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# Alternative complement pathway of channel catfish (*Ictalurus punctatus*): Molecular characterization, mapping and expression analysis of factors Bf/C2 and Df

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ABSTRACT

The complement system is important in both innate and adaptive host defense against microbial infection in vertebrates. It contains three pathways: the classical, alternative, and lectin pathways. Complement component factors B and D are two crucial proteases in the alternative pathway. In this study, the genes of complement factors Bf/C2 and Df from channel catfish, *Ictalurus punctatus* were identified and characterized. Two complement factor B-related genes, Bf/C2A and Bf/C2B, and factor D gene Df were identified. Phylogenetic analysis suggested that Bf/C2A and Bf/C2B is likely orthologous to factor B and factor C2, respectively. Southern blot results suggested that these three genes are all single-copy genes in the catfish genome. The catfish Bf/C2A, Bf/C2B and Df genes were genetically mapped on linkage group 3, 20 and 29, respectively. Bf/C2A and Bf/C2B are highly expressed in liver and kidney, while Df is highly expressed in gill and spleen. After infection with *Edwardsiella ictaluri*, the expression of Bf/C2A, Bf/C2B and Df genes were found to be remarkably induced in the gill, liver, spleen and kidney at some sampling times, indicating that these three complement factors play a pivotal role in immune responses after the bacterial infection in catfish.



## 1. Introduction

The complement system consists of more than 30 serum and cell surface proteins, and plays a crucial role in both innate and adaptive immune response pathways in vertebrates. Its functions include microbial killing, phagocytosis, inflammatory reactions, immune complex clearance, and antibody production [1]. The complement system can be activated through one or more distinct yet overlapping pathways: the classical, alternative, and lectin pathways (CP, AP and LP). These three pathways merge at a common amplification step involving the formation of key protease complexes, named C3 convertases [C3($H_2O$)Bb] [2]. The C3 convertases promote further generation of C3b and are required for the formation of the C5 convertases (C4b2aC3b), which initiate formation of the membrane attack complex (MAC) [3]. The complement factor B (Bf) and factor D (Df) are important components of the AP. Upon activation of the AP, C3 is hydrolyzed spontaneously to yield C3($H_2O$), which is then capable of interacting non-covalently with Bf. Thereafter, Df present in the plasma cleaves Bf into Ba and Bb fragments, resulting in the formation of the alternative C3 convertase [4]. In mammals, factor B and C2 are serine proteases that act as the catalytic subunits of the C3/C5-convertases in the AP and CP, respectively. Bf and C2 have the same domains: each contains three complement control protein (CCP) modules at the N-terminal, one Von Willebrand factor A (VWA) domain and a serine protease domain at the C-terminal. Linkage analysis showed that Bf and C2 together with C4 gene reside in close proximity within the MHC class III region in mammals [5].

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Molecules similar in sequence to mammalian factor B and C2 have been cloned from several teleost species including medaka (*Oryzias latipes*) [6], zebrafish (*Danio rerio*) [7,8], carp (*Cyprinus carpio*) [9], rainbow trout (*Oncorhynchus mykiss*) [10] and large yellow croaker (*Pseudosciaena crocea*) [11]. Based on the sequence information and structure domains, it was hard to determine whether these molecules were *bona fide* factor B or C2 proteins, so they were described as factor Bf/C2 [12].

Some studies revealed that Bf in the AP is homologous to its counterpart C2 in the CP and the homology extends to gene organization and intron-exon structure, suggesting that the C2 and Bf genes arose by duplication from a common ancestor [13]. After the duplication, the C2 component evolved significantly faster than Bf [14]. C2 has thus far been found only in mammals, while Bf (or Bf/C2) has been reported not only in mammals [15] but also in birds [14], amphibians [16], teleost fishes [6,7,9–11] and some invertebrates, such as sea urchin [17], ascidians [18] and horseshoe crab [19]. Df in teleosts has been purified in trout, carp, zebrafish, medaka, olive flounder (*Paralichthys olivaceus*) and halibut (*Hippoglossus Stenolepsis*) [10,20–22] and. It appears that a kallikrein-like molecule that was cloned in brook trout is highly homologous to the primary sequence of Df [23]. In mammals, Df is the rate-limiting enzyme in the AP of complement activation [24]. So far, multiplication of the complement genes has been found in all bony fish species analyzed. The high diversity of certain complement components like C3 and factor B (Bf) and high AP activity in fish serum compared with mammals, suggesting that AP is very important in the defense system of fish [4,10,25,26].

Although the complement system has been studied extensively in mammals, considerably less research is conducted in lower vertebrates, in particular teleost fish [4]. In teleost fish, most investigations have focused on gene cloning and structural studies of Bf/C2, only in a few studies were conducted on the immune response of Bf/C2 genes. In rainbow trout and large yellow croaker, it was reported that Bf/C2 gene was up-regulated in response to inactivated *Vibrio anguillarum* or live *V. anguillarum* strain [11,27]. In carp, transcriptional upregulation of Bf was observed in the skin following *Ichthyophthirius multifiliis* infection [28]. In zebrafish, Bf and Bf/C2B was up-regulated in fins after infection by immersion with viral haemorrhagic septicemia virus (VHSV), but Bf and Bf/C2B have the different expression patterns in various organs [29]. Much fewer studies have been conducted with Df in teleost fish, particularly unknown with its regulation after bacterial infection.

Channel catfish is not only the top aquaculture species in the United States but also a research model for comparative immunology. Its immune system is among the best characterized in teleost fish [30–42]. However, genes involved in its complement system have not been well studied. Here we report the isolation, characterization, mapping and expression analysis of Bf/C2A, Bf/C2B and Df from the channel catfish.

## 2. Materials and methods

### 2.1. Identification and sequence analysis of Bf/C2 and Df genes

BLAST searches were used to identify partial cDNAs for Bf/C2 and Df using zebrafish Bf, LOC563828 (similar to complement factor Bf/C2B) and Df as queries against the channel catfish expressed sequence tags (ESTs) from previous sequencing efforts [43–45]. All channel catfish ESTs were assembled into contiguous sequences (contigs) using the sequence assembly program CAP3 (http://deepc2.psi.iastate.edu/aat/cap/cap.html). Contigs assembled with CAP3 to Bf/C2 and Df homologous were aligned with the genomic sequence contigs (unpublished) using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) to infer the gene structures. The full-length cDNAs of Bf/C2A, BfC2B and Df were obtained from the genomic sequence. The full-length cDNAs were also obtained by sequencing fragments amplified using primers designed according to the EST contigs. The PCR products were sequenced using the BigDye Terminatorv3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols with modifications [46] on an ABI 3130XL automated DNA sequencer (Applied Biosystems).

For sequence analysis, the Bf/C2A, Bf/C2B and Df amino acid sequences were either identified by simple key word searches, or with BLASTP searches using zebrafish Bf, Bf/C2 and Df amino acid sequences at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Protein sequences retrieved from public database were used for open reading frame (ORF) and domain searches, alignment, and phylogenetic reconstruction. ORF were predicted using Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), signal peptides and functional domains were identified by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de).

### 2.2. Multiple sequence alignment and phylogenetic analysis

Sequences similar to Bf, Bf/C2 and Df retrieved from databases were aligned using ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic trees were constructed using the neighbor-joining method based on the deduced full-length amino acid sequences within the Molecular Evolutionary Genetics Analysis (MEGA 4.0) package [47]. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the trees was evaluated by 10,000 bootstrap replications.

### 2.3. Genetic mapping of Bf/C2A, Bf/C2B and Df genes

To locate the Bf/C2A, Bf/C2B and Df genes on the linkage map of catfish, we searched catfish genomic DNA contigs from our ongoing whole genome sequencing project using Bf/C2 and Df cDNA sequences as queries by *blastn*. Microsatellites associated with these genes were then identified from matching contigs. Microsatellite primers of these three genes were designed using FastPCR program (Table 1). Progenies of a backcross family, F1-2 × Ch-6 were used for constructing a linkage map [48]. A tailed primer protocol [49] was used to amplify microsatellite alleles. The PCR conditions used followed Kucuktas et al. [48]. Polymorphic markers were assigned to the linkage groups using JoinMap 4.0 (Kyazma BV, Wageningen, Netherlands). To know the genes linked with Bf/C2A, Bf/C2B and *Df* genes, all the BAC-end sequences in the contigs including the markers tightly linked with Bf/C2A, Bf/C2B and Df genes were blasted with zebrafish genome sequences using blastx program at NCBI.

### 2.4. Southern blot analysis

To determine the genomic copy number of Bf/C2 and Df genes in channel catfish, Southern blot analysis was conducted as previously described [50]. Briefly, genomic DNA was isolated from three individual adult channel catfish and 10 μg was digested with 30 U of the restriction endonucleases *Eco*RI, *Hin*d III or *Pst* I respectively (New England Biolabs, Beverly, MA) in a 25 μL reaction at 37 °C. The digested DNA samples were electrophoresed on a 0.7% agarose gel with 1× TE buffer. *Hin*d III-digested λ DNA was used as molecular weight marker. The gel was submerged in 0.25 N HCl for 15 min, then in denaturation (0.5 M NaOH, 1.5 M NaCl) and neutralization buffer (0.5 M Tris–HCl, 3.0 M NaCl, pH 7.0) for 30 min, respectively. The DNA was transferred to an Immobilon positively-charged nylon

**Table 1**
PCR primers used in this study.

| Gene | Primer | Forward primer sequence (5′-3′) | Reverse primer sequence (5′-3′) |
|---|---|---|---|
| Bf/C2A | For qRT-PCR | TAGAGCAAGCCTGCGCCTCAC | TAGCATCGGTTGCCACAAGTCC |
| | For southern blot | TGTCCTAATCAGACAGGGGCA | AGTCATGTTGCTGATCCGGAA |
| | For linkage map | CTTTATCAGCCAGGGGTA | CTGTGAATTTGATCCAGAGTC |
| Bf/C2B | For qRT-PCR | TGCAAAGACATTCTTTGCCAGC | TGGAAGGGTACCAGTCCAGTGG |
| | For southern blot | ACTGCTAGCCTTATACGCAAGTTGAG | AGAAACATGCCAAGGCCTGGTA |
| | For linkage map | CCAAGCATTATGCCTTGA | ACTCACCATGCTGAAGT |
| Df | For qRT-PCR | AAACCACGCAAGGACACCTGC | GGTCATAGTGCGGGTGATCCA |
| | For southern blot | AGAGCGATGCAGTGAAGCCTCTG | GAGCTAAAGCAAGTAACCTGTCACACC |
| 18S RNA | For qRT-PCR | GAGAAACGGCTACCACATCC | GATACGCTCATTCCGATTACAG |

membrane (Millipore, Bedford, MA) by capillary transfer for 18 h using 20× SSC buffer. The DNA was fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA) with the auto-crosslink setting. The membrane was hybridized with cDNA probes amplified using primers listed in Table 1. After pre-hybridized for 2 h with 100 μg/ml salmon sperm DNA (Sigma, Santa Clara, CA) and hybridized with a $^{32}$P-dCTP labeled probe at 63 °C for 16 h, the membrane was washed twice with wash buffer 1 (2× SSC, 0.1% SDS buffer) and one time with wash buffer 2 (0.5× SSC, 0.1% SDS buffer) at 60 °C and exposed to X-ray film at −80 °C for 20 h.

### 2.5. Bacterial challenge and tissue sampling

All experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University. Bacterial challenge was conducted as previously described [50]. Channel catfish, with an average body weight of 6.1 g and average body length of 9.5 cm, were kept at 27 °C in a flow-through system utilizing heated, dechlorinated municipal water were used for the challenge. Fish were treated in each of two groups: (1) control group (phosphate-buffered saline, 100 μL PBS (pH 7.4) injected); (2) *Edwardsiella ictaluri* challenged group (injection). To inoculate bacteria for the challenge, a single colony of *E. ictaluri* grown on brain heart infusion (BHI) agar plate was isolated and cultured in BHI broth at 28 °C overnight. The bacterial culture was diluted with PBS, and $1 \times 10^5$ CFU of bacteria in 100 μL PBS were injected intraperitoneally into each fish. Injections were carried out under anesthesia using tricaine methanesulfonate (MS 222) at 100 mg $L^{-1}$.

Before dissection, fish were euthanized by MS 222 exposure at a concentration of 300 mg $L^{-1}$. Samples of 11 tissues including brain, gill, heart, head kidney, trunk kidney, intestine, liver, muscle, skin, spleen and stomach of channel catfish from the control group were dissected and immediately immersed in RNA*later* (Invitrogen, Carlsbad, CA) to determine expression in various healthy catfish tissues. Similarly, gill, liver, spleen, and trunk kidney tissues from 45 fish (3 pools of 15 fish each) at 4 h, 24 h, 3 d and 7 d post-treatment in each group of the bacterial challenge experiment were pooled and stored respectively in RNA*later* (Invitrogen) for RNA extraction. Correspondingly, uninfected control samples were taken at each time interval. All samples were stored at −80 °C until RNA extraction.

### 2.6. Quantitative real-time RT-PCR analysis

Total RNA was extracted using the RNeasy Plus kit (Qiagen, Germantown, MD) following manufacturer's instructions with modifications previously described [50]. First strand cDNA synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc, CA) was used for determination of gene expression by Quantitative real-time RT-PCR (qRT-PCR). qRT-PCR was performed in triplicate using the CFX Real Time PCR Detection System (Bio-Rad) in 10 μl reactions containing the following components: 250 ng of cDNA, 5 μl iQ™ SYBR® Green Supermix (Bio-Rad), and 5 pmol of each primer. The real-time PCR profile was as following: One cycle of 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 58 °C for 5 s, followed by 65–95 °C, 5sec/step. The primers used in qRT-PCR were designed using FastPCR 6.1 software and listed in Table 1. Optimal primer pairs were selected based on their amplification specificity by melting curve analysis. In addition, the amplicons were checked by agarose gel with a 100 bp ladder in order to confirm the correct amplicon sizes. The 18S rRNA gene was used as an internal control for normalization of expression levels [30,32,33,35]. To assess the relative expression of Bf/C2 and Df in various healthy catfish tissues, expression of these genes were arbitrarily presented relative to that in the spleen.

The fold induction of the channel catfish Bf/C2 and Df genes after *ESC* challenge was quantified in the gill, liver, spleen and trunk kidney. Cycle threshold (Ct) values were generated and converted to fold differences by the relative quantification method using the Relative Expression Software Tool 384 v.1 (REST) [51]. The fold change after different treatments was made into a graphical representation; differential regulation was considered significant when $p < 0.05$.

## 3. Results

### 3.1. Identification of the channel catfish Bf/C2 and Df genes

Initially, Bf/C2 and Df cDNAs were identified from the catfish ESTs. *In silico* analysis of extensive EST collections indicated the presence of two distinct clusters of Bf/C2 (herein referred to as Bf/C2A and Bf/C2B) in the channel catfish genome. In order to obtain complete coding sequences, alignments of the EST contigs were made against the partial assembly of the whole genome sequences obtained using a doubled haploid fish [52]. The catfish Bf/C2A gene had 18 exons, while Bf/C2B has 19 exons. The sizes of Bf/C2 exons appeared to be similar among vertebrate genes (Fig. 1a). They have an open reading frame (ORF) of 2247 bp and 2553 bp, and encode for 749 amino acids and 851 amino acids, respectively. Only one transcript contig was assembled from ESTs for Df gene. Its ORF is 750 bp long encoding 250 amino acids. Comparison of the cDNA sequences with the genomic sequences indicated the presence of 5 exons (Fig. 1b). The channel catfish Bf/C2A, Bf/C2B and Df cDNA sequence has been deposited in GenBank with the accession number of JN995600, JN995601 and JN995602.

Analysis of domain regions indicated that Bf/C2A consists of a signal peptide, three complement control protein (CCP) molecules, a von Willebrand factor type A (VWA) domain, and a Trypsin-like serine protease (Tryp_SPc) domain (Fig. 2a). The Bf/C2B has a similar domain with one more CCP domain (Fig. 2b). The Df consists only a signal peptide and a Tryp_SPc domain (Fig. 2c).

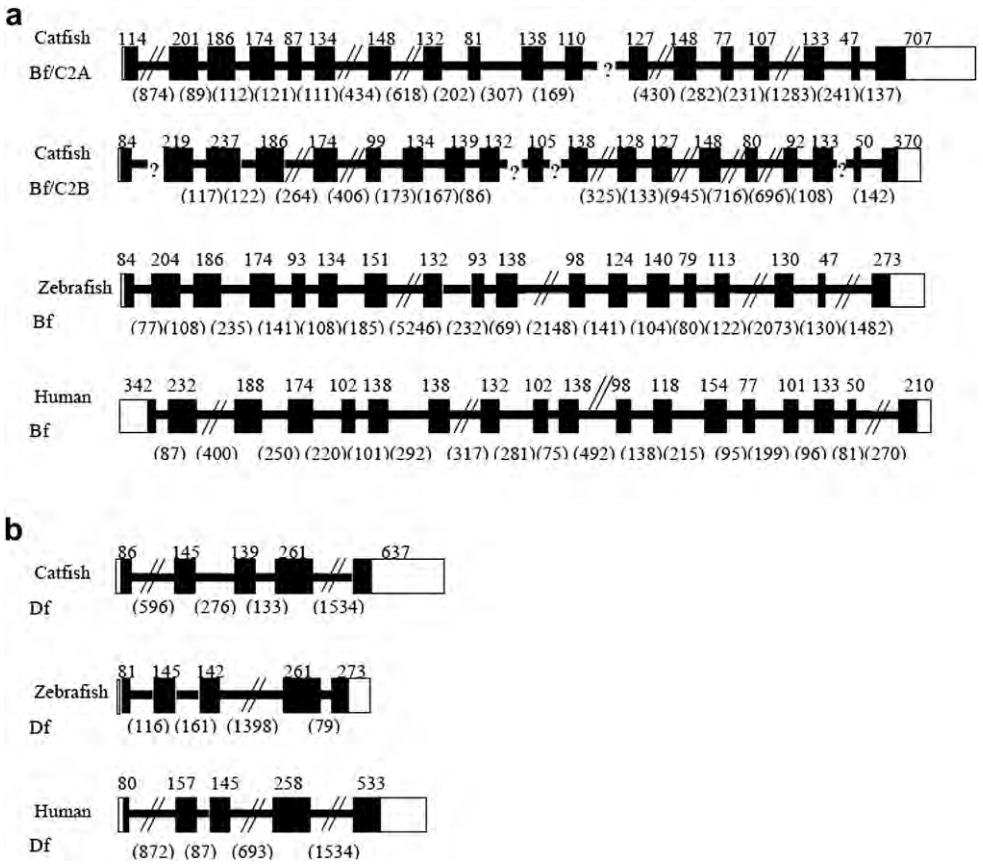



**Fig. 1.** Schematic diagram of complement factor B (a: Bf/C2A and Bf/C2B) factor D (b: Df) gene structures. Exons are represented by black boxes, whereas the white boxes indicate non-coding regions. Values on the top of boxes are the length of the region in base-pairs. The length of introns (in base-pairs) is represented in parentheses below each gene structure. Double slashes indicate non-proportional representation of the introns and question mark indicates unknown intron lengths.

Sequence analysis indicated low similarities between the two catfish Bf/C2 genes, amino acid identities within the ORF was 31%. Similarly, the catfish Bf/C2 genes had low sequence similarities with Bf, C2 and Bf/C2 genes from other mammals and fish, amino acid identities ranged from 32 to 58% (data not shown). The catfish Bf/C2A seemed a little bit more (2%) close to human Bf while the Bf/C2B is equally close to human Bf and C2 (34%). The catfish Df amino acids showed 56–68% identities with those of other teleost fish and 37–39% identities with that of mammals.

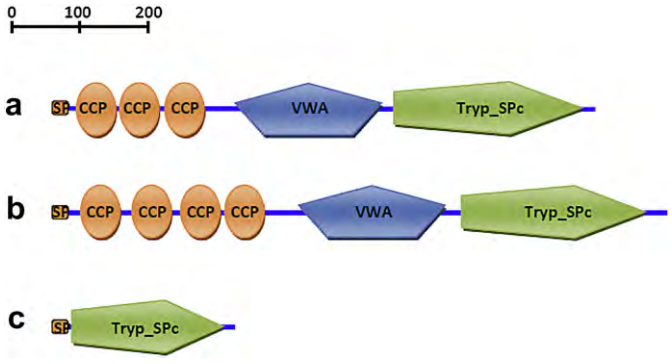



**Fig. 2.** Domain structure of the catfish complement factor B (a: Complement factor Bf/C2A; b: Complement factor Bf/C2B) and factor D (Df). Different shapes represent different domains. SP represents signal peptides, CCP represents complement control protein, VWA represents von Willebrad factor type A domain, Tryp_SPc represents Trypsin-like serine protease. The scale on the top margin is measured by amino acid number.

Phylogenetic analysis was conducted based on amino acid sequences retrieved from the GenBank. The two catfish Bf/C2 sequences fell in different clades: Bf/C2A is more similar to the fish Bf/C2A and mammalian Bf; while Bf/C2B is more similar to that of other fish Bf/C2B and mammalian C2. The catfish Bf/C2 were most closely related to zebrafish, with high bootstrap support (Fig. 3a).

The catfish Df gene is most similar to the zebrafish Df gene and all teleost Df genes fell into the same clade, neighboring with the frog Df gene, which is clustered with the mammalian Df genes' clade first, all consistent with the evolutionary relations (Fig. 3b).

### 3.2. Copy number analysis of the channel catfish Bf/C2B and Df genes

Genomic Southern blot analysis was conducted to determine the copy number of the Bf/C2 and Df genes in the channel catfish genome. As shown in Fig. 4, only a single band was detected for Bf/C2A, Bf/C2B, and Df, respectively, suggesting the presence of a single-copy gene encoding Bf/C2A, Bf/C2B and Df respectively.

### 3.3. Location of Bf/C2 and Df genes

Microsatellites were identified from catfish Bf/C2A and Bf/C2B genes. Genotyping analysis indicated that the gene-associated microsatellites were polymorphic, allowing their mapping genetically by genotyping the mapping resource family. For Bf/C2A and Bf/C2B genes, segregation of the polymorphic microsatellites within the two loci allowed them to be mapped. Bf/C2A and Bf/C2B genes were mapped to genetic linkage group 3 and linkage group 20,

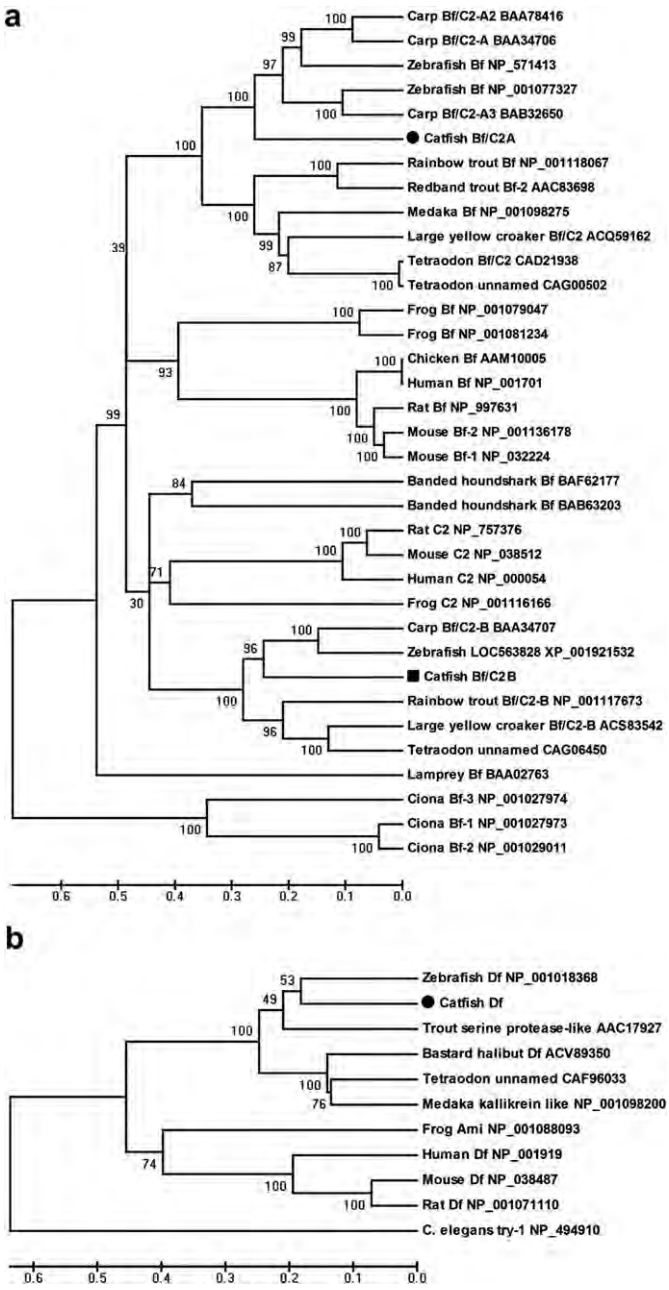



**Fig. 3.** a: Phylogenetic analysis of catfish complement factor B (Bf/C2A and Bf/C2B) with related genes. b: Phylogenetic analysis of catfish complement factor D (Df) with related genes. The phylogenetic trees were constructed using the neighbor-joining method, based on a ClustalW multiple sequence alignment of amino acid sequences. The topological stability of the trees was evaluated by bootstrapping; bootstrap values are indicated by numbers at the nodes. GenBank accession numbers for the retrieved sequences are listed in the figure.

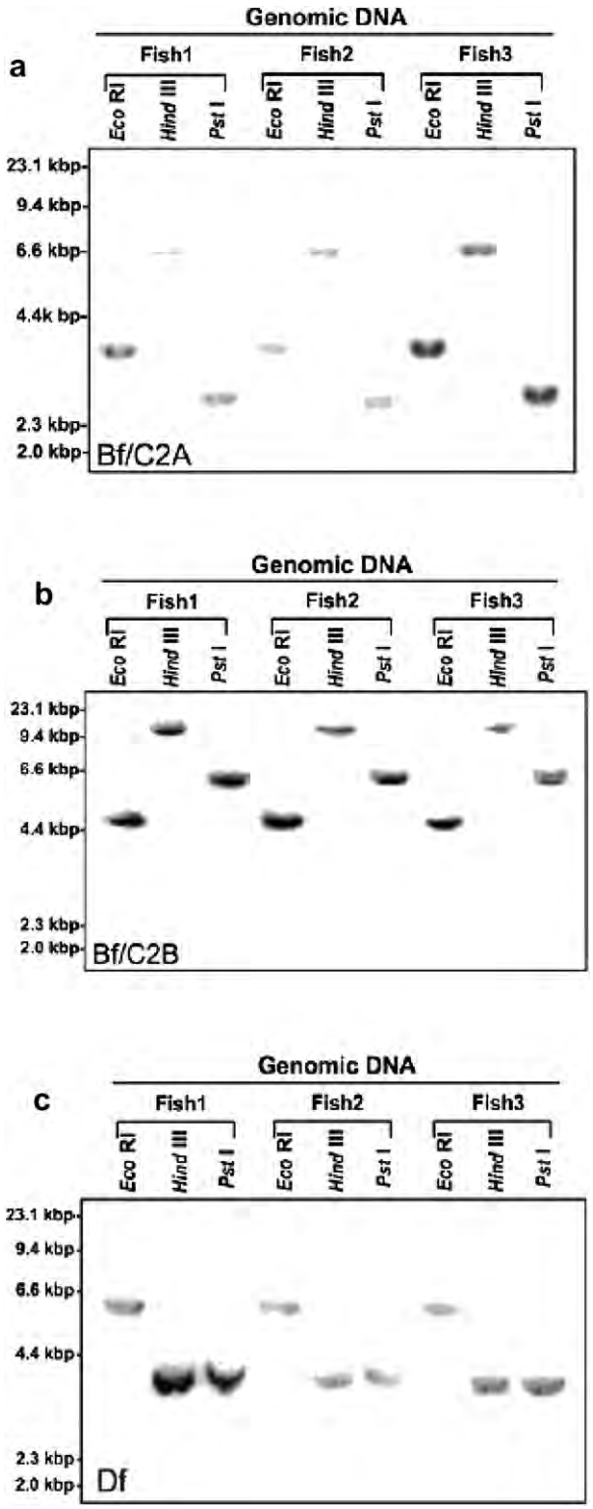



**Fig. 4.** Southern blot analysis of catfish Bf/C2A, Bf/C2B and Df genes using genomic DNA of three channel catfish individuals. DNA markers (kb) are indicated on the left margin.

respectively (Fig. 5). For Df gene, BLAST analysis indicated its physical map location in contig750 of catfish physical map [53]. Mapping of BAC-end sequences associated with the contig allowed its mapping to linkage group 29.

### 3.4. Tissue expression of the channel catfish Bf/C2 and Df genes

qRT-PCR was used to determine tissue expression patterns of the Bf/C2A, Bf/C2B and Df genes in channel catfish. The Bf/C2A was expressed at a very high level in the liver, followed by trunk kidney and head kidney, but at very low levels in the gill and skin, although it was detected in all tested tissues (Fig. 6a). Bf/C2B was detected in all tested tissues, but was also expressed at a very high level in the liver, followed by spleen; much lower levels of expression were detected in other tested tissues (Fig. 6b). In contrast, Df was highly expressed in the gill and spleen, and very low in brain tissues (Fig. 6c).

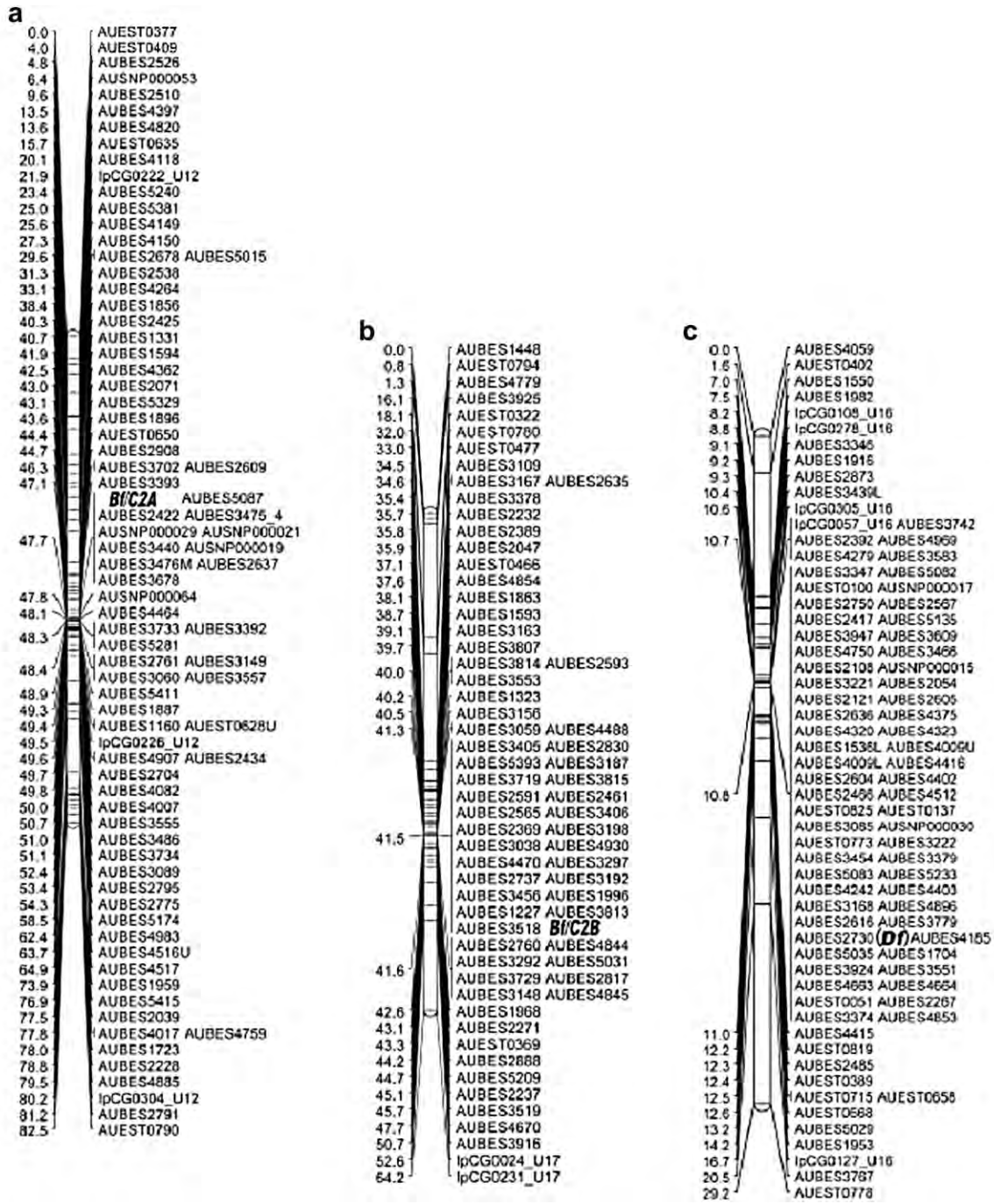



**Fig. 5.** The location of Bf/C2A, Bf/C2B and Df on the linkage groups of the catfish genetic linkage map (a: Linkage group 3; b: Linkage group 20; c: Linkage group 29).

### *3.5. Expression of the channel catfish Bf/C2 and Df genes after bacterial infection*

To investigate the immune response to disease infection with the catfish pathogen and Gram-negative intracellular bacterium *E. ictaluri*, qRT-PCR was used to determine relative tissue expression patterns of the catfish Bf/C2A, Bf/C2B and Df genes in infected gill, liver, spleen and trunk kidney tissues. The gene expression levels after injecting with *E. ictaluri* were apparent in different tissues and at different sampling times (Fig. 7). It is apparent that all three genes were significantly induced in the gill after the bacterial infection, with Bf/C2A gene expression being up-regulated about 7.0 fold at 24 h after infection and Bf/C2B gene expression being up-regulated about 3.0 fold at 4 h and 8.0 fold at 24 h after infection. The induced high expression in the gill did not last at day 3 after infection, and expression of Bf/C2A and Bf/C2B was very low at day 3 after infection (down ~2.8-fold and ~16-fold), and then they were elevated again at day 7 after infection. Df gene was also significantly expressed in the gill, but the induced expression was slow (at day 7 after infection) and much less dramatic (~3.7 fold) as compared with Bf/C2A or Bf/C2B genes (Fig. 7).

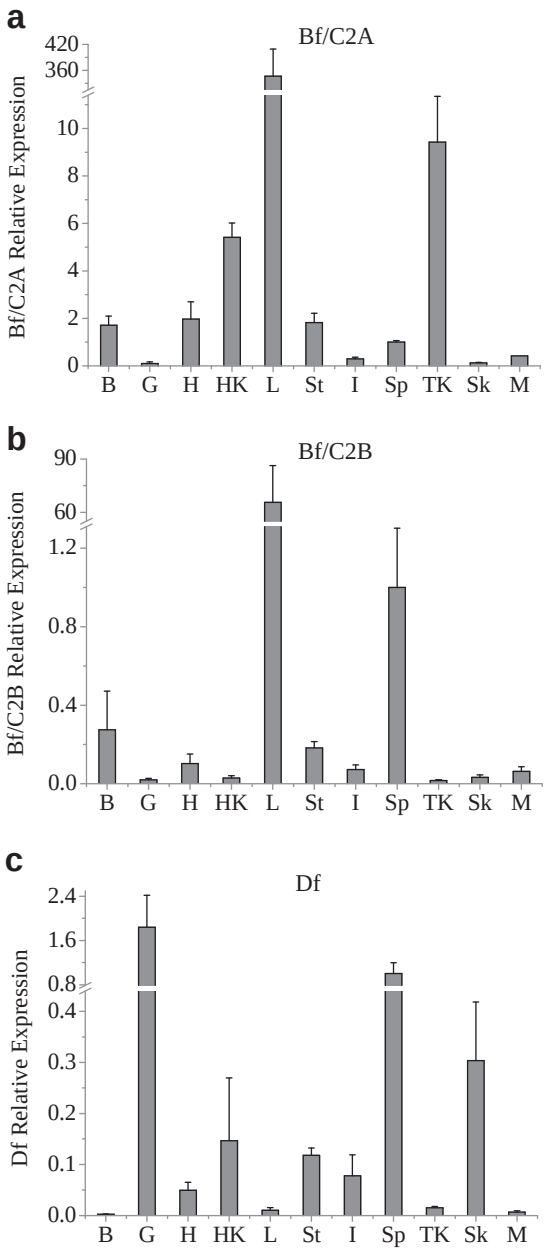



**Fig. 6.** Relative expression of Bf/C2A, Bf/C2B and Df genes in channel catfish determined using qRT-PCR. Tissue RNA samples are labeled along the *X*-axis. Expression levels in all tissues are presented relative to that in the spleen tissue (1×). The tissues are as the following from left to right: B: brain, G: gill, H: heart, HK: head kidney, L: liver, St: stomach, I: intestin, Sp: spleen, TK: trunk kidney, Sk: skin, M: muscle.

In addition to the gill tissue, Bf/C2A was significantly up-regulated in the liver, whereas Bf/C2B was significantly up-regulated in the spleen. No significant differences in expression were observed for any of the three genes in the trunk kidney (Fig. 7).

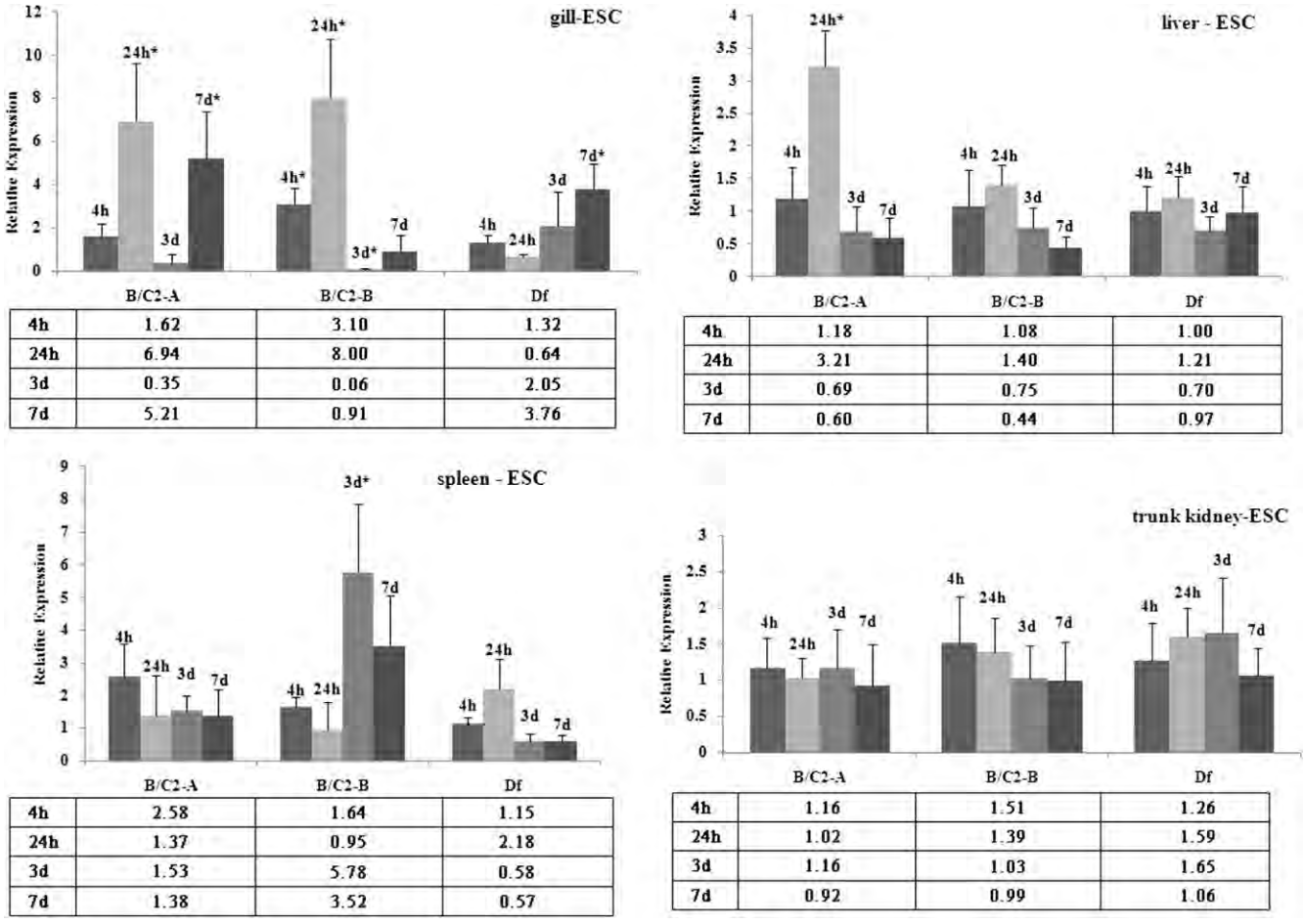
## 4. Discussion

In the present study, we identified and characterized three genes involved in the alternative complement pathway of catfish: two Bf/C2-like (Bf/C2A and Bf/C2B) genes and one Df gene. Their gene structures, organization, sequences, copy number, and genetic linkage map locations were determined; their tissue expression profile in normal tissues and expression profile after bacterial infection were analyzed.

We named the two Bf/C2-like genes as Bf/C2A and Bf/C2B, following the nomenclature of existing literature for teleosts. However, sequence analysis indicated low similarities between the two catfish Bf/C2 genes, only 31% identity at the amino acid level. Clearly, higher levels of similarity even exist between the catfish genes with their phylogenetic counterparts (32–58%). In addition, phylogenetic analysis clearly placed these two genes into two distinct clades, with Bf/C2A falling under the clade containing all the mammalian Bf genes and Bf/C2B falling under the clade containing all the mammalian C2 genes (Fig. 3). This suggested that the teleost Bf/C2A is probably the ortholog of complement factor B; and the teleost Bf/C2B is probably the ortholog of complement factor C2.

In order to determine the orthologous relationships of the catfish Bf/C2A and Bf/C2B genes with those in teleost fish, a comparative genome analysis was conducted. Conserved syntenies were found between catfish and zebrafish. For instance, linkage analysis suggested that catfish Bf/C2A is closely linked with calneuron 1 (AUBES3060), suppressor of Ty 6 (AUBES3060) and ubiquitin specific peptidase 28 (AUBES4464) genes on linkage group 3, and they are also closely linked with Bf gene on the genome sequence assembly of zebrafish. Similarly, catfish Bf/C2B is mapped to linkage group 20, along with sacsin (AUBES3187), transcription elongation regulator foggy and tyrosylprotein sulfotransferase1 (AUBES3719) genes; the same genes were found to be closely linked on the zebrafish genome sequence. For Df gene, the catfish Df was mapped to linkage group 29 and it was found to be tightly linked with a gene named methionyl aminopeptidase 1(AUBES3168), and this linkage was found to be true also in zebrafish, suggesting orthologies as well. In other teleost fish such as trout [10], zebrafish [7], carp [9,54] and large yellow croaker [11], multiple Bf/C2 genes have been reported. It is likely that in these cases as well, the genes actually represented factor B and C2. However, orthologies cannot be established between the teleost genes with the mammalian genes. In mammals, the complement Bf gene is closely linked to the C2 and C4 genes, and resides in the MHC class III region [13]. The amphibian *Xenopus* Bf gene is also linked to the MHC class I or II genes [55]. In contrast, the teleost Bf gene is not linked to the MHC class I or II genes in either zebrafish [56] or medaka. Moreover, the medaka Bf gene is not linked to the C4 gene [57]. This suggested the absence of a linkage between the class III complement genes themselves. In channel catfish, *MHC* and C4 genes are not linked with Bf/C2 genes [58,59].

In mammals, it has been proven by *in vivo* using specific inhibitors and gene-targeted mice that components of the complement system that are unique to the AP are factor B, factor D and properdin [60]. Some studies suggested that AP activation is so important *in vivo* even when initiation appears to depend upon one of the other pathways. In contrast to CP and LP activation, the AP is mainly triggered by the certain structures on microbial surface in an antibody-independent manner. Cleavage of C3b-bound factor B (Bf) by Df leads to the formation of the C3 convertase of the AP [61]. The AP can also be initiated as an "amplification loop" when fixed C3b that is generated by CP or LP activation. The amplification loop is much more robust *in vivo* than previously appreciated. Indeed, an in vitro serum study indicates that under certain experimental conditions, amplification by the AP may account for 80% of the C5a and MAC generated by CP activation, much more than has been thought traditionally [62]. It seems to be generally believed that the AP in fish is more active than its mammalian counterpart [10,25,26]. Moreover, fish AP is reported to be even more active than CP [10,26,63]. The research in the larvae of rainbow trout [64] and zebrafish [65] showed that Bf and Df transcripts representing the AP were expressed at much higher levels than C4 that is central to CP, suggested that the complement operating via the AP is already competent and may be more significant than the CP during




| | B/C2-A | B/C2-B | Df |
|---|---|---|---|
| 4h | 1.62 | 3.10 | 1.32 |
| 24h | 6.94 | 8.00 | 0.64 |
| 3d | 0.35 | 0.06 | 2.05 |
| 7d | 5.21 | 0.91 | 3.76 |

| | B/C2-A | B/C2-B | Df |
|---|---|---|---|
| 4h | 1.18 | 1.08 | 1.00 |
| 24h | 3.21 | 1.40 | 1.21 |
| 3d | 0.69 | 0.75 | 0.70 |
| 7d | 0.60 | 0.44 | 0.97 |

| | B/C2-A | B/C2-B | Df |
|---|---|---|---|
| 4h | 2.58 | 1.64 | 1.15 |
| 24h | 1.37 | 0.95 | 2.18 |
| 3d | 1.53 | 5.78 | 0.58 |
| 7d | 1.38 | 3.52 | 0.57 |

| | B/C2-A | B/C2-B | Df |
|---|---|---|---|
| 4h | 1.16 | 1.51 | 1.26 |
| 24h | 1.02 | 1.39 | 1.59 |
| 3d | 1.16 | 1.03 | 1.65 |
| 7d | 0.92 | 0.99 | 1.06 |

**Fig. 7.** Fold induction of channel catfish Bf/C2A, Bf/C2B and Df genes after *E. ictaluri* treatment in the liver, gill, spleen and trunk kidney. Relative gene expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Asterisks indicate statistical significance at the level of $P < 0.05$ relative to appropriate control.

embryonic and post-hatch development. Though these research results were not all consistent by some studies [66,67], we can see the importance of AP in fish and some research results and deductions still need to be further demonstrated.

In the present study, the relatively high expression levels of catfish Bf/C2 genes in liver also demonstrated that Bf/C2 products may be synthesized mainly in the liver. In trout, two Bf/C2-like isotypes have been illustrated are synthesized exclusively in the liver [10]. In gill, the high constitutive expression levels of channel catfish Df gene may be related to a fast activation of the AP in this organ [28]. Bf/C2A and Bf/C2B had lower expression levels in gill, but Bf/C2 gene expression was up-regulated and quickly reached a significant level at 24 h after infection (Fig. 7). This was most likely due to an influx and accumulation of granulocytes and monocytes from the blood stream to the site of infection. Migration of these cell types to the inflammation site has previously been observed after infection [68].

In previous studies [69], three C3 isoforms, C4, and two components of the membrane attack complex C7 and C9 were all found to be highly up-regulated in the liver of blue catfish after *E. ictaluri* challenge. Complement factor H, a regulator factor of AP that inactivates C3b in the AP [4] was also highly induced after infection [69]. This suggested that two complement pathways AP and CP were all involved in the immune response to *E. ictaluri* infection in blue catfish. Df mRNA in carp was constitutively detected, but its expression was not significantly changed by infection with the parasite *I. multifiliis* [28], suggested that the expression of Df is modulated by complex mechanisms in the host defense system. In rainbow trout, studies showed Df was not the limiting factor in fish, in contrast to the situation in humans; as little as 0.2 mg/ml of Df was capable of cleaving the same amount of Bf-2 as at physiologic concentration of Df (25–50 mg/ml) in trout serum [10]. In channel catfish, *Df* expression was not changed significantly at most sampling intervals after infection, supporting this notion. Bf/C2 and Df gene expression patterns of channel catfish indicated that they are involved in the immune response to the bacterial pathogen although their immune response mechanisms are unknown at present. Further research is warranted to elucidate the role of these complement factors in immune response of channel catfish.

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