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Characterization, expression, and evolutionary analysis of new TLR3 and TLR5M genes cloned from the spiny eel *Mastacembelus armatus*

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ABSTRACT

Toll-like receptors (TLRs) play an important role in innate and adaptive immunity. Here, we identify two new TLRs from the spiny eel *Mastacembelus armatus* (TLR3 and membrane TLR5M). Both MaTLR3 and MaTLR5M were expressed in all tested tissues; expression was highest in liver and spleen, respectively. After infection with *Vibrio parahaemolyticus*, expression of both TLRs fluctuated and differed significantly from controls at several time points. The predicted three-dimensional model of the MaTLR3 and MaTLR5M proteins indicates that most sites under positive selection were located in the extracellular domains of TLRs. Evolutionary analysis detected positively selected sites in the ancestral lineages of vertebrates, amphibians and reptiles. Multiple ML methods recovered 10 positively selected sites in teleost TLR3 and 24 in TLR5M, and most sites were located in leucine-rich repeat domain, possibly related to an "arms-race" co-evolution with pathogens.

1. Introduction

The modern vertebrate immune system includes innate and adaptive immune systems. In fish, the innate immune system acts as the primary defense against aquatic microbial pathogens (Rauta et al., 2014). PRRs are critical to the innate immune system, and play an important role in the recognition of PAMPs including lipopolysaccharids, lipopeptides, flagllins, and dsRNAs (Akira and Takeda, 2004; Akira et al., 2006). In addition, the interaction between PRRs and PAMPs is known to activate a well-coordinated signaling pathway and trigger the adaptive immune response (Akira et al., 2001). PAMPs are highly conserved: they are functionally critical, so any loss or mutation within the PAMP is typically fatal to the pathogen (Areal et al., 2011). Therefore, only a limited number of PRRs are required to recognize pathogenic infections (Medzhitov and Janeway, 1997).

TLRs are an important group of PRRs with three typical motifs: an extracellular LRR domain, a TM domain, and an intracellular TIR domain. As TLRs were first identified in fruit fly embryos (Anderson et al., 1985), the location and function of TLRs in other species is an ongoing focus of immunological research. To date, approximately 28 TLRs have been identified in various taxa including fish, amphibians, birds, reptiles, mammals, and invertebrates (Wang et al., 2016a). TLRs in fish are found in more types of genes than in other vertebrates, and several TLRs, including TLR18-20 and TLR23-28, are known to be fish specific (Wang et al., 2015, 2016a, 2016b). As sequencing technology has advanced, several full length TLRs have been identified in a variety of fish species (e.g. zebrafish, common carp, channel catfish), and the TLR signaling cascade in some teleost fishes has become increasingly well understood (Zhang et al., 2014).

TLRs are also good candidates for evolutionary analysis because, while they lie directly at the host-pathogen interface and are prone

Abbreviation: TLR, toll-like receptors; PRR, Pattern-recognition receptor; PAMP, pathogen-associated molecular pattern; LRR, leucine-rich repeat; TM, transmembrane; TIR, Toll/IL-1 receptor; PBS, phosphate buffered saline; ORFs, open reading frames; PCR, polymerase chain reaction; qPCR, quantitative PCR; ML, maximum likelihood; 3D, three-dimensional; lnL, log-likelihood; LRTs, likelihood ratio tests; SLAC, single likelihood ancestor counting model; FEL, fixed-effect likelihood model; REL, random effect likelihood model; FUBAR, fast unconstrained Bayesian approximation model; MEME, mixed effects model of evolution; UTR, untranslated region; PDB, protein databank.

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to adaptive selection (Hamblin et al., 2002), they are also somewhat conserved due to strong functional constraints (Roach et al., 2005). Several recent works has thus focused on the evolutionary dynamics of TLR genes in fish. Chen et al. (2008) first analyzed teleost TLR9 genes and suggested that they had been subject to positive selection. However, a subsequent study, using more suitable models, found no such positively selected sites in TLR9 (Zhu et al., 2013). Selection has also been shown at various sites in other fish TLRs including TLR4 and TLR21 (Tong et al., 2015; Sundaram et al., 2012).

TLR3, which has been widely identified in both fish and mammals (Jacobs and Langland, 1996; Huang et al., 2011), recognizes the double-stranded RNA produced during viral infection (Alexopoulou et al., 2001). It has also been shown that TLR3 defends against bacterial infection in several taxa including mice and some fish (Kadowaki et al., 2001; Huang et al., 2011). TLR5, a member of MyD88-dependent TLR signaling family, is important in bacterial-flagellin recognition and is widely expressed in various cell types across species (Sebastiani et al., 2000; Basu et al., 2012). After flagellin recognition, TLR5 triggers the MyD88-dependent signaling pathway, which activates the transcription factor NF-κB, inducing transcription of pro-inflammatory cytokines such as IL-1 and TNF-α (Didierlaurent et al., 2004). In teleosts, there are two types of TLR5s: the membrane form (TLR5M) and the soluble form (TLR5S). TLR5M is orthologous to mammal TLR5 and contains an LRR domain, a TM domain, and a TIR domain; TLR5S lacks a TM domain and an intracellular TIR domain (Oshiumi et al., 2003).

The spiny eel *Mastacembelus armatus* (Synbgranchiformes: Mastacembelidae) is primarily distributed in tropical and subtropical streams and rivers (Froese and Pauly, 2016; Cakmak and Alp, 2010). This inland water species is economically important due to its high market demand in southern China where its good taste and high nutritional value make it a popular eating fish (Li et al., 2016). Indeed, wild populations of *M. armatus* have declined in recent years due in part to overfishing (Hossain et al., 2015). As artificial propagation methods have become more successful, farming of *M. armatus* has increased, especially in the Guangdong and Guangxi provinces of China. However, as little is known about the growth and immune system function of *M. armatus*, aquaculture is challenging. This species thus makes an ideal model organism with which to examine function of TLR3 and TLR5M, not only to increase understanding of immune system function in *M. armatus* itself, but also to investigate selection pressure on these genes in general.

Our study thus had two aims: 1) to identify and characterize the genes TLR3 and TLR5M in *M. armatus*; 2) to detect the positive selection pressures imposed on ancestral lineages of various vertebrate taxa and determine positively selected sites in extant teleost and their domain location.

2. Materials and methods

2.1. Sample collection and cDNA synthesis

We purchased 33 healthy juvenile specimens of *M. armatus* (average body weight, 20.5 g) from the Huangsha fish market (Guangzhou, China). We reared the fish in aerated water tanks at room temperature for about 2 weeks, feeding them bloodworm. After acclimatization, fish were randomly divided into a control group and an infection group. Each fish in the infection group were injected intraperitoneally with a 100 μL bacterial suspension (1.0×10^8 CFU/ml *Vibrio parahaemolyticus* in 1 × PBS). Each fish in the control group was injected in an identical manner with 100 μL of PBS.

At 6, 12, 24, 48, and 72 h post-injection, we anaesthetized three fish from each group with MS-222 (Sigma, USA) and euthanized them. After euthanasia, we collected the primary immune tissues (liver, spleen, and kidney) from each fish and immersed them immediately in RNA Keeper Tissue Stabilizer (Vazyme, China). Samples were kept overnight at 4 °C and then moved to −20 °C for longer-term storage. To detect baseline distribution of TLR3 and TLR5M, we collected ten tissues (liver, spleen, kidney, intestine, brain, muscle, gill, eye, fin, and stomach) from 3 healthy fish before grouping following the procedure described above.

We obtained total RNA from all collected tissues using the RNA isolator Total RNA Extraction Reagent (Vazyme, China), following the manufacturer's instructions. We checked total RNA quality with a spectrophotometer and gel electrophoresis. We synthesized cDNA using the HiScript Q RT SuperMix for qPCR with gDNA wiper (Vazyme, China) following the manufacturer's protocol, and stored the cDNA at −20 °C.

We used BLAST (v2.6.0; Altschul et al., 1990) to search the *M. armatus* transcriptome (unpublished results) using the TLR3 and TLR5M gene sequences from the fish *Danio rerio*, *Miichthys miiuy*, and *Monopterus albus* (Table S1). We predicted the ORFs of the resulting sequences (hereafter referred to as MaTLR3 and MaTLR5M) with ORF finder (https://www.ncbi.nlm.nih.gov/orffinder; accessed on DATE) and validated the predicted ORFs with BLASTP (v2.6.0; Altschul et al., 1990).

We then designed six novel primer pairs to amplify the full-length cDNAs of MaTLR3 and MaTLR5M using Primer 3 (v4.0.0; http://primer3.ut.ee/, accessed on DATE; Table S2). Our PCRs contained 2 μL cDNA (25 ng/μL), 2 μL of each primer, 20 μL 2 × Taq PCR master mixture (GenStar, China), and 14 μL of ddH_2O: a total reaction volume of 40 μL. Thermal cycling conditions were: initial denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 50–60 °C for 30 s, 72 °C for 120 s; and a final extension at 72 °C for 10 min. PCR products were separated on 1% agarose gel and purified with a TIANgel Midi Purification Kit (TIANGEN, China). We cloned the PCR products into a pGM-T vector (TIANGEN, China), and propagated them in competent *Escherichia coli* DH5a cells. We sequenced positive clones with an ABI 3730 DNA analyzer (Applied Biosystems, USA).

2.2. Expression analysis of MaTLR3 and MaTLR5M genes

We measured the expression of MaTLR3 and MaTLR5M in ten tissues obtained from 3 healthy fish and three immunity-related tissues (liver, spleen, and kidney) from 15 infected fish and 15 control fish at 5 time points using real-time qPCR as follows. We first designed three pairs of novel primers (TLR3-RT-F/R, TLR5M-RT-F/R, and β-actin-RT-F/R; Table S2) to amplify TLR3, TLR5M, and β-actin fragments. We used *M. armatus* β-actin (GenBank accession number: MF092893) as an internal control to normalize the expression levels of TLR3 and TLR5M. We ran our qPCRs on a LightCycle 480 Thermal Block Cycler (Roche, Germany) using the AceQ qPCR SYBR Green Master Mix (Vazyme, China), following the manufacturer's instructions. The thermal cycling conditions of the qPCR were denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s. We used a dissociation curve analysis to confirm the target specificity of the qPCR reaction. All qPCRs were performed in triplicate. After normalization to β-actin, the expression levels of MaTLR3 and MaTLR5M were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001), and were shown as mean ± standard error. We tested for significant differences among groups using a one-way analysis of variance, followed by Duncan's multiple tests, with SPSS (v20.0; IBM, USA). We considered differences between means statistically significant at 95% confidence ($P < 0.05$).