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Short communication

A non-mammalian Toll-like receptor 26 (TLR26)gene mediates innate immune responses in yellow catfish *Pelteobagrus fulvidraco*

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ABSTRACT

In this study, we identified a fish-specific Toll-like receptor (TLR) in *Pelteobagrus fulvidraco*, an economically important freshwater fish in China. This TLR, *Pf*TLR26, was shown to be encoded by a 3084 bp open reading frame (ORF), producing a polypeptide 1027 amino acids in length. The *Pf*TLR26 protein contains a signal peptide, eight leucine-rich repeat (LRR) domains, two LRR_TYP domains in the extracellular region, and a Toll/interleukin (IL)-1 receptor (TIR) domain in the cytoplasmic region, consistent with the characteristic TLR domain architecture. This predicted 117.1 kDa protein was highly homologous to those of other fish, with phylogenetic analysis revealing the closest relation to TLR26 of *Ictalurus punctatus*. Real-time quantitative reverse transcription-PCR (qRT-PCR) analysis showed that the *Pf*TLR26 gene was expressed in all tissues tested, with the highest expression levels seen in the head kidney and blood, and the lowest seen in muscle. *Pf*TLR26 exhibited significant upregulation in liver, spleen, head kidney, and blood at different time points following challenge with the common TLR agonists lipopolysaccharide (LPS) and polyriboinosinic polyribocytidylic acid (Poly I:C). Taken together, these results suggest that *Pf*TLR26 may be an important component of the *P. fulvidraco* innate immune system, participating in the transduction of TLR signaling under pathogen stimulation.

1. Introduction

The fish innate immune system is an efficient first line of defense against various microbes, including bacteria, viruses, fungi and parasites [1]. Among the various components of the innate immune system, Toll-like receptors (TLRs) play an essential role in the detection and activation of innate immunity in response to various pathogens [2]. TLRs function as pattern recognition receptors (PRRs) capable of activating signaling cascades upon binding to conserved pathogen-associated molecular patterns (PAMPs) [3]. Following recognition of a cognate PAMP, such as polyriboinosinic polyribocytidylic acid (Poly I:C), a synthetic dsRNA, is often used in models of viral infection to study the immune response against viral pathogens [4]. TLRs change their conformation, triggering the activation of TIR domain-containing adaptor molecules (i.e., TRIF, MyD88, and TRAM) and nuclear factor-κB (NF-κB), resulting in the transcription of numerous genes including antimicrobial peptides (AMPs) and pro-inflammatory cytokines [5].

As one of the most widely studied PRRs, TLRs play an essential role in innate immune signaling and the activation of the innate immune system [6]. From a structural standpoint, all TLRs are type I transmembrane proteins consisting of three parts: an extracellular N-terminus with a leucine-rich repeat (LRR) domain, a transmembrane domain, and an intracellular C-terminus with a Toll/interleukin (IL)-1 receptor (TIR) domain [7]. Since the first report of Toll in *D. melanogaster*, countless TLRs have been identified in both vertebrates and invertebrates, including 11 in human, 13 in mouse, and 17 in fish [8].

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Based on amino acid similarity, genomic structure, and ligand properties, TLRs have been classified into seven major families: TLR1, TLR3, TLR4, TLR5, TLR7, TLR11, and TLR21 [9]. Among the teleosts, the TLR21 family includes TLRs 13, 19–23, 25, and 26. TLR21, a common non-mammalian TLR, has been found in birds [10], amphibians [11], and teleosts [12]. To date, at least 20 TLR types (TLRs 1–4, 5 M, 5S, 7–9, 13, 14, 18–26) have been found in teleost species [13], where TLR26 appears to be specific to channel fish [14]; however, details regarding the expression and sequence structure of TLR26 in *P. fulvidraco* are not known.

Yellow catfish, *Pelteobagrus fulvidraco* (Siluriformes: Bagridae), has emerged as an important commercial freshwater fish species in China owing to its desirable flavor and high market value [15]. Given this increase in commercial interest, *P. fulvidraco* has been subject to increasing research interest, with significant investigations being conducted into its breeding habits, development, lipid metabolism, and toxicology [16–19]; however, litter is known regarding the structure and function of its immune system. Such an understanding of piscine immunology is of increasing importance, as farmed *P. fulvidraco* have proven susceptible to a variety of pathogenic diseases, resulting in significant economic losses. Here, we identify the open reading frame (ORF) for TLR26 in *P. fulvidraco* using PCR methods and investigated its expression patterns in different tissues following challenge with lipopolysaccharide (LPS) and polyriboinosinic polyribocytidylic acid (Poly I:C). Together, these data provide important insights into the role of TLR26 in *P. fulvidraco*.

2. Materials and methods

2.1. Experimental fish

P. fulvidraco (average weight, 50 ± 10 g) were collected from the tigerbridge market, Yancheng, Jiangsu province, China, on May 2017 and acclimated at 24 °C before commencing with the experiment. Thirteen tissues were dissected for analysis, including blood, brain, gill, head kidney, intestine, liver, heart, muscle, ovary, spleen, testis, and trunk kidney. Sixty individuals were equally distributed among three PVC tanks at room temperature. Randomly selected tanks were then treated with 100 μL of LPS (5 mg/L; L-2654, Sigma), poly I:C (poly I:C, 50 mg/L, P9582, Sigma), or phosphate-buffered saline (PBS) as control. After treatment, four tissue samples (blood, head kidney, liver, and spleen) were collected at 3, 6, 12, 24, 36, and 48 h, frozen in liquid nitrogen, and then stored at −80 °C.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Sangon, China) according to the manufacturer's instructions. RNase-Free DNase I was used to remove contaminating genomic DNA (Promega, USA). RNA integrity and DNA contamination were assessed on a 1% formaldehyde gel. The concentration of RNA in the samples was measured using a NanoDrop 2000c spectrophotometer (NanoDrop, USA). Only samples with 260 nm/280 nm absorbance ratios (A260/A280) ranging from 1.8 to 2.0 were used. First-strand cDNA synthesis was performed using 1 μg of total RNA per sample with a TRUEscript cDNA Synthesis Kit (Aidlab, China).

2.3. Cloning of the PfTLR26 gene

Expressed sequence tags (ESTs) of TLR26 homologs in *P. fulvidraco* were isolated by random EST sequencing and transcriptome analysis [20–23]. Oligonucleotide primers for the ORF were designed using Primer Premier 5.0 software (Table 1). The primers were used for RT-PCR with a program consisting of 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 180 s. PCR products were then analyzed on 1% agarose gels (Axygen, USA), after which the purified PCR products were ligated into the pTOPO vector (Aidlab, China) and sequenced (General Biosystems, China).

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Purpose
RT-F1	ATGTTTCGGTTCCTCTCCATGCT	RT-PCR
RT-R1	TCAGAAAGGCTTAGGTGCGAGCT	RT-PCR
F1	CCATCATCCATGACCGCAGAG	qRT-PCR
R1	CGACCACCGACAGAAAGAACG	qRT-PCR
Actin-F	GCACAGTAAAGGCGTTGTGA	qRT-PCR
Actin-R	ACATCTGCTGGAAGGTGGAC	qRT-PCR

2.4. Sequence analysis of PfTLR26

BLAST searches were performed at http://www.ncbi.nlm.nih.gov/blast.cgi. Translation of the cDNA was performed using the Expert Protein Analysis System (http://au.expasy.org/). Putative signal peptide prediction was performed using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The deduced amino acid sequence was analyzed using DNASTAR software (DNASTAR, USA). A motif scan was performed at http://hits.isb-sib.ch/cgi-bin/motif_scan. The functional domain was predicted using SMART (http://smart.embl-heidelberg.de/). The isoelectric point (pI) and molecular weight (MW) of the deduced amino acid sequences were predicted using the Compute pI/MW Tool at the Expert Protein Analysis System (ExPASy) site (http://web.expasy.org/compute_pi/).

2.5. Homologous alignment and phylogenetic analysis

Homology searches were performed using BLASTn and BLASTp by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The amino acid sequences of TLR26 from different organisms used for phylogenetic analysis were downloaded from the GenBank database. Multiple sequence alignments were carried out using Clustal X software [24]. A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 [25]. The data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining (NJ) trees was evaluated by 1000 bootstrapping replications.

2.6. Quantitative reverse transcription-PCR (qRT-PCR) analysis of expression patterns of PfTLR26

qRT-PCR was performed to determine mRNA expression of *PfTLR26* in several tissues, with and without pathogen infection; actin (GenBank accession number: EU161065) was used as an internal reference. qRT-PCR was performed on a Mastercycler ep realplex machine (Eppendorf, Germany) using the 2 × SYBR Green qPCR Mix kit (Aidlab, China). Reaction mixtures (20 μL) consisted of 10 μL 2 × SYBR Green qPCR Mix, 1 μL forward and reverse primers, 1 μL cDNA, and 7 μL RNase-free H_2O. The PCR procedure was as follows: 95 °C for 10 s followed by 40 cycles of 95 °C for 15s, 56 °C for 15s, and 72 °C for 20s. At the end of the reaction, a melting curve was produced by continuous monitoring of fluorescence while slowly heating the sample from 60 to 95 °C. Each independent experiment was conducted in triplicate and the relative expression levels were determined as described previously [26].

2.7. Data analysis

Data are presented as the mean ± standard error of the mean (SEM). SPSS software version 21.0 was used to analyze data by one-way analysis of variance (ANOVA). Statistical analyses were performed with P values < 0.05 considered statistically significant.

3. Results and discussion

3.1. Sequence analysis of the PfTLR26 gene

The putative ORF of *PfTLR26* was obtained using RT–PCR, which revealed a 3084 bp product encoding 1027 amino acids containing both LRR and TIR domains. Protein signal peptide prediction revealed a putative signal peptide in *P. fulvidraco*, suggesting that it is a secretory protein; similar results were reported for *Ictalurus punctatus* [14]. Based on the entire amino acid sequence, the predicted MW and pI of *Pf*TLR26 were 117.1 kDa and 5.19, respectively. Motif-scan results indicated that the *Pf*TLR26 protein contained a variety of functional sites. Conserved domain prediction analysis showed that the *Pf*TLR26 protein contains eight LRR domains and two LRR_TYP domains in the extracellular region, a pattern commonly found in innate immune surveillance proteins, as well as a TIR domain in the cytoplasmic region acting as a TLR signaling molecule, consistent with the characteristic TLR domain architecture [27,28].

3.2. Homologous alignment and phylogenetic analysis

To assess the relatedness of the TLRs (Fig. 1), the deduced amino acid sequence of *Pf*TLR26 was aligned with several other known and predicted TLR26 proteins using Clustal X. Clustal alignment revealed that *Pf*TLR26 was highly similar to that of other fish species. In addition, sequence alignment and prediction of functional domains revealed that the amino acid sequences for the conserved features of *Pf* TLR26 were nearly identical, with high sequence identity and similar lengths and positions. Among these regions, the N-terminal LRR domain plays an important role in signal transduction, regulating apoptosis and inflammatory responses [29], while the C-terminal TIR domain is involved in inflammatory innate immune responses [30]. Meanwhile, three highly conserved boxes [box 1 (YDAFVIØ), box 2 (LC-RD-(A/P)G), and box 3 (FWXRLR), where "Ø" refers to any hydrophobic amino acid and "X" to any amino acid], found in the TIR domain were highly conserved among all tested animals. Box 1 is involved in mediating the coupling of molecules in the family to inflammation signaling pathways, box 2 is involved in pathogens recognition, and box 3 is involved in directing the localization of the receptor, possibly through interactions with cytoskeletal elements [31]. Further studies will be necessary to characterize the relationship between the structural factors and immune activities of TLR26 in fish.

To determine the molecular position of TLR26, a total of 39 representative TLR sequences were used to reconstruct their phylogenetic relationships based on amino acid sequences. Evolutionary history was inferred using the neighbor-joining method, with complete deletion of gaps and 1000 bootstrap interactions. As shown in Fig. 2, the TLRs clustered into three main groups, with *Pf*TLR26 being most closely related to TLR26 of *I. punctatus*; fish-specific TLRs clustered into a single group, indicating that they may represent a novel TLR protein superfamily. Overall, the phylogenetic tree was consistent with other known phylogenetic relationships, confirming the identity of *Pf*TLR26 based on strong orthology to its counterparts in other species. Taken together, these results suggest that TLR proteins are highly conserved throughout the evolution of animals [32].

3.3. Expression of TLR26 in P. fulvidraco

Next, qRT-PCR was used to investigate the basal transcription level of *Pf*TLR26 in selected tissues of healthy yellow catfish. Gene expression in each tissue was normalized to that of β-actin. Expression fold differences were calculated based on the expression levels in muscle, to assess tissue-specific expression profiles. As shown in Fig. 3, the results revealed that the *Pf*TLR26 gene was expressed in all tested tissues, with the strongest expression seen in the head kidney, a vital immune organ for excretion in teleosts; sporadic reports have suggested this organ acts

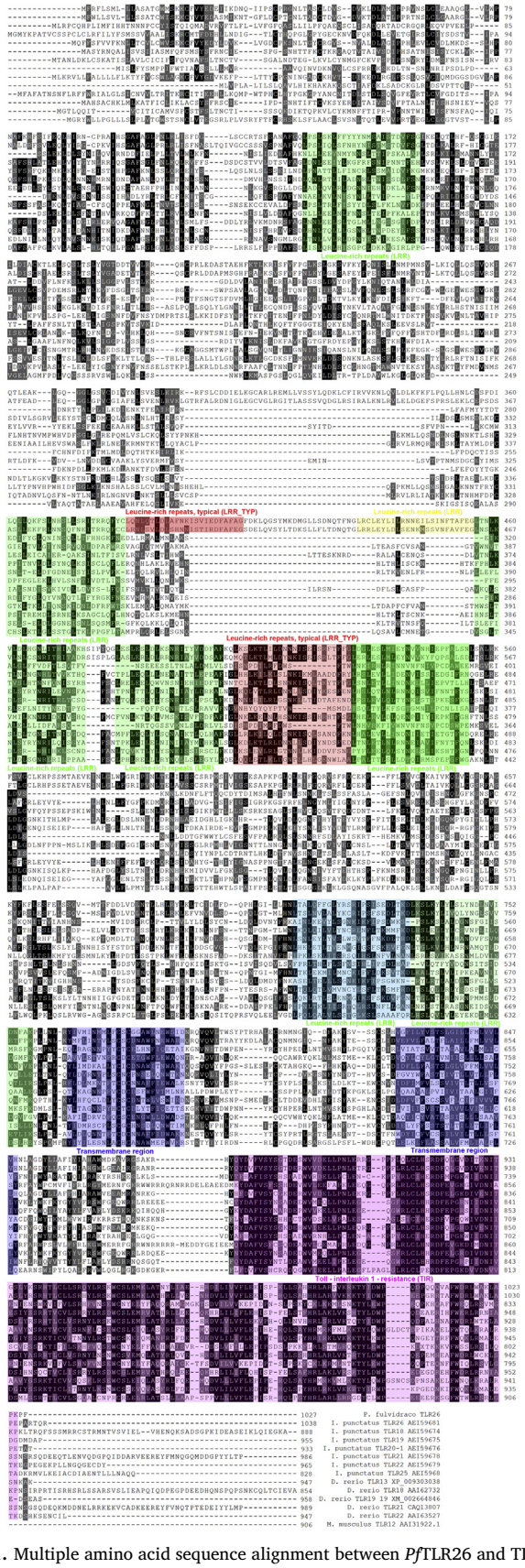

Fig. 1. Multiple amino acid sequence alignment between *Pf*TLR26 and TLRs of other vertebrates. Identical amino acids are highlighted in black, similar amino acids are highlighted in gray.

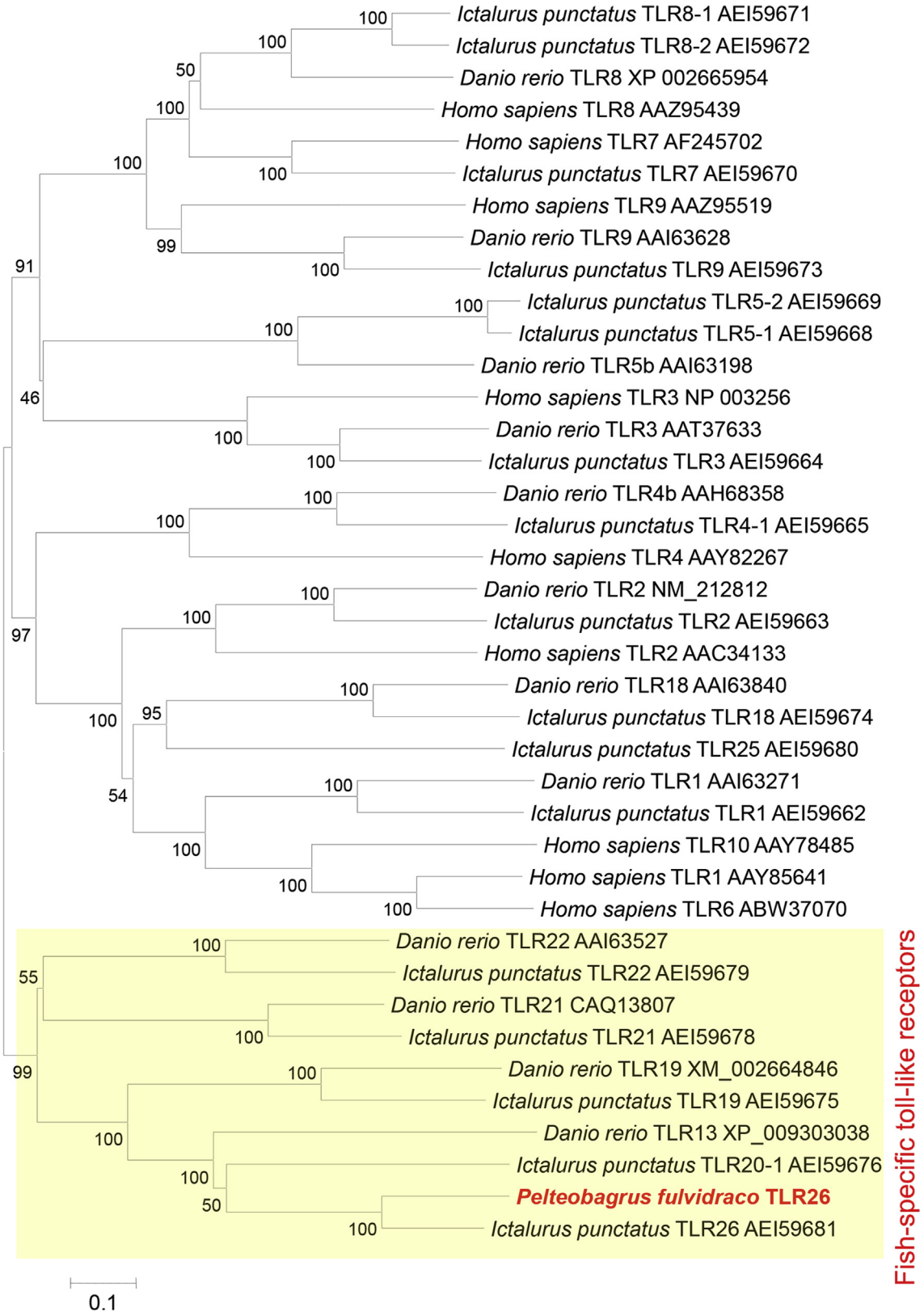

Fig. 2. Phylogenetic tree constructed by MEGA using the neighbor-joining analysis method. Bootstrap p-values (1000 repetitions) of branches are indicated at nodes. Numbers at nodes are bootstrap *P* values.

as a center for pathogen processing. High expression was also seen in blood and spleen, while the lowest expression was seen in muscle, suggesting that *Pf*TLR26 plays an important role throughout the entire life cycle of the fish. Similar results have also been seen in other fish, with TLR expression observed across a wide range of tissues; the highest expression occurs in immune-related tissues, indicative of their importance in the immune system [8]. Moreover, TLR26 expression in *I. punctatus* was also shown to be highest in the head kidney, with

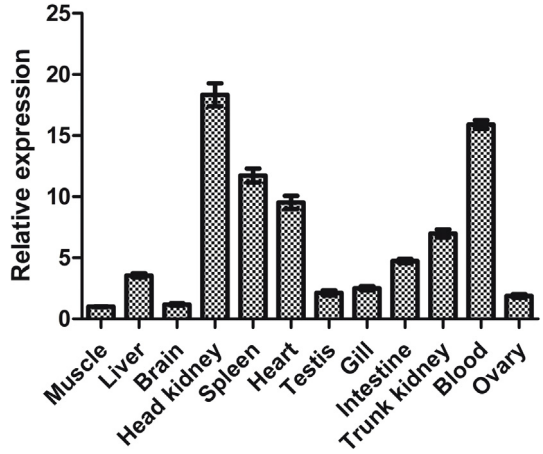

Fig. 3. Expression analysis of *Pf*TLR26 gene in various tissues. Relative expression levels were analyzed by qRT-PCR and the actin gene was used as an internal standard. The lowest gene expression level was set to 1.0. The data were expressed as the mean fold change (means ± SE, n = 3) relative to the untreated group.

minimal expression in the gonads and gills [14]. High expression was consistently detected in immune-related tissues, such as in the spleen and head kidney, which are major sites of innate and adaptive immune responses in fish and may play a role in the regulation of fish mucosal immunity and innate immune responses [33]. Taken together, the results showed that TLR26 was primarily located in the immune organs, including the trunk kidney, spleen, and head kidney, all of which serve as sites of initial interaction between host and pathogen.

3.4. Expression levels of *Pf*TLR26 gene in response to pathogen infections

To further understand the immune function of TLR26 in *P. fulvidraco*, qRT-PCR was used to assess the expression of *Pf*TLR26 in immune tissues following treatment with the common TLR agonists LPS and Poly I:C. Four tissues were selected at early time points based on their involvement in the immune response or pathogen attachment. As shown in Fig. 4, following LPS challenge, *Pf*TLR26 showed different expression patterns among the four tissues, with peak *Pf*TLR26 expression seen at 3 h in the spleen and head kidney. *Pf*TLR26 was also highly expressed in other tissues, with peak expression being observed at 36 and 24 h in the liver and blood, respectively.

Following Poly I:C challenge, the expression of *Pf*TLR26 reached a peak at 3 h in the spleen, blood, and head kidney, and at 36 h in the liver (Fig. 5). Similar results were also seen in large yellow croaker, with expression of TLR21 significantly upregulated in the spleen from 6 to 72 h following trivalent bacterial vaccine challenge [34]; meanwhile, *Oplegnathus fasciatus* TLR21 expression significantly increased from 3 to 48 h in the spleen upon *Streptococcus iniae*, rock bream iridovirus (RBIV), and *Edwardsiella tarda* injections [35]. TLR21 mRNA transcript levels in Grass carp were also upregulated in the spleen following *A. hydrophila* and Aquareovirus challenge [36], as was *Epinephelus coioides* TLR21, which was significantly upregulated following *Cryptocaryon irritans* challenge [37]. In contrast, TLR22 transcripts were inhibited during the early stages post-infection in a grass carp kidney cell line, followed by strong upregulation thereafter (peaking at 24 h post infection), and then by decreasing expression [38]. When challenged with poly (I:C) or *A. hydrophila* bacteria, CcTLR22 expression was significantly upregulated in a variety of common carp tissues [39]. These results suggest that fish-specific TLRs serve as essential adaptor

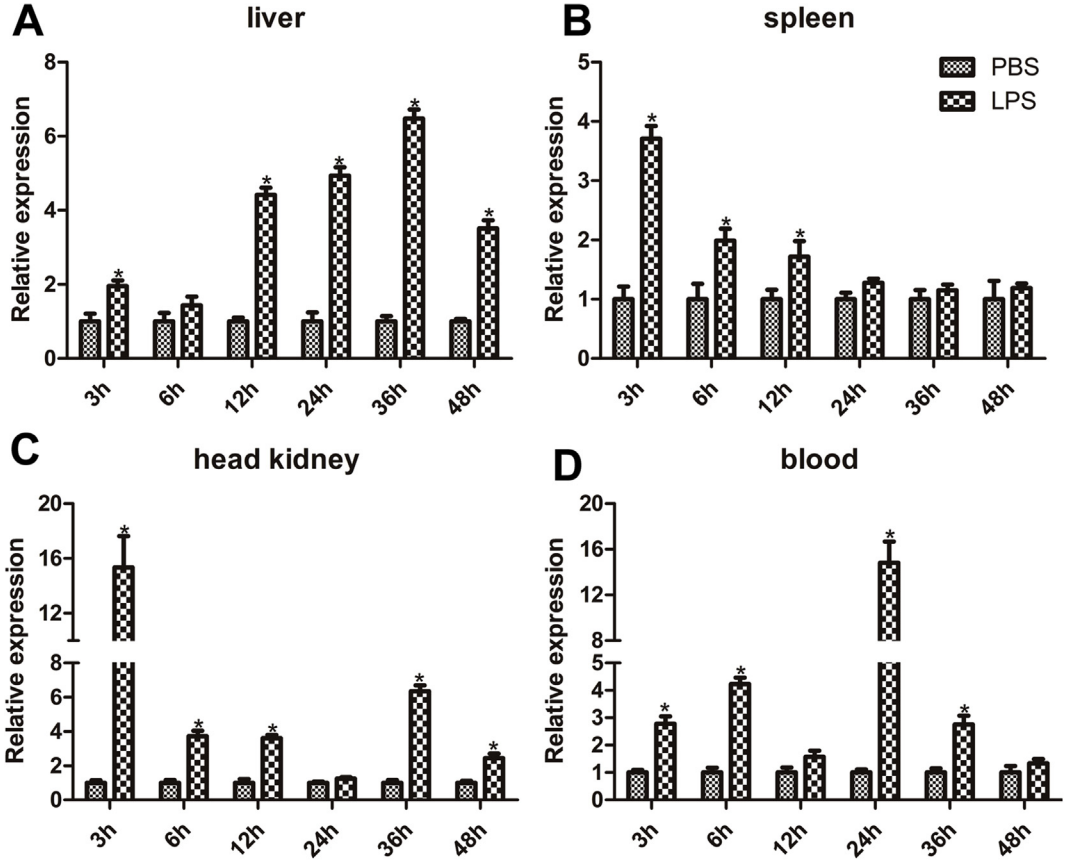

Fig. 4. Relative mRNA expression levels of *Pf*TLR26 in response to LPS in four tissues by qRT-PCR. The actin gene was used as an internal standard. Gene expression level in the control group was set to 1.0. The data were expressed as the mean fold change (means ± SE, n = 3) relative to the untreated group. The values were significantly different to the control at the same time point when marked with asterisks (*P < 0.05).

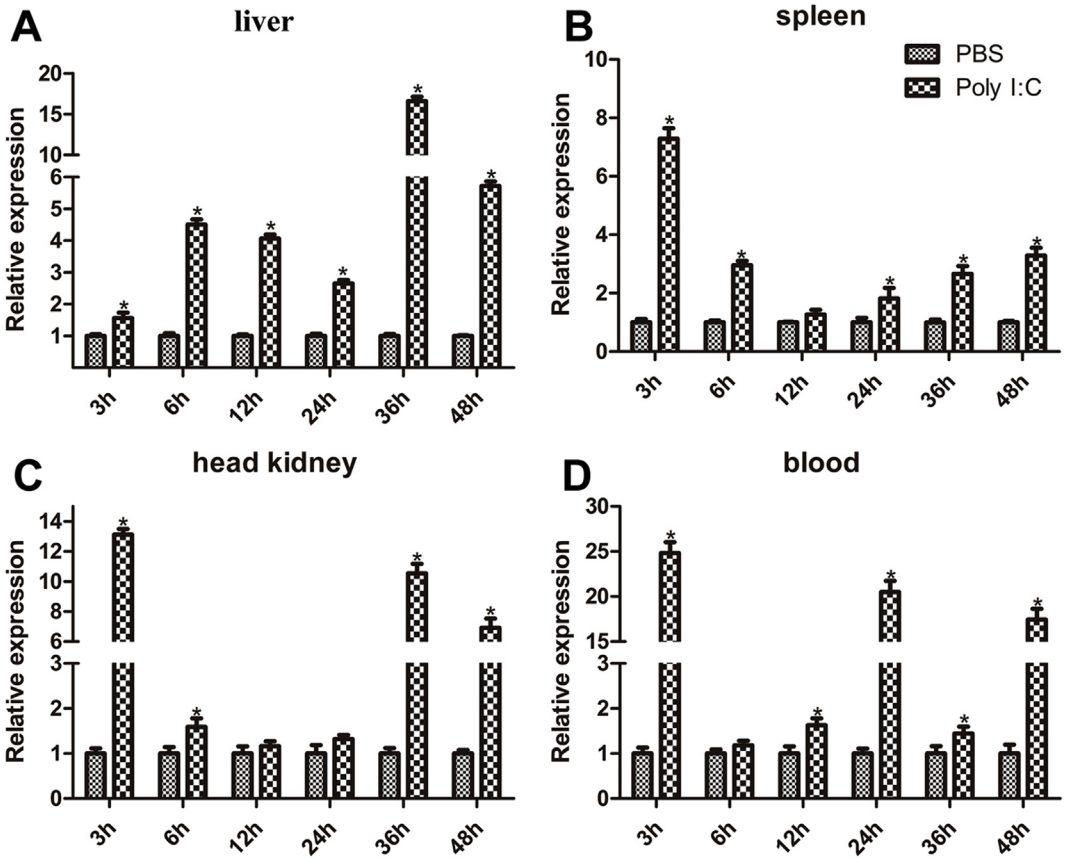

Fig. 5. Relative mRNA expression levels of *Pf*TLR26 in response to Poly I:C in different tissues by qRT-PCR. The actin gene was used as an internal standard. Gene expression level in the control group was set to 1.0. The data were expressed as the mean fold change (means ± SE, n = 3) relative to the untreated group. The values were significantly different to the control at the same time point when marked with asterisks (*P < 0.05).

proteins, and may play an important role in the host defense against pathogen infection.

In conclusion, this study provides the first detailed description of the *Pf*TLR26 gene, including its isolation and cloning, along with phylogenetic analysis and expression profiling results following challenge with different pathogens. The results suggested that *Pf*TLR26 might play an important mediating role in the innate immune response to pathogen infection. Expression analyses should provide valuable insights into the molecular mechanisms underlying immune responses in fish.

Declaration of competing interest

The authors declare no competing interests.

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