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Molecular Cloning and Expression Patterns of Two Tumor Necrosis Factor Alpha Genes in Crucian Carp (*Carassius carassius*)[1]

R. A. Kajungiro, L. Xue, and M. Aynealem

College of Marine Sciences, Laboratory of Molecular Biology, Ningbo University, Ningbo, Zhejiang 315211, P.R. China; e-mail: xueliangyi@nbu.edu.cn

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Abstract—Tumor necrosis factor (TNF[31]) is a potent inflammatory cytokine produced during inflammation. In this study, two crucian carp *TNF*-α genes, *TNF*α-1 and *TNF*α-2, were cloned and sequenced. The *TNF*α-1 is 720 bp long and consists of a 699 bp opening reading frame (ORF) encoding 232 amino acids and *TNF*α-2 is 793 bp long and contains an ORF of 687 bp, which encodes 228 amino acids. The genomic structure of both genes consists of 4 exons and 3 introns similar to the other known *TNF*-α genes. The amino acid sequences of the crucian carp TNFα-1 and TNFα-2 share the highest identity with common carp, goldfish and the lowest with flounder and humans. The phylogenetic analysis grouped crucian carp TNFα-1 and TNFα-2 with other cypriniformes, which are more closely related to goldfish and common carp TNFα-1 and TNFα-2. Real time PCR analysis showed a constitutive expression of crucian carp *TNF*α-1 and *TNF*α-2 in all seven tissues examined. The *TNF*α-1 mRNA was expressed significantly higher in the liver and kidney than those of *TNF*α-2, while *TNF*α-2 in turn was expressed significantly higher in the muscle. The *Aeromonas hydrophila* BSK-10 strain upregulated the expression level of both *TNF*α-1 and *TNF*α-2 in all of the tested tissues. At 6 h, the expression levels of *TNF*α-1 were increased significantly higher in the muscles, skin, and liver, while the expression levels of *TNF*α-2 were increased significantly higher in the muscles and gills. *TNF*α-1 was expressed much stronger than *TNF*α-2. At 12 h the expression levels started to decline and were even lower at 24 h. These results imply that both *TNF*α-1 and *TNF*α-2 mRNA are distributed differently in tissues and are implicated in the immune response to bacterial infection.

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INTRODUCTION

TNF-α is a key member of the TNF ligand superfamily, which plays a crucial role in the host immune responses and inflammatory processes [1]. TNF-α is known as a pleiotropic and potent proinflammatory cytokine, which is produced by several cell types including macrophages, monocytes, polymorphonuclear leukocytes, mast cells, and smooth muscle cells [2] in response to inflammation, infection, and other physiological challenges [3]. TNF-α has been shown to upregulate the production of other inflammatory cytokines [4] and to eliminate bacterial and viral pathogens by enhancing a variety of cellular responses, including phagocytosis, chemotaxis, and production of reactive oxygen and nitrogen intermediates [4, 5]. Two forms of TNF (TNF-α and TNF-β) have been reported in mammals, but only one form of TNF[35], similar in structure and genomic organization to mammalian TNF-α, is believed to be found in fish [7]. In teleost fish, *TNF*-α was first identified in the Japanese flounder (*Paralychthys olivaceus*) [8], since then *TNF*-α has been cloned and sequenced from a variety of bony fishes [9–24].These genes were found to be similar to mammalian *TNF*-α and consisted of 3 introns and 4 exons [25]. As with mammalian *TNF*-α, in the Japanese flounder TNF-α was found to exist as a single copy gene [8]. Recently multiple isoforms of TNF-α have been identified in some fish, for example four isoforms were found in common carp [13–15] and two isoforms in trout, goldfish, and bluefin tuna respectively [10, 23, 24, 26, 27]. Studies revealed that TNF-α isoforms differ in the expression patterns, for example, in goldfish *TNF*α-2 mRNA is expressed higher than *TNF*α-1 in all examined tissues [23], while in bluefin tuna it is the other way around in all tested tissues *TNF*α-1 mRNA shows higher expression than *TNF*α-2 [24]. Studies have reported that expression of *TNF*-α was up regulated in various fish species after stimulation with bacteria [15, 18] and viruses [19].

Crucian carp is a commercially important freshwater fish species, which is cultured in Europe and throughout the northeastern region in countries such as Japan, China, and Korea [28]. The major problem affecting their production is the outbreak of infectious diseases caused by bacteria, which result in the loss of stock and, hence, economic losses. Bacterial disease

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Primers used to amplify crucian carp *TNF*a-1 and *TNF*a-2 and for the expression study

Name	Nucleotide sequence, 5' → 3'	Use
T1F	CGAGGACTAATAGACAGTGATG	Cloning of *TNF*α-1 cDNA and genomic DNA
T1R	ATGGCAGCCTTGGAAGTGAC	
T1FF	CGTCACTTCCAAGGCTGCCATC	
T1RR	CATCATAAAGCAAACACCCCG	
TNF2F	TGAAGTGATAMACTGCACTGAGG	Cloning of *TNF*α-2 cDNA and genomic DNA
TNF2R	ACACCCCAAARAAGGTCTTTCC	
TNFα2R	TCATAAAGCAAACACCCCCRAAG	
T1QF	GCTGTCTGCTTCACGCTCAAC	
T1QR	CCGCCTGAAGTGAAAGCC	
T2FQ2	GCCGCTGTCTGCTTCACATTCAAC	Primers for expression studies
T2RQ2	CAAGCCACCTGAAACAAAAGCCTG	
REF	GGATGGGACAGAAGGACAGC	For reference genes
RER	CTACAACGAGCTGCGTGTTG	

caused by the Gram-negative bacterium *Aeromonas hydrophila* is responsible for the serious morbidity and mortality in marine and freshwater aquacultures [29]. *Aeromonas hydrophila* causes motile aeromonad septicaemia, affecting a wide variety of freshwater fish species e.g. carps, catfish, and, occasionally, marine fish, such as salmonids [30].

To understand the interaction of the *TNF*-α gene and the bacterial pathogen of the *Aeromonas hydrophila* BSK-10 strain in crucian carp, two TNF-α isoforms were cloned and their expression patterns were studied in various tissues. The study of *TNF*-α expression in different tissues and its interaction with the pathogen is important for understanding the degree of the early immune response.

EXPERIMENTAL

Fish. Crucian carp, *Carassius carassius* (300g) were purchased live from a local fish market in Ningbo, Zhejiang Province, China. The fish were kept in 100 L tanks supplied with aerated fresh water and fed with a commercial pelleted diet twice a day. The temperature was kept at 20–25°C. The fish were acclimatized to laboratory conditions for at least 7 days prior to bacterial infection.

Cloning and sequencing of crucian carp *TNF*-α genes. Total RNA was extracted from crucian carp kidneys using RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA using reverse the PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions. The cDNA was used in degenerative PCR amplification with primers: T1F/T1R for *TNF*α-1 and TNF2F/TNF2R for *TNF*α-2 (Table 1), which were designed against the regions conserved between common carp *TNF*α-1 (AJ3111800) and *TNF*α-2 (AJ311801), goldfish *TNF*α-1 (EU069817) and *TNF*α-2 (EU069818), and grass carp *TNF*-α (HQ696609) sequences using Vector NTI 7 and primer 5 programs. Polymerase Chain Reaction (PCR) conditions were : 1 cycle at 94°C for 2 min, 30 cycles of 94°C for 30 s, annealing temperatures specific to each primer pair (50–60°C) for 30 s, and 72°C for 30 s, followed by 1 cycle at 72°C for 10 min. An agarose gel DNA purification kit (Sangon, China) was used to purify the PCR product which was then ligated into the pMD19–T vector (Takara) and transformed into *Escherichia coli* TG1 competent cells, positive colonies were picked and then sequenced (Invitrogen, China).

Partial mRNA sequences of 267 bp for *TNF*α-1 and 771 bp for *TNF*α-2 including the start codon were obtained. The sequences were blasted and showed strong homology with other known *TNF* sequences and were used to design new primers (Table 1). For *TNF*α-1, the forward primer (T1FF) was designed from the obtained partial mRNA sequence and the reverse primer (T1RR) was designed based on the conserved regions of other known *TNF*α-1 sequences, which included the stop codon, and for *TNF*α-2 the reverse primer (TNFα2R) was designed based on other known *TNF*α-2 sequences to obtain the full ORF.

Genomic DNA was isolated from crucian carp liver using the Phenol-Chloroform method described in [31], with reagents from (Invitrogen). PCR was performed with the total genomic DNA template using primers T1F and T1RR for *TNF*α-1 and TNF2F and

TNFα2R for *TNF*α-2 (Table 1). PCR conditions were: 1 cycle at 94°C for 3 min, 30 cycles of 94°C for 30 s, annealing temperatures specific to each primer pair (50–60°C) for 1 min, and 72°C for 2 min, followed by 1 cycle at 72°C for 10 min. The obtained PCR products were ligated into the pMD19–T vector (Takara) and transformed into *Escherichia coli* TG1 competent cells (Takara). Recombinant plasmids from positive clones were then sequenced (Invitrogen). The genomic structures of *TNF*α-1 and *TNF*α-2 were determined by comparing the cDNA and DNA sequences.

Sequence and phylogenetic analysis. Nucleotide and amino acid sequence data were analyzed to identify the other sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments of derived amino acid sequences were generated using the CLUSTALW program. The transmembrane region predictions were made using the TREMPRED program [32]. The protein family signature was predicted using the PROSITE database [33]. The phylogenetic tree was constructed with MEGA4 using the neighbor-joining method basing on the amino acid sequences of the known TNF molecules. The reliability of the trees was assessed by bootstrapping using 10000 bootstrap replications.

Bacteria culture. The bacterial *Aeromonas hydrophila* BSK-10 strain was provided by the Zhejiang Institute of Freshwater Fisheries and was stored at –80°C until it was used. Before introducing into crucian carp, some bacteria were reactivated and cultured on LB agar plates for 12 h at 37°C. An inoculum from a single colony of this culture was grown in liquid medium for 18 h and the bacterial cells were collected by centrifugation (6000 rpm) for 10 min. The obtained pellet was resuspended in PBS to a concentration of 5×10^7 cells/mL. The bacteria were quantified by measuring absorbance at OD_{600} using a UV/Visible spectrophotometer (Ultrospec 1100 pro, Amersham Biosciences).

Bacterial challenge. Fifteen crucian carps were maintained in three tanks and used for this study. Twelve fish were intraperitoneally injected with 0.1 mL of the *Aeromonas hydrophila* BSK-10 strain (5×10^7 cells/mL). Three fish were kept in a separate container untreated as a control group. Three fish from each tank were randomly sampled at 6, 12, and 24 h post the bacterial injection. Tissues (kidney, liver, skin, muscles, gill, heart and spleen) were isolated from each fish (stimulated and non stimulated) under sterile conditions and stored at –80°C for further experiments.

Expression analysis of *TNF*-α. Three pairs of primers were designed according to the complete ORF sequence of crucian carp *TNF*α-1, *TNF*α-2, which were obtained from the procedure described above and β-actin (JN006052). The primers used were T1QF and T1QR for *TNF*α-1, T2QF and T2QR for *TNF*α-2 and REF and RER for β-actin (Table 1). Fragments of 200, 207 and 150 bp of *TNF*α-1, *TNF*α-2 and β-actin respectively, were PCR amplified and inserted into the pMD19–T vector (Takara) and transfected into the TG1 strain. Plasmid DNA was extracted from the positive colonies using the SanPrep Column Plasmid Mini-Preps Kit (Sangon). The concentration of each plasmid DNA was measured using a spectrophotometer and then serially diluted (10-fold dilutions) and used to generate standard curves for the amplification efficiencies of the real-time PCR assays.

The total RNA was extracted from the target tissues of stimulated and non stimulated fish as described above. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA). Two microlitres of total RNA were reverse transcribed into the cDNA using PrimeScript RT Reagent Kit with gDNAEraser (Takara) according to the manufacturer's instructions. cDNA was used for real time PCR analysis using the SYBR Premix Ex Taq II (Perfect Real Time) kit (Takara). A total volume of 25 μL in every reaction contained 12.5 μL of SYBR Premix Ex Taq II, 0.1 μL of forward primer, 0.1 μL of reverse primer (10 μM), 11.3 μL of RNase free water and 1 μL cDNA. Controls lacking cDNA were included. PCRs were performed using an Eppendorf Realplex Mastercycler instrument according to the following protocol: initial denaturation cycle at 95°C for 2 min, 40 cycles of denaturation at 95°C for 10 s, annealing at primer specific temperature (58–64°C) for 30 s, and extension at 72°C for 20 s. Melting curve analysis at 95°C for 15 s, 55°C for 15 s and at 95°C for 15 s was performed to ensure the particularity of amplification.

All samples were run in triplicates and *TNF*-α expression was normalized against the crucian carp β-actin. For *TNF*-α expression in different tissues, the target gene expression was quantified relatively to that of β-actin. To compare the changes in gene expression between the untreated and treated groups, the $2^{-\Delta\Delta Ct}$ method [34] was used. The data are presented as the fold change in *TNF*-α expression normalized to β-actin and relative to the untreated control, as mean ± SE of 3 fish.

Statistical analysis. The resulting data represent the means and standard errors (SE) of triplicate samples. The obtained data were analyzed for statistical significance by one way ANOVA, followed by the Duncan test to determine the differences between groups. All statistical tests were conducted using the computerized SPSS v.16.0 software and the graphs were plotted by Origin 8 software. Differences were considered significant at $P < 0.05$.

RESULTS

Cloning and Sequencing Analysis of Crucian Carp *TNF-α*

720 bp and 793 bp cDNA fragments were amplified and named *TNF*α-1 (KF500408) and *TNF*α-2

(KC771269), respectively. The *TNF*α-1 cDNA contained an ORF of 699 bp, which encoded 232 amino acids, and *TNF*α-2 contained an ORF of 687 bp, which encoded 228 amino acids. The acquired genomic DNA sequence of *TNF*α-1 was 1723 bp (KJ923252) in length, had three introns (109, 116, and 777 bp) and four exons (178, 28, 72, and 440 bp). *TNF*α-2 genomic DNA contained 2160 bp (KJ923253) and also had three introns (109, 93, and 1165 bp) and four exons (253, 28, 72, and 440 bp). The intron splicing followed the classical 'GT–AG' rule.

Both the predicted *TNF*α-1 and *TNF*α-2 translated sequences contained all the basic elements of the TNFα-1 family features of fish and higher vertebrate TNFs. These included the consensus motifs IIIPTDGIYFVYSQV for TNFα-1 and IIIPYDGIYFVYSQV for TNFα-2, which are similar to the TNF family signature [LV]-x-[LIVM]-x3-G-[LIVMF]-Y-[LIVMFY]2-x2-[QEKHL]-[LIVMGT]-x-[LIVMFY] (with an exception of I instead of V or L at position 1). The trans-membrane domains located at position ^{32}VCGVLLAVALCAAAAVCFTL^{51} of TNFα-1 and at ^{28}VCGVLLAVALCAAAAVCFTF ^{47} of TNFα-2 were analyzed using TMPRED software. Multiple comparisons with other known vertebrate TNF-α amino acid sequences showed that many signature residues are conserved in crucian carp TNF-α (Fig. 1). Two conserved cysteine residues which are important for the maturity of TNF-α were found to be conserved in both TNF-α sequences. Conserved Thr-Leu (TL) residues of the putative TNF-α converting enzyme (TACE) cleavage site were also found at positions 67 and 68 in TNFα-1 and at 63 and 64 in TNFα-2 (Fig. 1). The amino acid sequence alignment of TNF-α by Clustalo (UniProt) identified that, TNFα-1 was by 98% identical to goldfish and by 81% identical to common carp TNFα-1, while TNFα-2 was also by 98% identical to goldfish and by 86% identical to common carp TNFα-2. Crucian carp TNFα-1 and TNFα-2 share 94% amino acid sequence identity.

Phylogenetic Analysis

The salmoniforme (trouts) and the perciforme (cichlids) were branched separately from those of the cypriniforme species (cyprinids). Crucian carp TNFα-1 and TNFα-2 showed a close relationship between the goldfish and common carp TNF-α isoforms (Fig. 2).

Expression Patterns of Crucian Carp *TNF*α-1 and *TNF*α-2 Before Bacterial Injection

Both *TNF*α-1 and *TNF*α-2 mRNAs were found to be expressed constitutively in all tested tissues of healthy fish (non-stimulated). Both *TNF*α-1 and *TNF*α-2 mRNA showed a significant difference in all examined tissues ($P < 0.05$); *TNF*α-1 mRNA was expressed significantly higher in the liver and very low in the muscle, while *TNF*α-2 mRNA was expressed significantly higher in the spleen (Fig. 3). Two isoforms showed a significant difference in their expression patterns. In the liver and kidney, *TNF*α-1 mRNA levels were significantly higher than those of *TNF*α-2, while in the muscle *TNF*α-2 mRNA was expressed significantly higher than that of *TNF*α-1 (Fig. 3).

Expression Patterns of Crucian Carp *TNF*α-1 and *TNF*α-2 After Bacterial Infection

After injection with bacteria *Aeromonas hydrophila* BSK-10, the expression levels of *TNF*α-1 and *TNF*α-2 mRNA were up regulated and increased significantly in all examined tissues. The expression levels of both *TNF*α-1 and *TNF*α-2 were the highest 6 h post injection and began to reduce at 12 h. At 6 h, *TNF*α-1 expression was significantly induced to high peak in skin (~8.5 fold) followed by liver (~8 fold) and muscle (~3.7 fold) as compared to the untreated control fish (0 h) (Fig. 4), while *TNF*α-2 expression level was significantly increased in the gill (~3.2 fold) followed by muscle (~3 fold) as compared to the untreated control fish (0 h) (Fig. 5). The expression levels of *TNF*α-1 and *TNF*α-2 were even more reduced at 24 h.

DISCUSSION

In the presented study, two types of the *TNF*-α gene (*TNF*α-1 and *TNF*α-2) were cloned from crucian carp. Both *TNF*α-1 and *TNF*α-2 genes consisted of four exons and three introns, which is a typical feature of *TNF*-α in mammals and other fish [35]. Only one *TNF*-α gene was identified in Japanese flounder [8]. In this study we obtained two *TNF*-α genes: *TNF*α-1 and *TNF*α-2. Our results are similar to those reported for rainbow trout [10, 26], goldfish [23], and bluefin tuna [24], but different from common carp, in which four types of *TNF*-α were identified [13–15]. Multiple isoforms of *TNF*-α in different fish might be due to genome duplications [36].

TNFα-1 and TNFα-2 share more than 90% identity with goldfish and 80% identity with common carp respectively. Crucian carp TNFα-1 and TNFα-2 share 94% amino acid sequence identity. High homology between TNFα-1 and TNFα-2 has been reported earlier: at 92.5% in rainbow trout [9], 91.5% in atlantic salmon [27], and 78.1% in common carp [13], respectively. High identity between *TNF*α-1 and *TNF*α-2 in fish suggests that there is a high probability that the two genes may share many common functions.

Similar to TNF-α molecules in mammals and other teleosts, the two TNF-α in crucian carp had two conserved cysteine residues involved in *TNF*-α maturation, a transmembrane domain and a TNF family signature. A protease cleavage site was also found between threonine and leucine residues, also present in gilthead seabream, flounder, rainbow trout, common carp, and goldfish. These features support that

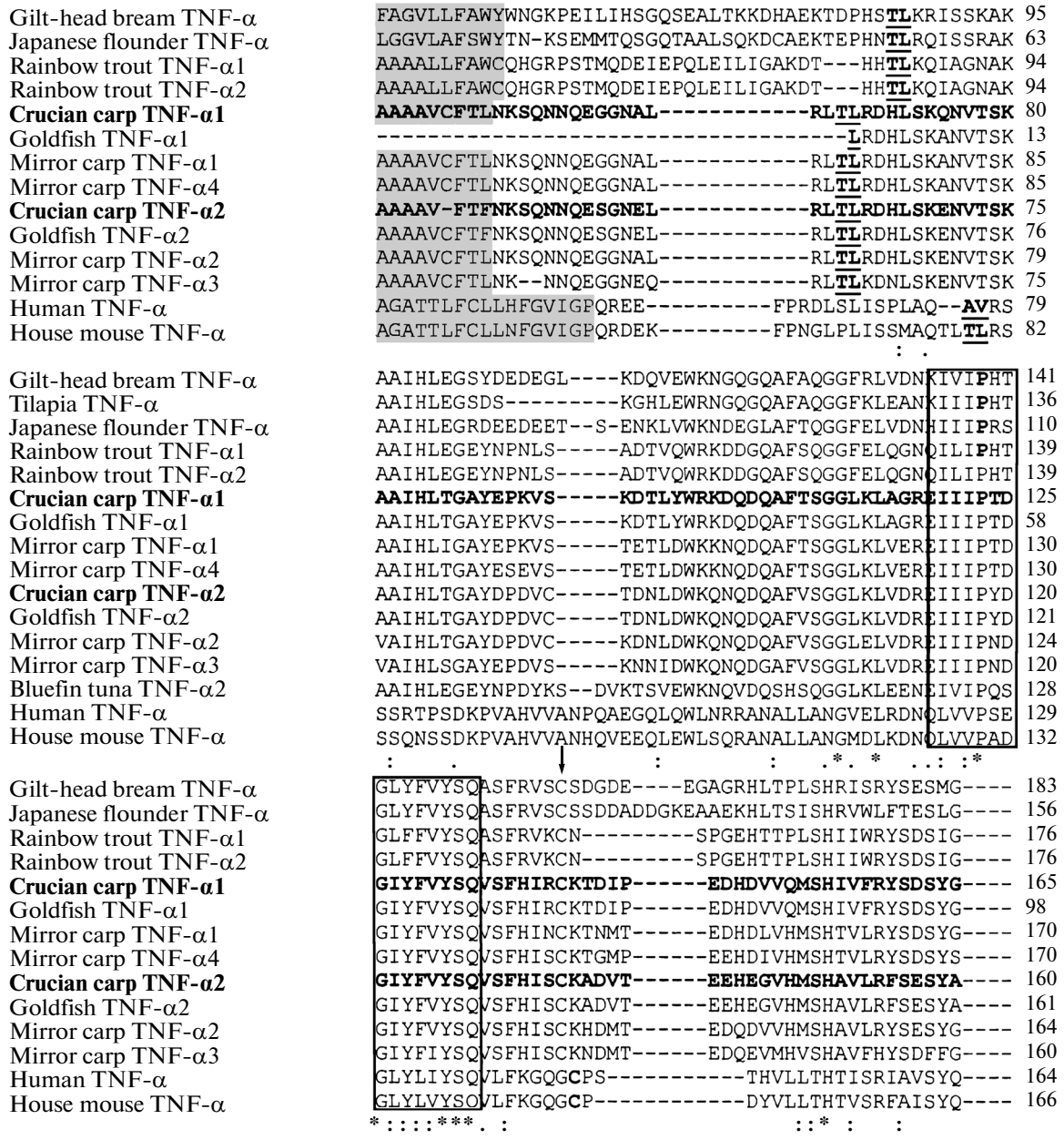

Fig. 1. Multiple alignments of the crucian carp TNFα-1 and TNFα-2 amino acid sequences with other fish and higher vertebrate species TNF-α proteins. Sequence alignment was performed using Clustal W. Dashes indicate gaps introduced to optimize the similarity between sequences. Fully conserved residues are indicated by an asterisk (*), partially conserved and semi-conserved substitutions are represented by (:and.) respectively. Conserved cysteines responsible for the TNF-α tertiary structure are shown by arrows. TNFα-1 converting enzyme (TACE) cleavage site Thr-Leu (AV in Human) is underlined. The TNF family signature is boxed and the predicted trans-membrane domain is shaded.

crucian carp TNFα-1 and TNFα-2 belong to the TNF family.

Phylogenetic analysis clustered crucian carp *TNF*-α genes together with other teleosts and apart from mammalian *TNF*-α and *TNF*-α. Within the order cypriniformes, crucian carp *TNF*α-1 and *TNF*α-2 were grouped more closely with goldfish and common carp *TNF*-α isoforms, than with grass carp and zebrafish and far apart from the Perciformes and Salmoniformes. This implies that mammals and teleosts have one ancestral *TNF* gene and the divergence might be due to gene duplication, which has occurred in fish in the recent past.

The constitutive expression of *TNF*-α in teleosts has been shown to vary with fish species and tissues being analyzed. Constitutive expression of *TNF*-α in different tissues has been reported in seabream [11], catfish [16], tilapia [17], mandarin fish [19], and sea

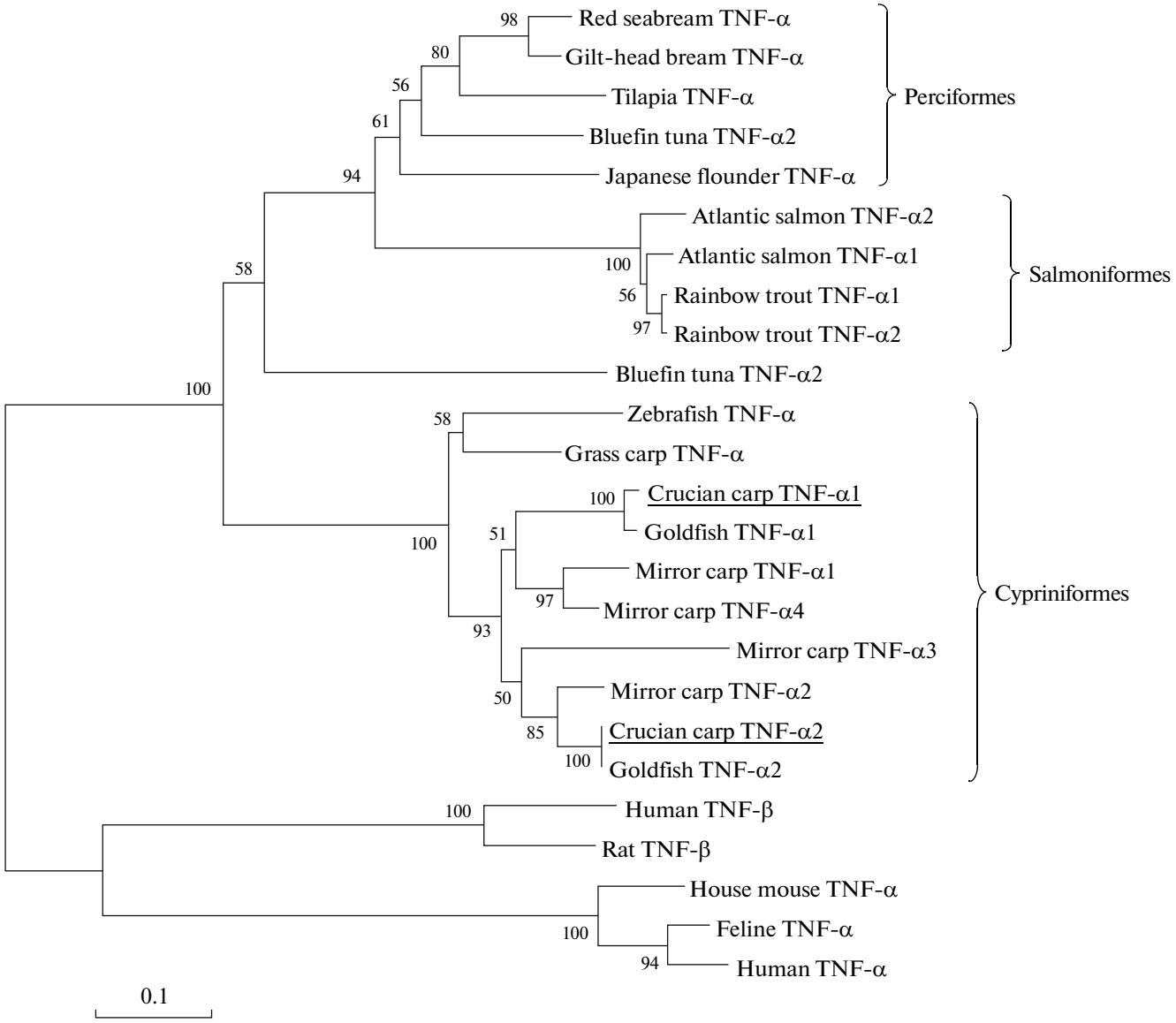

Fig. 2. Phylogenetic tree of crucian carp TNFα-1 and TNFα-2 isoforms with other fish and mammals based on the mature *TNF* proteins. Amino acid sequences were aligned using Clustal W. The phylogenetic was constructed with the MEGA4 program using the NJ method, based on the amino acid alignment. The numbers at the relevant branches refer to bootstrap values of 10000 replications.

bass [20]. In the presented study, both *TNF*α-1 and *TNF*α-2 mRNA showed constitutive expression in all of the examined tissues of healthy crucian carp, and our results are consistent with those observed in goldfish [23] and bluefin tuna [24].

Some studies have reported a limited pattern of tissue expression for example in common carp, in which *TNF*α-3 was constitutively expressed in head kidney, gill, spleen, intestine and muscle from normal tissues, while *TNF*α-1 and *TNF*α-2 were expressed only in the gills of healthy fish [14]. In rainbow trout constitutive expression was only shown in the gill and kidney [10]. Interestingly, no constitutive *TNF*-α expression was detected in Japanese flounder and turbot in all of the examined tissues [8, 18].

A differential expression pattern of the two isoforms has been shown in tissues from healthy fish, for example in bluefin tuna *TNF*α-2 was expressed significantly higher in the blood, while *TNF*α-1 was expressed at a same level in all tissues [24]. We observed that *TNF*α-1 was expressed significantly higher in the liver and lower in the muscle. The relatively low expression of *TNF*-α in the muscle has also been reported in mandarin fish [19]. We also observed that *TNF*α-2 was expressed significantly higher in the spleen than in other tissues. Our results are similar to those obtained in goldfish. In crucian carp *TNF*α-1

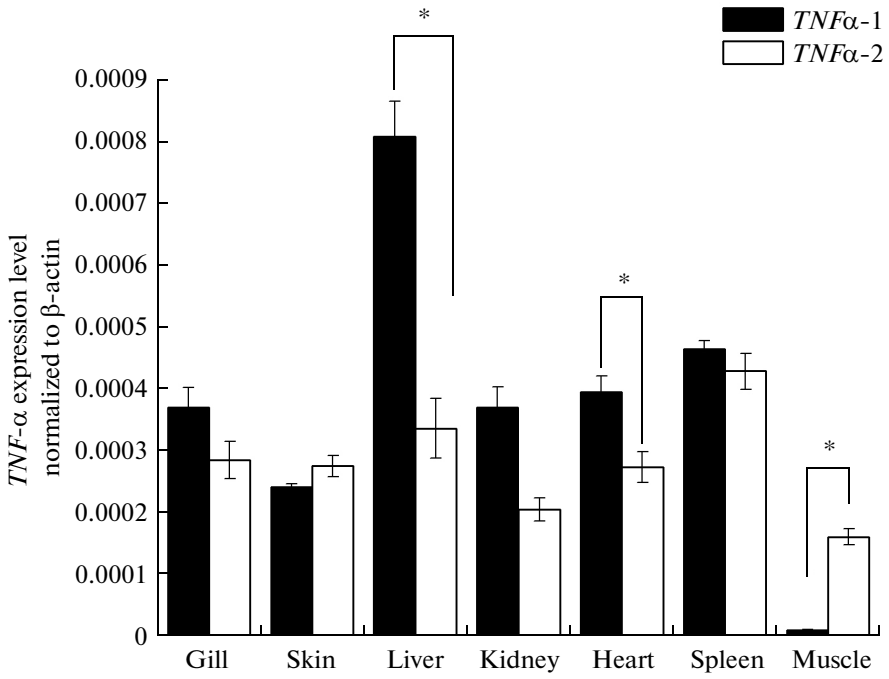

Fig. 3. Expression patterns of *TNF*α-1 and *TNF*α-2 mRNAs in various tissues of un-stimulated crucian carp. Gene expression is relative to β-actin. Data are presented as mean ± SE; $n = 3$. Asterisks indicate significant differences ($P < 0.05$).

mRNA was expressed significantly higher than that of *TNF*α-2 in the liver and kidney, while *TNF*α-2 was expressed significantly higher in the muscle. Both *TNF*α-1 and *TNF*α-2 genes showed the same expression level in the gill, this is contrary to goldfish in which in the gill *TNF*α-2 was expressed significantly higher than *TNF*α-1 [23]. This suggests that the two genes in fish are differently expressed in different tissues.

Inducible expression of *TNF*-α has been revealed in various fish species after stimulation with different agents. Lipopolysaccharides (LPS) upregulated *TNF*-α expression in flounder PBLs [8], rainbow trout head-kidney leukocytes and macrophages [10] and in catfish peripheral blood lymphocytes (PBL) and B cells [16]. Induction of *TNF*-α expression by bacteria has also been demonstrated in some fish. *Vibrio pelagius* strain Hq222 upregulated *TNF*-α expression in head kidney and in the liver of turbot [18]. Aeromonas *hydrophila* Ahcs01 induced the expression of *TNF*α-4 in common carp's leukocytes. While most studies have reported on the regulation of *TNF*-α in head kidney, leukocytes, macrophages and cells, in this study the expression patterns of *TNF*-α genes in seven tissues of crucian carp at 6, 12, and 24 h post stimulation with *Aeromonas hydrophila* BSK-10 are reported. The expression levels of *TNF*α-1 and *TNF*α-2 in all tissues examined at 6 h after injection with *Aeromonas hydrophila* BSK-10 were upregulated. In contrast, in sea bream the non-virulent strain of *Vibrio anguillarum* failed to increase *TNF*-α expression in tissues [11]. At 6 h the expression level of *TNF*α-1 increased more rapidly in the skin, liver, and muscle respectively as compared to the untreated control, while the expression level of *TNF*α-2 increased in the muscle and gill, but in the first case weaker and in the other stronger than *TNF*α-1. This implies that *TNF*α-1 and *TNF*α-2 respond differently toward bacterial infection in crucian carp: *TNF*α-1 being quick to respond and drastically upregulated in many tissues as compared to *TNF*α-2. *TNF*-α expression early after stimulation indicates at a pro-inflammatory response of this cytokine to the pathogen.

Previous studies show that the *TNF*α-2 gene could be more significantly induced than the *TNF*α-1 gene after stimulation with different stimuli, for example in bluefin tuna, LPS, phytohemagglutinin (PHA), ConA, and phorbol myristate acetate (PMA) in vitro induced the expression of *TNF*α-2 in PBL, while the expression level of *TNF*α-1 was not induced [24]. In common carp the *TNF*α-2 gene was higher induced than *TNF*α-1 in head kidney phagocytes after stimulation with LPS and the protozoan blood flagellate *Trypanoplasma borreli* [13]. However in the present study induction of the two genes showed that *TNF*α-1 is stronger expressed than *TNF*α-2. These observations suggest that the two *TNF*-α genes may play different roles in fish species and respond differently to different stimuli.

Differential expression patterns at different times have been observed in fish. For example in rainbow trout, *TNF*α-1 and *TNF*α-2 expression was induced in macrophages at 4 h and then decreased at 8 h after

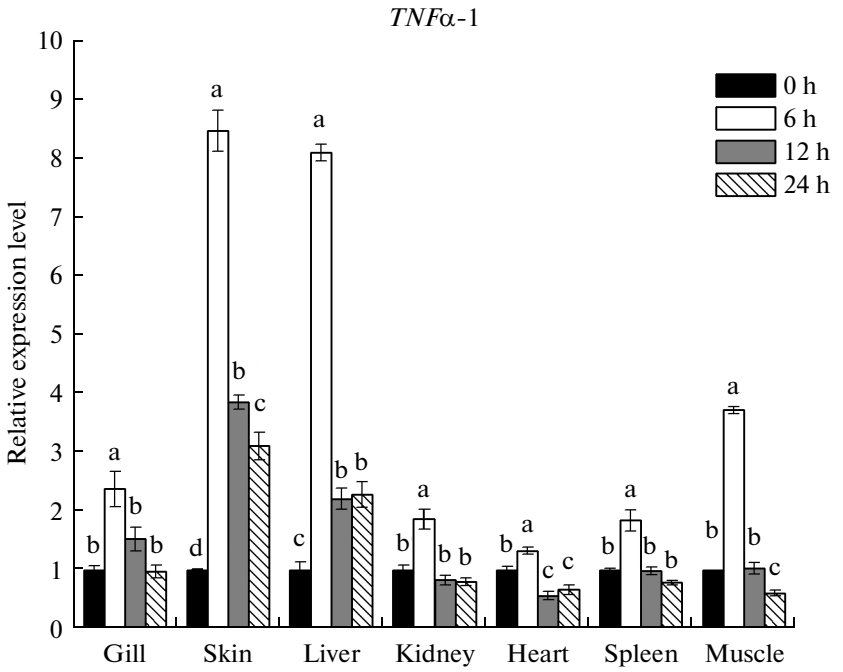

Fig. 4. Relative expression of *TNF*α-1 mRNA in various tissues of Crucian carp 6, 12, and 24 h after stimulation with *Aeromonas hydrophila* BSK-10 (5×10^7 cells/mL). Gene expression is normalized against β-actin and relative to the untreated fish. Data are presented as mean ± SE; $n = 3$. Different letters above the columns indicate significant differences ($p < 0.05$).

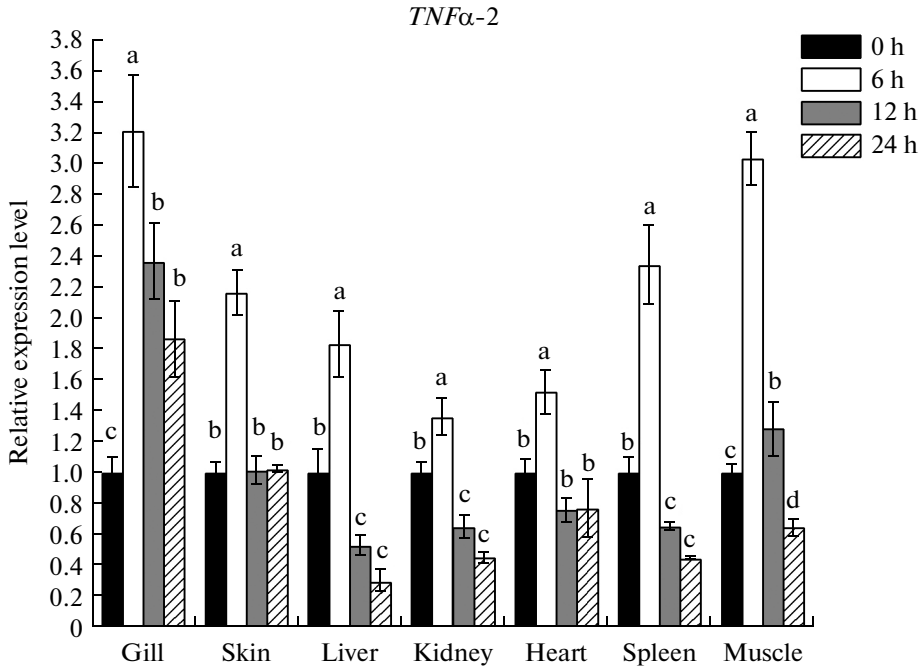

Fig. 5. Relative expression of *TNF*α-2 mRNA in various tissues of Crucian carp 6, 12, and 24 h after stimulation with *Aeromonas hydrophila* BSK-10 (5×10^7 cells/mL). Gene expression is normalized against β-actin and relative to the untreated fish. Data are presented as mean ± SE; $n = 3$. Different letters above the columns indicate significant differences ($P < 0.05$).

stimulation with LPS. *TNF*α-2 was expressed higher than *TNF*α-1 and *TNF*α-2 expression was detected earlier and lasted longer [9]. *Aeromonas hydrophila* Ahcs01 induced the expression of *TNF*α-4 in leokocytes in Common carp. The expression was the highest at 12 h and declined at 24 h [15]. The Bacterial *V. pelagius* strain Hq222 in turbot induced *TNF*-α expression in the liver 24 h post infection while VHSV induced the expression in the liver and kidney by 8 h and the expression began to decrease after that [18]. In crucian carp, *TNF*α-1 and *TNF*α-2 also showed a decline in the expression levels in tissues after 12 h. The reduction of the expression levels with time may be due to the rapid clearance of the bacteria by phagocytes.

In conclusion, in the presented study, two genes *TNF*α-1 and *TNF*α-2 have been cloned in crucian carp. *TNF*α-1 and *TNF*α-2 have different expression patterns: *TNF*α-1 being highly expressed in the liver and weakly in the muscle, while *TNF*α-2 was higher expressed in the spleen than other tissues. *TNF*α-1 and *TNF*α-2 respond differently to the bacterial pathogen *Aeromonas hydrophila* BSK-10, *TNF*α-1 being quicker to respond than *TNF*α-2. The findings showed that *Aeromonas hydrophila* BSK-10 upregulates the expression levels of *TNF*α-1 and *TNF*α-2 early after invasion and *TNF*α-1 and *TNF*α-2 play critical roles in the pro-inflammatory responses to pathogens. The information obtained in this study can be applied for the development of new vaccines against bacterial infection. These duplicate genes show high similarity identity but different expression patterns. Further study of the two isoforms is needed to understand their function in the immune response of fish.

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