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Molecular characterization and transcriptional analysis of non-mammalian type Toll like receptor (TLR21) from rock bream (*Oplegnathus fasciatus*)

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ABSTRACT

Toll-like receptors (TLRs) are a large family of pattern recognition receptors, which are involved in triggering host immune responses against various pathogens by detecting their evolutionarily conserved pathogen associated molecular patterns (PAMPs). TLR21 is a non-mammalian type TLR, which recognizes unmethylated CpG DNA, and is considered as a functional homolog of mammalian TLR9. In this study, we attempted to identify and characterize a novel TLR21 counterpart from rock bream (*Oplegnathus fasciatus*) designated as *RbTLR21*, at molecular level. The complete coding sequence of *RbTLR21* was 2919 bp in length, which encodes a polypeptide of 973 amino acids with a predicted molecular mass of 112 kDa and a theoretical isoelectric point of 8.6. The structure of the deduced RbTLR21 protein is similar to that of the members of typical TLR family, and includes the ectodomain, which consists of 16 leucine rich repeats (LRRs), a transmembrane domain, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain. According to the pairwise sequence analysis data, RbTLR21 was homologous to that of the orange-spotted grouper (*Epinephelus coioides*) with 76.9% amino acid identity. Furthermore, our phylogenetic analysis revealed that RbTLR21 is closely related to *E. coioides* TLR21. The *RbTLR21* was ubiquitously expressed in all the tissues tested, but the highest expression was found in spleen. Additionally, upon stimulation with *Streptococcus iniae*, rock bream iridovirus (RBIV), and *Edwardsiella tarda*, *RbTLR21* mRNA was significantly up-regulated in spleen tissues. Collectively, our findings suggest that RbTLR21 is indeed an ortholog of the TLR21 family and may be important in mounting host immune responses against pathogenic infections.

1. Introduction

Innate immunity is a less-specific immune strategy that serves as the first line defense in a host organism against various disease-causing pathogens (He et al., 2012). These invading microbial pathogens are recognized through highly conserved microbial structures known as pathogen associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs) (Janeway, 1989; Subramaniam

Abbreviations: TLR, Toll-like receptors; PAMPs, pathogen associated molecular patterns; ORF, open reading frame; DNA, deoxyribonucleic acid; cDNA, complementary deoxyribonucleic acid; LRRs, leucine rich repeats; TIR, Toll/interleukin-1 receptor; RBIV, rock bream iridovirus; RNA, ribonucleic acid; mRNA, messenger ribonucleic acid; PRRs, pattern recognition receptors; NOD, nucleotide-binding oligomerization domain; RbTLR21, rock bream Toll-like receptor 21; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor kappa B; MAPK, mitogen activated protein kinase; ILs, interleukins; TNFs, tumor necrosis factors; IFNs, type I interferon; RNA, ribonucleic acid; ssRNA, single-stranded ribonucleic acid; CpG DNA, CpG deoxyribonucleic acid; TLR5S, Toll-like receptor 5 soluble form; CpG-ODN, CpG oligodeoxynucleotides; *E. tarda*, *Edwardsiella tarda*; *S. iniae*, *Streptococcus iniae*; BAC, bacterial artificial chromosome; gDNA, genomic deoxyribonucleic acid; PCR, polymerase chain reaction; AA, amino acid; SMART, Simple Modular Architecture Research Tool; NJ, neighbor joining; MEGA, Molecular Evolutionary Genetic Analysis; BDGP, Berkeley Drosophila Genome Project; PBS, phosphate buffered saline; CFU, colony forming unit; qPCR, quantitative real time polymerase chain reaction; UTR, untranslated region; μl, micro-liter; U, units; dNTPs, Deoxynucleotide triphosphates; pmol, pico-molar; °C, degrees of Celsius; s, seconds; ng, nano-gram; rpm, revolutions per minute; h, hours; nm, nano-meters; SD, standard deviation; NCBI, National Center for Biotechnology Information; Bp, base pair; kDa, kiloDalton; EcTLR21, *Epinephelus coioides* TLR21; EcTLR21-B, *Epinephelus coioides* TLR21-B; TrTLR21, *Takifugu rubripes* TLR21; IpTLR21, *Ictalurus punctatus* TLR21; DrTLR21, *Danio rerio* TLR21; GgTLR21, *Gallus gallus* TLR21; D, aspartic acid; Y, tyrosine; Asp, aspartic acid; Tyr, tyrosine; Phe, phenylalanine; F, phenylalanine; L, leucine; G, glycine; Pro, proline; P, proline.

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et al., 2004). Major PRR classes were recently identified, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) like receptors, and RIG-I-like receptors (Franchi et al., 2009). Among these, TLRs are the key components of the innate immune system and play a critical role by triggering downstream signaling cascades to initiate immune responses against invading pathogens (Schnare et al., 2001). Typically, TLRs are comprised of three distinct domains: an extracellular leucine rich repeat (LRR) region, a transmembrane domain, and a cytoplasmic Toll/interleukin-1 receptor (TIR) signaling domain (Palti, 2011). The extracellular LRR region is responsible for ligand recognition, whereas a cytoplasmic TIR domain is necessary for binding adaptor molecules involved in signal transduction and localization of TLR (Akira et al., 2006; Hacker et al., 2006; Xu et al., 2000). Activation of TLRs by the appropriate ligand can initiate two different intracellular downstream signaling pathways, known as the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and the MyD88-independent pathway (McGettrick and O'Neill, 2004; Palti, 2011). Ultimately, both pathways activate nuclear factor kappa B (NF-κB) or mitogen activated protein kinase (MAPK) and subsequently induce pro-inflammatory cytokines like interleukins (ILs), tumor necrosis factors (TNFs), and type I interferon (IFNs) (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2010; Silverman and Maniatis, 2001).

The cell surface and nucleic acid sensing subfamilies are two major TLR subfamilies that have recently been identified in humans. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 belong to the cell surface subfamily and are able to recognize microbial lipids, sugars, and proteomes (Hajjar et al., 2001; Takeuchi et al., 2001; Underhill et al., 1999; Werts et al., 2001). In mammals, the nucleic acid sensing subfamily is comprised of TLR3, TLR7, TLR8, and TLR9, which are prominently found in various intracellular compartments, including the endoplasmic reticulum and lysosome-like vesicles (Akira et al., 2006; Alexopoulou et al., 2001). Mammalian TLR3 primarily detects double stranded RNA whereas TLR7 and TLR8 detect single stranded RNA (ssRNA). TLR9 is able to recognize bacterial non-methylated CpG DNA (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). Recently, 17 different TLR types, including TLR1, TLR2, TLR3, TLR4, TLR5, TLR5S (Toll-like receptor 5 soluble form), TLR7, TLR8, TLR13, TLR14, TLR18, TLR19, TLR20, TLR21, TLR22, and TLR23 have been identified in teleost fish (Rebl et al., 2010). From the abovementioned TLRs, TLR5S, TLR14, TLR19, TLR20, TLR21, TLR22, and TLR23 are novel non-mammalian, fish specific TLRs (Hirono et al., 2004; Rebl et al., 2007; Tsukada et al., 2005). However, TLR1, TLR2, TLR3, TLR5, TLR8, and TLR9 have been identified as prominent mammalian TLR orthologs (Hirono et al., 2004; Rebl et al., 2007; Tsukada et al., 2005).

TLR21 is a newly identified non-mammalian TLR type that is almost restricted to the fish species. To date, TLR21 has been identified in several teleost fish species including, puffer fish (*Takifugu rubripes*), zebrafish (*Danio rerio*) (Jault et al., 2004; Meijer et al., 2004; Oshiumi et al., 2003), catfish (*Ictalurus punctatus*) (Baoprasertkul et al., 2007), Agnatha lamprey (*Petromyzon marinus*) (Kasamatsu et al., 2010), orange-spotted grouper (*Epinephelus coioides*) (Li et al., 2012), and grass carp (*Ctenopharyngodon idella*) (Wang et al., 2013). Interestingly, TLR21 has also been identified in amphibians and birds, including African clawed frogs (*Xenopus laevis*) (Ishii et al., 2007) and chickens (*Gallus gallus*) (Temperley et al., 2008). Emerging experimental evidence has revealed that there is a functional similarity between the chicken and zebrafish TLR21 with mammalian TLR9 in detecting CpG oligodeoxynucleotides (CpG-ODN) (Yeh et al., 2013) (Brownlie et al., 2009; Keestra et al., 2010). The CpG-DNA motifs are major components of bacterial genomes and genomes of DNA viruses that are recognized by TLR9, ultimately activating inflammatory cytokines and inducing the host immune response against invading bacterial and viral pathogens (Akira et al., 2006; Hemmi et al., 2000; Hochrein et al., 2004; Krug et al., 2004; Lund et al., 2003; Tabeta et al., 2004). To trigger the host immune response against invasion by nucleic acid sensing TLRs, bacterial or viral DNA must be transported into the intracellular vesicles. In the intracellular vesicles, bacterial or viral double stranded DNA undergoes degradation into the multiple single stranded CpG-DNA motifs through the highly acidic and reducing environment of the intracellular compartments. Afterwards those single stranded CpG-DNA motifs are recognized and trigger the downstream signaling cascade by TLR9 (Ahmad-Nejad et al., 2002; Akira et al., 2006; Latz et al., 2004). This phenomenon has previously been seen by inhibiting endosomal maturation, which then leads to the inhibition of the TLR-mediated signaling cascade (Hacker et al., 1998). Moreover in vitro and in vivo studies showed that chicken and some teleost TLR21 expression could be upregulated by live pathogens and by CpG-DNA (Brownlie et al., 2009; Keestra et al., 2010; Li et al., 2012; Wang et al., 2013).

Rock bream is one of the economically important fish species in South Korean aquaculture industry and is prominently distributed in the Pacific and Indian Oceans. Recently, the fish farming industry has been suffering from drastic economical loss due to various pathogenic infections caused by viruses and bacteria (Li et al., 2011; Oh et al., 2006). Therefore, extensive investigation of the rock bream immune system is important in order to improve and facilitate the disease management strategies and to facilitate the long-term sustainability of the aquaculture industry. In this study, we identified and characterized a TLR21 counterpart from rock bream (*RbTLR21*) at the transcriptional level. Furthermore, quantitative real-time PCR (qPCR) was carried out to analyze basal level tissue distribution and temporal expression of *RbTLR21* under live pathogen stress mounted by rock bream iridovirus (RBIV), *Edwardsiella tarda* (*E. tarda*), and *Streptococcus iniae* (*S. iniae*).

2. Materials and methods

2.1. Identification of rock bream TLR21 (RbTLR21) partial cDNA sequence

A rock bream cDNA sequence database was established using the Roche 454 Genome Sequencer FLX System (GS-FLX™). Constructions of cDNA library, normalization and GS-FLX sequencing have been described in our previously published report (Whang et al., 2011). Using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/), the partial cDNA sequence with the highest homology to known TLR21 was identified from the corresponding sequence database of this library.

2.2. Identification of RbTLR21 genomic sequence and complete coding sequence

A random sheared bacterial artificial chromosome (BAC) genomic DNA (gDNA) library was custom constructed (Lucigen, USA) using rock bream genomic DNA from blood. Polymerase chain reaction (PCR) was carried out to screen the gDNA library in order to identify the respective clone bearing the gene, according to the manufacturer's instructions, using gene specific primers (Table 1), designed based on the previously identified partial *TLR21* cDNA sequence (Section 2.1). PCR was conducted using a TaKaRa PCR Thermal Cycler in 20 μl reaction volume containing 0.5 U of Ex Taq polymerase (Takara Bio Inc., Japan), 75 ng of template, 2 μl of 10× Ex Taq buffer, 1.6 μl of 2.5 mM dNTPs, and 10 pmol of each primer. The PCR cycle profile was as follows: initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 30 s, and final elongation at 72 °C for 2 min. The PCR products were analyzed by electrophoresis on 1% agarose gel and the exact location of the BAC clone in the library was detected. The identified BAC clone was subsequently sequenced and the genomic DNA sequence of *RbTLR21* was obtained. Thereafter the complete coding sequence of RbTLR21 was predicted using BLASTx server and DNAssist 2.2 software. The predicted coding sequence was amplified using gene specific primers (13-214 and 13-215) (Table 1) and cloned into pMD19vector (TaKaRa, Japan) following manufacturer's instructions. After sequencing the coding sequence of *RbTLR21* was confirmed.

Table 1
Oligomers used in this study.

Name	Purpose	Sequence (5′–3′)
Rab 027	BAC library screening and qPCR of RbTLR21	TTCCTAAGCAGCGAGTGGTGTTCT
Rab 028	BAC library screening and qPCR of RbTLR21	AGATTGGCTCCAGAAACACGAGCA
Rb-βF	qPCR for rock bream β-actin gene	TCATCACCATCGGCAATGAGAGGT
Rb-βR	qPCR for rock bream β-actin gene	TGATGCTGTTGTAGGTGGTCTCGT
13-214	Coding sequence amplification	ATGGGAAGTCTACCTTTTCGTCTGTTG
13-215	Coding sequence amplification	TTAAGGTAGCAAGTAATGGTTTTCATCTGACG

2.3. Sequence analysis

The open reading frame (ORF) and its deduced amino acid (AA) sequence along with several physiochemical properties of the RbTLR21 protein were determined using DNAssist 2.2 software. The characteristic protein motifs and anticipated domain architecture were predicted by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de). Multiple sequence alignment and pairwise sequence alignment were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and EMBOSS Needle servers (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), respectively. NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict putative protein N-glycosylation sites and signal peptide was determined using SignalP 4.1 online server (http://www.cbs.dtu.dk/services/SignalP/). The phylogenetic tree was constructed by the neighbor joining (NJ) method using Molecular Evolutionary Genetic Analysis (MEGA) software version 5.0 (Tamura et al., 2011). Furthermore, the secondary structure and protein folding pattern of RbTLR21 were predicted by FoldIndex®, online bioinformatics tool (http://bip.weizmann.ac.il/fldbin/findex) (Prilusky et al., 2005).

2.4. Experimental animals

Healthy rock breams, weighing around 50 g, were obtained from the Ocean and Fisheries Research Institute (Jeju, Republic of Korea). Rock breams were acclimatized to laboratory conditions in 400 l aerated tanks in an aquarium at 24 ± 1 °C with salinity of 34 ± 1‰ and a pH of 7.6 ± 0.5 for one week prior to the experiment.

2.5. Tissue collection

In order to determine the *RbTLR21* transcriptional distribution among the different tissues, gill, liver, heart, spleen, intestine, head kidney, kidney, skin, muscle, and brain tissues were dissected from three healthy rock breams. Meanwhile, blood samples (~1 ml per fish) were collected from the caudal fin and cells were subsequently separated by centrifugation at 3000 rpm for 10 min at 4 °C. Collected tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until use for the RNA extraction.

2.6. Immune challenge experiments

To investigate the transcriptional responses of *RbTLR21* upon pathogen and mitogen stimulation, an immune challenge experiment was designed and performed as described in a previous study (Whang et al., 2011). Rock bream iridovirus (RBIV), *E. tarda*, and *S. iniae* were used as immune stimulants (Whang et al., 2011). For the viral challenge, 100 μl of RBIV in PBS was intramuscularly injected into healthy rock breams. For the bacterial challenge, 100 μl of *E. tarda* (5×10^3 CFU/μl) in PBS and 100 μl *S. iniae* (1×10^5 CFU/μl) in PBS were intraperitoneally injected. Meantime, the same volume of PBS (100 μl) was injected into another group of healthy fish to use as a control. Three fish were dissected at 3, 6, 12, 24, and 48 h post-injection and spleen tissues were subsequently collected from the challenged and control group as described in Section 2.5. The collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C.

2.7. Total RNA extraction and cDNA synthesis

Total RNA was extracted from collected tissues using Tri-Reagent™ (Sigma-Aldrich, St. Louis, MO, USA) as described previously (Whang et al., 2011). Absorbance was measured at 260 nm and 280 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA) in order to determine the concentration and purity of the extracted RNA samples. Purified RNA samples were diluted to 1 μg/μl and cDNA was subsequently synthesized using PrimScript™ first strand cDNA synthesis kit (Takara Bio Inc.) according to the manufacturer's instructions.

2.8. Quantitative real-time PCR (qPCR) analysis

The tissue specific expression profile and temporal gene expression of *RbTLR21* upon pathogenic infection were investigated by qPCR in Dice™ Real Time System (TP800; Takara Bio Inc.). The reaction was carried out in a total volume of 15 μl, containing 7.5 μl of TaKaRa Ex-Taq™ SYBR Green real-time master mix, 0.6 μl of each gene specific primer (Rab 027 and Rab 028) (Table 1), 4 μl of the diluted cDNA, and 2.3 μl of Molecular Biology Grade water, using the following temperature profile: one cycle at 95 °C for 10 s, 35 cycles at 95 °C for 5 s, 58 °C for 10 s, 72 °C for 20 s, and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Rock bream β-actin (Genbank ID: FJ975145) gene was amplified as an invariant control gene to calibrate measurement of *RbTLR21* transcription using gene specific primers (Table 1) and using the same thermo cycling profile used to amplify *RbTLR21*. The expression level of *RbTLR21* was analyzed by the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). The base line was automatically set by Dice™ Real Time System software (version 2.00) to maintain consistency. To determine the fold change after pathogenic injection (*E. tarda*, *S. iniae*, and RBIV), relative expression of *RbTLR21* was normalized with the corresponding transcript levels of the PBS injected control group and compared with the mRNA expression levels of non-injected fish as the basal transcript level.

2.9. Statistical analysis

All data on *RbTLR21* transcription were presented as relative mRNA expressed as means ± standard deviation (SD). The two-tailed unpaired Students t-test was used to determine statistical significance ($p < 0.05$).

3. Results and discussion

3.1. Sequence analysis

The complete open reading frame (ORF) of *RbTLR21* was 2919 bp in length. Sequence information was deposited in NCBI-GenBank database under the accession number: KJ004565. The ORF encodes 973 amino acid residues forming a 112 kDa protein with a theoretical isoelectric point of 8.6. The deduced RbTLR21 protein bears a typical TLR domain architecture, comprising an ectodomain with 16 LRRs (residues 81–676), a transmembrane domain (residues 744–766), and a cytoplasmic

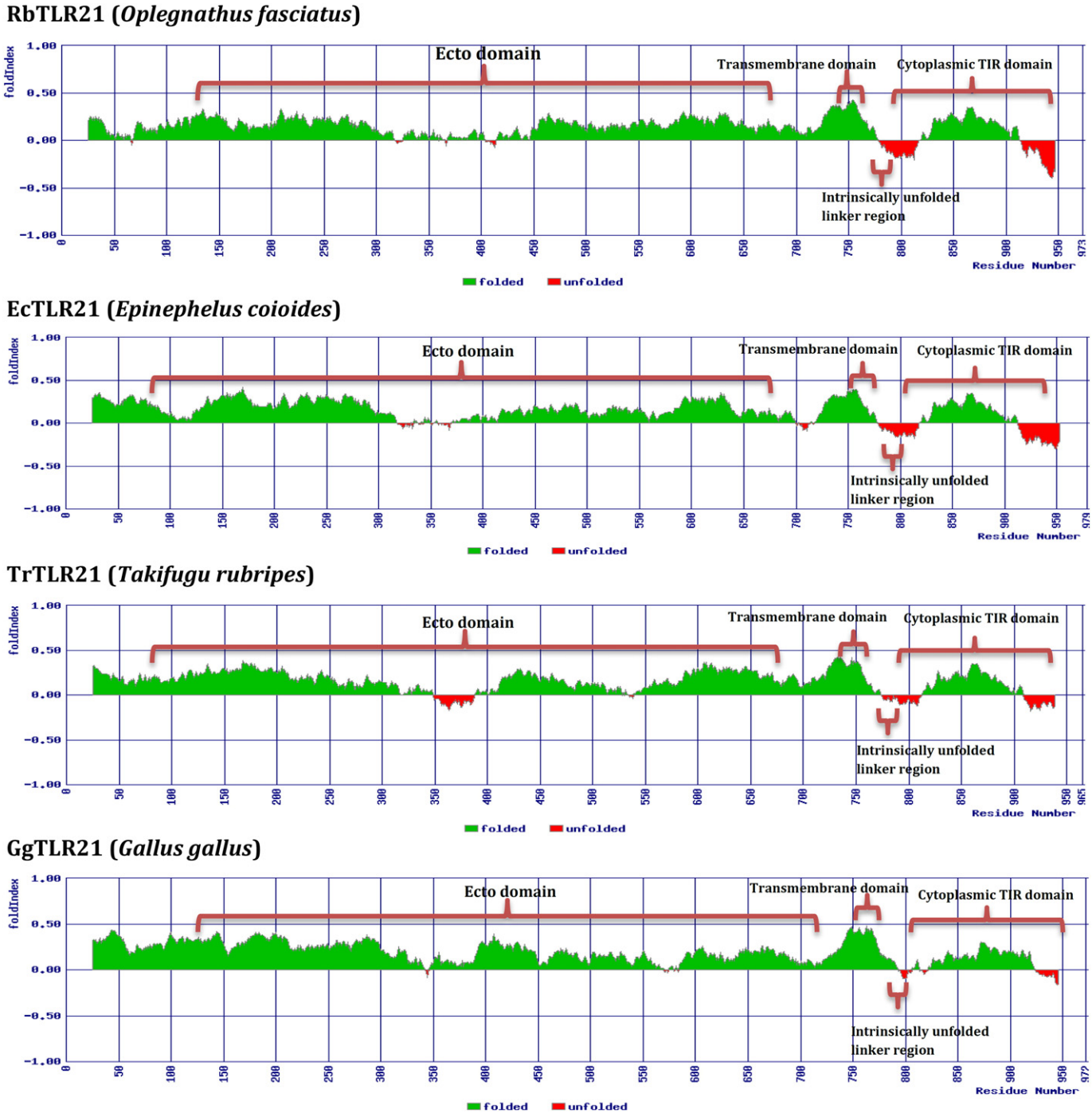

Fig. 1. Secondary structure and protein folding prediction of RbTLR21 (*Oplegnathus fasciatus*), EcTLR21 (*Epinephelus coioides*; AEK49148), TrTLR21 (*Takifugu rubripes*; AAW69371), and GgTLR21 (*Gallus gallus*; NP_001025729). Green areas indicate the folded regions whereas unfolded areas are represented by red.

TIR domain (residues 794–943). A signal peptide with 23 AA residues representing a cleavage site between residues 23 and 24 was identified by SignalP (version 4.1) online software. In addition, 15 putative N-glycosylation sites (at positions 62, 112, 117, 168, 191, 199, 270, 288, 558, 478, 644, 652, 701, 802, and 820) were detected using NetNGlyc 1.0 Server. The TLR21 protein structures of EcTLR21 (*E. coioides*; AEK49148), EcTLR21-B (*E. coioides*; AEK49147), TrTLR21 (*T. rubripes*; AAW69371), IpTLR21 (*I. punctatus*; AEI59678), and DrTLR21 (*D. rerio*; NP_001186264) were predicted using SMART server and compared them with that of RbTLR21 (results are not shown).

Table 2
Percentage of intrinsically unfolded regions in each domain of TLR21 counterparts.

Species	Disordered/intrinsically unfolded percentage (%)		
	Ectodomain	Transmembrane domain	TIR domain
Oplegnathus fasciatus	2.18	0	33.5
Epinephelus coioides	3.36	0	35.5
Takifugu rubripes	6.9	0	35.5
Gallus gallus	0	0	22.5

According to the comparison, most of the fish TLR21 counterparts have 16–17 LRRs, except those from *D. rerio* (13) and *I. punctatus* (21). The LRR region is responsible for binding PAMPs and ligands to the respective TLRs; hence the variation of LRR number would change the broad range capacity for recognition of different PAMPs and ligands (Matsushima et al., 2007) of piscine TLR21s.

3.2. Secondary structure and protein folding prediction

Protein folding pattern of RbTLR21 secondary structure was predicted using FoldIndex® online bioinformatics tool and compared with that of EcTLR21, TrTLR21, and GgTLR21 (*G. gallus* TLR21). The middle section of the ectodomain (approximately 300–400 amino acid residues) in TLR21 counterparts of all the fish species considered herein, including RbTLR21, exhibited intrinsically unfolded regions (Fig. 1) (Prilusky et al., 2005). However, here we observed a moderately structured and folded ectodomain in RbTLR21, while GgTLR21 shows a substantially structured ectodomain compared to fish counterparts, encompassing lesser number of unfolded regions in its ectodomain (denoted by red color in Fig. 1) (Table 2). As depicted in Fig. 1 and Table 2, a transmembrane domain of RbTLR21 is not contained intrinsically unfolded regions. Moreover, its fish counterparts and GgTLR21's transmembrane domains appeared as highly structured and folded manner (Fig. 1) (Table 2). Intriguingly, a large part of the cytoplasmic TIR domain in RbTLR21 and its counterparts from *E. coioides*, *T. rubripes*, and *G. gallus* appeared well-structured and folded manner, even though the initial and final segments of TIR domain are predicted to be intrinsically unfolded in structure (Fig. 1) (Table 2). The presence of well-structured and folded domains in protein may facilitate some of the vital modifications, like oligomerization to acquire their optimum activity. Oligomerization is a key feature of TLRs (especially on TIR domain) which plays an eminent role in specific ligand recognition and regulation of TLR mediated downstream signaling cascade (Godfroy et al., 2012). According to the prediction we can infer that, all the TLR21s considered herein have tightly regulated and efficient downstream signal transduction mechanism due to the higher percentage of highly folded regions throughout the protein. However further studies are required to elucidate the relationship between oligomerization and folding patterns of TLR domains.

3.3. Homology alignment

Pairwise sequence alignment revealed that the entire amino acid sequence of RbTLR21 shows the highest percent identity (76.9%) to EcTLR21, followed by EcTLR21-B (76.8%), TrTLR21 (66.5%), IpTLR21 (57.8%), DrTLR21 (55.0%), and GgTLR21 (41.7%) (Table 3). Moreover, the TIR domain of RbTLR21 exhibited the greatest identity with the TIR domain from EcTLR21 (98.7%) (Table 3). According to the resultant data, the TIR domain from different TLR21 counterparts evidently showed greater amino acid identity and similarity with RbTLR21 than the rest of the amino acid sequence (Table 3). This may be attributed to the fact that the extracellular LLR region tends to undergo more rapid evolutionary changes with respect to their amino acid composition than cytoplasmic TIR regions (Johnson et al., 2003).

ClustalW multiple sequence alignment was performed to compare the RbTLR21 amino acid sequence with known TLR21 sequences from different species (Fig. 2). Our alignment study illustrated that the TIR regions were more highly conserved than the LRR regions from all the TLRs of all the vertebrate species tested. Previous findings demonstrated that the two amino acid residues (D^{535} and Y^{537}) in the LRR region of murine TLR9, which is involved in recognition and interaction with CpG DNA (Rutz et al., 2004), were conserved in DrTLR21, CiTLR21, and GgTLR21 (Fig. 2). However, these findings were slightly different for EcTLR21, EcTLR21-B, PoTLR21, TrTLR21, and RbTLR21 (D^{443} and Y^{445}), where Asp (D) was conserved in all the deduced TLR21 amino acid sequences, but Tyr (Y) was replaced by another amino acid residue, Phe (F). When the aligned TIR region was considered alone, three highly conserved active motifs: BOX 1 (YD#F#SYN), BOX 2 (LCLHHRDF#LG), and BOX 3 (FW##L) were identified (Fig. 2). BOX 1 and BOX 2 are pivotally involved in regulation of the downstream signal transduction pathway, whereas BOX 3 has been implicated in cellular localization of receptor (Slack et al., 2000). Furthermore, the three essential amino acid residues (F^{732}, L^{742}, and G^{743}) which are known to participate in ligand-induced NF-κB and IFN-β promoter activation in human TLR3 (Funami et al., 2004) were also identified in the deduced amino acid sequence of RbTLR21 (F^{824}, L^{835}, and G^{836}) (Fig. 2). These three amino acid residues were well conserved in RbTLR21 and the other fish species tested with exception of DrTLR21 and GgTLR21. In fugu (*T. rubripes*) and chicken (*G. gallus*) counterparts, F and G were conserved but L was replaced by another amino acid residue Pro (P) (Fig. 2). However, the function and importance of these three amino acid residues in RbTLR21 need to be verified.

3.4. Phylogenetic relationship of RbTLR21

In order to reveal the molecular evolutionary relationship of RbTLR21 with other TLR sequences, a phylogenetic tree was constructed using the neighbor joining method. As expected, fish specific TLRs (TLR20, TLR21, TLR22, and TLR23) and TLR 9 were separated into two major branches (Fig. 3). As depicted in Fig. 3, the amino acid sequences of TLR20, TLR21, TLR22, and TLR23 separately clustered in fish specific TLR branches. Interestingly, murine TLR11 and TLR12 also clustered within the main fish specific TLR branch, because they share such high sequence similarity with fish specific TLR family (Palti, 2011). According to the previous studies, TLR11 and TLR12 have evolved fast and appear as pseudogenes in other mammals including humans and dogs. These findings suggest that TLR11 and TLR12 are distantly related with other TLRs, nevertheless they are orthologs of fish specific TLR21 and TLR22 (Roach et al., 2005). Moreover, the tree diagram revealed that, the members of TLR21 share prominent homology with fish specific TLR counterparts, such as TLR20, TLR22, and TLR23 (Fig. 3). Interestingly, RbTLR21 was positioned in the piscine TLR21 clade, exhibiting a closest homology with the TLR21 isoform from *E. coioides* with the support of significant bootstrapped value (95). Collectively, this clustering pattern affirms that RbTLR21 has evolved from a common vertebrate ancestor.

Table 3
Sequence identity and similarity of RbTLR21 with respect to the entire sequence and TIR domain sequence to other known TLR21 counterparts at protein level.

Name	Accession no.	Entire sequence			TIR sequence		
		Identity (%)	Similarity (%)	Amino acids	Identity (%)	Similarity (%)	Amino acids
Epinephelus coioides (orange-spotted grouper)	AEK49148	76.9	86.1	979	94.0	98.7	150
Epinephelus coioides (orange-spotted grouper)-TLR 21B	AEK49147	76.8	86.1	975	93.3	98.7	150
Takifugu rubripes (Fugu)	AAW69371	66.5	78.3	965	87.3	93.3	149
Ictalurus punctatus (catfish)	AEI59678	57.8	70.8	986	78.0	90.0	147
Danio rerio (zebrafish)	NP_001186264	55.0	69.7	989	82.0	92.0	147
Gallus gallus (chicken)	NP_001025729	41.7	57.3	972	62.7	80.0	142

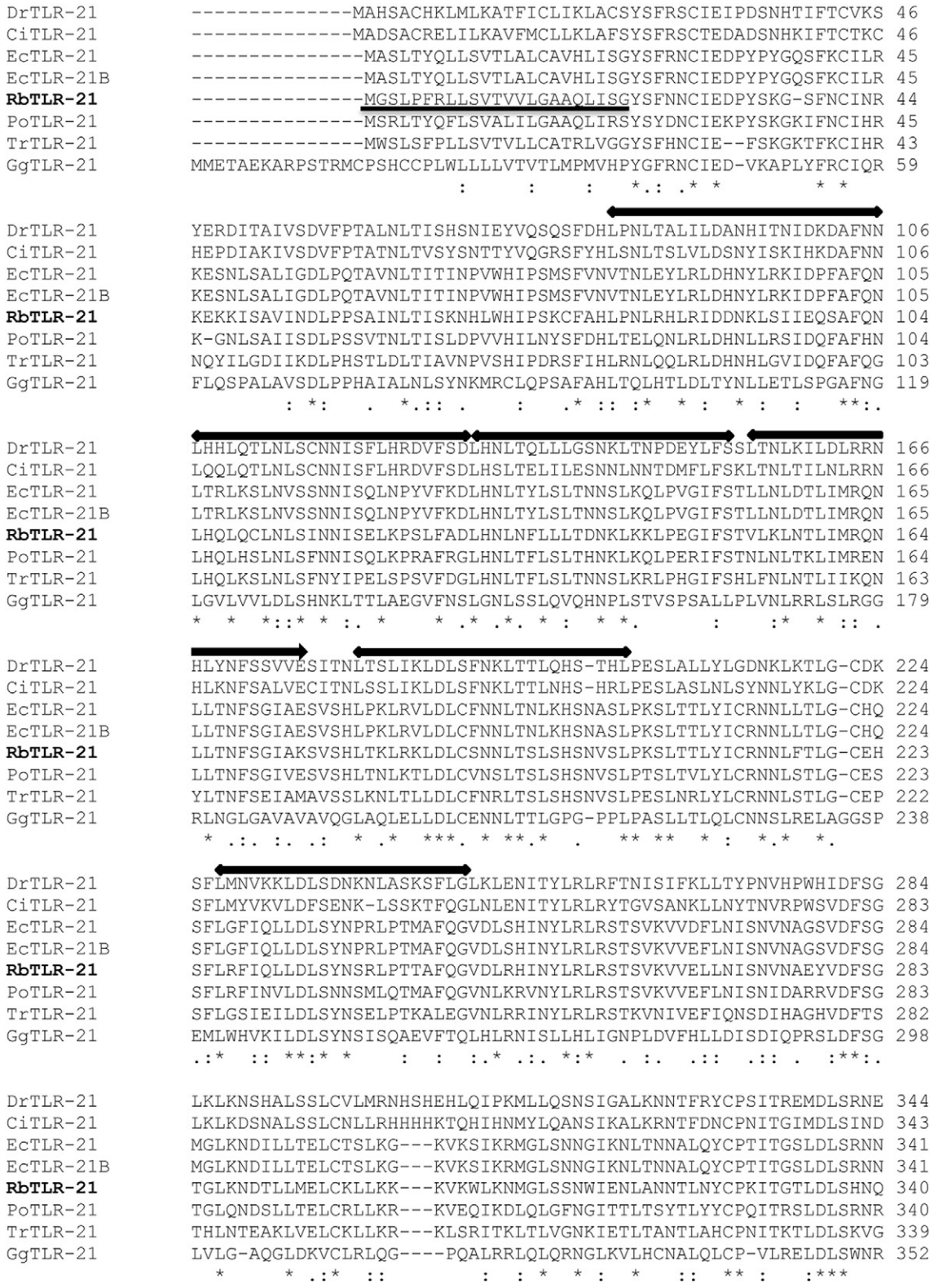

Fig. 2. ClustalW2 multiple sequence alignment of *Oplegnathus fasciatus* (RbTLR21) with other known TLR21 sequences. Completely conserved (100%) amino acid residues are depicted by asterisks (*), and semi-conserved residues are denoted by semicolons (:) and dots (.). The signal peptide sequence of RbTLR21 is underlined. Leucine rich repeats (LRRs) are indicated by black solid double-headed arrows, and the transmembrane domain is represented by unfilled double headed arrows. The intracellular TIR domain is indicted by a pattern filled double headed arrow. The active motifs in the TIR domain are boxed and depicted as BOX 1 (YD#F#SYN), BOX 2 (LCLHHRDF#LG), and BOX 3 (FW##L). Completely conserved (100%) amino acid residues in the three active motifs are highlighted with light gray. The three amino acid residues involved in ligand-induced NF-κB and IFN-β promoter activation are shaded in black. The two amino acid residues involved in CpG DNA recognition are shaded in red. The GenBank accession numbers are as follows: RbTLR21 (*Oplegnathus fasciatus*; KJ004565), (*Epinephelus coioides*; AEK49148), EcTLR21-B (*Epinephelus coioides*; AEK49147), TrTLR21 (*Takifugu rubripes*; AAW69371), IpTLR21 (*Ictalurus punctatus*; AEI59678), DrTLR21 (*Danio rerio*; NP_001186264), PoTLR21 (*Paralichthys olivaceus*; AFW04263), and GgTLR21 (*Gallus gallus*; NP_001025729).

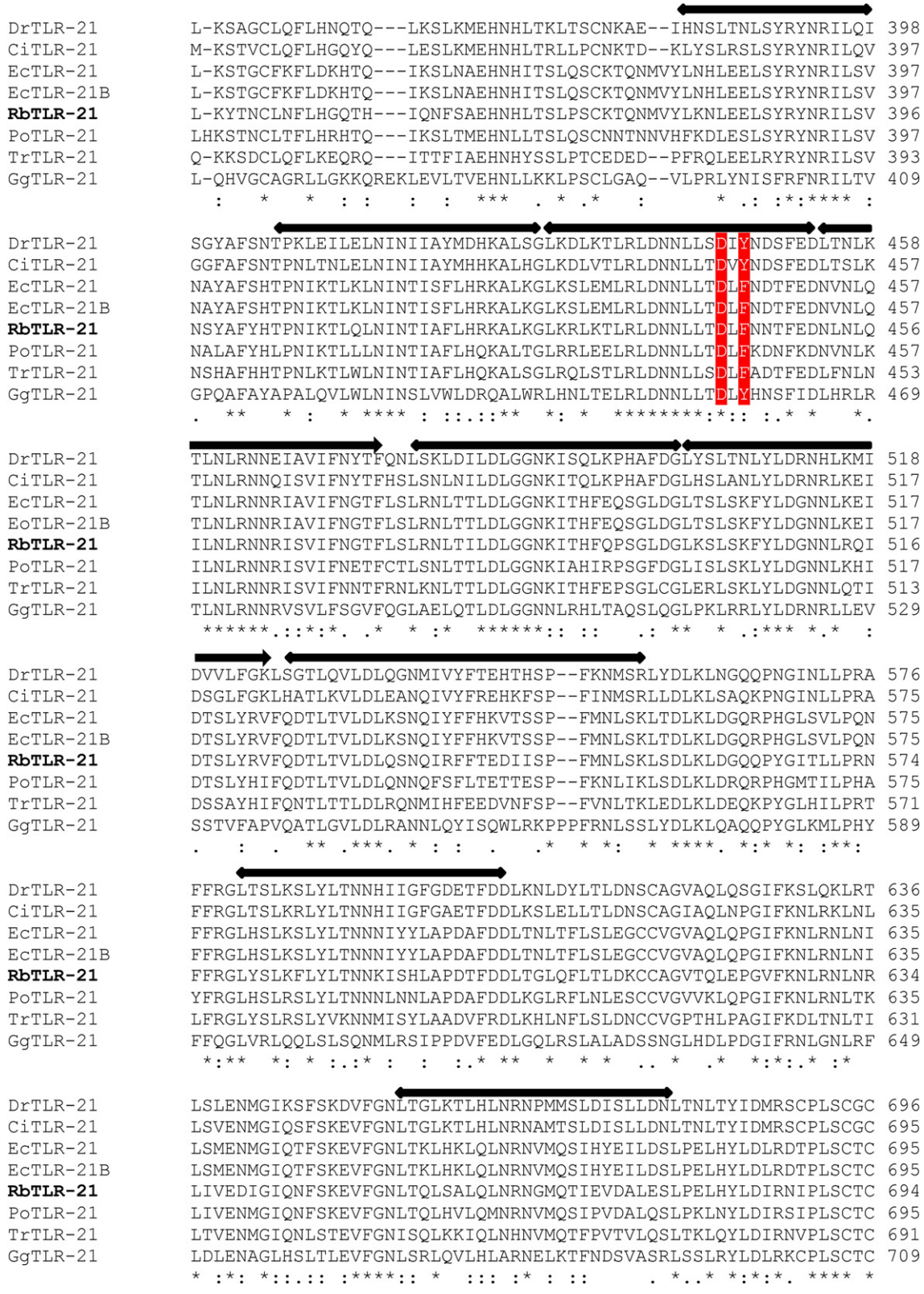

Fig. 2 (*continued*).

3.5. Tissue distribution of RbTLR21

The tissue specific mRNA expression profile of *RbTLR21* was examined by quantitative real-time PCR using β actin as a reference gene which did not show any significant expressional variation in each tissue under provided experimental conditions. The *RbTLR21* transcripts were ubiquitously expressed in the muscle, brain, intestine, skin, head kidney, gill, heart, kidney, blood, liver, and spleen of healthy fish. As shown in Fig. 4, the most prominent expression was in the spleen followed by the liver and blood. In contrast, the kidney, heart, gill, head kidney,

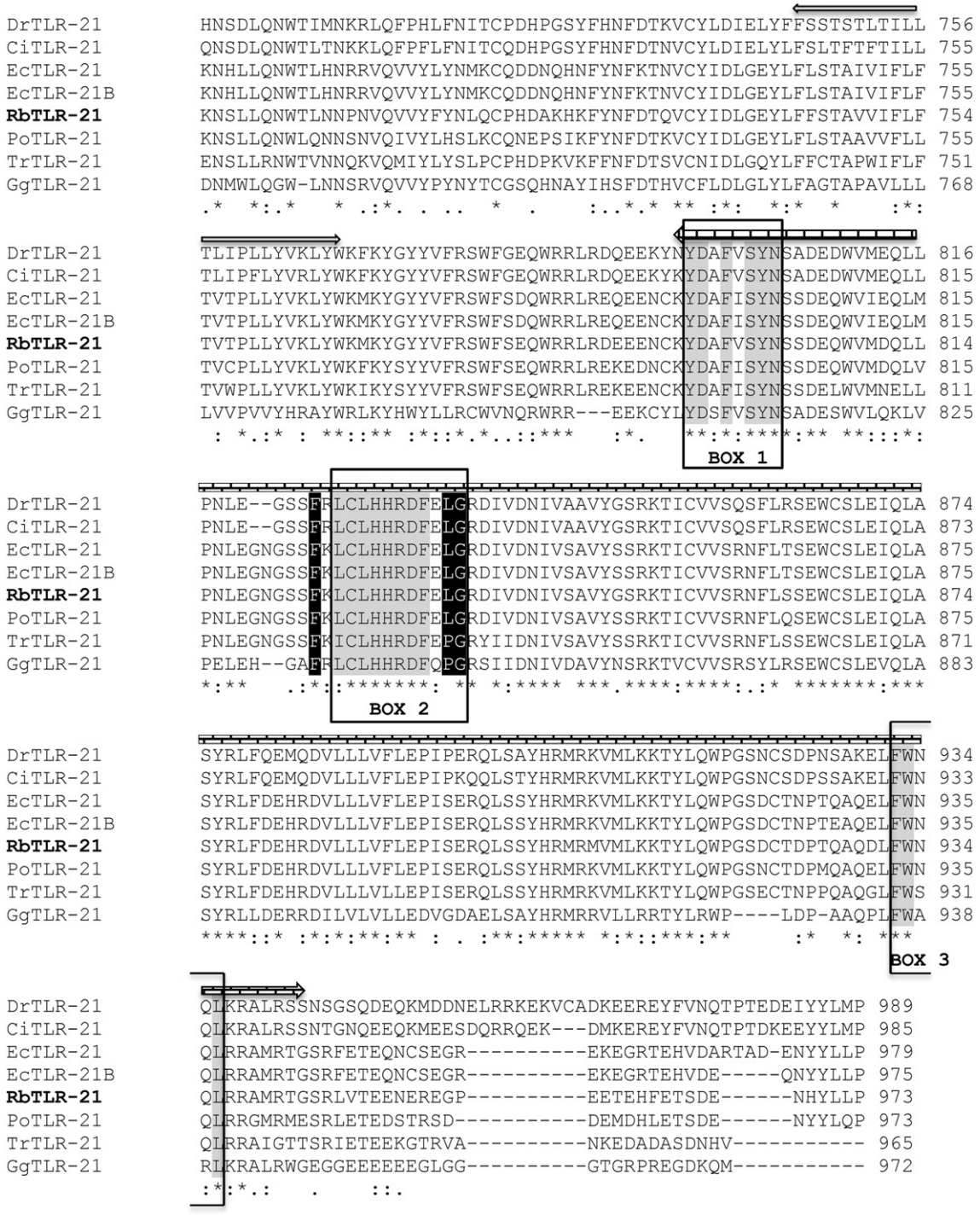

Fig. 2 (*continued*).

and skin had relatively low expression levels of *RbTLR21* compared to the expression levels in the spleen. Furthermore, very weak expression level of *RbTLR21* was found in the intestine, brain, and muscle. Similarly, a recent finding of *TLR21* from *Paralichthys olivaceus* (olive flounder) revealed that the greatest expression levels were detected in the spleen and gill, slightly in the head kidney, liver, and blood (Gao et al., 2013). Moreover, in *I. punctatus* (catfish), the spleen, intestine, stomach, liver, trunk kidney, ovary, and brain had the greatest expression of *TLR21*, whereas expression in the muscle was not detected (Baoprasertkul et al., 2007). The *C. idella* (Grass carp) showed predominant expression of *TLR21* in the skin, spleen, and head kidney, and only slightly in the muscle and trunk kidney (Wang et al., 2013). Furthermore, the *TLR21* expression in the *E. coioides* (orange spotted grouper) was highest in the head kidney, trunk kidney, and spleen and the lowest in the skin and rhombencephalon (Li et al., 2012). These results imply that the teleost *TLR21* is predominantly expressed in the main immune related tissues, including the spleen. Nevertheless, tissue specific distribution profiles of *TLR21* vary depending on the fish species due to the genetic background and developmental stage of the fish (Renshaw et al., 2002). However, in *Oplegnathus fasciatus* the most abundant expression of *TLR21* was detected in the spleen followed by the liver and are considered the most potent immune related and lymphoid rich tissues

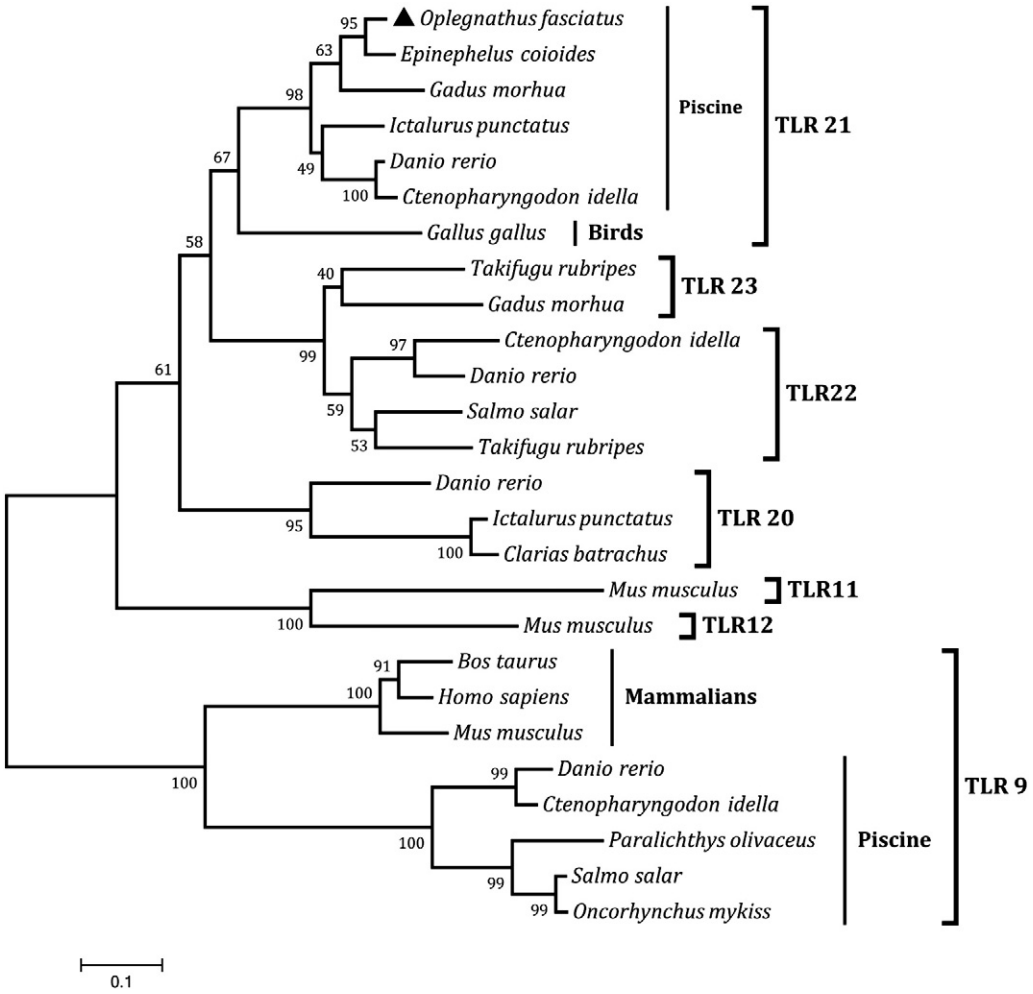

Fig. 3. Phylogenetic reconstruction of RbTLR21 (*Oplegnathus fasciatus*) with other known TLRs by the neighbor joining method available in MEGA version 5. The bootstrap confidence values based on 1000 replications are indicated at the nodes of the tree. The accession numbers for TLR homologs are as follows: TLR21 (*Epinephelus coioides*: AEK49148; *Gadus morhua*: AFK76484; *Ictalurus punctatus*: AEI59678; *Danio rerio*: NP_001186264; *Ctenopharyngodon idella*: AGM21642; *Gallus gallus*: NP_001025729); TLR23 (*Takifugu rubripes*: AAW70378; *Gadus morhua*: AFK76497); TLR22 (*Ctenopharyngodon idella*: ADX97523; *Danio rerio*: NP_001122147; *Salmo salar*: AEE38255; *Takifugu rubripes*: NP_001106664); TLR20 (*Danio rerio*:AAI63785; *Ictalurus punctatus*: ABF74621; *Clarias batrachus*: ADR81933); TLR11 (*Mus musculus*: NP_991388); TLR12 (*Mus musculus*: NP_991392); and TLR9 (*Bos taurus*: NP_898904; *Homo sapiens*: NP_059138; *Mus musculus*: NP_112455; *Danio rerio*: NP_001124066; *Ctenopharyngodon idella*: ADB96920; *Paralichthys olivaceus*: BAE80690; *Salmo salar*: NP_001117125; *Oncorhynchus mykiss*: NP_001123463.1).

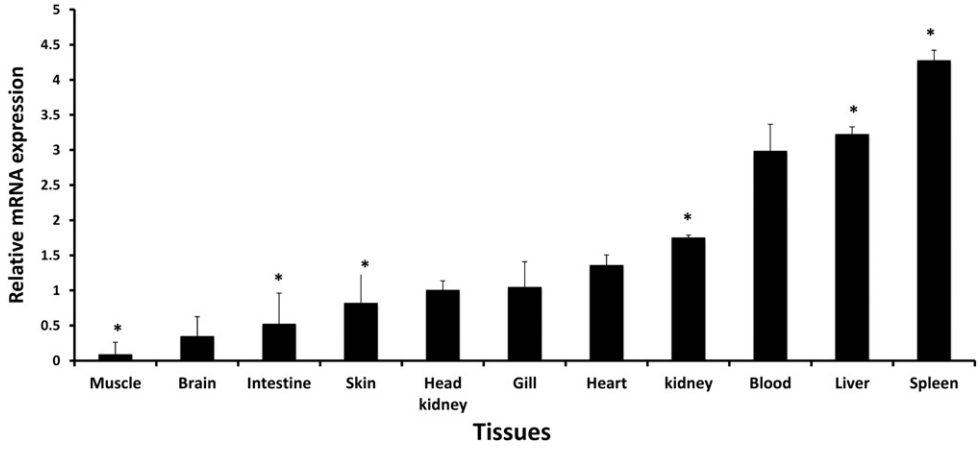

Fig. 4. Tissue specific expression analysis of *RbTLR21* mRNA in healthy *Oplegnathus fasciatus* by quantitative real-time PCR. The mRNA expression level of each tissue is expressed relative to the mRNA expression in the head kidney. Each bar represents the standard deviation (SD) of triplicates (n = 3). Asterisks (*) represent the significant difference in expression of *RbTLR21* against the expression of *RbTLR21* in the head kidney ($p < 0.05$).

involved in host defense against pathogenic attack (Fang et al., 2012; Seki et al., 2000). Moreover, macrophages, dendritic cells, T cells and B cells are considered as pivotal sources of the TLRs which are abundantly found in immunologically important tissues like the spleen (Akira et al., 2006; St Paul et al., 2011). Based on our observations (Fig. 4) we can infer that the predominant expression of *RbTLR21* in the spleen may be attributed to its importance in host immunity, since spleen tissues show wide prevalence of the macrophages, dendritic cells, T cells and B cells.

3.6. Expression profile of RbTLR21 upon pathogenic challenge

In order to evaluate the putative importance of *RbTLR21* in innate immune responses against various pathogenic infections, quantitative real time PCR was used to examine the temporal mRNA expression of *RbTLR21* in the spleen upon challenged with rock bream iridovirus (RBIV), *E. tarda* and *S. iniae*. As expected, β-actin did not show any significant expressional variation in this experiment under any of the provided condition affirming its suitability to be used as an internal reference. In this study, rock bream spleen was selected to investigate the transcriptional responses of *RbTLR21*, since the greatest tissue specific mRNA expression level of *RbTLR21* was observed in the spleen under a normal physiological condition; moreover, the spleen plays a significant role in innate immune defense in vertebrates.

As shown in Fig. 5, the relative mRNA expression level of *RbTLR21* in the spleen was significantly up-regulated at 3 h (2.1 fold, $p < 0.05$), 6 h (2.2 fold, $p < 0.05$), 12 h (2.6 fold, $p < 0.05$), 24 h (1.6 fold, $p < 0.05$), and 48 h (4.7 fold, $p < 0.05$) post-injection (p.i.) of *S. iniae*. After injection with *E. tarda*, *RbTLR21* basal transcript level was up-regulated throughout the experiment, with significant inductions at 3 h (2.7 fold, $p < 0.05$) and 6 h (2 fold, $p < 0.05$) p.i. (Fig. 5). Interestingly, the *RbTLR21* transcript induction folds were reduced along with the time p.i. after stimulation with *E. tarda*. Upon stimulation with RBIV, *RbTLR21* expression in the spleen was up-regulated at the early phase of post-induction period even though *RbTLR21* expression level was fluctuated at the latter phase of post-induction period; furthermore, significant elevations were detected at 3 h (2.5 fold, $p < 0.05$), 6 h (2.5 fold, $p < 0.05$), 12 h (3.4 fold, $p < 0.05$) and 48 h (2.3 fold, $p < 0.05$) p.i. (Fig. 5).

Recent findings showed that, inductive responses of *TLR21* at transcriptional level of *C. idella* upon bacterial (*Aeromonas hydrophila*) and viral (Aquareovirus) infection in their spleen tissues. Interestingly, at the early phase of p.i. after the viral infection *TLR21* mRNA transcripts were gradually up-regulated in the spleen tissue of *C. idella* (Wang et al., 2013). Likewise, we observed gradual up-regulation of *RbTLR21* mRNA transcripts in the spleen at the early phase of p.i. upon the RBIV challenge in this study (Fig. 5). Moreover, Gao et al. (2013) reported that significant up-regulations of *P. olivaceus TLR21* mRNA transcripts in the spleen tissue after the bacterial (*Vibrio anguillarum*) infection. Furthermore, post-*Cryptocaryon irritans* infection could significantly induce the *E. coioides TLR21* expression in the spleen (Li et al., 2012).

Recent studies have demonstrated that the chicken TLR21 and zebrafish TLR21 are functional homologs of mammalian TLR9 that sense bacterial genomic DNA or synthetic CpG DNA (Keestra et al., 2010). Chicken genome does not encode the TLR9; however both TLRs (TLR9 and TLR21) have been identified in some piscine genomes (Gao et al., 2013; Han et al., 2010). Herein we provided evidence of the up-regulation of *TLR21* expression in the spleen tissues of rock bream upon bacterial and viral infection. Overall, considering previous experimental results together with our observations on transcriptional modulation of *RbTLR21*, we can speculate that the up-regulation of *RbTLR21* mRNA transcripts might be due to the detection of DNA, like CpG DNA in *E. trada*, *S. iniae*, and RBIV. In addition, the up-regulation of *RbTLR21* could be mediated independent from RbTLR21 activation, since it has been described that a specific PAMP for one specific TLR can modulate the expression of other TLRs (Sepulcre et al., 2007). Our findings suggest that RbTLR21 may play a critical immune role against various pathogens (bacterial and virus) by activating TLR21 mediated downstream signaling cascade. However, further studies are required to be able to elucidate the exact mechanism of action of RbTLR21 upon pathogen invasion.

4. Conclusion

In summary, a TLR21 from rock bream, designated as RbTLR21, was identified and characterized at the transcriptional level. It contained an ectodomain with 16 LRRs, a single transmembrane domain, and a

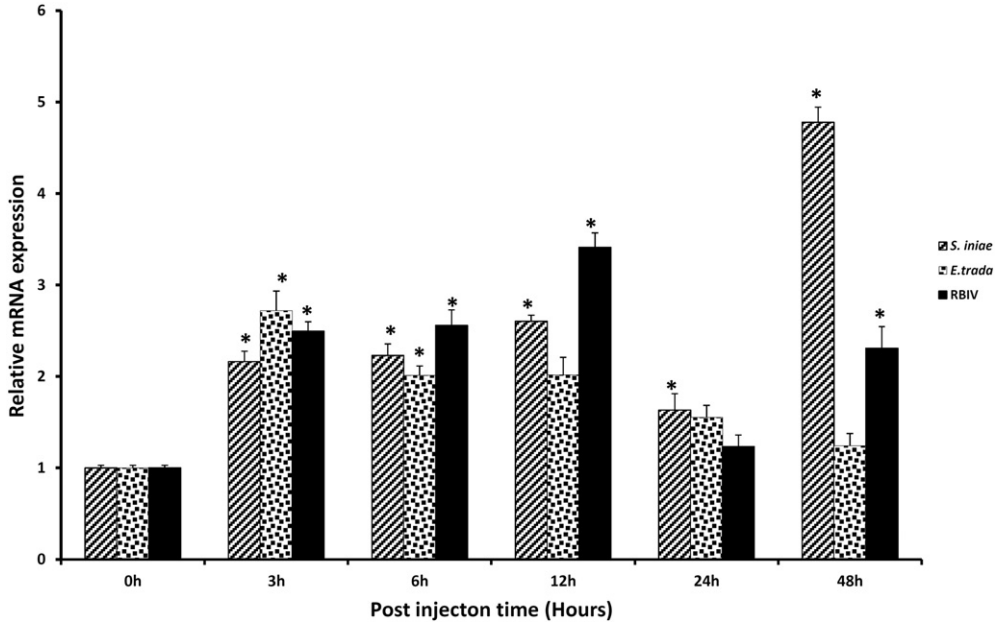

Fig. 5. Relative mRNA expression pattern of *RbTLR21* in spleens after the stimulation with *Streptococcus iniae*, *Edwardsiella tarda*, and rock bream iridovirus. *RbTLR21* expression analysis was determined by SYBR green quantitative real-time PCR. The Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using rock bream β actin as the internal control gene. Each bar represents the standard deviation (SD) of triplicates (n = 3). Asterisks (*) represent the significant difference in expression against the non-injected control ($p < 0.05$).

cytoplasmic TIR domain. Herein, we found that RbTLR21 was closely related with *E. coioides*, affirming it as an ortholog of TLR21. The *RbTLR21* was ubiquitously expressed in all the tissues tested. Up-regulation of basal transcription of *RbTLR21* was detected upon stimulation with *S. iniae*, *E. tarda*, and rock bream iridovirus. Collectively, our findings suggest that RbTLR21 is indeed a homolog of the TLR21 family and that RbTLR21 may be involved in host immune responses against bacterial and viral infections.

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