Molecular Characterization and Expression Analysis of Toll-like Receptor 9 Gene in Turbot, *Scophthalmus Maximus*

Guanjie Wang [1,2] , **Xuepeng Li** [1,2] , **Qiuming Liu** [1] , **Xianzhi Dong** [3*] , **Guobin Hu** [1,2*]

[1] College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

[2] Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China

[3] Institute of Biophysis, Chinese Academy of Sciences, Beijing 100101, China

Email: xzdong@ibp.ac.cn (X. Dong), huguobin@ouc.edu.cn (G. Hu)

Abstract: Toll-like receptors (TLRs) have emerged as key sensors of invading microbes by recognizing pathogen-associated molecular patterns and activating innate immune responses. TLR9 functions as a pattern-recognition receptor that recognizes unmethylated CpG motifs in bacterial and viral DNA. In this study, the full cDNA and genomic sequences of tlr9 in *Scophthalmus maximus* (*smtlr9*) were identified and characterized. The full-length cDNA of *smtlr9* was of 3754 bp encoding a polypeptide of 1066 amino acid residues. The genome sequence of smtlr9 was composed of 4083 nucleotides, including three exons and two introns. The putative Smtlr9 protein was characterized by a signal peptide sequence, a leucine-rich repeat domain (LRD) containing 15 leucine-rich repeats (LRRs), a transmembrane region, and a Toll/interleukin-1 receptor (TIR) domain. The putative protein shows the highest sequence identity (36.0~73.6%) to human and fish TLR9s. Phylogenetic analysis grouped Smtlr9 with other teleost Tlr9s. The *smtlr9* mRNA was constitutively expressed in all tissues examined, with the highest level in the head kidney and kidney. The smtlr9 transcription level was up-regulated by ODN2395 in the muscle, head kidney, spleen, and gills.

Keywords: Scophthalmus maximus, tlr9, molecular characterization, ODN2395, TRBIV, poly I: C, gene expression

1. Introduction

Toll-like receptors (TLRs) are type Ⅰ transmembrane proteins that contain an extracellular N-terminus with leucine-rich repeat domain (LRD) and an intracellular C-terminus with a Toll/interleukin (IL)-1 receptor (TIR) domain. In the LRD, the leucine-rich repeat (LRR) senses a variety of ligands including lipopolysaccharide (LPS), flagellin, dsRNA, and unmethylated CpG DNA [1, 2]. The TIR domain is involved in the signaling and the localization of TLRs [3, 4]. TLRs are at the top of the immune system [5, 6], which emerged as key sensors of invading microbes. To date, 13 TLRs have been identified from mammals and 29 from fish species [7, 8]. Although many studies reveal that fish TLRs have a similar function to their mammalian counterparts, some specific features have been discovered for them [7], and the detailed functions of various fish TLRs differed greatly [2, 9].

TLR9 is a TLR member recognizing unmethylated CpG DNA of bacterial and viral genomic DNAs (Hemmi et al. 2000). The binging of TLR9 with its ligands triggers the myeloid differentiation factor 88 (MyD88)-dependent signaling pathway to activate innate immune effectors such as inflammatory factors and interferons (IFNs) [10]. In mammals, the *tlr9* gene is expressed primarily on antigen-presenting cells (APCs), such as dendritic cells (DCs), an immune cell type playing crucial roles in antigen recognition in peripheral tissues and naive T lymphocyte activation in lymphoid tissues [11]. The newly produced TLR9 is retained in the endoplasmic reticulum and trafficked to endocytic vesicles mediated by UNC-93B, a 12-membrane-spanning protein, for sensing microbial CpG DNAs [12]. Until now, fish TLR9 has been cloned and characterized in puffer fish (*Takifugu rubripes*) [13], zebrafish (*Danio rerio*) [14], gilthead seabream (*Sparus aurata*) [15], Japanese flounder (*Paralichthys olivaceus*) [16], large yellow croaker (*Pseudosciaena crocea*) [17], rainbow trout (*Oncorhynchus mykiss*), [18], silver pomfret (*Pampus argenteus*) [19], spiny eel (*Mastacembelus armatus*) [20] and darkbarbel catfish (*Pelteobagrus vachellii*) [21].

Turbot (Scophthalmus maximus) is one of the most economically important mariculture species in North China. In recent years, it has suffered serious diseases caused by viral, bacterial, and parasitic infections, leading to catastrophic economic losses. Since the *tlr9* signaling plays an important role in the host's immune responses against infection of pathogens, the study of *tlr9* in S. maximus may contribute to developing strategies for the control of diseases of this economically important species. Here, we report the molecular characterization, mRNA tissue distribution, and expression analysis of a *tlr9* from S. maximus (*smtlr9*). The gene expression study was performed following challenges with ODN2395, a type of artificially synthetic oligodeoxynucleotides, Polyinosinic-polycytidylic acid (poly I: C), a synthetic

double-stranded RNA (dsRNA) analog, and turbot reddish body iridovirus (TRBIV), a DNA virus causing a turbot reddish body disease in cultured turbot.

2. Materials and methods

The studies presented in this manuscript were approved by the Experimental Animal Care Ethics Committee of the Chinese Centre for Disease Control and Prevention (experimental approval number:120; date of approval: 09-03-2011).

2.1 Fish, ODN2395

Turbot (53.4 ± 2.8 g, n =170) were purchased from a local mariculture farm and 10 fish were stocked in each 96-L tank over 1 week under natural photoperiod before use. Water in the tanks was continuously circulated, aerated, and regulated at 18°C. ODN2395 (class C, 50-T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G-30, phorothioate modifications are marked with*, Coley Pharmaceutical Group, Ottawa, USA) was dissolved in TE buffer (10mM Tris, 1mM EDTA, pH 8.0)..

2.2 Stimulation by ODN2395

Three groups of turbot were intraperitoneally injected with ODN2395 (0.15 mg/mL, 95μL per fish). Control fish from other three replicate tanks for poly I: C or TRBIV -treated group was injected with PBS, while those for ODN2395-treated group were injected with TE buffer. The volume of PBS or TE buffer used was the same as that of the corresponding stimulant.

2.3 Collection of fish samples

For gene expression assay, turbots were anesthetized with 0.1% (w/v) tricaine methanesulfonate (Sigma, MO, USA) at various time points (0h, 3h, 6h, 12h, 24h, 48h, 72h, and 120h) after injection. Tissues including muscle, head kidney, spleen, and gills were surgically isolated, placed in Sample Protector for RNA/DNA (TaKaRa, Dalian, China) at -80°C until homogenization. For tissue distribution analysis, eight tissues including skin, heart, kidney, liver, head kidney, brain, stomach, and intestine were collected from the untreated healthy turbot.

2.4 Extraction of RNA and genomic DNA

Before RNA extraction, samples were homogenized using mortar and pestle under liquid nitrogen. Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The purity and concentration of total RNA were determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, WA, USA) with A_{260}/A_{280} ration=1.9. RNA integrity was evaluated on 1.2% agarose/formaldehyde gel. The genomic DNA was isolated from the spleen by a standard phenol/chloroform extraction procedure [22].

2.5 Cloning of cDNA and genomic sequences

From 1 μg of total RNA extracted from the head kidney of turbot, a double-strand cDNA pool was constructed using a SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). Based on the conserved sequences of known fish *tlr9*, degenerate primers were designed (Table 1), and then, a core cDNA sequence of *smtlr9* was obtained by a combination of first-round and nested homology PCRs. The 5′- and 3′-end fragment were obtained using the Rapid amplification of cDNA ends (RACE) method with a combination of gene-specific primer and universal adaptor primer provided by the SMART cDNA Library Construction Kit (Clontech).

2.6 Sequence analysis

The sequence result of *smtlr9* was compared with the GenBank database by basic local-alignment search tool (http://blast.genome.ad.Jp/). The nucleotide sequence was translated into a protein sequence by the ExPASy translate tool (http://web.expasy.org/translate/). The signal peptide sequence was predicted by SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP). The LRRs were identified according to a method [23]. The protein domains were predicted by the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The multiple alignment of protein sequences was generated by the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis of the overall amino acid sequences was performed by software MEGA v7.

2.7 Quantitative real-time PCR (qPCR)

1.0 μg of total RNA from each tissue (5 individuals for each time point) was reverse-transcribed into cDNA by random primers using Superscript First Strand Synthesis System (Invitrogen, CA, USA). Primer pair TLR9-qF/TLR9-qR was used for the amplification of *smtlr9* while pair 18S rRNA-qF/18S rRNA-qR for reference gene, turbot 18S rRNA

(GenBank accession no.: EF126038) (Table 1). PCR was conducted in 20μL volume contained 1×UltraSYBR Mixture (with ROX Ⅰ) (CWBiotech, Beijing, China), each of specific forward and reverse primers (Table 1), and diluted cDNA (10 ng/μL) in an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems, CA, USA). PCR conditions were 94℃ for 4 min, followed by 43 cycles of 94℃ for 20 s, 61.5℃ for 30 s, 72℃ for 20 s. All the samples were amplified in triplicates. Fluorescent detection was performed after each extension step. The expression levels of *smtlr9* were normalized to 18S rRNA and further expressed as fold change relative to the expression level in control according to the $2^{-\Delta\Delta CT}$ method [24] in the analysis of gene expression upon immune challenges.

Table 1. Primer sequences used in this study

Primer name	Sequence (5′→3′)	Target gene	Application
TLR9-cF1	AACACNATHTTYAARGCNTGGGC	*smtlr9*	First round homology PCR
TLR9-cR1	TCYCTYTCCATNGGRTTNGGNGG		
TLR9-cF2	GATGARCCNGAYCCNGCNAARTG		Nested homology PCR
TLR9-cR2	ATCARSCCNCKRTCCATNACRTC		
TLR9-3′F1	TTGGGCAGGACATAAAGGGTATTC		3′-RACE PCR
TLR9-3′F2	GGACTCCAGGCCTTTCATGTATTG		
TLR9-5′R1	ATGGTCAGGTTCGCAAATGCT		5′-RACE PCR
TLR9-5′R2	CTCTGAAAACCCTCTCACTGATG		
TLR9-gF1	GTGGTTGTGACAGACTTGAC		Genomic PCR of intron 1
TLR9-gR1	ATGGCTTCAGACGGATCCT		
TLR9-gF2	TGAGGATCCGTCTGAAGCCA		Genomic PCR of intron 2
TLR9-gR2	CTTTGGTTCCGTCGTATTTCA		
TLR9-qF	GGATGAGAAGATGGATGCAG		qRT-PCR
TLR9-qR	CATCATCAATAACTTTCCACC		
18rRNA-qF	CACAGTGCCCATCTATGAG	*18rRNA*	
18rRNA-qR	CCATCTCCTGCTCGAAGTC		

Note: qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends.

2.8 Statistical analysis

All data were expressed as the mean ± standard error (S. E.), n = 3. Differences in the data were compared by one-way analysis of variance (ANOVA) followed by Duncan's post hoc test for multiple comparisons using Statistical Package for Social Science software v14 (Chicago, IL, USA). Significant differences were declared at P < 0.05.

3. Results

3.1 Molecular characterization of smtlr9 cDNA

The full-length cDNA of *smtlr9* (Genbank accession no. KU746969) consists of 3754 bp with an open reading frame (ORF) of 3201 bp that encodes a protein of 1066 amino acid residues. The 5'- and 3'- untranslated regions (UTR) are 124 bp and 429 bp, respectively. In the 3'-UTR, four ATTTA mRNA instability motifs and two downstream polyadenylation signals (ATTAAA, AATAAA) were found. A potential signal peptide locates at positions 1-28, a LRD containing 15 LRRs at positions 121-845, a transmembrane region at positions 847-869, and a TIR domain in the C-terminal region at positions 903-1045 were found in the predicted *Smtlr9* (Figure 2). Two conserved amino acid residues Asp^{563} and Tyr^{565}, and two CXXC motifs separated by six amino acid residues were found in the extracellular region of *Smtlr9* (Figure 1). A typical TIR architecture comprising three motifs highly conserved among teleosts: BOX1 (YDAFVVFD), BOX2 (LCLEERDWTPG), and BOX3 (FWNRM) was identified in the intracellular region of *Smtlr9* (Figure 1). BLASTP search revealed that *Smtlr9* shares a sequence identity of 73.6%, 71.6%, 63.5%, 58.9%, 52.0%, 51.1% and 36.0% with the Tlr9 sequences of Japanese flounder, large yellow croaker, red seabream, fugu rubripes, Atlantic salmon, channel catfish, zebrafish, and human, respectively (Table 2). The amino acid sequence homology in the TIR domain is approximately 15% higher than those in the entire sequence (Table 2).

TATCTTGAAGACTGCCTCATGTTCCGCCTCTGGTATGAATATTATATGCGCTCTACAAGCAATG*gt*aagtgagaatattaaattgttta
                                        M N I I C A L Q A M

1   aactttgttatatacatgtgataaaaaggtcttgctggaaaggctgaataagccaaagtgatttattcagtttttttgaagccacctca
1   ctctaggatgcatagggtgatatttttagaatgacagaatgaaccgtggaccgtatactaaaatgtaaaaaatgaaagaaaagaaaago
1   atggtaaaaaatgagatgaatttggtttcttgtcttatctc*ag*GCTATGCTGAGAAATATCCTCATCATTTGTCAGCTTATTCCATT(
                                        A M L R N I L I I C Q L I P F

1   CGAGGACCCGAAATATCAAATTCTTCCCATGTGAGACGGATGAGAACGTCACCACGGTAGACTGCTACGAGAGACCAATCAGGAATAT(
     A R T R N I K F F P C E T D E N V T T V D C Y E R P I R N I
1   CAGTCATCACGTTTACCACAGTGGTGTCACTCAATTTAAATCCGATGAAGATACGGCAAGTTGGGCAGCATGCTTTTGCAGATGCCCC/
     P V I T F T T V V S L N L N P M K I R Q V G Q H A F A D A P
1   ACCTTCTCACTCTGAAAATAATGGGGACTTGTCAACCAGGTCAACTGAGAGCCATAAAGGACCAGTCATGCAAAATGAAGATCCACCC1
     N L L T L K I M G T C Q P G Q L R A I K D Q S C K M K I H P
1   ACGGCCTTCAAAAGTCTACTTAGTCTTCAATCACTGTACCTATCTGGAAATAGCCTCACCTCCATACCCTGGTTACCTGAAACCCTGAG(
     H A F K S L L S L Q S L Y L S G N S L T S I P W L P E T L R
1   TTCTCGATCTACAGAACAATTGCATCTTCCACATTACAGACCCTTTAAACACTCCAAATCTTGAAATGCTCTTCCTCACCAAGAACTG(
     V L D L Q N N C I F H I T D P L N T P N L E M L F L T K N C
1   TTTTATGCAAACCCTTGCAACCAGTCTTTTTACATCAGTGAGAGGGTTTTCAGAGAGCTCCGTAATCTCAAAAACCTGACGTTGGGGTA(
     F Y A N P C N Q S F Y I S E R V F R E L R N L K N L T L G Y
1   ACAACTTGACAGCTGTCCCCAATGGACTGCCACCCTCACTGCTGACCATGCTCAAAAAATTGAATTTGGAGTGGAACTGCCAGCGTTGT
     N N L T A V P N G L P P S L L T M L K K L N L E W N C Q R C
81   ACCATGCATCGAGGCCCTGTTTTCCTTGCCCTAATAATCACTCTCTTTACCTCCATCCAAACTCATTCTATGCTGAGAACAGCTCGAT(
     D H A S R P C F P C P N N H S L Y L H P N S F Y A E N S S I
71   CCTTCCTAAGCTTGAGGGGCAACTCTTTAAAAACCTTCCCAGAGGGCCTTTCAGACCTTTGACTAATTTAAAGAAACTGGACCTCTCT
     T F L S L R G N S L K T F P E G L F R P L T N L K K L D L S
61   ACAACCTCCTGGCATATGCTATACAAAATGGCACCTTCTTCACAGAGCTGAGGGGCCTCACTTGGATGAGTCTAATCTACAACTATGA/
     D N L L A Y A I Q N G T F F T E L R G L T W M S L I Y N Y E
51   CGCTGAAGACATTCCAGGAACTTAGTCTTTCATCACATATTGGCAATATATCTGGTCTACAACAGCTCCTCCTCAGTGGTAACTTTTT(
     P L K T F Q E L S L S S H I G N I S G L Q Q L L L S G N F F
41   ACAGTCTTTCCCCTCAGAGCCTTAGTGTTCTGTCCAAACTCAGACATTTAAAAACACTAGAGCTGAGAATGAACTTCATTAGAAATTGT
     H S L S P Q S L S V L S K L R H L K T L E L R M N F I R N C
31   ACTTGACAGCTCTGAAACAGTTACCATCTCTAATTGAAATTGACCTCTCCCAAAACATGCTTTCTTTCCTTCCTTGTCCATCATCTGA/
     N L T A L K Q L P S L I E I D L S Q N M L S F L P C P S S E
21   TTTTGGCACAACATAGATGTCAGAACCAGAATTTGTACACGAATGATTTCTGTGACCAACCCATTATTGTAGTTGATCGCGAAGTCAC/
     I L A Q H R C Q N Q N L Y T N D F C D Q P I I V V D R E V T
11   CAGGAGATGGGATATGGGAACCCAACCAATCAAAGTTGCTGGAAACGCTAAAGGACAACGTGTCACCATTTCCATCACTATGGGATTT1
     S G D G I W E P N Q S K L L E T L K D N V S P F P S L W D F
01   GAACCTATTTCTGCCAAAATAATTTAACGTTTGACCTGTCTCAAAATGACATTCTGTCTCTTAACAAACATGTGTTTCTAGGCATGGA(
     R T Y F C Q N N L T F D L S Q N D I L S L N K H V F L G M E
91   ATGCTGTTTGTTTAGACCTTTCCTTTAATTACATGAACCAGGCATTGAGGAGAGGACAATTTAGTAGCACAAAAAATTTAGTTTTTCT/
     N A V C L D L S F N Y M N Q A L R R G Q F S S T K N L V F L
81   ATTTGTCATACAATAGGTTTGATCTTTATTATACAGATGCGTTCAGTGAGCTTAAAACTACTCTGAAGGTATTAGATGTTAGTAACAA(
     N L S Y N R F D L Y Y T D A F S E L K T T L K V L D V S N N
71   ATTTTCACTTCAGAATGAGGGGCATGGGCCATCGTTTCGAGTTTCTTCAAAATCTGACTAACTTGGAAGTCCTAAGTCTCGCAAACAAT
     D F H F R M R G M G H R F E F L Q N L T N L E V L S L A N N
61   GCATTGGAATGAGAATAGATCAAATGCTGATCAGCAGCTCAGTAAAGTACCTCTACTTCTATGGAAATCACCTTAACATTATGTGGGA/

2251 CTGGCAACAACCAGTACACTAAATTCTTCCAAAACCTGACAAACCTCATCTACCTGGACATCTCTGACAACGATCTGACCTCAATCACAC
651   S G N N Q Y T K F F Q N L T N L I Y L D I S D N D L T S I T
2341 CGGACGTACTGTGTCACCTCCCAGCAAGCATTGAATCCCTCAGCATCAGCAACAATCTGCTGAACTATTTTCCATGGCAGAACATGTCGG
681   P D V L C H L P A S I E S L S I S N N L L N Y F P W Q N M S
2431 CACTCAGCAACTTGTGTCACCTGAACCTCAGTCAAAATTATCTTTACTATTTACCTTATAGAGTCATAGAATTCGGAGCCAATTTTTCTC
711   A L S N L C H L N L S Q N Y L Y Y L P Y R V I E F G A N F S
2521 TCTTGGACCTCAGTCACAATCGCTTTAGTGTTATTCCTGAGAATTCTTCAGAAAGGCAAAATCAATGCAATATCTGTATCTCAGCAACA
741   L L D L S H N R F S V I P E N F F R K A K S M Q Y L Y L S N
2611 ATCAGATCAAAGAGTTGGACCATCAGTATGTACCTACCCCTTTTAAAAATGGTAGTGCCCTCAAAATACTCACCCTGCATGCCAACCCCT
771   N Q I K E L D H Q Y V P T P F K N G S A L K I L T L H A N P
2701 TTAAATGTGATTGTTCTACATCTTGGTTTGCAGACTTCTTGAGCACTACTCCAGTAAACATTCCTCATCTCACCACACGCATACATTGTG
801   F K C D C S T S W F A D F L S T T P V N I P H L T T R I H C
2791 AATATCCAGAGTCCAAACAGGGTCAGAGTATACTCTCTATGGATCAGAAATCCTGCCAGGATGTATATGTAGCTTAGCTTTCCTCGTCT
831   E Y P E S K Q G Q S I L S M D Q K S C Q D V Y G S L A F L V
2881 GTTCTTTCTTGGCTCTGACAATCACTGCTCTGCCCCTACTGAAGCACCTCTATGGCTGGGACATGTGGTATTGCATACGGTGCTTTGGG
861   C S F L A L T I T A L P L L K H L Y G W D M W Y C I Q V L W
2971 CAGGACATAAAGGGTATTCCCAGTTGGCAGGCTCTGATTCACATAATCACTACGATGCCTTTGTGGTGTTGACACCACAAACCAGGCCG
891   A G H K G Y S Q L A G S D S H N H Y D A F V V F D T T N Q A
3061 TGAGGGATTGGGTCTACAACGAGTTAACTGTCAACCTGGAGAACTTTGGTCCAAGGAGATTTTGCCTCTGTTTGGAAGAGAGGGACTGGA
921   V R D W V Y N E L T V N L E N F G P R R F C L C L E E R D W
3151 CTCCAGGCCTTTCATGTATTGGAATCTACACAGTGCTGTCTACAACAGTGTGAAGACGGTGTTTGTGCTGTCCAGCTGTTTGAATGGTG
951   T P G L S C I E N L H S A V Y N S V K T V F V L S S C L N G
3241 GCGAGACGGCGAACGGCGTGATCCGCCAGGCGTTCTACATGGTTCAACAGCGGCTCCTGGATGAGAAG*gt*cagtgaaattgtacttcact
981   G E T A N G V I R Q A F Y M V Q Q R L L D E K
3331 tttatgtgcattttgtgggatgtcaatatcgccacttctgttttcacaacacaaaattacctctgattttatccaaaca*ag*ATGGATGCA
1004   M D A
3421 GCTGTGCTGGTTCTGTTGGATGAGATGTTTCCCAAATTGAAGTACCTTCAGCTGAGGAAGAGGTTGTGCAGAAAGTCTGTGTTGACCTGG
1007 A V L V L L D E M F P K L K Y L Q L R K R L C R K S V L T W
3511 CCGAGAAACCCAAAGGCTCAACCCCTTTTCTGGAATCGAATGAGAATGGCGTTGTCGTCGGATAACCTCAAATTATATGACAACAACATG
1037 P R N P K A Q P L F W N R M R M A L S S D N L K L Y D N N M
3601 AGTGAAAGTTTTATATGATGGAGAGCAGTTGGTGGAAAGTTATTGATGATGTGACACACACACACACACACACACACACACACACACAC
1067 S E S F I –
3691 ACACACACACACACAATGATACTTGATTAATGTTTTTAATATGTTAATTTACACAATTGATTAAATCGTAGCAGCTTTTGTGCTCTGT
3781 TGATTTATCTGTAGGGTGATTTAATTGATATTGTACTTTTTATATATTTGACATCGTCGTGTGTTCTTTGTTTTCCATCCCGATTTTATT
3871 TACTGTCGTTTTTGAGTTAGTCCTTGTCTTCAGAGAACTTGAGTATGAAGTCTGAAAAAGGTAAAGCACGGTTAACTTAATGTATGGGCA
3961 TTGTACATGATGATACTTTGATATTGGACCATTCAAATAAACCTTTAAAGAGACCTAACTTCA

Figure 1. The *smtlr9* gene and its predicted proteins

Note: Exons are represented by capital letters and introns are represented by lower case letters. The splice donor/acceptor (gt/ag) sequences are in italics. The structure domain is represented by gray shadow, in which the green letter represents transmembrane region, red represents LRRs, and blue represents TIR (BOX 1, 2, 3 as shown in the box). Two conserved amino acid residues Asp^{563} and Tyr^{565} are in orange letters. Two CXXC motifs separated by six amino acid residues are underlined.

Table 2. Comparison of TLR9 amino acid sequences of turbot with those of other teleosts and a human

Species	Category name	Amino acid residues	Number of LRR[a]	Identity(Similarity) of TLR9[b]	Identity(Similarity) of TIR domain[b]	GenBank numbers
Turbot (*Scophthalmus maximus*)	Pleuronectiformes	1066	15	—	—	AMQ35506
Japanese flounder (*Paralichthys olivaceus*)	Pleuronectiformes	1065	15	73.6 (82.8)	88.1 (92.7)	BAM11216
Red seabream (*Pagrus major*)	Perciformes	1063	14	71.6 (82.7)	86.8 (92.7)	ABY79217
Fugu rubripes (*Takifugu rubripes*)	Tetraodontiformes	1045	17	63.5 (76.1)	79.7 (88.9)	AAW69377
Atlantic salmon (*Salmo salar*)	Salmoniformes	1074	17	58.9 (75.6)	79.4 (87.7)	ABV59002
Channel catfish (*Ictalurus punctatus*)	Siluriformes	1061	13	52.0 (68.2)	71.1 (87.5)	AEI59673
Zebrafish (*Danio rerio*)	Cypriniformes	1057	15	51.1 (67.6)	71.5 (84.8)	AAI63628
Human (*Homo sapiens*)	Mammals	1032	19	36.0 (52.1)	50.0 (65.8)	AAQ89443

[a]Predicted LRR domain based on results from the SMART program.

[b]Sequence identity and similarity identified by EMBOSS Pairwise Alignment Tools for Global Sequence Alignment EBI.
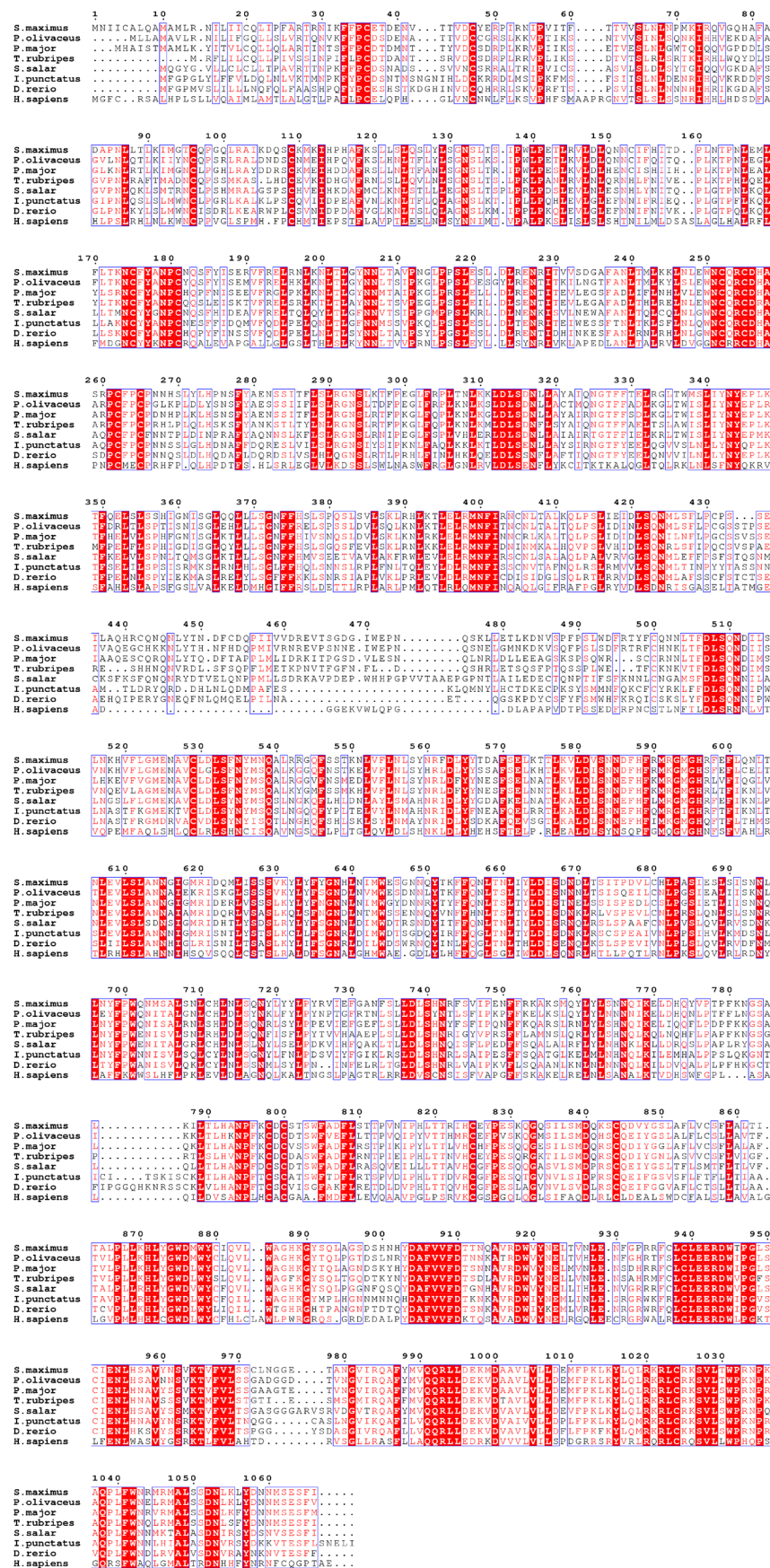
Figure 2. The multiple alignment of Tlr9 protein sequences (GenBank accession numbers in Table 2)

3.2 Genomic structure of smtlr9

The *smtlr9* genomic sequence (Genbank accession no. KU746968) spans 4083 bp, with a structure of three exons-two introns. The two introns locate in the region of the ORF. The splice donor/acceptor (GT/AG) sequence is conserved at the 5'- and 3'- ends of the introns (Figure 3). The tlr9 gene structure of turbot is consistent with that of Japanese flounder (which belongs to the Pleuronectiformes), as well as that of Perciformes and Cyprinodontiformes, both of which have two introns, while the tlr9 gene of Salmoniformes has three introns. The tlr9 gene of Siluriformes and Cypriniformes have only one intron, like the mammals.
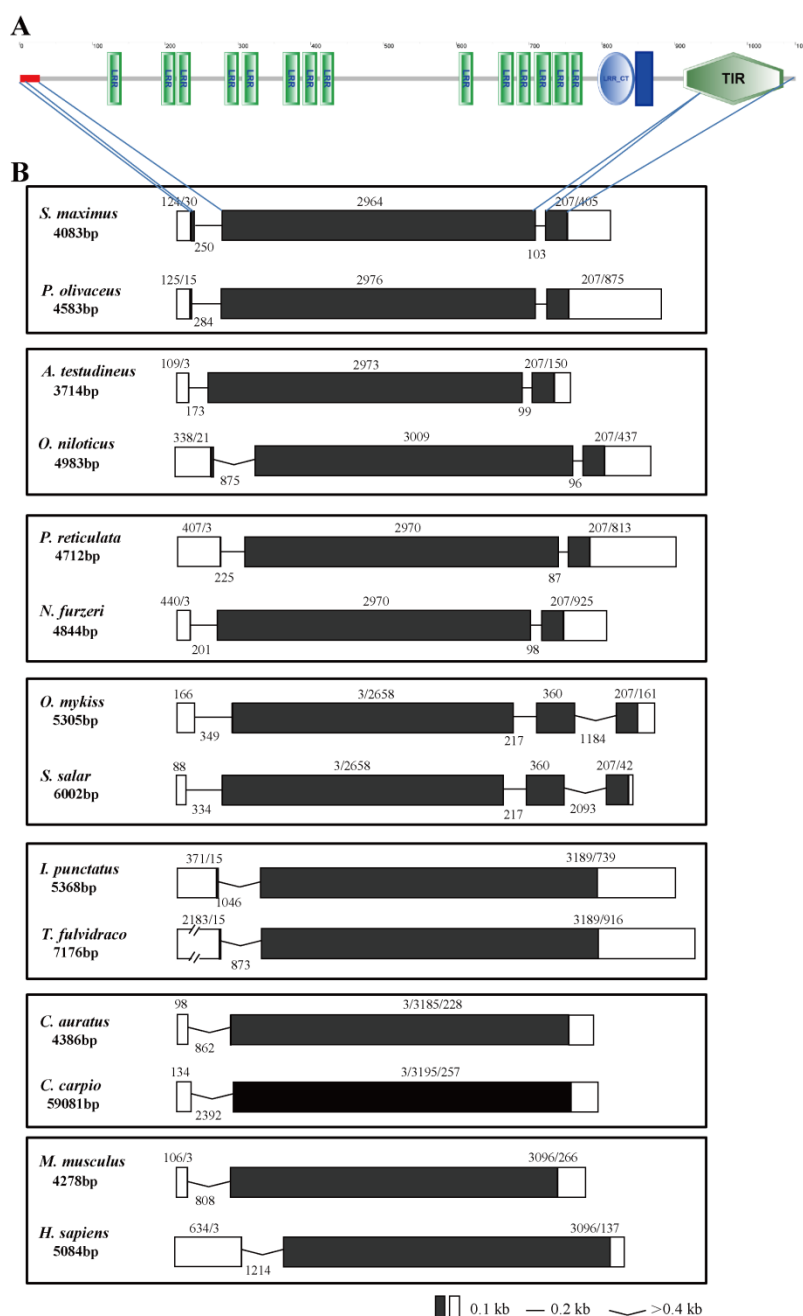
Figure 3. Schematic comparative domain organization of smtlr9 (A) and Comparison of tlr9s genome structure (B)

Note: The signal peptide is red; LRRs are light green; LRR_CT is blue circle; transmembrane domain is blue square; TIR is dark green. Genbank accession numbers: Turbot (Scophthalmus maximus), KU746968; Japanese flounder (Paralichthys olivaceus), AB234024; Climbing perch (Anabas testudineus), NW_020535999.1; Nile tilapia (Oreochromis niloticus), NC_031984.2; Guppy (Poecilia reticulata), NC_024337.1; Turquoise killifish (Nothobranchius furzeri), NW_015448355.1; Rainbow trout (Oncorhynchus mykiss), NC_035092.1; Atlantic salmon (Salmo salar), NC_027312.1;

Channel catfish (Ictalurus punctatus), NC_030420.1; Yellow catfish (Tachysurus fulvidraco), NW_020847974.1; Common carp (Cyprinus carpio), NW_017539660.1; Goldfish (Carassius auratus), NC_039250.1; House mouse (Mus musculus), NC_000075.6; Human (Homo sapiens), NC_000003.12.

3.3 Phylogenetic analysis of Tlr9

To assess the phylogenetic position of *Smtlr9*, overall amino acid sequences of 36 Tlr9s were collected to reconstruct a phylogenetic tree using the Neighbor-Joining method (Figure 4). In the tree, *Smtlr9* is shown the closest phylogenetic position with Tlr9 from another fish in Pleuronectiformes, Japanese flounder, and next to the fishes in Perciformes. A far position was seen between *Smtlr9* and fishes in Cypriniformes, crucian carp (Carassius carassius).

Figure 4. Construction of phylogenetic tree of Tlr9 proteins in fish

Note: Turbot (Scophthalmus maximus) is marked with a black triangle.

3.4 Tissue distribution of smtlr9

The mRNA expression of *smtlr9* was examined by qPCR in various tissues including skin, heart, kidney, liver, head kidney, brain, stomach and intestine. The results showed that *smtlr9* was broadly expressed in all examined tissues. Higher expression was detected in the head kidney and kidney, followed by the brain and heart. Low levels were observed in the intestine, spleen and skin. An extremely weak expression was detected in the gills, stomach, liver, gonad and muscle (Figure 5).
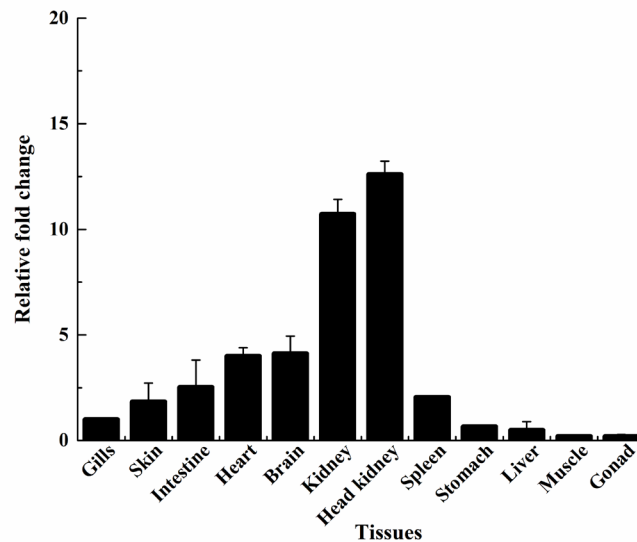
Figure 5. Relative expression of smtlr9 mRNA in different tissues

3.5 Gene expression of smtlr9 upon ODN2395 challenges

After ODN2395 stimulation, the tlr9 transcription level was up-regulated in the muscle, head kidney, spleen, and gills. The maximum values in muscle and spleen appeared at 72 h, while those in head kidney and gill appeared at 12 h and 120 h respectively (Figure 6).
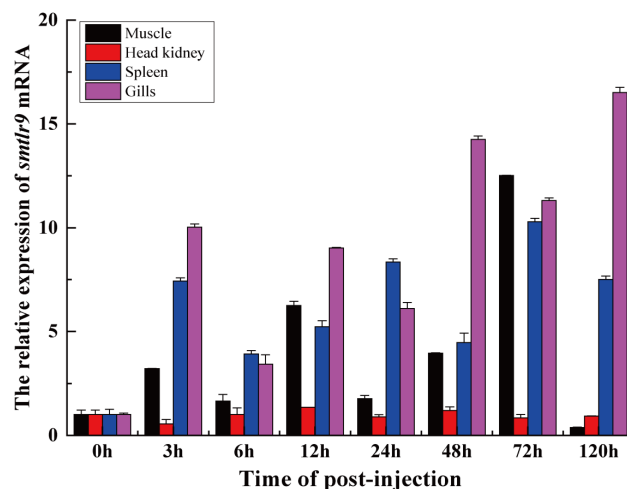

Figure 6. Relative expression of smtlr9 mRNA after stimulate by OND2395

4. Discussion

In this study, we cloned and characterized the full-length cDNA and genomic sequences of a tlr9 homolog in turbot. Based on the TLR-like structure of the deductive peptide and homology with fish tlr9 orthologs, the cloned sequence was identified as *smtlr9*. The phylogenetic analysis further confirmed the authenticity of the nomenclature for *Smtlr9* by clustering it into the group of teleost Tlr9. Similar to other fish TLR mRNA, four ATTTA mRNA instability motifs, which is a characteristic of inflammatory mediator genes, were found in the 3'-UTR of *smtlr9* suggesting a transient expression feature of *smtlr9* [25].

When analyzed with SMART program, 14 leucine-rich repeats (LRRs) and one LRR C-terminal domain (LRR_CT) structure were identified in the LRD of *Smtlr9* (Figure 2). The LRRs are supposed to mediate specific PAMP recognition [26]. Almost all LRRs in *Smtlr9* are similar to those of Japanese flounder, large yellow croaker, gilthead seabream, rainbow trout, zebrafish, and human Tlr9s, but some of the LRRs are excluded (Figure 2). Similar to Japanese flounder Tlr9, the

two functional amino acid residues Asp563 and Tyr565 in *Smtlr9* (corresponding to Asp563 and Tyr565 in Japanese flounder Tlr9) don't locate in an LRR, while other Tlr9s possess the LRR that contains these two residues which supposed to bind directly to unmethylated CpG dinucleotide sequences [26]. The difference of Tlr9s specificity for unmethylated CpG ODNs has been confirmed in several species [27]. Therefore, the differences in LRRs may explain why the CpG motif recognition is species-specific. The typical TIR architecture of *Smtlr9* has high sequence identities to the TIR domains of other known Tlr9s which are essential for cellular signaling and intracellular localization [3, 4].

The *smtlr9* gene is composed of three exons and two introns. Such a gene structure is the same with the structures of Japanese flounder [16] and gilthead seabream tlr9s [15], but different from those in salmon [28], fugu [13], rainbow trout, zebrafish, and human (Figure 2). This result suggests that the genomic organization of tlr9 is not conserved among vertebrates even within the teleost group.

It was reported that tlr9 was mainly expressed in dendritic cells and B lymphocytes in mammals [29]. In this study, *smtlr9* was found to be predominantly transcribed in the brain and head kidney, but relatively low or very weakly transcribed in other examined tissues. This tissue expression pattern is similar to that of other teleosts on the whole [15] [14] [13] [16] [17] [30] [18]. However, rainbow trout and Japanese flounder tlr9s were highly expressed in the spleen where *smtlr9* was expressed at a relatively low level [16, 18]. This difference may be due to the fact that tlr9-expressing cell populations differ among various species. It was reported that the myeloid lineage cells don't express tlr9 in humans, but they express tlr9 in mice [31]. Similar discrepancies in tissue expression profiles among fish species were previously found for tlr1, tlr3, tlr7, and tlr8 [32, 33]. These differences may be due to species variation, immunological status, developmental stage, or genetic background.

The *smtlr9* transcription level was up-regulated by ODN2395 in all four test issues, the muscle, head kidney, spleen, and gills. It is consistent with the previous report that TLR9 in fish was up-regulated by killed Vibrio. Parahaemolyticus, Mycobacterium marinum, synthetic CpG ODN, and IFN-γ [17, 28, 34]. It was also reported that the expression level of TLR9 didn't significantly change in the spleen of gilthead seabream after exposure to a low virulent bacterial pathogen [15] and even down-regulated after treatment with killed V. parahaemolyticus in the liver of large yellow croaker [17]. Similar to the findings in mammals, the TLR9 signaling in fish species is also MyD88-depended and eventually leads to the production of inflammatory factors including tumor necrosis factor (TNF)-α, IL6, and type Ⅰ IFNs [16]. As the response of salmon macrophages to CpGs can be clogged by chloroquine, an inhibitor of endosomal acidification, it is deduced that the endosomal acidification is essential for the activation of fish TLR9 by CpGs as demonstrated in mammals [35]. In rainbow trout, a direct correlation was found between tlr9 transcripts and those of cluster of differentiation 83 (cd83) and CXC chemokine receptor 4 (cxcr4), the mammalian orthologs of which are the known marker for DCs, thus suggesting that fish dendritic-like cells are the TLR9-expressing cells [18].

5. Acknowledgments

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