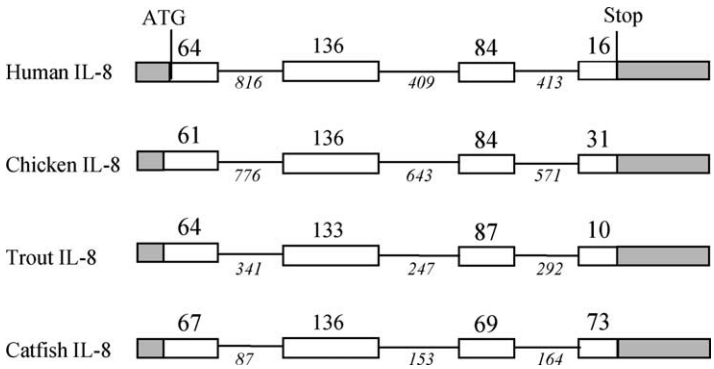

Fig. 2. Genomic structure of the channel catfish IL-8 like gene, compared with those of human, chicken, and rainbow trout. The four exons are indicated by rectangles and three introns by lines. The sizes of exons are indicated above the exons, and the sizes of introns are indicated below the introns. The translation start and termination codons are indicated. Shaded areas indicated untranslated regions (UTRs).

The rainbow trout IL-8 had a similar motif, DLR; in the catfish IL-8 like gene, ER was immediately before the CXC motif, but the leucine residue was not present. If fish IL-8 functions the same as in mammals, then the ELR motif may not be required in fish for the induction of angiogenic, angiostatic, and chemotactic activities [25].

3.3. Phylogenetic analysis

BLASTX similarity searches indicated that the catfish CXC chemokine was most similar to the rainbow trout VHSV-induced protein 7, an IL-8 motif containing CXC chemokine [38], followed by human IL-8 with very similar significance *P*-values. Phylogenetic analysis indicated that the catfish CXC chemokine was most related to the human IL-8 than to any other human CXC chemokine (Fig. 4). However, its concrete identity and orthology should wait as large numbers of fish chemokines are yet to be discovered. Caution should be exercised when assigning orthologies in absence of a large pool of fish chemokines. For instance, a close homologue could be erroneously interpreted as an orthologue today when an even closer homologue is found tomorrow.

3.4. Expression of the catfish IL-8 like gene

The catfish IL-8 like gene expression was analyzed by RT-PCR. As shown in Fig. 5, both alternative spliced forms were expressed, with the shorter transcripts being more abundant. The IL-8 gene

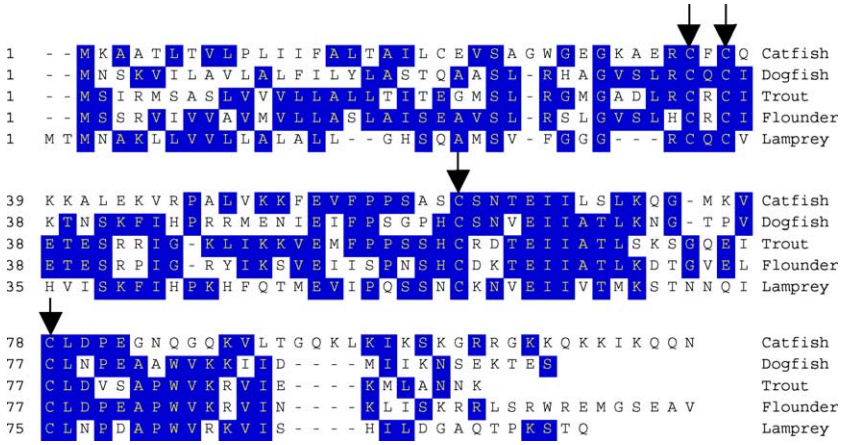

Fig. 3. Comparison of amino acid sequences of the channel catfish IL-8 like gene with IL-8 genes reported from flounder, lamprey, rainbow trout, and banded dogfish. The four conserved cysteine residues are indicated by arrows.

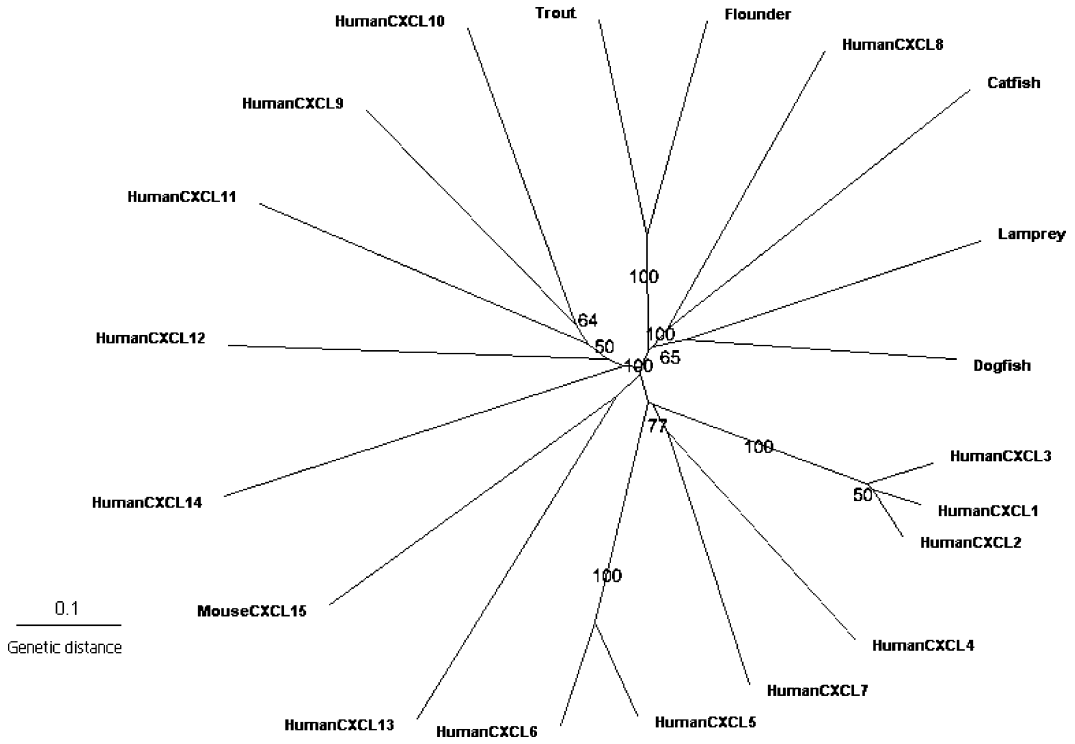

Fig. 4. An unrooted phylogenetic tree constructed with Neighbor-joining method from CLUSTAL generated alignments of all available fish IL-8 sequences and human CXCL1-14, and mouse CXCL15 chemokines. Bootstrap values were derived from 1000 replicates.

expression was detected in the head kidney, spleen, stomach, and gill, but not in the liver, muscle, skin, and heart (Fig. 5).

In order to analyze IL-8 expression following infection in catfish with *E. ictaluri*, IL-8 like transcripts were analyzed by real time RT-PCR. A susceptible strain Marion Select of channel catfish and a resistant strain D&B of blue catfish (*I. furcatus*) were used in the study. As shown in Fig. 6, overall, IL-8 gene expression was induced after challenge with *E. ictaluri*. IL-8 expression was up 3–5-fold in

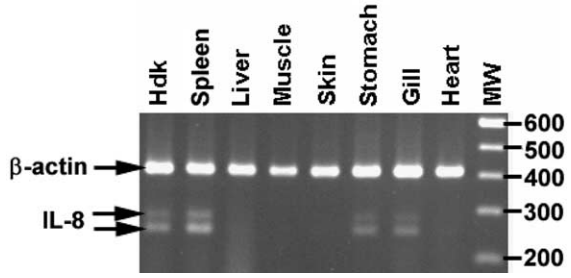

Fig. 5. RT-PCR analysis of the catfish IL-8 like gene expression. Two bands amplified from the alternatively spliced transcripts of the catfish IL-8 like gene are indicated by double arrows. β-actin was used as an internal control. RT-PCR products were loaded from left to right when using total RNA from head kidney (Hdk), spleen, liver, muscle, skin, stomach, gill, and heart. Molecular weight (MW) markers were 100-bp ladders as indicated on the right margin.

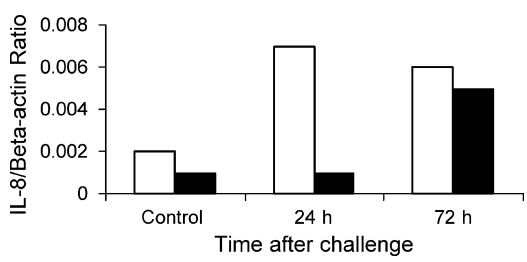

Fig. 6. Changes of IL-8 chemokine mRNA levels in spleen of channel catfish and blue catfish as analyzed by real time RT-PCR. Data is expressed as the ratio of IL-8 RNA and β-actin RNA at various times: control, immediately before challenge; 24 and 72 h after challenge when the tissue sample was collected. Open bar, Marion Select strain of channel catfish (*Ictalurus punctatus*); solid bar, D&B strain of blue catfish (*I. furcatus*).

both strains, but the induction of elevated IL-8 gene expression was detected earlier in the susceptible strain Marion Select than in the resistant strain D&B. This could be in part due to the speed of the disease progression. In the susceptible strain Marion Select, enteric septicemia disease may have progressed more rapidly than in the resistant strain D&B. As IL-8 was known to attract neutrophils to inflammatory sites, the elevated expression of IL-8 after infection of *E. ictaluri*was expected. Induction of another CXC chemokine, CXCL10, was also found after infection of catfish with the same bacterial pathogen during the same period [36]. However, in the case of CXCL10, very different expression profiles were observed between channel catfish and blue catfish [36], in contrast to the situation here with the IL-8 like gene. The expression analysis was conducted with pooled samples of 10 fish. Though pooled samples did not provide opportunities for the analysis of expression variation among individuals, they should represent averages of the expression from the 10 fish individuals used in the study. It should be interesting to assess variations of the chemokine expression among individuals in future studies.

Acknowledgements

This project was supported by a grant from USDA NRI Animal Genome Basic Genome Reagents and Tools Program (USDA/NRICGP 2003-35205-12827), and also in part by a specific cooperative agreement with the USDA-Agricultural Research Service. We appreciate the support of Auburn University Experiment Station Foundation and of Auburn University Office of the Vice President for Research BioGrant Program. We would also like to thank Drs Dongfeng Cao, Arif Kocabas, Attila Karsi, and Zhenlin Ju for their contribution in the EST analysis.

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