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Pathogen recognition receptors in channel catfish: III Phylogeny and expression analysis of Toll-like receptors

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ABSTRACT

Toll-like receptors (TLRs) were the earliest characterized and the most extensively studied pathogen recognition receptors (PRRs). The majority of tetrapod TLR orthologs have been found in teleost fish. In addition, a group of "fish-specific" TLRs have been identified. In catfish, a number of TLR-related sequences have been reported, but systematic phylogenetic analyses have not been conducted. In this study, we conducted phylogenetic and comparative analysis of 20 catfish TLR genes against their counterparts from various species. TLR25 and TLR26 are TLRs identified only in channel catfish. Phylogenetic analyses suggested that four catfish TLR genes have duplicated copies in the genome, i.e., TLR4, TLR5, TLR8, and TLR20. Six fish-specific TLRs were identified, and the vast majority of these belong to the TLR11 subfamily. In healthy catfish tissues, most of the tested TLR genes were ubiquitously expressed although expression levels varied among the 11 tested tissues. We tested nine TLRs for their expression in response to *Edwardsiella ictaluri* infection. They were significantly up-regulated in the spleen and liver, but down-regulated in the head kidney, suggesting their involvement in the immune responses against the intracellular bacterial pathogen in a tissue-specific manner in catfish, perhaps through rapid migration of phagocytes to infection sites.

1. Introduction

Innate immune system is the primary defense mechanism in lower vertebrates such as fishes. Primarily, it recognizes the conserved pathogen-associated molecular patterns (PAMPs) on the invading pathogens through pathogen recognition receptors (PRRs). After sensing the PAMPs, PRRs trigger the activation of signaling transduction pathways which result in effective immune responses to the infection (Akira et al., 2006; Uematsu and Akira, 2006).

Three major groups of PRRs have been identified, i.e., Toll-like receptors (TLRs), NOD-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). They were so classified mostly based on their structural characteristics. For instance, TLRs are type I transmembrane receptors characterized by the presence of leucine-rich repeats (LRRs) in their extracellular domain and by the presence of a Toll/interleukin-I receptor domain (TIR domain) in the C-terminal in the cytosol that initiates signal transduction (Akira et al., 2006), while the typical NLRs are characterized by the presence of a N-terminal protein–protein binding or effector domain, a central nucleotide oligomerization (NACHT) domain, and a C-terminal leucine-rich repeat (LRR) domain (Benko et al., 2008; Chen et al., 2009). TLRs were the earliest characterized and also the most extensively studied PRRs in both vertebrates and invertebrates (Hansen et al., 2011). They are known to recognize PAMPs of bacteria, viruses, fungi and protozoa at the cell membrane or the endosomal region (Uematsu and Akira, 2006).

The numbers of TLR genes vary among various organisms. Thirteen TLRs (TLR1–13) have been identified in mammals, and functionally these receptors recognize and respond to a wide range of exogenous as well as endogenous ligands. Of the 13 mammalian TLRs, TLR11, 12, and 13 were identified only in the murine genome (Hopkins and Sriskandan, 2005). In teleost fish, orthologs of TLR1–5, 7–9 have been identified, while various reports indicated that TLR6 and TLR10 do not exist in teleost fish (Roach et al., 2005; Rebl et al., 2010; Palti, 2011). In addition to the orthologs of TLRs in mammals, 'fish-specific' TLRs have been reported including TLR18, TLR19, TLR20, TLR21, TLR22, and TLR23 (Roach et al., 2005; Huang et al., 2008; Oshiumi et al., 2008; Rebl et al., 2010;

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Hansen et al., 2011; Palti, 2011). However, all these fish TLRs and their signaling cascade factors represent high structural similarity to the mammalian TLR system (Palti, 2011).

Many studies have been conducted with TLRs in teleost fish including channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), gilthead seabream (*Sparus aurata*), pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*), Japanese flounder (*Paralichthys olivaceus*), rare minnow (*Gobiocypris rarus*) and half-smooth tongue sole (*Cynoglossus semilaevis*) (Oshiumi et al., 2003; Jault et al., 2004; Meijer et al., 2004; Phelan et al., 2005; Bilodeau and Waldbieser, 2005; Bilodeau et al., 2006; Tsoi et al., 2006; Tsujita et al., 2006; Baoprasertkul et al., 2006, 2007a,b; Chaves-Pozo et al., 2008; Wu et al., 2008; Su et al., 2009a, 2009b; Sullivan et al., 2009; Yu et al., 2009; Hwang et al., 2010; Palti et al., 2010; Kongchum et al., 2010; Star et al., 2011; Holen et al., 2012; Huang et al., 2012; Sundaram et al., 2012; Yang et al., 2012). Channel catfish *I. punctatus*, a prominent aquaculture species in the United States, is one of the species whose immune system is well characterized. We previously characterized several catfish TLRs including TLR2, TLR3, TLR5S, TLR20a and TLR21 (Baoprasertkul et al., 2006, 2007a,b). Quiniou and Waldbieser (2011) have submitted a complete set of channel catfish TLR sequences recently released from the NCBI database. The objective of this study was to conduct a phylogenetic and comparative analysis of catfish TLRs against those identified from various other species, and examine their expression after bacterial infection. Here we report phylogenies of the 20 catfish TLR genes, their duplication status in the catfish genome, and their expression in normal tissues and after infection with the bacterial pathogen *Edwardsiella ictaluri*, the causative agent for the enteric septicemia of catfish (ESC). This paper is the third of the serial publications related to pathogen recognition receptors in channel catfish including NLRs (Rajendran et al., 2012b), RLRs (Rajendran et al., 2012a) and TLRs (this communication).

2. Materials and methods

2.1. Database mining and phylogenetic analysis

To identify the TLR genes, the catfish databases (Lu et al., 2011) containing ESTs (Li et al., 2007; Wang et al., 2008), RNA-seq (Liu et al., 2011, 2012) and the whole genome (our unpublished data) database of channel catfish were searched using available teleosts (*D. rerio*, *Carassius auratus*, *C. carpio*, *Oncorhynchys mykiss*, *P. olivaceus*, *S. salar*, *S. aurata*, *T. rubripes*, *Gasterosteus aculeatus*, *Oryzias latipes*, *T. nigroviridis*, *G. morhua*, *Oreochromis niloticus*), chicken and mammalian (human, mouse and cattle) TLRs as queries. In order to pull all potential TLRs, the cutoff value was set at intermediately stringent level of e-10 such that sequences with conserved domains are captured initially for additional analysis. The retrieved sequences were translated using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and GENSCAN (Burge and Karlin 1997). Further, the predicted ORFs were verified by BLASTP against NCBI non-redundant protein sequence database. The Simple Modular Architecture Research Tool (SMART) was used to predict the conserved domains based on sequence homology and further confirmed by conserved domain prediction from BLAST. LRRfinder 2.0 (Offord and Werling, 2012) was used to identify LRRs using their Toll-like receptor LRR database. TMHMM 2.0 was applied to predict transmembrane domain in TLRs. The TIR domains and LRR domains were used in the phylogenetic analysis, respectively. Multiple protein sequence alignments were conducted using the ClustalW program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura et al., 2011).

2.2. Expression analysis of catfish TLRs

Reverse-transcriptase PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) were conducted to study the mRNA expression of selected TLR genes. To analyze the expression of these genes in healthy fish, 11 tissues/organs including blood, skin, muscle, gill, heart, liver, spleen, intestine, head kidney, trunk kidney and brain were collected from 15 individual fish, with five fish each pooled into three separate pools. The tissues were snap-frozen in liquid nitrogen and immediately subjected to RNA extraction using RNeasy Mini Kit (Qiagen, USA) following the manufacturer's protocol. The extracted total RNA was quantified using a UV-spectrophotometer and an aliquot (1 μg) of RNA was treated with 1 U of RNase-free DNase (Qiagen) prior to reverse transcription. A uniform quantity of DNA-free RNA was reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) following manufacturer's protocol.

PCR was carried out using Platinum Taq DNA polymerase (Invitrogen). The 20 μl PCR reaction mixture contained 2.0 μl of 10× buffer, 1.0 μl of $MgCl_2$ (25 mM), 1.0 μl of dNTP (10 mM), 0.4 μl of Taq polymerase (1 U), 1 μl (10 pmol/μl) of each primers, 2 μl cDNA and 12.6 μl PCR-grade water. Gene-specific primers and internal reference 18S rRNA-specific primers were used, respectively in the PCR amplification. Amplification was performed on a Bio-Rad PCR system for 35 cycles of denaturation at 95°C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min. The PCR products were resolved on a 2% agarose gel.

2.3. Bacterial challenge and quantitative real-time PCR

Healthy channel catfish (Marion strain) were exposed to a Gram negative bacterium, *E. ictaluri* via immersion challenge following the procedure reported by Peatman et al. (2007). Briefly, 100 fish (9.3 cm, mean length and 11.2 g, mean weight) were acclimatized in the laboratory for 3 days with water temperature maintained at 27 °C. A virulent strain of *E. ictaluri* was cultured in a brain heart infusion (BHI) medium by incubating in shaking incubator at 28 °C overnight. The experimental fishes were immersed in 15 L aerated freshwater mixed with bacterial culture added to a concentration of 4×10^6 CFU/ml. During the immersion, water circulation was turned off for 2 h followed by continuous water flow-through. Another set of unexposed fish were maintained as the control group. The fishes exposed to *E. ictaluri* showed typical clinical signs of enteric septicemia including appearance of small circular red spots over the body, bloody areas on base of fins, white circular spots, raised reddish area on top of head, protruding eyes, ulcerated areas on top of the head (so-called hole-in-the-head), bloated fluid-filled belly, and heavy signs of hemorrhages of organs and tissues. Behavioral signs included erratic swimming, swirling, and hanging head up and tail down in the pond (Chappell, 2008), while unchallenged fish stayed healthy without any similar symptoms of the disease. Further, the experimental fishes showed large-scale mortality after 5 days of infection, whereas control fishes remained healthy. Fifteen fish were sacrificed at each time point (4 h, 24 h, 4 day and 6 day post-infection) from both experimental and control groups. The same tissues from five individual fish were pooled, and RNA was prepared as described above.

Real time PCR was conducted for the analysis of TLR expression with nine TLR receptors including TLR3, TLR4, and seven non-mammalian "fish-specific" TLR genes (TLR18–TLR22, TLR25 and TLR26). Three biological replicate RNA samples (five fish in each replicate) from head kidney, spleen and liver in both normal and infected fish at different time points were analyzed for gene

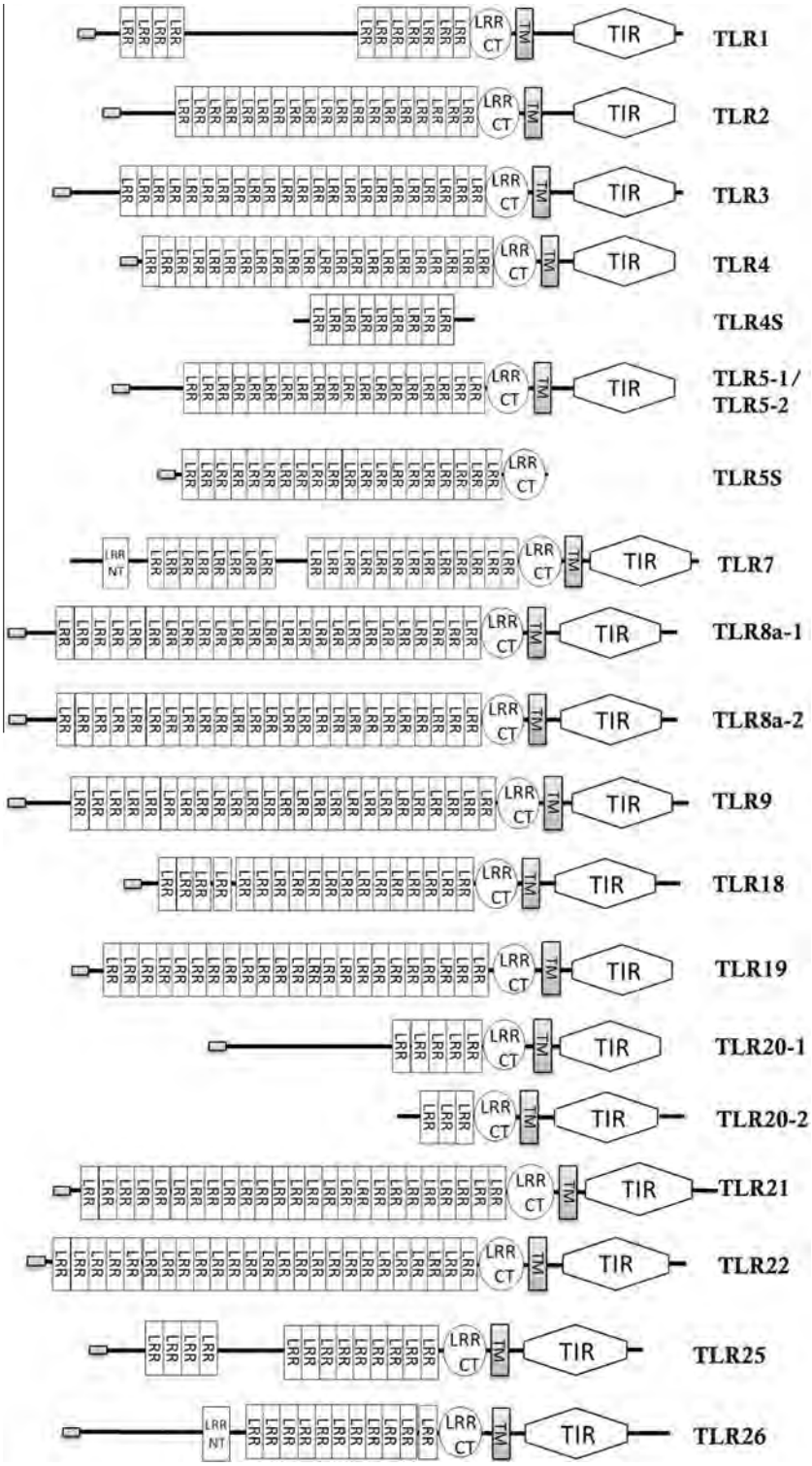

Fig. 1. Schematic representation of the domain architecture of TLRs in channel catfish. Domains were determined using SMART program, LRRfinder 2.0 and TMHMM 2.0. *Abbreviations*: LRR: leucine rich repeats; LRR CT: leucine rich repeats C-terminal capping motif; LRR NT: leucine rich repeats N-terminal capping motif; TIR: TIR domain, TM: transmembrane domain. Note that the TM domains in several TLRs (TLR7, TLR9, TLR19, and TLR20-1) were predicted to be weak using TMHMM 2.0.

expression profiles using qRT-PCR as we previously reported (Rajendran et al., 2012a,b; Liu et al., 2010), with 18S rRNA as the internal reference because the 18S rRNA is stably expressed under ESC infection (Small et al., 2008). After master mix was prepared, each sample was divided into three PCR replicates. Each qRT-PCR reaction consisted of a total volume of 10 μl containing 5.0 μl Sso-Fast EvaGreen Supermix (Bio-Rad, USA), 0.5 μl of each primer (5 pmol/μl), 2 μl cDNA and 2 μl PCR-grade water. The thermal cycling was carried out on a C1000 Thermal Cycler (Bio-Rad, USA) using the cycling conditions: denaturation, 95 °C/30s, 40 cycles of 95 °C/5s, 57 °C/5s, and 72 °C/5s followed by dissociation curve analysis to verify the specificity of amplified products. The qRT-PCR data were exported into a Microsoft Excel Sheet for analysis. The relative expression ratio of target gene in experimental group

and the control group was calculated using the $2^{-\Delta\Delta}$CT method. The data generated were further analyzed statistically using one-way ANOVA.

3. Results

3.1. Identification of TLR-related genes

TLR protein sequences reported from teleosts, chicken and mammalian species were used as query sequences to blast against the catfish EST, RNA-seq and genome databases to identify an initial pool of TLR-related sequences for the analysis of the repertoire of the catfish TLRs. A total of 20 TLR genes were identified in catfish with high levels of similarities to TLR1, TLR2, TLR3, TLR4, TLR4S, TLR5-1, TLR5-2, TLR5S, TLR7, TLR8a-1, TLR8a-2, TLR9, TLR18, TLR19, TLR20-1, TLR20-2, TLR21, TLR22, TLR25 and TLR26.

TLR genes are characterized by possession of several structural features such as their conserved functional domains of leucine rich repeats (LRR), transmembrane domain (TM), and Toll/interleukin-I receptor domain (TIR). As shown in Fig. 1, the 20 TLR genes in catfish harbor various numbers of LRR domains, ranging from four LRR to 26 LRRs. The vast majority of the TLRs harbor a C-terminus LRR (LRR CT in Fig. 1); six TLRs do not have a C-terminus LRR domain, i.e., TLR4S, TLR8a-2, TLR19, TLR20-1, TLR20-2, and TLR26 (Fig. 1). The transmembrane domain TM exists in most TLRs except in TLR5S. All TLRs have the TIR domain with the exception of TLR4S and TLR5S (Fig. 1). It is noteworthy that the similar domain organizations may not indicate their relatedness, and phylogenetic analysis is required to identify their identities.

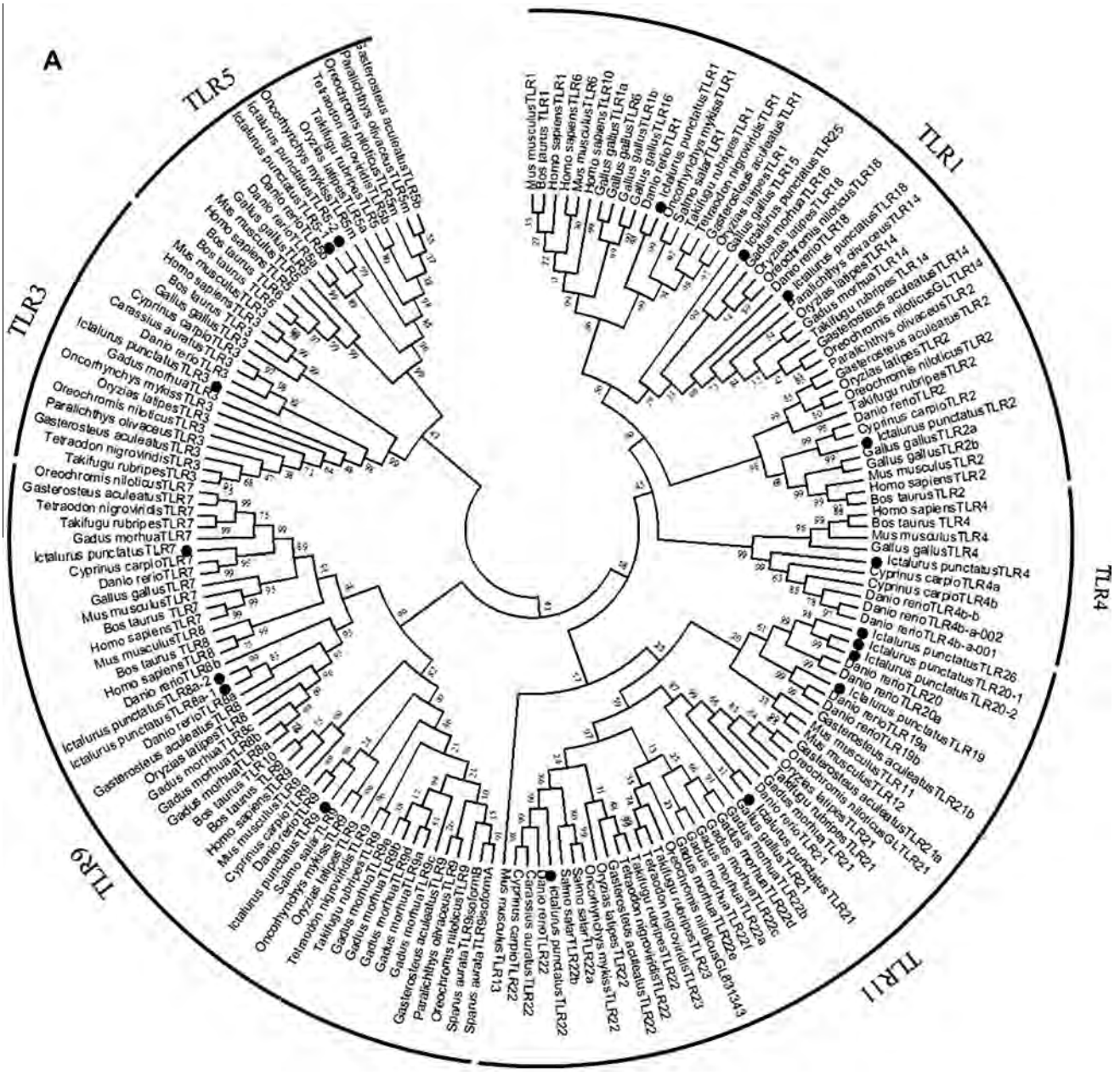

Fig. 2. Phylogenetic relationship of catfish and other vertebrate TLRs. Phylogenetic analysis of catfish and other vertebrate TLRs using TIR domains (A) or LRR (B). ClustalW alignments of all the amino acid sequences were used to generate a neighbor-joining tree using MEGA5 software. The tree is shown with 10,000 replicates from the bootstrap test and the percentage of bootstrap values were given next to the branches.

3.2. Phylogenetic analysis of TLRs

Various annotations have been documented in the literature or in the databases concerning the identities of various TLR genes. However, it is important to conduct phylogenetic analysis to determine the identities of these genes. We have used all available TLR sequences in selected species (amino acids) for the construction of the phylogenetic tree. As shown in Fig. 2A and B, phylogenetic analysis provided strong support for the identities of many of the catfish TLRs, but ambiguous situations still exist (Table 1). For instance, strong bootstrap support was obtained with the identities of TLR1, TLR2, TLR3, TLR4, TLR5 (teleost fish appear not to have TLR6), TLR7, TLR8, TLR9 (teleost fish appear not to have TLR10, TLR11, TLR12, and TLR13), TLR14 (though catfish TLR14 was not found), TLR19, TLR20, TLR21, TLR22, TLR23 (though catfish TLR23 was not found) (teleost fish appear not to have TLR24). As detailed in Table 1, the identities of a number of teleost TLR genes are uncertain at this point. For instance, TLR16 was annotated in Atlantic cod, but it is highly related to TLR18 in medaka (Fig. 2A and B). Two TLR genes, TLR25 and TLR26 were only found in catfish. While the catfish TLR25 appeared to be quite unique in its sequence as compared with other TLR genes although it is related to Atlantic cod TLR16 and medaka TLR18. In contrast, the catfish TLR26 is highly related to its TLR20 genes (Fig. 2A and B).

3.3. Copy numbers of TLR genes in catfish

A total of 26 TLR genes have been annotated across a broad spectrum of species in the literature including those of catfish (Table 1). Of these 26 genes, 15 distinct genes were identified in catfish: TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, TLR18, TLR19, TLR20, TLR21, TLR22, TLR25, and TLR26. Among these TLRs found in catfish, four TLR genes are duplicated. These are TLR4, TLR5, TLR8 and TLR20. In birds, there were two copies in TLR1, TLR2

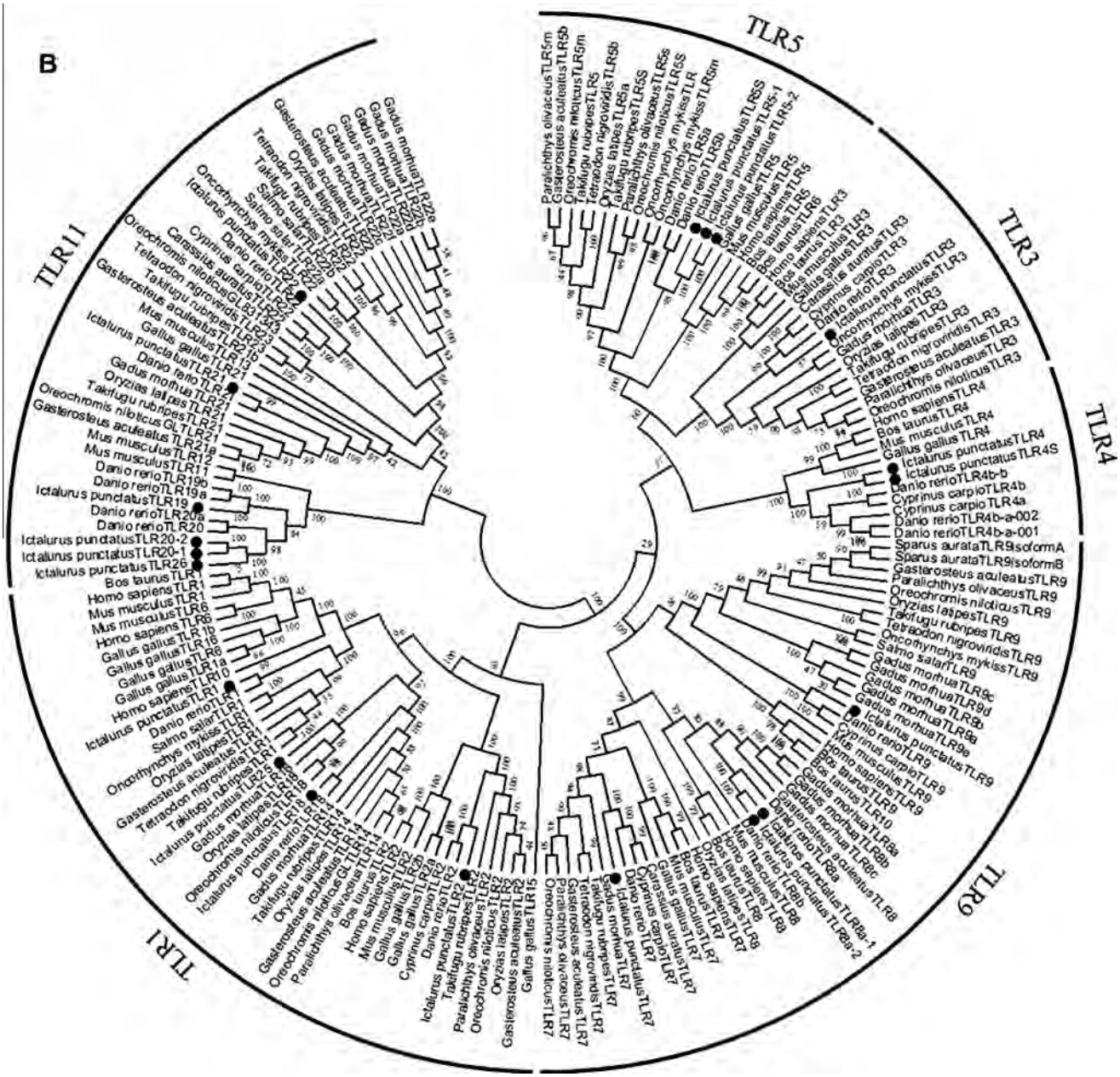

Fig. 2. (*continued*)

Table 1
Comparison of TLR genes among mammals, birds, jawless fish and or amphibians, and teleost fish with focus on zebrafish and catfish. Blue shading indicates TLRs that were not found in teleost fish; pink shading indicates TLRs that were found only in teleost fish; yellow shading indicates TLRs that were not found in mammals but were not fish-specific; and orange shading indicates TLRs that were found only in catfish.

TLR Genes	Copy # Human/ Murine	Copy # Bird	Copy # Amphibian/ jawless fish	Copy # zebrafsih	Copy # Catfish	Note	Strong phylogenetic clade
TLR 1	1/1	2	1/0	1	1		Yes
TLR 2	1/1	2	2/0	1	1		Yes
TLR 3	1/1	1	1/1	1	1		Yes
TLR 4	1/1	1	0/0	3	2		Yes
TLR 5	1/1	1	1/1	2	3		Yes
TLR 6	1/1	0	2/0	0	0		Yes
TLR 7	1/1	1	1/1	1	1		Yes
TLR 8	1/1	0	2/1	2	2		Yes
TLR 9	1/1	0	1/0	1	1		Yes
TLR 10	1/0	0	0/0	0	0		Yes
TLR 11	0/1	0	0/0	0	0	Murine only	No
TLR 12	0/1	0	1/0	0	0		No
TLR 13	0/1	0	1/0	0	0	Related to fish TLR 21	No
TLR 14	0/0	0	4/4	1	0		Yes
TLR 15	0/0	1	0/0	0	0	Birds only	
TLR 16	0/0	1	0/0	0	0	Named TLR 16 in cod, related to fish TLR 18	No
TLR 17	0/0	0	0/0	0	0		No
TLR 18	0/0	0	0/0	1	1	Related to TLR 14	Yes
TLR 19	0/0	0	0/0	2	1		Yes
TLR 20	0/0	0	0/0	2	2	Related to TLR 26	Yes
TLR 21	0/0	2	1/3	1	1		Yes
TLR 22	0/0	0	1/1	1	1		Yes
TLR 23	0/0	0	0/0	0	0		Yes
TLR 24	0/0	0	0/4	0	0	Lamprey only	
TLR 25	0/0	0	0/0	0	1	Related to medaka TLR 18 and Atlantic cod TLR 16	No
TLR 26	0/0	0	0/0	0	1	Related to TLR 20	No

and TLR21. While in amphibians, multiple copies were reported for TLR2, TLR6, TLR8 and TLR14. In Lamprey, only TLR14, TLR21 and TLR24 have multiple copies. Teleost fish appeared not to have the following TLRs: TLR6, TLR10, TLR11–13, TLR15, TLR17, and TLR24 (Table 1). Of the 18 TLRs identified from various teleost fish species, 15 were found in catfish. TLR14, TLR16, and TLR23 have not been found in catfish, whereas TLR25 and TLR26 were found only in catfish (Table 1).

3.4. Expression of TLR genes

Tissue expression of all TLR genes, except TLR4S and TLR20-2 because of difficulties in primer design for specific amplification, was determined by RT-PCR. Eleven healthy catfish tissues (blood, skin, muscle, gill, heart, liver, spleen, intestine, head kidney, trunk kidney and brain) were used. As shown in Fig. 3, TLR genes are mostly constitutively expressed in all tested tissues with a few notable exceptions: (1) TLR4 appears not expressed in the liver (see below, it was not detected with real time qPCR either); (2) As shown in Fig. 3, several TLR genes such as TLR4, TLR18, TLR25, and TLR26 appeared to exhibit tissue preference, but nonetheless are also expressed in all tissues. For instance, TLR4 was expressed most highly in the gill (Lane 4) and intestine (Lane 8); TLR18 was expressed most highly in the gill and trunk kidney; TLR26 was most highly expressed in head kidney, and in blood, muscle, heart, liver, and brain tissues, TLR25 was expressed at low levels.

3.5. Expression of selected TLR genes after bacterial infection

Expression of a set of nine TLR genes was analyzed after bacterial infection in three tissues, head kidney, spleen, and liver. The nine genes include TLR3, TLR4, TLR18, TLR19, TLR20-1, TLR21, TLR22, TLR25, and TLR26. As shown in Fig. 4, the expression of these nine genes after bacterial infection can be characterized in the following general patterns: (1) All the nine genes responded to bacterial infection; (2) The nine genes responded drastically differently in different tissues; (3) In the head kidney tissue, expression levels of all nine genes were down after bacterial infection, although the levels of down-regulation and the exact time of down-regulation varied depending on the genes, but in general, the expression levels were downward within 6 days after infection; (4) In contrast to the situation in the head kidney, expression levels of the nine genes in the spleen was initially drastically induced and then slowly returned to almost the base level of

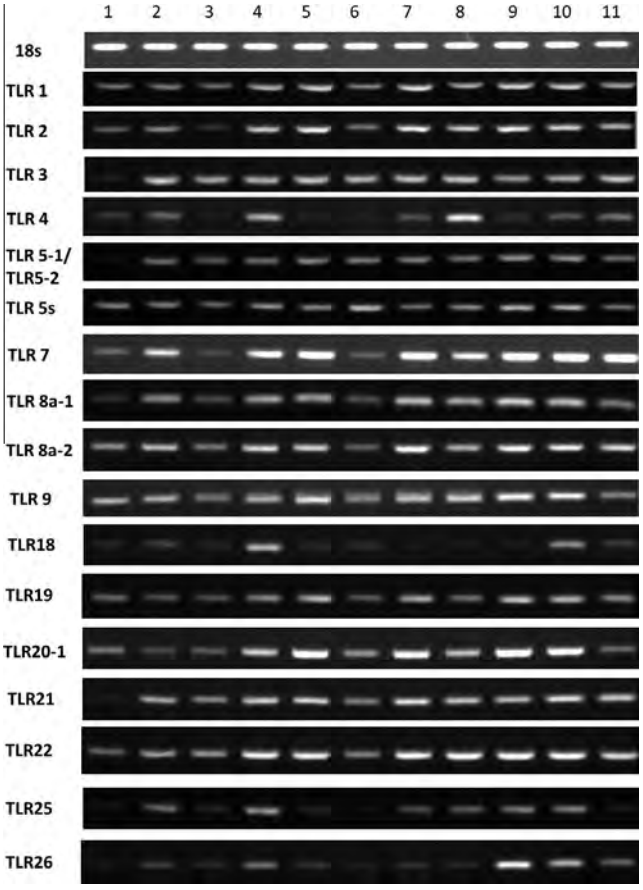

Fig. 3. RT-PCR-based expression analysis of selected members of TLRs in different tissues of healthy channel catfish. Tissues from five animals were pooled for RNA extraction. 1, blood; 2, skin; 3, muscle; 4, gill; 5, heart; 6, liver; 7, spleen; 8, intestine; 9, head kidney; 10, trunk kidney; 11, brain. 18S rRNA was used as an internal control and gene names are indicated on the left of the panel.

expression; (5) In the liver, the situation was very similar to that in the spleen except that the level of induction was even more dramatic, and that TLR4S was not expressed in the liver.

The kinetics of up- or down-regulation of the nine TLR genes exhibited a similarity of general trend, but differed in the timing and extent of regulation. In head kidney, all the nine TLR genes were down-regulated at 4 h ranging from twofold to fourfold. After 6 days of infection, TLR20-1 and TLR21 were observed as the most down-regulated genes with 31-fold and 161-fold reduction, respectively. In contrast, the dramatic up-regulation of all the nine TLR genes was observed at 4 h in spleen (ranging from twofold to sevenfold) and liver (ranging from fivefold to 18-fold). TLR18 (sevenfold) and TLR25 (fivefold) were found the two most up-regulated genes in spleen, while TLR18 (18-fold) and TLR26 (15-fold) were detected as the two most up-regulated genes in the liver.

4. Discussion

Pathogen recognition receptors play an important role in innate immune system. TLRs were among the earliest characterized and the most extensively studied pathogen recognition receptors in both vertebrates and invertebrates. Even within catfish, a number of studies have been conducted with TLRs (Bilodeau and Waldbieser, 2005; Bilodeau et al., 2006; Baoprasertkul et al., 2006, 2007a,b; Quiniou and Waldbieser, 2011). We previously reported complete sets of NLR and RLR receptors (Rajendran et al., 2012a,b), but a systematic analysis of TLR phylogenetics and expression was lacking. In this study, we identified a total of 20 TLRs *in silico* from EST, RNA-seq and genome databases. Such a repertoire of TLR genes, along with those from other species, allowed more thorough phylogenetic analysis.

The total number of TLR genes in catfish is yet to be determined, but we believe that the vast majority of its TLR genes have been found. A total of 26 TLR genes have been annotated across a broad range of organisms. Of these, TLR10 was present in various mammalian species except murine, but absent in teleosts; TLR11 was present only in murine species; TLR12 and TLR13 were present in murine and amphibians; TLR15 was found only in birds; TLR16 was present in birds, and it was so named in Atlantic cod as well, but the Atlantic cod gene may be more related to teleost TLR18 (see below); TLR24 was found only in lamprey, a jawless fish. Taken together, a total of 18 TLRs have been found in all teleost fish, and they are TLR1 to TLR5, TLR7 to TLR9, TLR14, TLR16, TLR18–23, TLR25 and TLR26. Teleost fish appeared not to have the following TLRs: TLR6, TLR10, TLR11–13, TLR15, TLR17, and TLR24 (Table 1). Of the 18 TLRs identified from various teleost fish species, 15 were found in catfish. TLR14, TLR16, and TLR23 have not been found in catfish, whereas TLR25 and TLR26 were found only in catfish.

The correctness of phylogenetic analysis depends much on the availability of sequences across a broad range of taxa. In this regard, there was strong support for the identities of the majority of TLR genes. These included TLR1–10 while TLR11 is murine specific. TLR12 and TLR13 were found in murine and amphibians, but the teleost TLR21 is highly related to TLR13; apparently, they make a sub-family of the TLRs, and they could be well evolutionarily related (Roach et al., 2005; Palti, 2011). The nomenclature of TLR16 in Atlantic cod, TLR18 in medaka and TLR25 in channel catfish can be questionable because they form a distinct clade within which the cod TLR16 is apparently highly related to the medaka TLR18 (Fig. 2A and B). The exact identities of these TLRs need to be further analyzed upon availability of additional sequences in the near future from various related taxa. The TLR18 annotated in zebrafish and channel catfish can also be questionable as they are more related to TLR14 of many teleost species than to TLR18 of medaka (Fig. 2A and B). Additionally, two duplicated genes were annotated as TLR20-1 and TLR20-2 by Quiniou and Waldbieser (2011), and they named a related gene TLR26 in channel catfish. However, it is apparent that TLR26 in channel catfish could be an ancient duplicated gene of TLR20 because a distinct subclade formed among the catfish TLR20-1 TLR20-2 and TLR26 within the clade of TLR20 (Fig. 2A and B).

Phylogenetic analysis apparently suggested the presence of a number of TLR subfamilies. Roach et al. (2005) suggested six TLR subfamilies: TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11. These subfamilies are still apparent, but with most of the teleost specific TLR genes falling under TLR11 subfamily (Fig. 2A and B), which included the catfish TLR19, TLR20-1, TLR20-2, TLR21, TLR22, and TLR26.

Four catfish TLR genes appeared to have duplicated gene copies; i.e., TLR4, TLR5, TLR8 and TLR20. This is similar to the situation in the zebrafish genome. In zebrafish, TLR4b-a1, TLR4b-a2 and TLR4b-b paralogs have been reported and annotated on chromosome 13 (Meijer et al., 2004; Jault et al., 2004; Sullivan et al., 2009); TLR5a and TLR5b paralogs are present on chromosome 20; For TLR8, two paralogs of zebrafish TLR8 have been annotated as TLR8a and 8b on chromosome 9 and 10, respectively; In regard to TLR19, the two copies located on chromosome 16; Two copies of zebrafish TLR20 appeared to be a recent duplicates located tandemly on chromosome 9. Of the duplicated catfish TLR genes, TLR4/TLR4S, TLR5S/TLR5-1/TLR5-2 and TLR20-1/TLR20-2 are tandem duplication in the catfish genome whereas TLR8 was identified in different linkage group (Quiniou and Waldbieser, 2011).

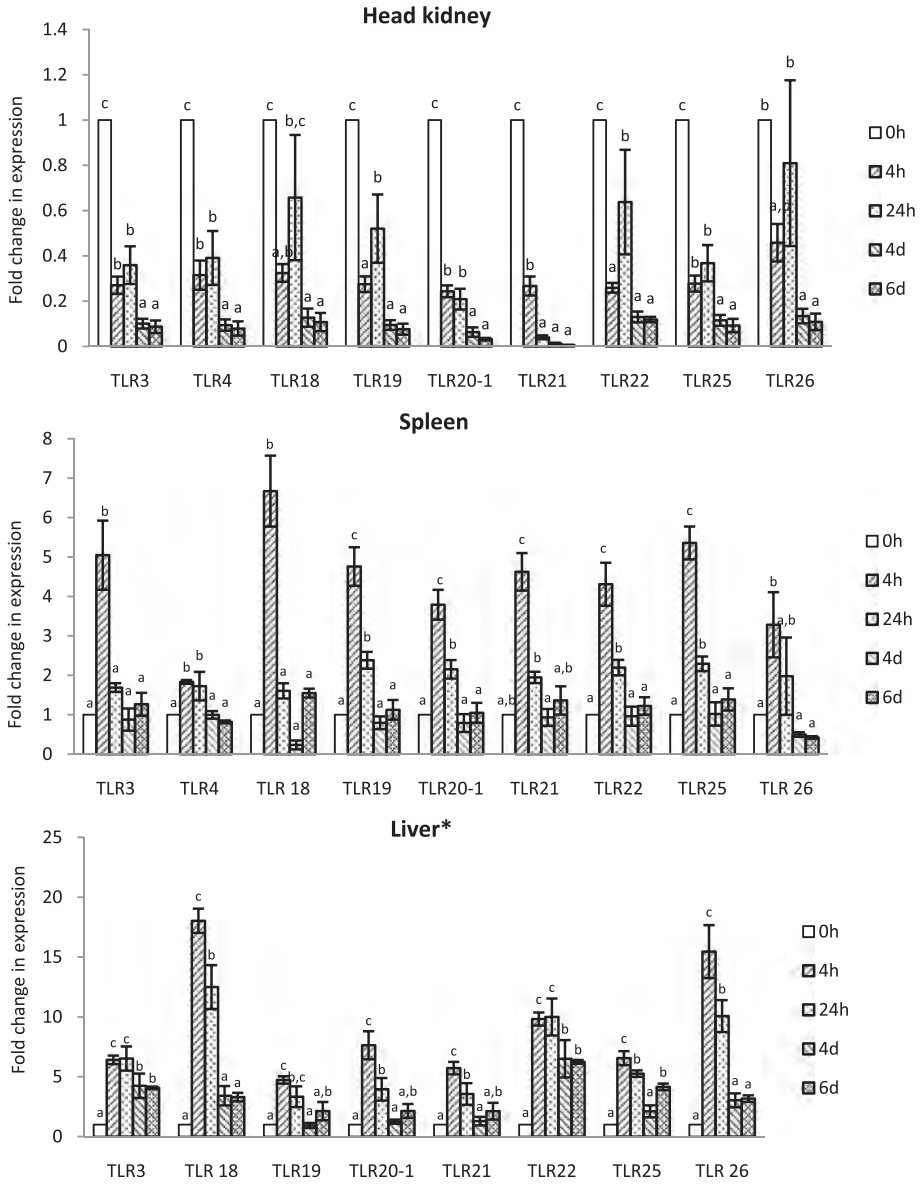

Fig. 4. qRT-PCR-based expression analysis of selected TLR genes in different tissues of channel catfish following bacterial infection. Healthy catfish were exposed to *E. ictaluri* through immersion. Tissues were collected at four different time points after bacterial challenge and RNA extracted from fifteen fishes (three pools of five each) were used in the qRT-PCR. Nine representative TLR genes were tested with 18SrRNA as internal reference. The results were expressed as mean ± standard error (bars) from three replicates. Significant difference ($p < 0.05$) among controls and various treatments are shown by a different letter, i.e., 'a' is statistically different from 'b', and 'b' is different from 'c' etc. *TLR4 was not evaluated because no expression was detected in the liver.

RT-PCR analysis of TLR expression indicated that most TLR genes are ubiquitously expressed in all tissues with the exception of TLR4 that is not expressed in the liver. Their expression levels vary depending on the tissues in healthy fish (Fig. 3). It is unknown at present the mechanisms behind the tissue preference, but it could be related with cell type specificity of TLR gene expression, and specific cell types may be represented very differentially in different tissues. For instance, several TLR genes appeared to be expressed at high levels in intestine, gill, and head kidney, all tissues heavily involved in immune responses.

However, their expression after bacterial infection exhibited drastic tissue differences. In the head kidney all the nine genes tested were down-regulated, while the same nine genes were drastically up-regulated in the spleen and liver. The two TLR genes with most drastic down-regulation in the head kidney were TLR20-1 and TLR21. They were down-regulated rapidly with four-fold down 4 h after infection, reaching very low expression (31-fold and 161-fold reduction, respectively) 4–6 days after infection. This could indicate that these TLR genes are the most responsive to bacterial infection as the subpopulation of phagocytes expressing these genes could rapidly migrate out of head kidney to infection sites. In comparison, TLR18 and TLR25 were the most highly induced in spleen, while TLR18 and TLR26 were the most induced in the liver. While it is difficult to speculate functional importance based on expression levels, it is apparent that all these TLRs are involved in immune responses after bacterial infection. Future research is warranted to understand the mechanisms of the expression regulation, particularly its relationship with regulation at the transcriptional level versus through cell migration.

The orthology of TLR4 genes has not been well established. In part, this was due to loss of ohnologs and perhaps followed by tandem gene duplications (Sullivan et al., 2009). For instance, TLR4

was not identified from pufferfish, but was identified from zebrafish (Jault et al., 2004; Meijer et al., 2004; Sullivan et al., 2009), common carp (Kongchum et al., 2010) and rare minnow (Su et al., 2009b) as well as catfish. However, based on the lack of responses to LPS signaling and the tandem arrangement of the TLR4ba and TLR4bb genes, Sullivan et al. (2009) thought the zebrafish TLR4 genes were ohnologs to the human TLR4 gene. In this study, phylogenetic analysis indicated the relatedness of TLR4 genes from zebrafish, carp, and catfish (Fig. 2B). We suspect that TLR4 genes from zebrafish, carp, and catfish are perhaps paralogous although syntenic analysis in these species is yet not possible at present. The catfish TLR4 genes apparently responded to infection with *E. ictaluri*, a Gram negative bacterium: It is up-regulated in the spleen and down-regulated in the head kidney, although it is not expressed at detectable level in the liver. Based on their expression after infection, we believe that it is involved in disease responses, but certainly additional functional analysis is required to obtain a solid conclusion. It is possible that sequence mutations through evolution in the promoter region made the TLR4 gene less functional as a pathogen recognition receptor. This later notion is supported by the low expression and low level of responses in the spleen and head kidney, and the lack of expression in the liver. Similar to the situation in catfish, TLR4 in rare minnow was activated by both viral and bacterial infection (Su et al., 2009b), suggesting it retained the functionality of LPS responses.

The strong contrast of down-regulation in the head kidney and up-regulation in the spleen and liver was also observed with NLR receptors (Rajendran et al., 2012b). The exact cause of such a strong contrast is unknown at present, but could be related to rapid migration of microphages and neutrophils out of the head kidney toward the inflammatory sites (Secombes and Fletcher, 1992; Press et al., 1994; Uribe et al., 2011). Baoprasertkul et al. (2006, 2007a) made similar observations with TLR2 gene in channel catfish, and with TLR3 gene in blue catfish (*Ictalurus furcatus*). Similarly, down regulation of TLR4 gene was reported in rainbow trout head kidney (Rodriguez et al., 2005). In all these cases, cell migration was discussed as a potential cause, but no experimental evidence yet is available to support the hypothesis.

Up-regulation of several TLR genes have also been reported in catfish kidney after *E. ictaluri* infection (Bilodeau and Waldbieser, 2005; Bilodeau et al., 2006). The major difference was the sampling time after infection. In the present study, we focused on early stages after infection, whereas the observations of Bilodeau and Waldbieser (2005) and Bilodeau et al. (2006) were made much later after infection. It is likely that the modulation of TLR expression depend largely on the pathogenesis of infection. At the earliest stages after infection, microphages and other immune response cells may migrate out of the melanomacrophage centers (MMCs) in the head kidney to the infection sites, while later in the pathogenesis, such cells could migrate back to the head kidney after clearance of the infectious bacteria. However, if this is true, the migration of macrophages out of head kidney is decoupled from inflammation as indicated by drastic induction in the head kidney of many inflammation response genes such as CC and CXC chemokines (Peatman et al., 2005, 2006; Baoprasertkul et al., 2004; Chen et al., 2005; Bao et al., 2006a), antimicrobial peptides (Bao et al., 2005, 2006b; Xu et al., 2005; Wang et al., 2006a), and proinflammatory cytokines (Wang et al., 2006b). In most cases, these genes have been shown patterns of gradual or dramatic up-regulation after ESC infection in head kidney. Clearly, future studies are needed to understand the mechanisms of gene regulation in relation to disease defense responses.

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Reference

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.
Bao, B., Peatman, E., Li, P., He, C., Liu, Z., 2005. Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection. Dev. Comp. Immunol. 29, 939–950.
Bao, B., Peatman, E., Xu, P., Baoprasertkul, B., Wang, G., Liu, Z., 2006a. Characterization of 23 CC chemokine genes and analysis of their expression in channel catfish (*Ictalurus punctatus*). Dev. Comp. Immunol. 30, 783–796.
Bao, B., Peatman, E., Xu, P., Li, P., Zeng, H., He, C., Liu, Z., 2006b. The catfish liver-expressed antimicrobial peptide 2 (LEAP-2) gene is expressed in a wide range of tissues and developmentally regulated. Mol. Immunol. 43, 367–377.
Baoprasertkul, P., Peatman, E., Abernathy, J., Liu, Z., 2007a. Structural characterization and expression analysis of Toll-like receptor 2 gene from catfish. Fish Shellfish Immunol. 22, 418–426.
Baoprasertkul, P., Peatman, E., Chen, L., He, C., Kucuktas, H., Li, P., Simmons, M., Liu, Z., 2004. Sequence analysis and expression of a CXC chemokine in resistant and susceptible catfish after infection of *Edwardsiella ictaluri*. Dev. Comp. Immunol. 28, 769–780.
Baoprasertkul, P., Peatman, E., Somridhivej, B., Liu, Z., 2006. Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*. Immunogenetics 58, 817–830.
Baoprasertkul, P., Xu, P., Peatman, E., Kucuktas, H., Liu, Z., 2007b. Divergent Toll-like receptors in catfish (*Ictalurus punctatus*): TLR5S, TLR20, TLR21. Fish Shellfish Immunol. 23, 1218–1230.
Benko, S., Phipott, D.J., Giardin, S.E., 2008. The microbial and danger signals that activate NOD-like receptors. Cytokine 43, 368–373.
Bilodeau, A.L., Peterson, B.C., Bosworth, B.G., 2006. Response to toll-like receptors, lysozyme, and IGF-I in back-cross hybrid (F1 male (blue x channel) x female channel) catfish challenged with virulent *Edwardsiella ictaluri*. Fish Shellfish Immunol. 20, 29–39.
Bilodeau, A.L., Waldbieser, G.C., 2005. Activation of TLR3 and TLR5 in channel catfish exposed to virulent *Edwardsiella ictaluri*. Dev. Comp. Immunol. 29, 713–721.
Burge, C., Karlin, S., 1997. Prediction of complete gene structures in human genomic DNA. J. Mol. Biol. 268, 78–94.
Chappell, J. 2008. Enteric Septicemia of Catfish. <http://www.aces.edu/dept/fisheries/aquaculture/documents/EntericSepticemiaofCatfish.pdf>.
Chaves-Pozo, E., Liarte, S., Fernandez-Alacid, L., Abellan, E., Meseguer, J., Mulero, V., Garcia-Ayala, A., 2008. Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream (*Sparus aurata* L.). Mol. Immunol. 45, 2998–3011.
Chen, L., He, C., Baoprasertkul, P., Xu, P., Li, P., Serapion, J., Waldbiesier, G., Liu, Z., 2005. Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with *Edwardsiella ictaluri*. Dev. Comp. Immunol. 29, 135–142.
Chen, G., Shaw, M.H., Kim, Y.G., Nunez, G., 2009. NOD-like receptors: role in innate immunity and inflammatory disease. Annu. Rev. Pathol. Mech. 4, 365–398.
Hansen, J.D., Vojtech, L.N., Laing, K.J., 2011. Sensing disease and danger: a survey of vertebrate PRRs and their origins. Dev. Comp. Immunol. 35, 886–897.
Holen, E., Lie, K.K., Araujo, P., Olsvik, P.A., 2012. Pathogen recognition and mechanisms in Atlantic cod (*Gadus morhua*) head kidney cells: bacteria (LPS) and virus (poly I:C) signals through different pathways and affect distinct genes. Fish Shellfish Immunol. 33, 267–276.
Hopkins, P.A., Sriskandan, S., 2005. Mammalian Toll-like receptors: to immunity and beyond. Clin. Exp. Immunol. 140, 395–407.
Huang, S., Yuan, S., Guo, L., Yu, Y., Li, J., Wu, T., Liu, T., Yang, M., Wu, K., Liu, H., Ge, J., Huang, H., Dong, M., Yu, C., Chen, S., Xu, A., 2008. Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. Genome Res. 18, 1112–1126.
Huang, R., Dong, F., Jang, S., Liao, L., Zhu, Z., Wang, Y., 2012. Isolation and analysis of a novel grass carp toll-like receptor 4 (tlr4) gene cluster involved in the response to grass carp reovirus. Dev. Comp. Immunol. 38, 383–388.
Hwang, S.D., Asahi, T., Kondo, H., Hirono, I., Aoki, T., 2010. Molecular cloning and expression study on Toll-like receptor 5 paralogs in Japanese flounder, *Paralichthys olivaceus*. Fish Shellfish Immunol. 29, 630–638.
Jault, C., Pichon, L., Chluba, J., 2004. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. Mol. Immunol. 40, 759–771.

Kongchum, P., Palti, Y., Hallerman, E.M., Hulata, G., David, L., 2010. SNP discovery and development of genetic markers for mapping innate immune response genes in common carp (*Cyprinus carpio*). Fish Shellfish Immunol. 29, 356–361.

Li, P., Peatman, E., Wang, S., Feng, J., He, C., Baoprasertkul, P., Xu, P., Kucuktas, H., Nandi, S., Somridhivej, B., Serapion, J., Simmons, M., Turan, C., Liu, L., Muir, W., Dunham, R., Brady, Y., Grizzle, J., Liu, Z., 2007. Towards the *ictalurid* catfish transcriptome: generation and analysis of 31,215 catfish ESTs. BMC Genomics 8, 177.

Liu, H., Takano, T., Peatman, E., Abernathy, J., Wang, S., Sha, Z., Kucuktas, H., Xu, D.H., Klesius, P., Liu, Z., 2010. Molecular characterization and gene expression of the channel catfish ferritin H subunit after bacterial infection and iron treatment. J. Exp. Zool. A Ecol. Genet. Physiol. 313, 359–368.

Liu, S., Zhang, Y., Zhou, Z., Waldbieser, G., Sun, F., Lu, J., Zhang, J., Jiang, Y., Zhang, H., Wang, X., Rajendran, K.V., Kucuktas, H., Peatman, E., Liu, Z.J., 2012. Efficient assembly and annotation of the transcriptome of catfish by RNA-Seq analysis of a doubled haploid homozygote. BMC Genomics 13, 595.

Liu, S., Zhou, Z., Lu, J., Sun, F., Wang, S., Liu, H., Jiang, Y., Kucuktas, H., Kaltenboeck, L., Peatman, E., Liu, Z., 2011. Generation of genome-scale gene-associated SNPs in catfish for the construction of a high-density SNP array. BMC Genomics 12, 53.

Lu, J., Peatman, E., Yang, Q., Wang, S., Hu, Z., Reecy, J., Kucuktas, H., Liu, Z., 2011. The catfish genome database cBARBEL: an informatic platform for genome biology of ictalurid catfish. Nucleic Acids Res. 39, D815–821.

Meijer, A.H., Gabby Krens, S.F., Medina Rodriguez, I.A., He, S., Bitter, W., Ewa Snaar-Jagalska, B., Spaink, H.P., 2004. Expression analysis of the Toll like receptor and TIR domain adaptor families of zebrafish. Mol Immunol. 40, 773–783.

Oshiumi, H., Matsuo, A., Matsumoto, M., Seya, T., 2008. Pan-vertebrate toll-like receptors during evolution. Curr. Genomics 9, 488–493.

Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., Seya, T., 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics 54, 791–800.

Offord, V., Werling, D., 2012. LRRfinder2.0: a webserver for the prediction of leucine-rich repeats. Innate Immun. http://dx.doi.org/10.1177/1753425912465661.

Palti, Y., Gahr, S.A., Purcell, M.K., Hadidi, S., Rexroad Iii, C.E., Wiens, G.D., 2010. Identification, characterization and genetic mapping of TLR7, TLR8a1 and TLR8a2 genes in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 34, 219–233.

Palti, Y., 2011. Toll-like receptors in bony fish: From genomics to function. Dev. Comp. Immunol. 35, 1263–1272.

Peatman, E., Bao, B., Baoprasertkul, P., Liu, Z., 2005. In silico identification and expression analysis of 12 novel CC chemokines in catfish. Immunogenetics 57, 409–419.

Peatman, E., Bao, B., Peng, X., Baoprasertkul, P., Brady, Y., Liu, Z., 2006. Catfish CC chemokines: genomic clustering, duplications, and expression after bacterial infection with *Edwardsiella ictaluri*. Mol. Genet. Genomics 275, 297–309.

Peatman, E., Baoprasertkul, P., Terhune, J., Xu, P., Nandi, S., Kucuktas, H., Li, P., Wang, S., Somridhivej, B., Dunham, R., Liu, Z., 2007. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Dev. Comp. Immunol. 31, 1183–1196.

Phelan, P.E., Mellon, M.T., Kim, C.H., 2005. Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (*Danio rerio*). Mol. Immunol. 42, 1057–1071.

Press, C.M., Dannevig, B.H., Landsverk, T., 1994. Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. 4, 79–93.

Quiniou, S.M., Waldbieser, G.C., 2011. Mapping of the Toll-like receptor family in channel catfish, *Ictalurus punctatus*. Anim. Genet. 42, 567–568.

Rajendran, K.V., Zhang, J., Liu, S., Peatman, E., Kucuktas, H., Wang, X., Liu, H., Wood, T., Terhune, J., Liu, Z., 2012a. Pathogen recognition receptors in channel catfish: II. Identification, phylogeny and expression of retinoic acid-inducible gene I (RIG-I)-like receptors. Dev. Comp. Immunol. 37, 381–389.

Rajendran, K.V., Zhang, J., Liu, S., Kucuktas, H., Wang, X., Liu, H., Sha, Z., Terhune, J., Peatman, E., Liu, Z., 2012b. Pathogen recognition receptors in channel catfish: I. Identification, phylogeny and expression of NOD-like receptors. Dev. Comp. Immunol. 37, 77–86.

Rebl, A., Goldammer, T., Seyfert, H.M., 2010. Toll-like receptor signaling in bony fish. Vet. Immunol. Immunopathol. 134, 139–150.

Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E., Aderem, A., 2005. The evolution of vertebrate Toll-like receptors. Proc. Natl. Acad. Sci. U.S.A. 102, 9577–9582.

Rodriguez, M.F., Wiens, G.D., Purcell, M.K., Palti, Y., 2005. Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics 57, 510–519.

Secombes, C.J., Fletcher, T.C., 1992. The role of phagocytes in the protective mechanisms of fish. Annu. Rev. Fish Dis. 2, 53–71.

Small, B.C., Murdock, C.A., Bilodeau-Bourgeois, A.L., Peterson, B.C., Waldbieser, G.C., 2008. Stability of reference genes for real-time PCR analyses in channel catfish (*Ictalurus punctatus*) tissues under varying physiological conditions. Comp. Biochem. Physiol. B Biochem .Mol. Biol. 151, 296–304.

Star, B., Nederbragt, A.J., Jentoft, S., Grimholt, U., Malmstrom, M., Gregers, T.F., Rounge, T.B., Paulsen, J., Solbakken, M.H., Sharma, A., Wetten, O.F., Lanzen, A., Winer, R., Knight, J., Vogel, J.H., Aken, B., Andersen, O., Lagesen, K., Tooming-Klunderud, A., Edvardsen, R.B., Tina, K.G., Espelund, M., Nepal, C., Previti, C., Karlsen, B.O., Moum, T., Skage, M., Berg, P.R., Gjoen, T., Kuhl, H., Thorsen, J., Malde, K., Reinhardt, R., Du, L., Johansen, S.D., Searle, S., Lien, S., Nilsen, F., Jonassen, I., Omholt, S.W., Stenseth, N.C., Jakobsen, K.S., 2011. The genome sequence of Atlantic cod reveals a unique immune system. Nature 477, 207–210.

Su, J., Jang, S., Yang, C., Wang, Y., Zhu, Z., 2009a. Genomic organization and expression analysis of Toll-like receptor 3 in grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 27, 433–439.

Su, J., Yang, C., Xiong, F., Wang, Y., Zhu, Z., 2009b. Toll-like receptor 4 signaling pathway can be triggered by grass carp reovirus and *Aeromonas hydrophila* infection in rare minnow *Gobiocypris rarus*. Fish Shellfish Immunol. 27, 33–39.

Sullivan, C., Charette, J., Catchen, J., Lage, C.R., Giasson, G., Postlethwait, J.H., Millard, P.J., Kim, C.H., 2009. The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. J. Immunol. 183, 5896–5908.

Sundaram, A.Y., Consuegra, S., Kiron, V., Fernandes, J.M., 2012. Positive selection pressure within teleost Toll-like receptors tlr21 and tlr22 subfamilies and their response to temperature stress and microbial components in zebrafish. Mol. Biol. Rep. 39, 8965–8975.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Tsoi, S., Park, K.C., Kay, H.H., O'Brien, T.J., Podor, E., Sun, G., Douglas, S.E., Brown, L.L., Johnson, S.C., 2006. Identification of a transcript encoding a soluble form of toll-like receptor 5 (TLR5) in Atlantic salmon during *Aeromonas salmonicida* infection. Vet. Immunol. Immunopathol. 109, 183–187.

Tsujita, T., Ishii, A., Tsukada, H., Matsumoto, M., Che, F.S., Seya, T., 2006. Fish soluble Toll-like receptor (TLR) 5 amplifies human TLR5 response via physical binding to flagellin. Vaccine 24, 2193–2199.

Uematsu, S., Akira, S., 2006. Toll-like receptors and innate immunity. J. Mol. Med. 84, 712–725.

Uribe, C., Folch, H., Enriquez, R., Morgan, G., 2011. Innate and adaptive immunity in teleost fish: a review. Vet. Med. 56, 486–503.

Wang, Q., Bao, B., Wang, Y., Peatman, E., Liu, Z., 2006a. Characterization of a NK-lysin antimicrobial peptide gene from channel catfish. Fish Shellfish Immunol. 20, 419–426.

Wang, Y., Wang, Q., Baoprasertkul, P., Peatman, E., Liu, Z., 2006b. Genomic organization, gene duplication, and expression analysis of interleukin-1beta in channel catfish (*ictalurus punctatus*). Mol. Immunol. 43, 1653–1664.

Wang, S., Sha, Z., Sonstegard, T.S., Liu, H., Xu, P., Somridhivej, B., Peatman, E., Kucuktas, H., Liu, Z., 2008. Quality assessment parameters for EST-derived SNPs from catfish. BMC Genomics 9, 450.

Wu, X., Xiang, L., Huang, L., Jin, Y., Shao, J., 2008. Characterization, expression and evolution analysis of Toll-like receptor 1 gene in pufferfish (*Tetraodon nigroviridis*). Int. J. Immunogenet. 35, 215–225.

Xu, P., Bao, B., He, Q., Peatman, E., He, C., Liu, Z., 2005. Characterization and expression analysis of bactericidal permeability-increasing protein (BPI) antimicrobial peptide gene from channel catfish *Ictalurus punctatus*. Dev. Comp. Immunol. 29, 865–878.

Yang, C., Su, J., Zhang, R., Peng, L., Li, Q., 2012. Identification and expression profiles of grass carp *Ctenopharyngodon idella* tlr7 in responses to double-stranded RNA and virus infection. J. Fish Biol. 80, 2605–2622.

Yu, Y., Zhong, Q., Li, C., Jiang, L., Yan, F., Wang, Z., Zhang, Q., 2009. Isolation and characterization of Toll-like receptor 9 in half-smooth tongue sole *Cynoglossus semilaevis*. Fish Shellfish Immunol. 26, 492–499.