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ORIGINAL PAPER

Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*

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Abstract Toll-like receptors (TLRs) are a family of transmembrane proteins that recognize specific pathogen-associated molecular patterns and use conserved signaling pathways to activate proinflammatory cytokines and type-1 interferons to fight infection. TLR3 in mammals is best known for its recognition of dsRNA as ligand and its MyD88-independent signaling. TLR3, upon recognition of dsRNA, recruits and binds its adaptor protein TIR domain-containing adapter molecule (TICAM) 1. Here we report the genomic sequences and structures of TLR3 and a TICAM adaptor from channel catfish (*Ictalurus punctatus*). Whereas a partial TLR3 cDNA sequence has been reported from channel catfish, and complete *TLR3* genes are known from other teleost fish species, a complete TICAM sequence has not been previously reported from a non-mammalian species. Analysis of catfish TLR3 and TICAM expression after infection with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), suggested a conserved TLR3-TICAM receptor–adaptor relation in catfish. Comparison of TLR3 and TICAM expression profiles in channel catfish with those from the closely related blue catfish species (*Ictalurus furcatus*), which exhibits strong resistance to ESC, revealed a striking pattern of species-specific expression. A dramatic down-regulation of *TLR3* and *TICAM* gene expression was observed in blue catfish head kidney and spleen, which we speculate may be the result of maturation and migration of different cell types to and from the lymphoid tissues following infection.

Keywords Catfish · Fish · TLR3 · TICAM · TRIF · Toll-like receptor · Disease · Innate immunity

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Introduction

Toll-like receptors (TLRs) are a family of transmembrane proteins first discovered in *Drosophila* (Belvin and Anderson 1996; Medzhitov et al. 1997). TLRs recognize pathogen-associated molecular patterns (PAMPs; Medzhitov and Janeway 2000) and use conserved signaling pathways to activate proinflammatory cytokines and type-1 interferons to fight infection (reviewed by Ishii et al. 2005; Tosi 2005). TLRs are characterized by the presence of a Toll/interleukin (IL) 1 receptor (TIR) domain in their cytoplasmic portion and a leucine-rich repeat (LRR) domain in their extracellular portion (Akira and Takeda 2004). At least 11 TLRs have been identified to date from mammals; TLR3 and TLR4 are the most thoroughly characterized family members. Roach et al. (2005), in their study of the evolution of vertebrate TLRs, identified six major subfamilies of TLRs, each recognizing a general class of PAMPs. Under this classification, individual TLRs recognize lipopeptides (TLR2), dsRNA (TLR3), lipopolysaccharide (LPS, TLR4), flagellin (TLR5), and nucleic acid and heme motifs (TLR7–9) (Roach et al. 2005). However,

continuing functional studies indicate that TLR PAMP recognition can differ considerably among host and pathogen species (Ishii et al. 2005; Baoprasertkul et al. 2006).

Toll-like receptors, upon recognition of microbial PAMPs, recruit and bind adaptor proteins to their TIR domains in the cytoplasm. These adaptor proteins appear to add another level of specificity to the host defense. Five TIR adaptor proteins have been identified from mammalian species (reviewed by O'Neill et al. 2003): a myeloid differentiation protein named MyD88; MAL (MyD88-adaptor-like protein or TIRAP); TRIF [TIR-domain-containing adaptor-inducing interferon (IFN) β] or TICAM-1 (TIR-domain-containing adapter molecule 1); TRAM (TRIF-related adaptor protein) or TICAM-2; and SARM (sterile α and HEAT-Armadillo motifs). All TLRs except TLR3 and TLR4 have been shown to require MyD88 for effective downstream signaling, allowing one to divide the TLR family between MyD88-dependent and MyD88-independent members (Ishii et al. 2005). More specifically, TLR4 can activate distinct MyD88-dependent and MyD88-independent pathways. Both TLR3 and TLR4 induce the activation of the IFN-β promoter, a function unimpaired by the knockdown of MyD88 (Takeda et al. 2003). The existence of MyD88-independent pathways for TLR3 and TLR4 predicted that additional TIR adaptors were yet to be discovered (O'Neill et al. 2003; Seya et al. 2005). Searches of sequences with TIR domains by Yamamoto et al. (2002) and use of the TIR domain of TLR3 as bait in a yeast two-hybrid experiment (Oshiumi et al. 2003a) both identified TICAM-1 or TRIF as the adaptor protein of TLR3 responsible for a powerful downstream induction of IFN-β. Subsequent studies by Oshiumi et al. (2003b) found an additional TIR adaptor, TICAM-2 (or TRAM), which was needed to bridge TLR4 and TICAM-1 in the TLR4 MyD88-independent signaling pathway (Fitzgerald et al. 2003).

The identification of TLR3 and elucidation of its dsRNA ligand recognition profile and signaling in mammalian species (Alexopoulou et al. 2001; Matsumoto et al. 2002) have been followed by identification and characterization of homologs in several teleost species, including zebrafish (*Danio rerio*) (Meijer et al. 2004; Jault et al. 2004; Phelan et al. 2005), fugu (*Takifugu rubripes*) (Oshiumi et al. 2003c), channel catfish and hybrid catfish (*Ictalurus* sp.) (Bilodeau and Waldbieser 2005; Bilodeau et al. 2006; Peterson et al. 2005), and rainbow trout (*Oncorhynchus mykiss*) (Rodriguez et al. 2005). In the case of catfish, only partial cDNA sequences have been identified. Whereas the TIR adaptor of TLR3, TICAM-1, has been reported to be present in draft fish genomes based on in silico searches (Jault et al. 2004; Meijer et al. 2004; Iliev et al. 2005), only a short partial cDNA sequence of TICAM-1 from zebrafish has been reported (AY389465). The genomic sequence and structure of *TICAM* genes in lower vertebrates and their phylogenetic relations with mammalian TICAM proteins remain unknown. Therefore, we have identified and characterized here the cDNA and genomic sequences of both TLR3 and a TICAM adaptor in channel catfish (*Ictalurus punctatus*), the primary aquaculture species in the USA. Additionally, we have mapped these two genes to clones of the catfish BAC library, conducted BAC-based Southern blots to determine genomic copy numbers, assessed their conservation through sequence and phylogenetic analysis, examined their expression patterns in various healthy tissues, and compared expression patterns after infection with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), in susceptible (channel) and resistant (blue) catfish (*Ictalurus furcatus*).

Materials and methods

Identification of catfish TICAM and TLR3

Two channel catfish ESTs (AUF_IpOva_20_g04 and AUF_IpOva_18_i01), generated in a recent expressed sequence tag (EST) sequencing project, were identified by BLASTX analysis as sharing highest identities with mammalian TICAM sequences. The complete sequences were obtained by primer walking sequencing. A partial catfish cDNA of TLR3 (AY741552) was obtained by tBLASTn search against the est_others database of the National Center for Biotechnology Information (NCBI) using human TLR3 as the query. Overgo primers were then designed to screen a BAC library for the identification of TLR3-containing BAC clones.

BAC library screening, genomic sequencing, and sequence analysis

Overlapping oligonucleotide probes or overgos based on the catfish TLR3 and TICAM cDNA sequences were used for screening channel catfish CHORI 212 BAC library. Two 24-base-long oligonucleotide primers were synthesized such that they had an 8-bp complementary region at their 3′ end. Upon mixing the two primers, they base-pair to form a complex structure that can be filled in with radioactively labeled nucleotides. High-density BAC filters containing CHORI212 BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA, USA). Overgo hybridization was conducted according to methods of a Web protocol (http://www.tree.caltech.edu/) with modifications (Baoprasertkul et al. 2005; Wang et al. 2006a; Bao et al. 2006). Briefly, overgo primers were purchased from Sigma Genosys (Woodlands, TX, USA) then labeled with ^{32}P-deoxyadenosine triphosphate (dATP) and ^{32}P-deoxycytidine triphos-

phate (dCTP) (Amersham, Piscataway, NJ, USA) in 10 mg/ml bovine serum albumin (BSA), overgo labeling reaction 1X buffer (Ross et al. 1999), and incubated for 1 h at room temperature with Klenow polymerase (Invitrogen, Carlsbad, CA, USA). Sequences of primers and probes used in this study are given in Table S1 in the supplementary information. Sephadex G50 spin columns were used to remove unincorporated nucleotides. Probes were denatured at 95°C for 10 min and added into hybridization tubes that had been under prehybridization for 2 h with the hybridization solution. The filters were hybridized at 50°C for 18 h in 50 ml hybridization solution (1% BSA, 1 mM EDTA at pH 8.0, 7% sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate, pH 7.2). The filters were washed at room temperature and exposed to X-ray film at −80°C for 24 h. Positive clones were identified according to the clone distribution pattern from CHORI. Positive clones were picked and cultured in 2X YT medium. After overnight culture, BAC DNA was isolated using a Perfectprep BAC 96 BAC DNA isolation kit (Brinkmann Instruments, Inc., Westbury, NY, USA).

A single BAC clone from catfish TLR3 (146-B23) and from TICAM (25-I2) was sequenced using primer-walking methods. Fourteen picomoles of each primer was used for sequencing reactions. BAC sequencing was performed in a 10-μl reaction using the BigDye Terminator v3.0 Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions and sequenced using an ABI 3130 automated DNA sequencer. BLAST searches were conducted to determine gene identities. The DNAS TAR software package was used for sequence analysis. MegAlign program of the DNASTAR package was used for sequence alignment of TLR3 and TICAM TIR domain using ClustalW (Serapion et al. 2004). NCBI's Spidey program was used for alignment of genomic and cDNA sequences.

5′-RACE of TLR3 and 3′-RACE of TICAM

For catfish TLR3, the identified clone contained the poly-A tail but was incomplete at the 5′ end. Therefore, 5′ RLM–RACE (RNA ligase-mediated rapid amplification of 5′ cDNA ends) was conducted using a GeneRACER kit with SuperScript III reverse transcriptase (RT) from Invitrogen. 5′ RLM–RACE reaction was performed following manufacturer's instructions. 5′ RLM–RACE products were separated by electrophoresis using 1% agarose gel and purified by using S.N.A.P. columns provided with the kit. The purified polymerase chain reaction (PCR) product was cloned into a TOPO TA cloning kit vector (Invitrogen) and sequenced using an ABI 3130 automated DNA sequencer.

3′ RLM–RACE (RNA ligase-mediated rapid amplification of 3′ cDNA ends) was conducted to obtain the complete poly-A tail of catfish TICAM cDNA, and the amplified fragment was cloned and sequenced as described for the 5′-RACE product.

Southern blot analysis

To characterize the gene copy number of catfish TLR3 and TICAM, Southern blot analysis was conducted using all BAC clones identified to contain the genes. BAC DNA was first digested with restriction endonuclease *Eco*RI and *Hin*dIII separately and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA, USA) by capillary transfer with 0.4 M NaOH overnight. DNA was fixed to the membrane by UV cross-linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) with the auto cross-link function. The membrane was washed in 0.5% SDS (w/v) at 65°C for 15 min and then prehybridized in 50% formamide, 5× SSC (Sambrook et al. 1989), 0.1% SDS (w/v), 5× Denhardt's, and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA (100 μg/ml) overnight. Hybridization was conducted overnight at 42°C in the same solution with appropriate probes added. The cDNA was used as the probe. The probe was prepared using the random primer labeling method (Sambrook et al. 1989) with a labeling kit from Roche Diagnostics (Indianapolis, IN, USA) and ^{32}P-dCTP. 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 65°C for 15 min each. In BAC-based Southern analysis, if the clones represent a single copy, the same hybridization pattern would be expected. If the clones represent different genomic copies (more than one gene) they will exhibit variations in the hybridization pattern with the rare exception of clones harboring the gene of interest at the end of their insert.

Phylogenetic analysis

The relevant sequences of TLR3 and TICAM were retrieved from GenBank for multiple amino acid sequence alignments using ClustalW. Phylogenetic trees were drawn by the minimum evolution method (Rzhetsky and Nei 1992) 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 trees was evaluated by 1,000 bootstrapping replications.

Fish rearing and bacterial challenge

Challenge experiments were conducted as previously described with modifications (Baoprasertkul et al. 2004;

Chen et al. 2005). Briefly, the 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), inoculated into brain-heart infusion (BHI) medium, and incubated in a shaker incubator at 28°C overnight. At the time of challenge, the bacterial culture was added to the tank to a concentration of 3×10^7 colony-forming units (CFUs)/ml. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/l. After 2 h of immersion exposure, 15 fish were randomly taken and placed into a rectangular trough containing pond water with constant water flow through. 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. The only difference was that ESC bacteria were not added. After 2 h, these control fish were incubated in a separate trough at the same density as the challenged fish.

Tissue sampling and RNA extraction

Eleven tissues were collected from healthy channel catfish, including head kidney, spleen, intestine, stomach, skin, muscle, liver, trunk kidney, ovary, brain, and gill. Head kidney and spleen were collected from challenged fish. Samples were collected from ten fish at each time point, including control (before challenge), 4, 24, and 72 h after challenge. Samples were also collected from dying fish during a period between days 4 and 7 after challenge. Moribund fish still had opercular movements, but had lost equilibrium in the water. The experimental fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg/l before tissues were collected. Samples of each tissue from ten fish were pooled to provide sufficient tissue samples for expression analysis. To obtain samples representing the average of the ten fish, the pooled tissue samples were ground with a mortar/pestle to fine powders and thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method (Chomczynski and Sacchi 1987) using the Trizol reagent kit from Invitrogen following manufacturer's instructions.

RT-PCR and quantitative real-time PCR analysis

RT-PCR reactions were conducted by two-step RT-PCR using M-MuLV RT (New England Biolabs, Ipswich, MA, USA). RT reactions were conducted in 40-μl reactions containing 4 μg DNase-I-treated RNA, 4 μl (40 uM) oligo dT primers, 8 μl (2.5 mM each) deoxynucleoside triphosphates (dNTPs), 1 μl RNase inhibitor, 1× RT reaction buffer, and 200 U RT. Detailed procedures followed the instructions of the manufacturer. After RT reaction, 1 μl of the RT products was used as a template for PCR using JumpStart Taq polymerase (Sigma, St. Louis, MO, USA). The reactions also included the gene-specific primers and primers of β-actin serving as an internal control. The reactions were completed in a thermocycler with the following thermoprofiles: denaturation at 94°C for 2 min followed by 38 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Upon the completion of PCR, the reaction was incubated at 72°C for an additional 10 min. The RT-PCR products were analyzed by electrophoresis on a 1.0 % agarose gel and documented with a gel documentation system (Nucleotech Corp., San Mateo, CA, USA).

Quantitative real-time RT-PCR (QRT-PCR) using a LightCycler (Roche) was carried out to validate the results obtained by standard RT-PCR. Concentration and quality of total RNA from each tissue were determined by spectrophotometry (optical density, 260/280 ratio) and electrophoresis. One-step QRT-PCR was carried out in the LightCycler using a Fast Start RNA Master SYBR Green I reagents kit (Roche) following manufacturer's instructions with modifications as previously described (Baoprasertkul et al. 2004). For head kidney tissue from the challenge, relative expression ratios were obtained by normalizing the expression of the target gene, as determined by mean crossing-point deviation, by that of a nonregulated reference gene, *β-actin*, using the Relative Expression Software Tool 384 v. 1 (REST) developed by Pfaffl et al. (2002; http://www.wzw.tum.de/gene-quantification/). Each reaction was carried out in triplicate. Expression ratio results were tested for significance by a randomization test built into the software. All ratios are relative to expression of the gene in 0 h (control) RNA samples. For comparison of healthy tissue expression profiles, standard curves from tenfold dilutions of each of the plasmid DNAs were constructed as previously described and included in each run (Baoprasertkul et al. 2004). Copy numbers for each reaction were calculated by the LightCycler software, and the values were normalized to their corresponding β-actin values to obtain relative copy numbers.

Results

Identification of TICAM and TLR3 from channel catfish

Two channel catfish ESTs sharing highest BLASTX identities with TICAM sequences from mammalian species were identified through analysis of catfish ESTs (Ju et al. 2000; Cao et al. 2001; Karsi et al. 2002; Kocabas et al. 2002). A complete 3′ cDNA sequence was obtained through 3′ RACE. An overgo probe designed from the

cDNA sequence was used to screen the catfish BAC library, resulting in 15 positive BAC clones (Table S2). A fragment of 6,290 bp was sequenced by primer walking on BAC 25–I02. BLAST analysis of the genomic sequence against catfish ESTs in NCBI's dbEST revealed a third catfish EST which spanned the 5′ untranslated region (UTR) and 5′ coding region of the catfish TICAM cDNA. Extension of the sequence contained in this clone (AUA_IpOva00065) allowed the formation of a cDNA contig with the previously identified catfish ESTs of 2,763 bp.

An overgo probe design based on a previously submitted partial cDNA sequence of catfish TLR3 (AY741552; Bilodeau and Waldbieser 2005) was used to screen the catfish BAC library. A total of 13 positive BAC clones were identified (Table S2). A fragment of 6,530 bp was obtained through primer walking on BAC 146-B23. The 5′ UTR of TLR3 was obtained by 5′ RACE. The putative catfish TLR3 cDNA consisted of 3,375 bp.

Sequence and structural analysis of the *TICAM* and *TLR3* genes of channel catfish

The channel catfish TICAM cDNA and gene were completely sequenced and deposited to the GenBank with accession numbers DQ423777 and DQ423778. The genomic structure of the catfish *TICAM* gene was determined by comparing the cDNA and genomic sequences. Catfish TICAM encodes a putative peptide of 520 amino acids within a single coding exon. A small noncoding exon contains a portion of the 5′ UTR (Fig. 1). A microsatellite sequence $(TG)_{28}$ was detected in the upstream genomic region that should be useful for genetic mapping of the *TICAM* gene to linkage maps. The same structural arrangement as catfish TICAM occurs in human isoform 2 of TICAM-1 and in murine TICAM-1. Human isoform 1 of TICAM-1 is a transcript variant with an alternative start codon and alternative splicing when compared with isoform 2. The important TIR domain, however, does not differ between the two isoforms. TICAM-2 in humans encodes a considerably smaller protein of only 232 amino acids in a single coding exon. Sequence conservation between species outside of the TIR domain of *TICAM* genes is low, and the size of the coding exon appears to vary considerably. TICAM-like sequences identified by BLAST searches in several species of fish (*D. rerio*, *T. rubripes*, *Tetraodon nigroviridis*) and the frog (*Xenopus tropicalis*) and chicken (*Gallus gallus*) encode proteins of approximately 450 to 550 amino acids.

A multiple sequence alignment of the TIR domains of the *TICAM* genes revealed high levels of similarity between all sequences (Fig. 2). A close examination indicated that fish TICAM sequences share some domain residues with mammalian TICAM-1 and others with mammalian TICAM-2. In fact, despite higher BLASTX identity with TICAM-1 sequences and a protein length more similar to mammalian TICAM-1, catfish TICAM shared 26% full-length amino acid sequence identity with human TICAM-2 compared with only 18% with human TICAM-1. Similar results were obtained in comparison to murine TICAM-1 and TICAM-2, raising interesting questions as to the phylogenetic relations of the vertebrate *TICAM* genes (see "Results" and "Discussion" below).

The channel catfish TLR3 cDNA and gene were completely sequenced and deposited to the GenBank with accession numbers DQ423775 and DQ423776. The *TLR3* gene from channel catfish encodes a putative peptide of 905 amino acids in four coding exons (Fig. 3). A small noncoding exon contains a portion of the 5′ UTR as in catfish TICAM. Two microsatellites, potentially useful for future genetic mapping, were detected in the 3′ UTR and downstream genomic region, a $(TA)_8$ at 5380 bp and an $(AC)_8$ at 5871 bp. Structural gene patterns and exon sizes are far more conserved in *TLR3* than in *TICAM* genes. The

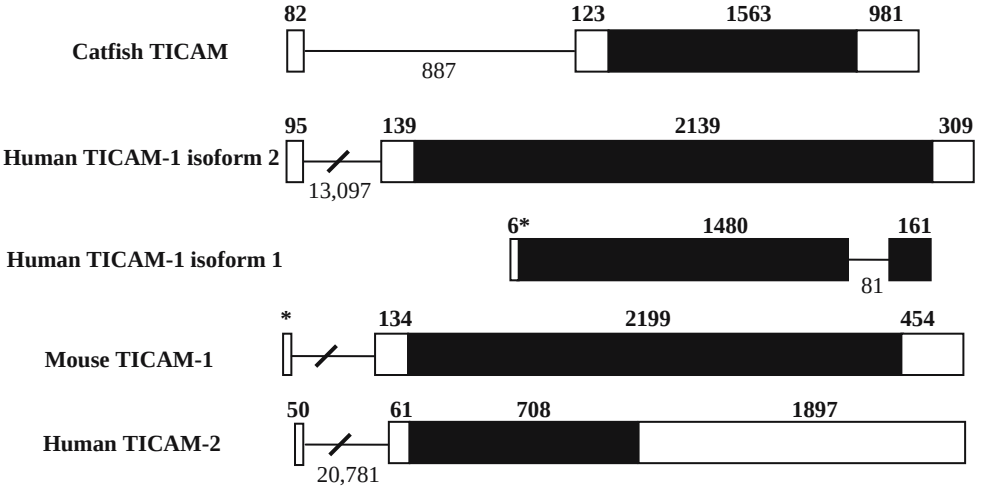


Fig. 1 A comparison of TICAM-1 and TICAM-2 genomic structure and organization from catfish, human, and mouse. Exons are represented by *boxes*; *solid boxes* represent the coding region of the gene; *white boxes* represent 5′-UTR and 3′-UTR. Their sizes in base pairs are shown on the *top of the boxes*. Introns are represented by a *line*, and the size is shown *below the line*. *The length and structure of a complete 5′ UTR has not been reported

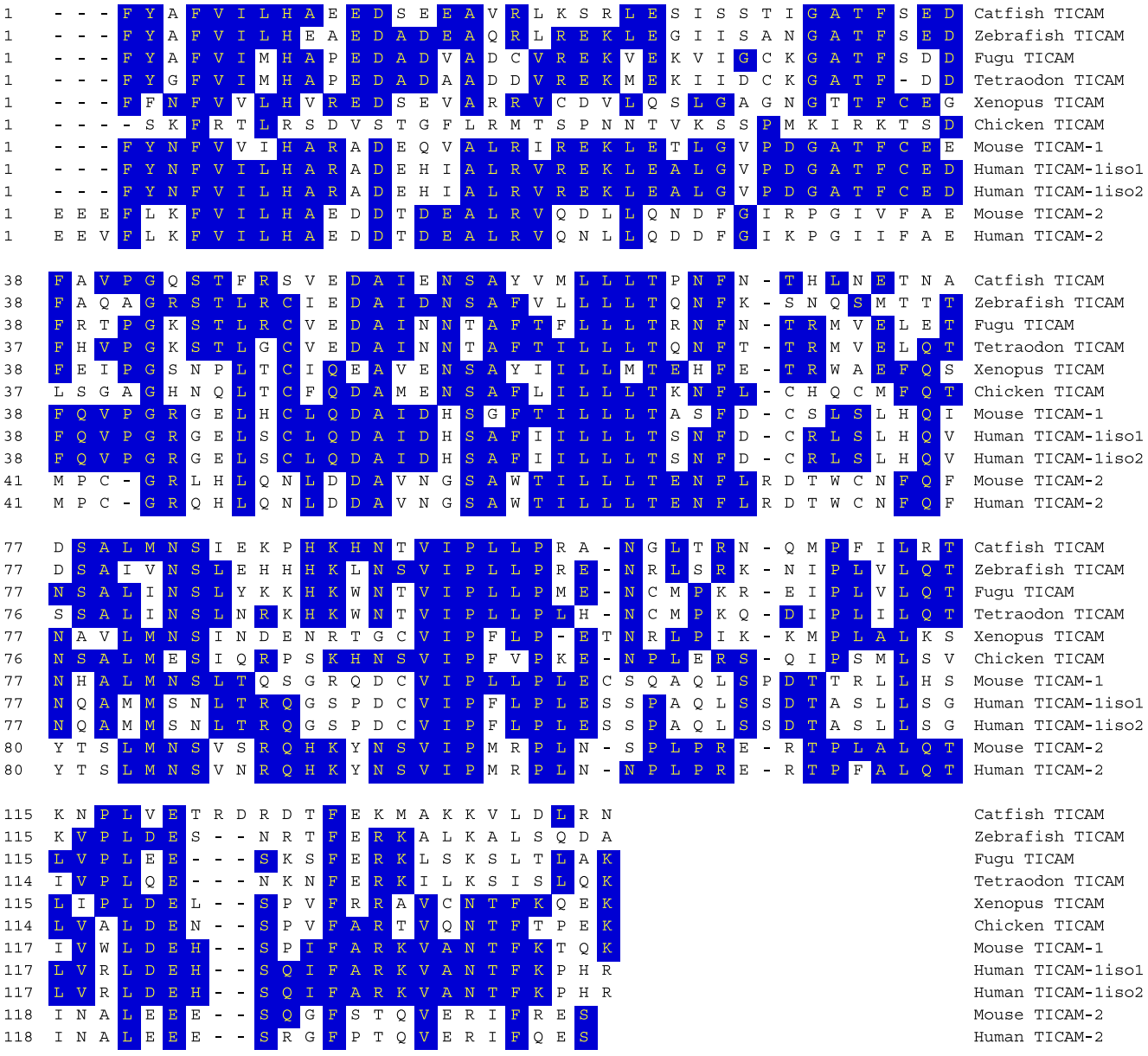


Fig. 2 Alignment of the catfish TICAM TIR domain with those of zebrafish, fugu, *Tetraodon*, *Xenopus*, and chicken, as well as with the TIR domain of *TICAM-1* and *TICAM-2* genes from mouse and human

four coding exons observed in channel catfish TLR3 concurred with previous reports from zebrafish, rainbow trout, and human, as did the presence of a noncoding exon containing a portion of the 5′ UTR. Exon sizes varied little among the vertebrate *TLR3* genes (Fig. 3).

A multiple sequence alignment of the TIR domains of the *TLR3* genes indicated high levels of amino acid sequence conservation (Fig. 4). Catfish TLR3 shared the highest percentage of identities with zebrafish (72.1%) and lowest with humans (51.4%) within the TIR domain. Percentage of identities shared with catfish in the less conserved full sequence ranged from 62.2 to 45.2% in zebrafish and humans, respectively. A comparison of the LRR distribution and the distribution of predicted N-linked glycosylation sites was made with human TLR3 for which the crystal structure is known (Choe et al. 2005; Bell et al. 2005). Both groups identified 23 LRRs in human TLR3 in addition to an N-terminal capping motif (LRR-NT) and a C-terminal cap (LRR-CT). This repeat structure appears well conserved in catfish, with all 23 LRRs and both caps easily identifiable (not shown). Bell et al. (2005) additionally reported four pairs of cysteine residues that form disulfide bonds, all of which are present in the catfish *TLR3* gene. We identified 13 potential sites for N-linked glycosylation, and eight of these align with positions observed to be glycosylated in human TLR3 (Bell et al. 2005).

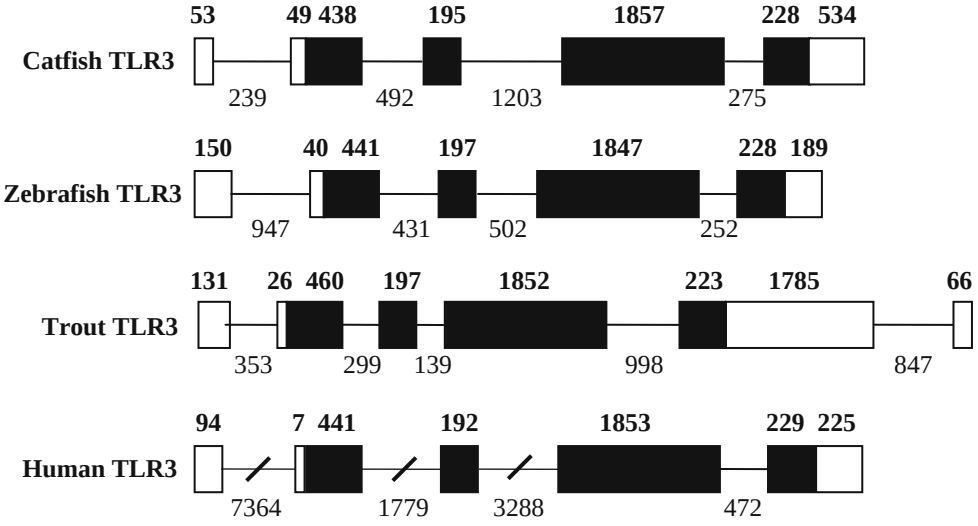


Fig. 3 A comparison of TLR3 genomic structure and organization from catfish, zebrafish, trout, and human. Exons are represented by *boxes*; *solid boxes* represent the coding region of the gene; *white boxes* represent 5′-UTR and 3′-UTR. Their sizes in base pairs are shown on the *top of the boxes*. Introns are represented by a *line*, and the size is shown *below the line*

Phylogenetic analysis of catfish TICAM and TLR3

To assess the evolutionary relations among vertebrate *TICAM* and *TLR3* genes, phylogenetic analysis was conducted using the minimum evolution method. Catfish TICAM was analyzed in the context of predicted TICAM sequences from zebrafish, fugu, *T. nigroviridis*, *X. tropicalis*, and *G. gallus* along with published mouse and human TICAM-1 and TICAM-2 sequences. All fish TICAM sequences formed a distinct clade, with tighter subclades between catfish and zebrafish and fugu and *Tetraodon* (Fig. 5). The *Xenopus* and chicken TICAM sequences were included in the clade of mammalian TICAM-1 sequences. Fish TICAM sequences grouped with mammalian TICAM-1 sequences; however, bootstrapping support was inconclusive, giving a value of 47 on the connecting branch. Mammalian TICAM-2 sequences formed their own distinct clade.

Catfish TLR3, analyzed along with TLR3 sequences from *Tetraodon*, fugu, rainbow trout, zebrafish, mouse, and human, was grouped strongly into a subclade with zebrafish TLR3. Fish and mammalian TLR3 sequences fell into

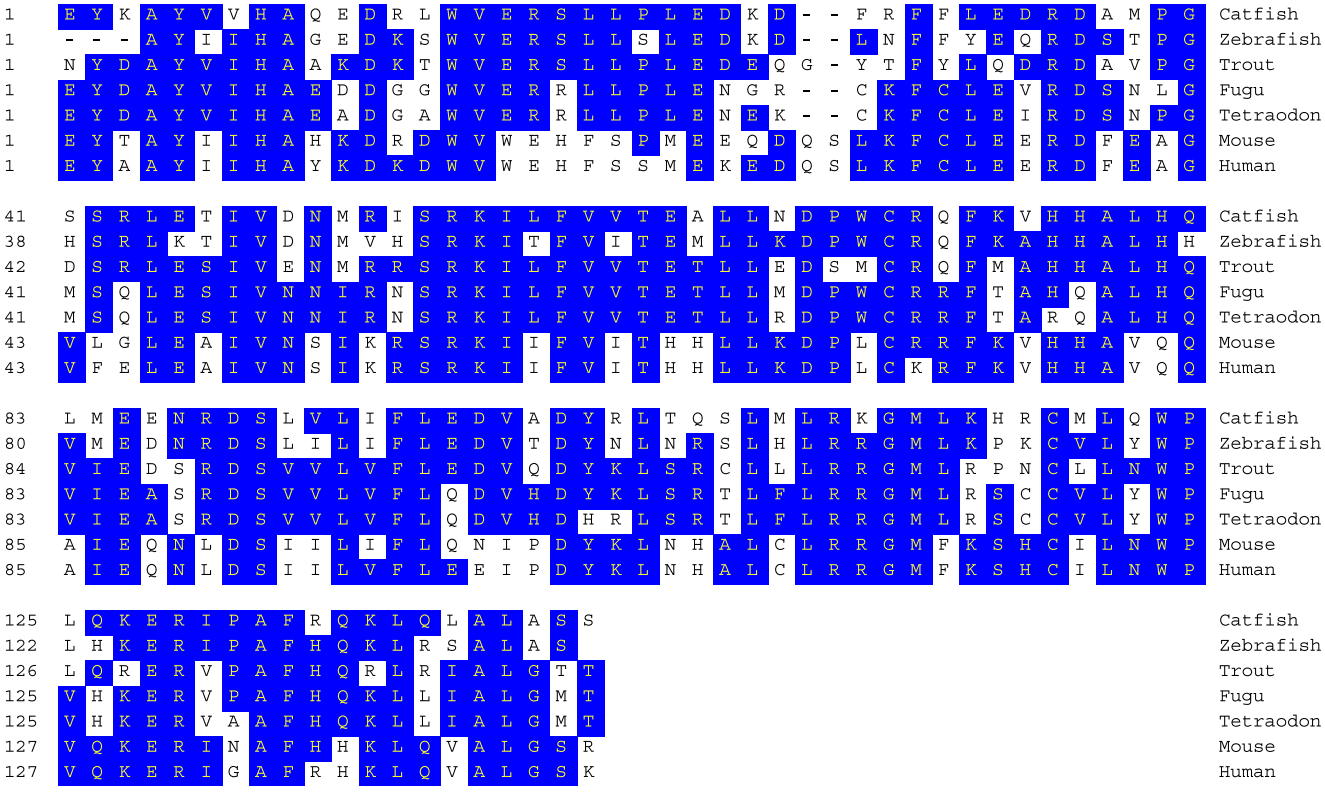


Fig. 4 Alignment of the catfish TLR3 TIR domain with those of zebrafish, trout, fugu, *Tetraodon*, mouse, and human genes

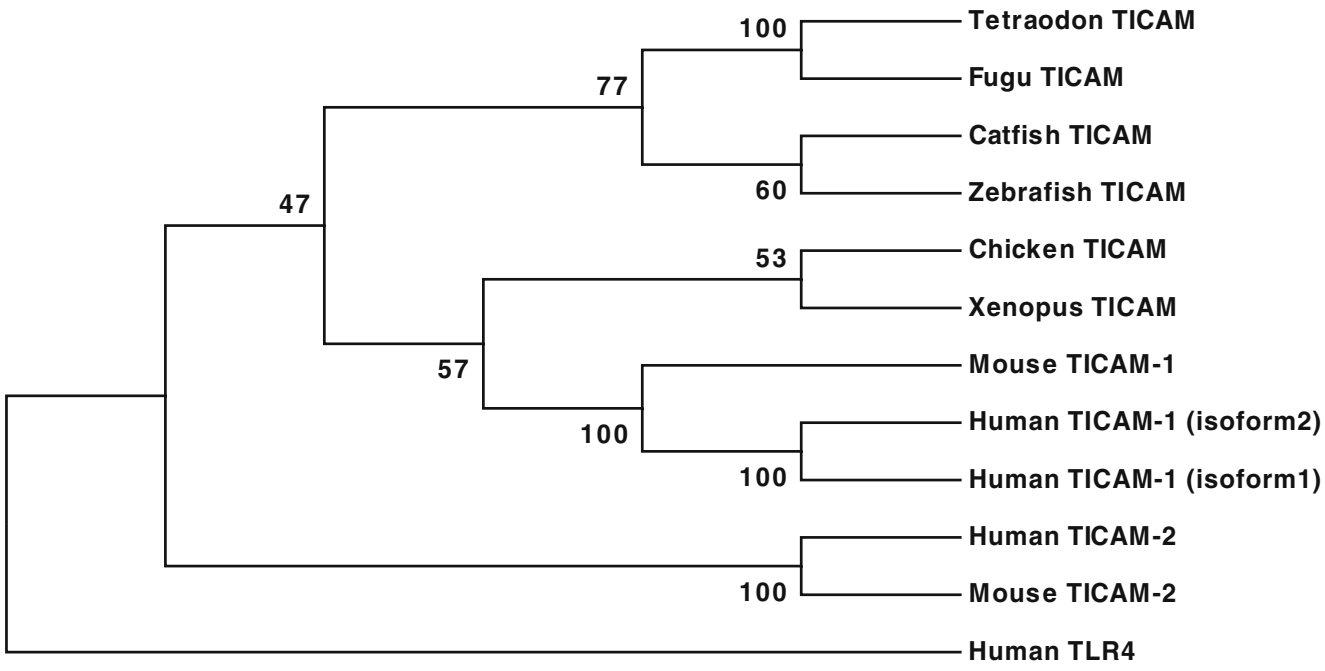


Fig. 5 Phylogenetic analysis of catfish TICAM. The phylogenetic tree was drawn from ClustalW-generated multiple sequence alignment of amino acid sequences using the minimum evolution method within the MEGA (3.0) package. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1,000 bootstrapping replications, and the bootstrapping values are indicated by *numbers* at the *nodes*. The sequences and their GenBank accession numbers or the genomic scaffolds from Ensembl containing TICAM sequences used are the following: catfish (DQ423777), fugu (FUGU4: scaffold_73), tetraodon (Tetraodon7: SCAF14992), zebrafish (Zv5_NA690), *Xenopus* (JGI4:scaffold_79), chicken (WASHUC1:28), mouse TICAM-1 (NP_778154), human TICAM-1 isoform 2 (NP_891549, Human TICAM-1 isoform 1 (NP_055076), human TICAM-2 (NP_067681), Mouse TICAM-2 (NP_775570), and human TLR4 (CAD99157) were used as an outgroup to root the phylogenetic tree

distinct clades but were clustered together based on outgroup analysis with human TLR4 (Fig. 6).

Copy numbers of TICAM and TLR3 in catfish

Fifteen and 13 BAC clones of the catfish BAC library were positive by overgo hybridization for TICAM and TLR3, respectively (Table S2). To determine the copy numbers of the *TICAM* and *TLR3* genes in the channel catfish genome, BAC DNA from the positive clones was isolated and subjected to Southern blot analysis after digestion with *Eco*RI and *Hin*dIII. As shown in Figs. 7 and 8, a single restriction pattern was observed with all BAC clones for both restriction enzymes in both TICAM and TLR3, indicating the existence of a single copy of each gene in the channel catfish genome. Faint bands in lane 10 of Fig. 7 were the result of a low concentration of isolated BAC DNA. The BAC-based Southern blot analysis was consistent with previous reports of a single copy of TLR3 in fugu (Oshiumi et al. 2003c), zebrafish (Meijer et al. 2004; Jault et al. 2004; Phelan et al. 2005), and rainbow trout (Rodriguez et al. 2005), as well as in mammalian species.

TICAM and TLR3 expression in healthy and infected tissues of blue and channel catfish

Quantitative real-time PCR analysis using total RNA from various healthy tissues of channel catfish indicated that catfish TICAM was expressed in all tested tissues. However, expression levels differed noticeably among the tissues, with expression highest in the ovary (Fig. 9a). TLR3 was also ubiquitously expressed, albeit at different levels. Highest expression of catfish TLR3 was found in the liver and muscle (Fig. 9b).

To assess the potential involvement of catfish TICAM and TLR3 during bacterial infection in catfish, RT-PCR was conducted using RNA isolated from the head kidney and spleen of blue and channel catfish at several time points after challenge with *E. ictaluri*, the causative agent of ESC. Channel catfish are highly susceptible to ESC infection, whereas blue catfish are resistant (Wolters et al. 1996). Drastically different expression profiles of TICAM and TLR3 were observed between channel and blue catfish, whereas the expression profiles of the two genes within each species were strikingly similar (Fig. 10a,b). TICAM expression in the head kidney of channel catfish was not

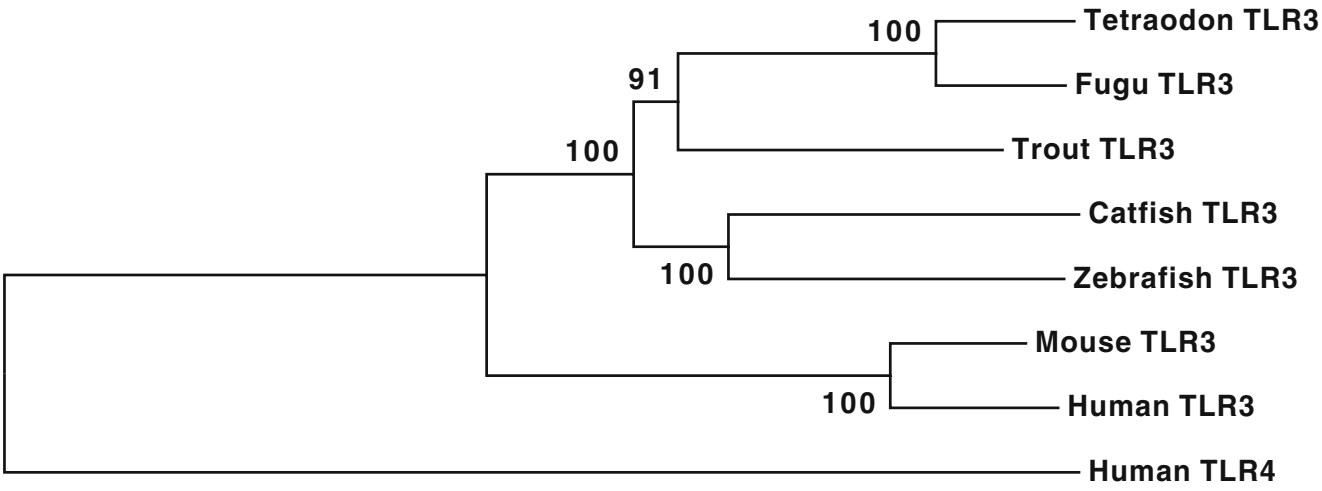


Fig. 6 Phylogenetic analysis of catfish TLR3. The phylogenetic tree was drawn from ClustalW-generated multiple sequence alignment of amino acid sequences using the minimum evolution method within the MEGA (3.0) package. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1,000 bootstrapping replications, and the bootstrapping values are indicated by *numbers* at the *nodes*. The sequences and their GenBank accession numbers used are the following: catfish TLR3 (DQ423775), fugu TLR3 (AAW69373), tetraodon TLR3 (CAF96592), trout TLR3 (AAX68425), zebrafish TLR3 (NP_001013287), human TLR3 (NP_003256), mouse TLR3 (NP_569054), and human TLR4 (CAD99157) was used as an outgroup to root the phylogenetic tree

noticeably altered after infection with ESC. Similarly, TLR3 expression in channel catfish head kidney did not appear to change considerably relative to the 0-h control, with the possible exception of the time point at 4 h after infection. In contrast, TICAM and TLR3 expression in the head kidney of blue catfish appeared to be drastically downregulated as early as 4 h postinfection. The faint bands of the gene products in head kidney made it somewhat difficult to judge the extent of this downregulation at 4 h. No bands were visible for either TLR3 or TICAM after the 4 h in head kidney of blue catfish.

Expression patterns of TICAM and TLR3 also showed similarities in infected spleen samples within each catfish species (Fig. 10a,b). In channel catfish, expression of TICAM appeared to decrease relative to the 0-h control starting at 4 h postinfection, with the lowest expression detected at 72 h postinfection. Higher expression levels were detected in moribund fish. A similar pattern of downregulation was evident for TLR3 in channel catfish spleen. No expression of the TLR3 transcript can be detected at 72 h, whereas a faint band can be seen in moribund fish. Although TICAM and TLR3 also appeared to be downregulated in the spleen of blue catfish, the pattern was more abrupt than in channel catfish. No expression of TICAM can be detected in spleen for any time points after the challenge. TLR3 expression decreased

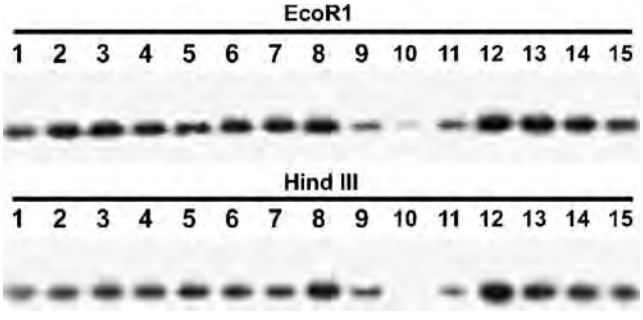


Fig. 7 Southern blot analysis of catfish TICAM using positive BAC clones. BAC DNAs were digested with *Eco*RI and *Hin*dIII, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to the TICAM cDNA probe. *Lanes 1–15* contained BAC DNA from 15 different BAC clones (Table S2). Only one pattern was observed, suggesting a single copy of the *TLR3* gene in the catfish genome

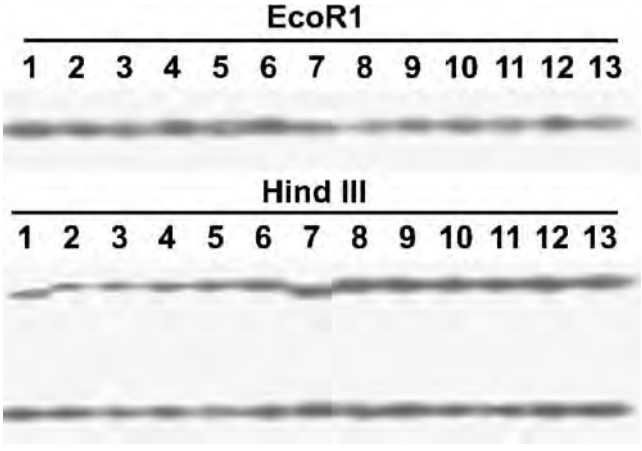


Fig. 8 Southern blot analysis of catfish TLR3 using positive BAC clones. BAC DNAs were digested with *Eco*RI and *Hin*dIII, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to the TLR3 cDNA probe. *Lanes 1–13* contained BAC DNA from 13 different BAC clones (Table S2). Only one pattern was observed, suggesting a single copy of the *TLR3* gene in the catfish genome. For *lanes 1* and *7*, the gene was found to be at the end of the BAC insert

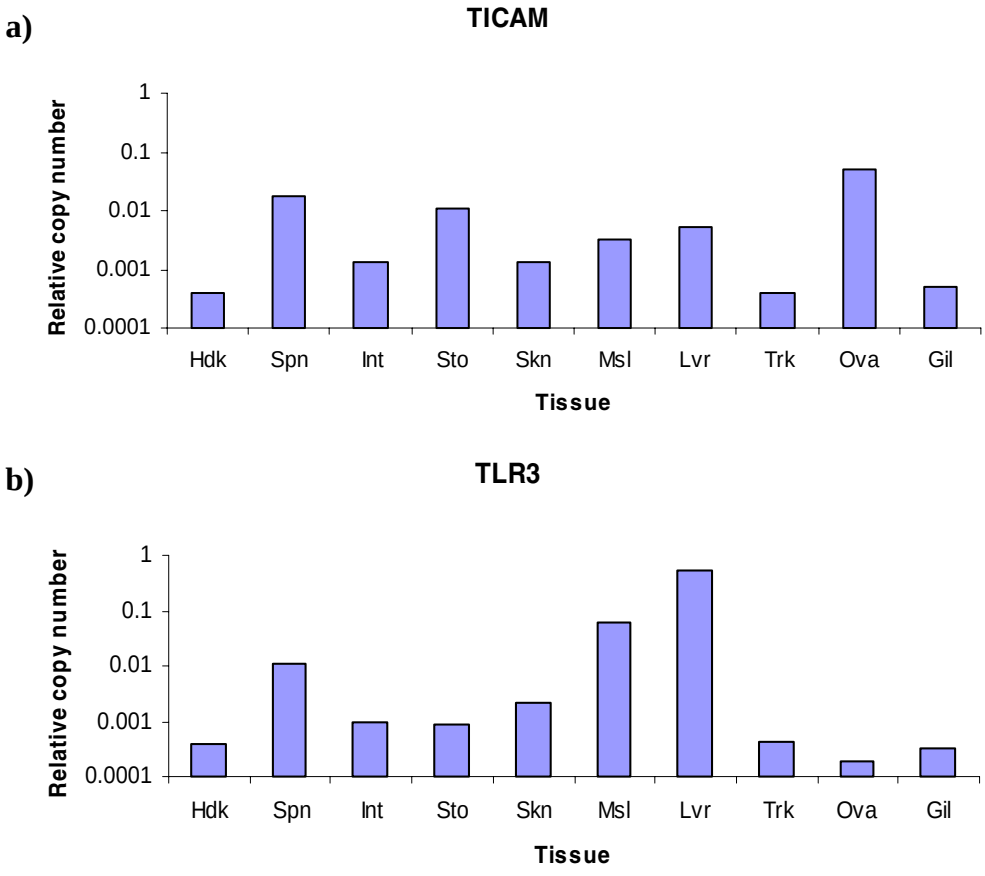


Fig. 9 Real-time PCR analysis of channel catfish TICAM (**a**) and TLR3 (**b**) mRNA expression in various healthy tissues: head kidney (*Hdk*); spleen (*Spn*); intestine (*Int*); stomach (*Sto*); skin (*Skn*); muscle (*Msl*); liver (*Lvr*); trunk kidney (*Trk*); ovary (*Ova*); and gill (*Gil*). Copy number was normalized to β-actin expression levels. Data are presented on a logarithmic scale

relative to the control sample at 4 h and was not observed in the other time points.

To validate the dramatic downregulation of TLR3 and TICAM observed following bacterial infection and quantify the striking differences in expression profiles between blue and channel catfish (particularly in head kidney) observed in the RT-PCR results, we conducted quantitative real-time PCR using the head kidney samples. Results confirmed the patterns observed from RT-PCR (Fig. 11). Expression changes after infection in channel catfish were small (most

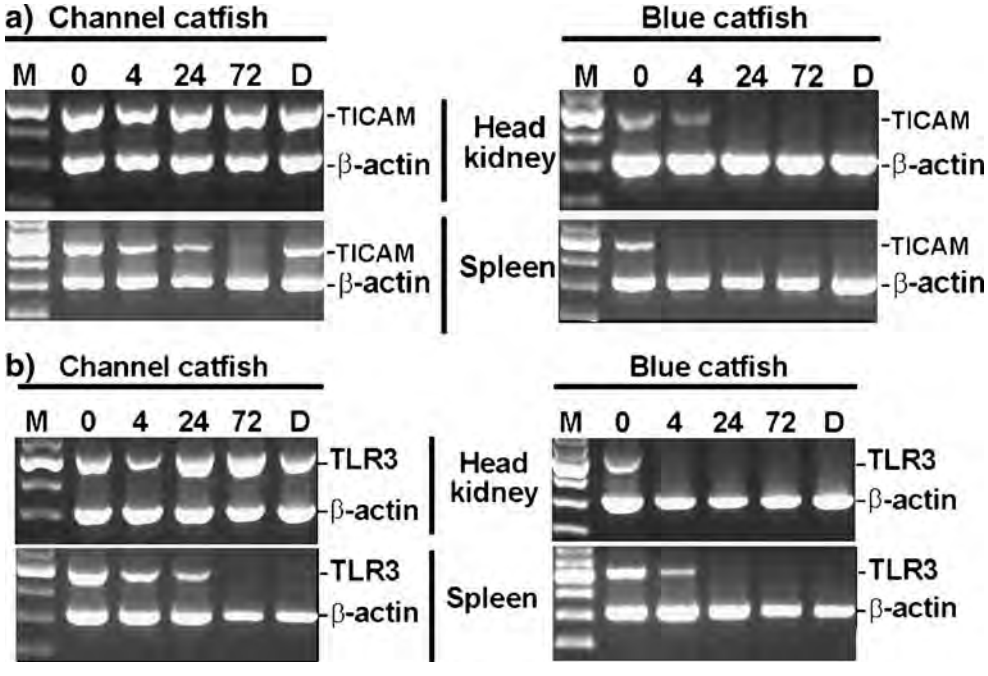
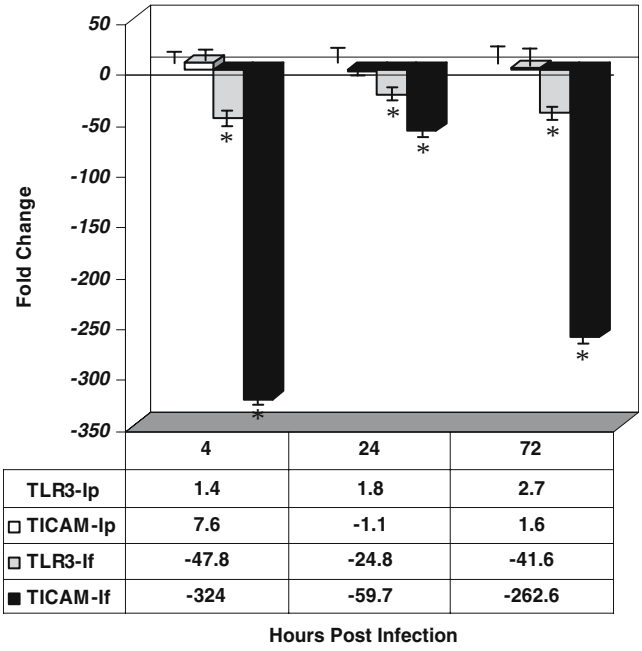


Fig. 10 RT-PCR analysis of catfish TICAM (**a**) and TLR3 (**b**) mRNA expression after bacterial challenge with *Edwardsiella ictaluri*. RT-PCR products were analyzed on agarose gels. *M* is 100 bp molecular weight marker. Samples of head kidney and spleen were collected at different time points: control (0 h), 4, 24, and 72 h after challenge, and moribund fish (*D*). Channel catfish samples are shown in the *left panel*, and blue catfish samples are shown in the *right panel*. The positions of the RT-PCR amplified bands of β-actin and TICAM or TLR3 are indicated on the *right margin*

less than twofold) and statistically insignificant. In contrast, expression changes in blue catfish for TICAM and TLR3 following infection were large and significant, and indicated an overall shutdown of gene expression. For example, TICAM was downregulated 324-fold relative to control (p=0.014; Fig. 11).

Discussion

In the present study, we have characterized the genomic structures of TLR3 and TICAM adaptor genes in channel catfish. Although a partial TLR3 cDNA sequence has been reported from channel catfish, and complete *TLR3* genes are known from other teleost fish species, a complete TICAM sequence has not been reported from a non-mammalian species. As a putative adaptor molecule of catfish TLR3, catfish TICAM likely represents an important component of the innate immune response to microbial pathogens. We have analyzed catfish TLR3 and TICAM expression together, therefore, to determine whether their expression patterns are suggestive of a conservation of the close receptor–adaptor relation seen in orthologous genes in mammals. Furthermore, we have compared their expression patterns in channel catfish with those from the closely related blue catfish species (*I. furcatus*), which exhibit greater resistance to the Gram-negative bacterium *E. ictaluri*. The results suggested a conserved TLR3–TICAM relation in catfish and revealed a striking pattern of species-specific expression potentially linked to disease resistance.



	4	24	72
TLR3-Ip	1.4	1.8	2.7
TICAM-Ip	7.6	-1.1	1.6
TLR3-If	-47.8	-24.8	-41.6
TICAM-If	-324	-59.7	-262.6

Fig. 11 Real-time PCR analysis of catfish TICAM and TLR3 expression in head kidney following infection with ESC. Fold change indicates the ratio of expression at the specified time after ESC exposure to that at 0 h before ESC exposure, as normalized with an internal reference gene of catfish β-actin. Three independent LightCyler reactions were run for each sample, and statistical analysis was conducted using the software REST. *Error bars* indicate standard error, and an *asterisk* indicates statistical significance. *Ip* and *If* signify channel catfish and blue catfish, respectively

Catfish TLR3 and TICAM differed noticeably in their conservation of gene sequence and structure. Although the TIR domain of both genes can be readily identified because of its high conservation, the regions outside this domain in TICAM are highly variable. These regions in human TICAM are proline-rich, including an eight-residue poly-proline track. In fact, hepatitis C virus has been reported to evade the TLR3/TICAM-1 signaling pathways by proteolysis of TICAM-1 within these proline-rich tracks (Ferreon et al. 2005). The proline content of murine TICAM, however, is significantly less, and high proline content was not observed in catfish TICAM. Outside the TICAM TIR domain, crucial for binding to the TIR domain of the TLR, there appears to have been little evolutionary pressure for structural conservation among vertebrate species. Protein length varies considerably. Catfish TICAM encodes 520 amino acids, whereas TICAM-1 in human and mouse are over 700 amino acids in length. A shorter human isoform encodes 546 amino acids (Fig. 1). TICAM sequences identified from the genomes of fish species, frog, and chicken by BLAST encode proteins of 450 to 550 amino acids. Catfish TLR3 sequence and structure (Fig. 3), on the other hand, showed little variation with reports from other vertebrate species.

The relation of catfish TICAM with mammalian *TICAM-1* and *TICAM-2* genes was examined. Initial results from BLASTX searches, and the longer length of catfish TICAM when compared with mammalian TICAM-2, supported naming the gene catfish TICAM-1. However, a comparison of amino acid identities within the TIR domain (Fig. 2) revealed a slightly closer similarity with TICAM-2. Others have reported finding TICAM-1 by in silico searches of teleost fish genomes, but a full sequence was not reported (Jault et al. 2004; Meijer et al. 2004; Iliev et al. 2005). When we conducted tBLASTn searches with human TICAM-1 as a query within the Ensembl (v. 37) server, a single genome region was hit for *D. rerio*, *T. nigroviridis*, and *T. rubripes*, as well as the frog (*X. tropicalis*) and chicken (*G. gallus*). A single long open reading frame (ORF) containing a TICAM-1-like gene was present in all species with the exception of chicken, which had a long run of "N"s within its sequence. Chicken ESTs present in NCBI's dbEST were used to span the area of unfinished sequence in the genome. Start codons were predicted based on the mammalian TICAM-1 hits of BLASTX searches. We concur with Iliev et al. (2005),

who found no TICAM-2 sequences within the fish genomes. All TICAM sequences identified by this method had higher BLAST matches with TICAM-1 sequences. A phylogenetic tree (Fig. 4) constructed with these sequences along with catfish and mammalian *TICAM* genes included the fish, frog, and chicken *TICAM* genes in a clade with TICAM-1. However, bootstrapping support of this clade was not conclusive. We also examined the genomic environs of the vertebrate *TICAM* genes for clues as to their identity. TICAM-1 in humans and mice is located between feminization 1 homolog a (FEM1A) and a mannose 6 phosphate receptor binding protein (TIP47) (Hardy et al. 2004). Examination of the genomic environs of the *TICAM* genes identified above found syntenic conservation of this region with *G. gallus*, strongly indicating that the gene identified from that species was a TICAM-1 ortholog. Similar neighboring genes were not identified from the genomic regions surrounding the other vertebrate *TICAM* genes.

It has been speculated that TICAM-1 and TICAM-2 in mammals arose from a duplication of a single ancestral gene and then evolved specific functions (Hardy et al. 2004). The theory of duplication within this region is supported by the presence of two additional closely related genes, FEM1A next to TICAM-1 and FEM1C next to TICAM-2. The apparent presence of a single fish *TICAM* adaptor gene, phylogenetically intermediate to TICAM-1 and TICAM-2, also supports such a theory. Interestingly, it appears that TLR4, which in mammals requires the TICAM-2 adaptor for MyD88-independent signaling, is not present in the genomes of *T. nigroviridis* or *T. rubripes* but is present in zebrafish (Meijer et al. 2004). Because a well-conserved *TLR3* gene has been identified in all teleost species studied to date, it seems more likely that the fish TICAM adaptor is functioning in the role of TICAM-1. In fish species with TLR4, we speculate the TICAM adaptor may be capable of performing the functions of both mammalian TICAM-1 and TICAM-2.

One preliminary method of assessing the functional conservation of catfish TLR3 and TICAM adaptor is expression analysis. We first examined the tissue expression profiles of the two genes in healthy channel catfish tissues (Fig. 9). Catfish TLR3 was expressed in a range of tissues as in zebrafish and rainbow trout (Rodriguez et al. 2005; Jault et al. 2004) and at high levels in liver and digestive organs (Bilodeau and Waldbieser 2005; Oshiumi et al. 2003c). TLR3 expression studies in mammals have focused on cell types rather than tissues (Muzio et al. 2000; Alexopoulou et al. 2001), making comparisons difficult. Catfish TICAM was also expressed in a range of tissues, with higher relative expression levels in the ovary, liver, stomach, and spleen. Similarly, Yamamoto et al. (2002) found highest TICAM-1 expression in human liver.

Expression analysis of catfish TLR3 and TICAM in tissues of fish infected with the bacterium *E. ictaluri* produced some of the most interesting results of the study (Figs. 10 and 11). Markedly different expression profiles of TICAM and TLR3 were observed when comparing between channel (susceptible) and blue (resistant) catfish, whereas the expression profiles of the two genes within each species were strikingly similar. Blue catfish are a closely related species sharing the same genus as channel catfish. Our previous studies indicated that their gene sequences were well conserved, with a divergence rate of 1.32 bp/100 bp (He et al. 2003). The expression results suggested a conserved TLR3–TICAM relation within each catfish species. The expression profiles of TLR3 and TICAM generally mirrored one another's trends in all the time points following infection.

The dramatic downregulation of *TLR3* and *TICAM* gene expression seen following infection in blue catfish head kidney and spleen and, to a lesser extent, channel catfish spleen, differed from the expression profiles of other catfish innate immune components, including CC and CXC chemokines (Peatman et al. 2005, 2006; Baoprasertkul et al. 2004; Chen et al. 2005), antimicrobial peptides (Bao et al. 2005, 2006; Wang et al. 2006a; Xu et al. 2005), proinflammatory cytokines (Wang et al. 2006b), and other TLRs (Baoprasertkul et al. 2006). These genes have generally shown patterns of gradual or dramatic upregulation, constitutive expression, or modest downregulation after ESC infection. However, transient downregulation of TLR3 has been observed in the head kidney of rainbow trout exposed to Gram-negative bacterium *Yersinia ruckeri* (Rodriguez et al. 2005). Comparison of our results with previous studies in channel and channel–blue backcross hybrids (Bilodeau and Waldbieser 2005; Bilodeau et al. 2006) is difficult because of different spacing of sampling time points after ESC infection. These studies, however, observed modest two- to threefold changes in expression of TLR3, similar to our real-time PCR results from channel catfish (Fig. 11). Significant upregulation of TLR3 was observed in adult zebrafish after infection with another Gram-negative bacterium, *Edwardsiella tarda* (Phelan et al. 2005). Taken together, these results suggest that TLR3 responds to bacterial and viral PAMPs in fish, but in a manner that differs considerably with time point, pathogen, and host species. Similarly, in mammalian species, human TLR3 responds solely to dsRNA, whereas murine TLR3 has been shown to also respond to LPS (Alexopoulou et al. 2001; Kadowaki et al. 2001).

The downregulation of catfish TICAM and TLR3 following infection can be explained by the maturation and migration of different cell types to and from the lymphoid tissues. Visintin et al. (2001) reported that expression of most TLRs, including TLR3, transiently

increased then nearly disappeared after immature dendritic cells were induced to mature by addition of LPS. Down-regulation of TLR3 expression in murine macrophages after LPS treatment has been described (Heinz et al. 2003). Macrophage populations play an important role in ESC infections of catfish. Resistant populations of channel catfish have been reported to have more macrophage aggregates in spleen and head kidney than in susceptible populations (Camp et al. 2000), and macrophages from resistant catfish more effectively kill the bacteria between 1 and 3 h after infection (Shoemaker et al. 1997). Additionally, Ruckdeschel et al. (2004) identified that TICAM-1 signals for apoptosis of murine macrophages infected with the Gram-negative bacterium *Yersinia*. Further cell-based studies are required to determine if the dramatic differences in expression of TLR3 and TICAM between resistant and susceptible catfish species can be explained by functional and temporal differences in macrophage activity.

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