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Short communication

Genome-wide identification and characterization of toll-like receptor genes in spotted sea bass (*Lateolabrax maculatus*) and their involvement in the host immune response to *Vibrio harveyi* infection

Hongying Fan, Lingyu Wang, Haishen Wen, Kuiqin Wang, Xin Qi, Jifang Li, Feng He, Yun Li*

The Key Laboratory of Mariculture (Ocean University of China), Ministry of Education, Qingdao, 266003, China

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ABSTRACT

Toll-like receptor (*TLR*) genes are the earliest reported pathogen recognition receptors (PRRs) and have been extensively studied. These genes play pivotal roles in the innate immune defense against pathogen invasion. In this study, a total of 16 *tlr* genes were identified and characterized in spotted sea bass (*Lateolabrax maculatus*). The *tlr* genes of spotted sea bass were classified into five subfamilies (*tlr1*-subfamily, *tlr3*-subfamily, *tlr5*-subfamily, *tlr7*-subfamily, and *tlr11*-subfamily) according to the phylogenetic analysis, and their annotations were confirmed by a syntenic analysis. The protein domain analysis indicated that most *tlr* genes had the following three major TLR protein domains: a leucine-rich repeat (LRR) domain, a transmembrane region (TM) and a Toll/interleukin-1 receptor (TIR) domain. The *tlr* genes in spotted sea bass were distributed in 11 of 24 chromosomes. The mRNA expression levels of 16 *tlr* genes in response to *Vibrio harveyi* infection were quantified in the head kidney. Most genes were downregulated following *V. harveyi* infection, while only 5 *tlr* genes, including *tlr1-1*, *tlr1-2*, *tlr2-2*, *tlr5*, and *tlr7*, were significantly upregulated. Collectively, these results help elucidate the crucial roles of *tlr* genes in the immune response of spotted sea bass and may supply valuable genomic resources for future studies investigating fish disease management.

1. Introduction

The innate immune system is the first line of defense by which all multicellular animals protect themselves from invading microbial pathogens [1]. This efficient and complex system is based on a set of pattern-recognition receptors (PRRs) that induce the host defense system and recognize conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) [2]. PAMPs are essential for both viral transmission and host survival and typically represent the molecular characteristics of one or more pathogens. The recognition of PAMPs by PRRs initiates a strategy of the innate immune response to eliminate invading microorganisms [3–5]. Toll-like receptors (*TLRs*) are PRRs that can recognize PAMPs to defend against bacterial invasion. TLRs are transmembrane proteins that consist of an extracellular N-terminus with a leucine-rich repeat region (LRR) and an intracellular C-terminus with a Toll–interleukin (IL)-1 receptor (TIR) domain. The LRR domains recognize conserved PAMPs, and the TIR domain activates downstream signaling pathways [6].

To date, in total, 27 members of the *TLR* family have been investigated in vertebrates [7]. For example, *TLR1-10* has been identified in humans (*Homo sapiens*), while *Tlr1-9* and *Tlr11-13* have been found in mice (*Mus musculus*). *TLR1-7, TLR15*, and *TLR21* have been characterized in chickens (*Gallus gallus*). In teleosts, at least 21 *tlr*s, including *tlr1-4, tlr5M, tlr5S, tlr7-9,* and *tlr13-14,* and several "non-mammalian" *tlrs,* including *tlr18-26*, have been identified. Different teleost species harboring distinct nonmammalian *tlr* genes, such as *tlr 18–22,* have been reported in zebrafish (*Danio rerio*); *tlr21-23* have been identified in miiuy croaker (*Miichthys miiuy*); and *tlr18-26* have been characterized in channel catfish (*Ictalurus punctatus*). All *TLR* genes are classified into six major subfamilies defined as the *TLR1*-subfamily (sometimes called the *TLR2-subfamily*), *TLR3*-subfamily, *TLR4*-subfamily, *TLR5*-subfamily, *TLR7*-subfamily (also known as the *TLR9*-subfamily), and *TLR11*-subfamily [8–10]. In mammals, the *TLR1*-subfamily consists of *TLR1, TLR2, TLR6* and *TLR10*, while no *tlr6* or *tlr10* gene has been identified in any fish species. Recently, members of this subfamily, including *tlr1, tlr2, tlr14* (also known as *tlr18*), and *tlr25*, have been found in fish [11,12]. Several studies have reported that the *tlr1* gene responds to lipopolysaccharide (LPS) and bacterial infection in several fish species, including zebrafish (*D. rerio*) [13], orange-spotted grouper (*Epinephelus coioide*) [14], large yellow croaker (*Pseudosciaena*

* Corresponding author.
E-mail address: yunli0116@ouc.edu.cn (Y. Li).

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crocea) [15], and pufferfish (*Tetraodon nigroviridis*) [16]. Evidence has shown that *Tlr2* could form heterodimers with *tlr1* that are responsible for the recognition of bacterial LPS or the synthetic triacylated lipoprotein (Pam3CSK4) in zebrafish (*D. rerio*) [17], common carp (*C. carpio*) [18] and channel catfish (*I. punctatus*) [19]. The fish *TLR3*-subfamily includes only the *tlr3* gene, while the *TLR4*-subfamily contains only the *tlr4* gene [20]. The *tlr4* gene in zebrafish (*D. rerio*) [21], rohu (*Labeo rohita*) [22], rare minnow (*Gobiocypris rarus*) [23] and channel catfish (*I. punctatus*) [24] retains the functionality of LPS and Gram-negative bacterial responses. The *TLR5*-subfamily in higher vertebrates is generally composed of a single *TLR5* gene [25], whereas this subfamily harbors two gene copies in teleosts, such as rainbow trout (*Onchorhynchus mikiss*) [26], Atlantic salmon (*Salmo salar*) [27] and Japanese flounder (*Paralichthys olivaceus*) [28], including a soluble form of *TLR5* (*tlr5S*) and a membrane-bound version of *TLR5* (*tlr5M*) [29]. The members of the *TLR7* subfamily include *TLR7, TLR8* and *TLR9* [6]. Several studies have shown that CpG-containing oligodeoxynucleotides (CpG ODN) are the ligands recognized by *tlr9* in fish species [30]. Notably, the *TLR11*-subfamily exhibits considerable diversity among species. For example, there are three gene members, i.e., *Tlr11*, *Tlr12*, and *Tlr13,* in mice, while no functional *TLR11*-subfamily gene has been found in humans. Notably, the gene expansion of *tlr11* has been reported in teleost species, such as *tlr13, tlr21, tlr22,* and *tlr23* in miiuy croaker (*M. miiuy*) [31]; *tlr19, tlr20, tlr21* and *tlr22* in common carp (*Cyprinus carpio*) and zebrafish (*D. rerio*) [12]; *tlr19* and *tlr21* in spotted gar (*Lepisosteus oculatus*) [32]; and *tlr19, tlr20, tlr21, tlr22* and *tlr26* in channel catfish (*I. punctatus*) [24]. *Tlr21* in zebrafish (*D. rerio*) recognizes CpG-oligodeoxynucleotides (CpG-ODNs) [30], and *tlr22* in torafugu (*Takifugu rubripes*) recognizes dsRNA [33]. Upon interaction with ligands, the TLR family members directly recruit adaptor protein myeloid differentiation primary response 88 (MYD88), except for the *TLR3* subfamily, which has an adaptor protein known as the Toll-like receptor adaptor molecule (TICAM).

The spotted sea bass (*Lateolabrax maculatus*) is among the largest marine commercial fishes in China with an annual output exceeding 150 thousand tons. However, in recent years, bacterial infection caused by high-intensity aquaculture consequently resulted in serious economic losses [39]. Furthermore, the immune system and underlying molecular mechanisms of spotted sea bass have not been thoroughly elucidated to date. In this study, we identified and characterized 16 *tlr* genes in genomic and transcriptomic databases of spotted sea bass and detected their expression patterns in the head kidney after infection with the bacterial pathogen *Vibrio harvey*. *V. harveyi* is a luminescent Gram-negative bacterium that is ubiquitous in the marine environment and exists as a free-living and common pathogen to many marine organisms [34–36]. Our systematic study of the *tlr* gene family in spotted sea bass provides fundamental genomic resources for obtaining a better understanding of the innate immune mechanism of host defense against infection caused by bacteria.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201). The field studies did not involve endangered or protected species.

2.2. Identification of tlr genes in spotted sea bass

To identify the *tlr* genes in spotted sea bass, the transcriptome database (SRR4409341 and SRR4409397) [37] and the whole genome database (Assembly: GCA_004028665.1 ASM402866v1) of spotted sea bass were searched using teleosts, such as zebrafish (*D. rerio*), turbot (*Scophthalmus maximus)*, common carp (*C. carpio*), miiuy croaker (*M. miiuy*) and channel catfish (*I. punctatus*), chicken (*G. gallus*) and mammals, including humans (*H. sapiens*), mice (*M. musculus*) and cattle (*Bos taurus*), TLRs as queries. All amino acid sequences from the selected species were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) or Ensemble (http://www.ensembl.org). TBLASTN was used to identify candidate *tlr*-family members in spotted sea bass with a cutoff E-value of 1e-5. The open reading frames (ORF) of spotted sea bass *tlr*s were identified by ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and further verified by Smart-BLAST against the NCBI nonredundant (NR) protein sequence database. The molecular weight and isoelectric point (pI) of the predicted *tlr* genes were calculated by ProtParam (https://web.expasy.org/protparam/). The subcellular localization of the *tlr* genes was predicted by ProtComp 9.0 (http://www.softberry.com).

2.3. Phylogenetic and syntenic analysis of tlr genes in spotted sea bass

A phylogenetic tree was constructed based on the predicted amino acid sequences of the *tlr*s in spotted sea bass and TLRs in several representative vertebrates, including human (*H. sapiens*), mouse (*M. musculus*), zebrafish (*D. rerio*), orange-spotted grouper (*Epinephelus coioides*), miiuy croaker (*M. miiuy*), and common carp (*C. carpio*). All reference sequence identifiers of TLRs are shown in Table S1. Multiple sequences were aligned by the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) program in MEGA7.0.26 with the default parameters [38]. The phylogenetic tree was generated using a maximum likelihood [39] analysis in MEGA7.0.26. The initial tree used for the heuristic search was obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the WAG + G mode. A discrete Gamma distribution was used to model the evolutionary rate differences among the sites. All positions containing gaps and missing data were eliminated, and the bootstrapping value was set as 1,000 replications. The tree was generated with Interactive Tree Of Life (iTOL, http://itol.embl.de/).

The syntenic analysis was conducted by comparing genomic regions that harbor *tlr* genes in spotted sea bass with those in selected teleost fishes. The neighboring genes of *tlr*s in spotted sea bass were identified from the spotted sea bass genome assembly by the Fgenesh program and verified by BLAST against the NCBI nonredundant database. The NCBI genome database was used to determine the conserved syntenic pattern of *tlr* genes among various teleosts, including large yellow croaker (*L. crocea*), yellowtail kingfish (*Seriola lalandi lalandi*), sheepshead minnow (*Cyprinodon variegatus*), greater amberjack (*Seriola dumerili)*, barramundi perch (*L. calcarifer*), and climbing perch (*Anabas testudineus)*.

2.4. Copy number analysis of tlr genes in spotted sea bass

A comparative analysis of the copy number of *tlr* genes in several vertebrates, including human (*H. sapiens*), mouse (*M. musculus*), cattle (*B. taurus*), sheep (*Ovis aries*), chicken (*G. gallus*), turkey (*Meleagris gallopavo*), painted turtle (*Chrysemys picta*), Chinese soft-shelled turtle (*Pelodiscus sinensis*), tropical clawed frog (*Xenopus tropicalis*), zebrafish (*D. rerio*), medaka (*Oryzias latipes*), common carp (*C. carpio*), channel catfish (*I. punctatus*), torafugu (*T. rubripes*), large yellow croaker (*Larimichthys crocea*), barramundi perch (*Lates calcarifer*) and miiuy croaker (*M. miiuy*), was conducted with genome information published in the NCBI and Ensemble databases.

2.5. Gene structure construction and protein domain analysis of tlr genes in spotted sea bass

The exon-intron structures of the *tlr* genes were constructed using the alignment of the complementary DNA (cDNA) sequences with their consistent genomic DNA sequences and then corrected by sequences from the spotted sea bass transcriptome databases. The schematic

diagrams of the exon-intron structures of the *tlr* genes were generated by the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/).

The conserved protein domains were identified and annotated by the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/) with the default parameters, and the prediction was further confirmed by BLAST, LRRfinder 2.0f, TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP server 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The domain distributions of the *tlr* genes in spotted sea bass were visualized by Illustrator for Biological Sequences (IBS) 1.0.3.

2.6. Chromosomal locations of tlr genes in spotted sea bass

The chromosome distributions of the *tlr* genes were determined based on the annotation information in the spotted sea bass genome database. The distribution map of the *tlr* genes in spotted sea bass was generated with MapChart 2.32 software [40].

2.7. Bacterial challenge experiment and fish sampling

To characterize the innate immune response of the *tlr* genes against bacterial infection in the spotted sea bass, the Gram-negative bacteria *V. harveyi* was selected to conduct a challenge experiment. The challenge experiment was conducted by intraperitoneal injection. The bacteria were inoculated in LB broth and incubated in a shaker (180 rpm) at 28 °C overnight. The concentration of the bacteria was determined using a colony forming unit (CFU) per mL by plating 1 mL of 10-fold serial dilutions onto plates.

The fish used in this study were healthy spotted sea bass (with an average body weight of 180 ± 6.38 g) obtained from Dongying Shuangying Aquaculture Company (Shandong Province, China) and transported to the fish breeding physiology and seed engineering lab of Ocean University of China. The fish were acclimated for 7 days in the laboratory (temperature 23.0 °C ± 1 °C, pH 6.9 ± 0.4, abundant dissolved oxygen) and fed a commercial feed daily. The fish were challenged in six 30-L tanks with 5 control and 5 treatment groups per challenge. The tanks were randomly divided into five sampling time points, i.e., 0 h, 12 h, 24 h, 48 h and 72 h postinfection, in both the control and treatment groups. The treated group was intraperitoneally injected with 0.1 mL (3.0×10^6 CFU/mL) of *V. harveyi* per fish. The control group was injected with an equal amount of physiological saline. No fish died during the challenge experiment. Head kidney tissues were collected from 30 fish (3 replicates of 10 fish each) at each time point from both the control and treatment tanks after being euthanized with 150 mg/L tricaine methane sulfonate (MS-222, Geruien, China). All collected samples were immediately frozen in liquid nitrogen and stored at −80 °C for the subsequent RNA extraction.

2.8. RNA extraction and qRT-PCR analysis of tlr genes

The total RNA was extracted from the head kidney tissue using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions and digested with RNase-free DNase I (Takara, Otsu, Japan) to remove genomic DNA contamination. The quantification and purity were assessed using Biodropsis BD-1000 spectrophotometric absorbance (Beijing Oriental Science and Technology Development Ltd., Beijing, China). The integrity and relative quantity of the RNA was checked by 1.5% agarose gel electrophoresis (AGE). Equal amounts of RNA from the head-kidney tissues of 9 individual fish from 3 replicated tanks obtained at the same time points were pooled into one sample. After the extraction, the RNA was reverse transcribed into cDNA by using a PrimeScript™ RT reagent Kit (Takara, Otsu, Japan) following the manufacturer's instructions. All gene-specific primers used for the qRT-PCR analysis were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA), and the primers are listed in Table S2. Quantitative real-time RT-PCR (qRT-PCR) was conducted in a 96-well optical plate, and the reactions were performed on a StepOne Plus Real-Time PCR system (Applied Biosystems) using TaKaRa SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Code No. RR820B). The 20 μl qRT-PCR reaction mixture consisted of 2 μl of template cDNA, 0.8 μl of each primer (10 μM), 10 μl of SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (2X), 0.4 μl of ROX Reference Dye (50x) and 6.0 μl of nuclease-free water. The terminal cycling qRT-PCR conditions were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The expression level was normalized against the spotted sea bass *18s ribosomal RNA* (*18s*) gene [41]. The expression levels of each gene were calculated using the $2^{-\Delta\Delta CT}$ method, and the correlation coefficient between the gene expression in the control group and that in the treatment group was determined by SPSS13.0. A one-way ANOVA followed by Duncan's multiple range tests was applied, and the differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Identification and characterization of tlr genes in spotted sea bass

In this study, we identified 16 *tlr* genes in the transcriptomic database and genomic database of spotted sea bass, including *tlr1-1, tlr1-2, tlr2-1, tlr2-2, tlr3, tlr5, tlr7, tlr8, tlr9, tlr13-1, tlr13-2, tlr13-3, tlr14, tlr21, tlr22* and *tlr23*. All sequence information of the identified *tlr* genes was deposited in the NCBI database under accession numbers MK273045-MK273060 (Table 1). The detailed information of each *tlr* gene in

Table 1
Characteristics of 16 *tlr* genes identified in spotted sea bass.

Gene name	Gene ID	mRNA (bp)	ORF (bp)	Number of amino acids	MW (kDa)	pI	Subcellular location
tlr1-1	MK273045	3311	2406	801	90.35	6.65	Plasma membrane
tlr1-2	MK273046	3542	2352	827	93.55	6.56	Plasma membrane
tlr2-1	MK273047	3042	2490	829	93.64	5.96	Plasma membrane
tlr2-2	MK273048	2151	2151	716	81.52	6.66	Plasma membrane
tlr3	MK273049	3366	2769	922	103.82	8.73	Membrane bound mitochondrial
tlr5	MK273050	3677	2442	813	92.40	6.14	Plasma membrane
tlr7	MK273051	4390	3162	1053	121.17	8.60	Plasma membrane
tlr8	MK273052	4024	3072	1023	116.71	6.88	Plasma membrane
tlr9	MK273053	3843	3183	1060	122.33	8.18	Plasma membrane
tlr13-1	MK273054	2419	1986	661	75.52	8.97	Membrane bound peroxisomal
tlr13-2	MK273055	2556	2556	855	96.38	6.24	Plasma membrane
tlr13-3	MK273056	2310	2310	769	87.65	5.87	Membrane-bound extracellular
tlr14	MK273060	3738	2634	877	101.18	7.96	Membrane-bound vacuolar
tlr21	MK273058	5013	2865	954	110.28	9.12	Plasma membrane
tlr22	MK273059	7144	2880	959	110.29	8.82	Plasma membrane
Tlr23	MK273057	2556	2556	855	85.40	6.30	Membrane bound mitochondrial

spotted sea bass is presented in Table 1. The mRNA lengths of the 16 *tlr* genes ranged from 2,151 bp (*tlr2-2*) to 7,144 bp (*tlr22*) with ORFs ranging from 1,986 bp (*tlr13-1*) to 3,183 bp (*tlr9*) and encoding proteins of 661 aa (*tlr13-1*) to 1,060 aa (*tlr9*) in length (Table 1). The results also showed that the maximum MW was 75.52 kDa (*tlr13-1*), the minimum MW was 122.33 kDa (*tlr9*), and the pI varied from 5.87 (*tlr13-3*) to 9.12 (*tlr21*) (Table 1). The predicted subcellular location suggested that most deduced TLR proteins in spotted sea bass, including *tlr1-1, tlr1-2, tlr2-1, tlr2-2, tlr5, tlr7, tlr8, tlr9, tlr13-2, tlr21,* and *tlr22,* were targeted to the plasma membrane. The other TLR proteins were located in membrane-bound organelles; for example, *tlr3* and *tlr23* were targeted to membrane-bound extracellular mitochondria, *tlr13-3* was targeted to membrane-bound extracellular mitochondria, *tlr13-1* was targeted to membrane-bound peroxisomes, and *tlr14* was targeted to membrane-bound vacuoles (Table 1).

3.2. Phylogenetic and syntenic analysis of tlr genes

To properly annotate the *tlr* genes in spotted sea bass, a phylogenetic tree was constructed by multiple sequence alignment of full-length amino acid sequences of spotted sea bass and five selected teleost species (Fig. 1). All 16 *tlr* genes were clustered into five subfamilies and named the *tlr1*-subfamily, *tlr3*-subfamily, *tlr5*-subfamily, *tlr7*-subfamily and *tlr11*-subfamily according to the nomenclature used in several teleosts, such as channel catfish (*I. punctatus*) [24], blunt snout bream (*Megalobrama amblycephala*) [42], common carp (*C. carpio*) [12], and Tibetan schizothoracine fish (*Gymnocypris eckloni*) [43]. In spotted sea bass, the members of the *tlr1*-subfamily included *tlr1-1*, *tlr1-2*, *tlr2-1*, *tlr2-2* and *tlr14*. Compared with the mammalian *TLR1*-subfamily, *tlr6* and *tlr10* were missing, but *tlr14* was detected in spotted sea bass. Such gene gain-or-loss is not unique to spotted sea bass, and there have been many similar reports in previous studies; for example, *tlr14* has been discovered in lamprey (*Lampetra japonica*), fugu (*T. rubripes*), and Japanese flounder (*P. olivaceus*). The *Tlr3*-subfamily and *tlr5*-subfamily included only one gene member each, namely, *tlr3* and *tlr5,* respectively. The *Tlr7*-subfamily comprised the *tlr7, tlr8* and *tlr9* genes. The *Tlr11*-subfamily had the largest number of *tlr* genes, including *tlr13-1, tlr13-2, tlr13-3, tlr21, tlr22* and *tlr23.* This analysis failed to detect the *tlr4*-subfamily in spotted sea bass and several fish species, such as three-spined stickleback (*Gasterosteus aculeatus*) [44] and torafugu (*T. rubripes*) [45]. The *Tlr13* gene had multiple copies and could not be accurately annotated based on the phylogenetic tree analysis; therefore, to further confirm the name of *tlr13,* the conservation of genes surrounding the *tlr13* gene was used for a comparative genomic locational distribution analysis. Based on their identity to orthologs of selected teleost fishes, we annotated the spotted sea bass paralogs as "*tlr13-1*", "*tlr13-2*", and "*tlr13-3*" following the nomenclature of zebrafish. The spotted sea bass, yellowtail kingfish (*S. lalandi lalandi*), greater amberjack *(S. dumerili),* and large yellow croaker (*L. crocea*) harbored several highly conserved genes surrounding *tlr13-1,* such as *psbp2, psmb4, rfx5* and *pi4kb* (Fig. 2A). Conserved synteny was found among spotted sea bass, large yellow croaker (*L. crocea*), yellowtail kingfish (*S. lalandi lalandi*), and sheepshead minnow (*C. variegatus*) in *tlr13-2*, which contains several highly conserved genes, including *nog2, ntn3, mgat5b, tbc1d24, atp6voc, prr35* and *metm* (Fig. 2B). Similar neighboring genes were found among spotted sea bass, yellowtail kingfish (*Seriola lalandi lalandi*), greater amberjack *(Seriola dumerili)* and climbing perch *(Anabas testudineus)* in *tlr13-3,* including *rnfl150, ppm1k, abcg2, cdkl2* and *anp32b* (Fig. 2c). In summary, the syntenic analysis provided sufficient evidence for the annotation and nomenclature of the three copies of the *tlr13* genes in spotted sea bass.

3.3. Gene copy number analysis of tlr genes

The copy numbers of the *tlr*-family genes varied among spotted sea bass and several other vertebrates. Overall, 16 *tlr* genes were identified in spotted sea bass, while 10 *tlr* genes were identified in human (*H. sapiens*), cattle (*B. taurus*), sheep (*O. aries*) and turkey (*M. gallopavo*); 11 *tlr* genes were identified in chicken (*G. gallus*); 12 *tlr* genes were identified in mouse (*M. musculus*) and torafugu (*T. rubripes*); 13 *tlr* genes were identified in medaka (*O. latipes*); 14 *tlr* genes were identified in miiuy croaker (*M. miiuy*); 15 *tlr* genes were identified in large yellow croaker (*L. crocea*); 16 *tlr* genes were identified in painted turtle (*C. picta*); 17 *tlr* genes were identified in Chinese soft-shelled turtle (*P. sinensis*) and barramundi perch (*L. calcarifer*); 18 *tlr* genes were identified in zebrafish (*D. rerio*); 19 *tlr* genes were identified in channel catfish (*I. punctatus*); 22 *tlr* genes were identified in tropical clawed frog (*X. tropicalis*); and 27 *tlr* genes were identified in common carp (*C. carpio*) (Fig. 3). A comparison of the *tlr*-family genes across several vertebrate species shows that the numbers of *tlr*-family genes exhibited considerable diversity in fish, which is likely due to genome duplication events or environmental adaptation [46]. The investigated results indicated that *TLR1-9* genes were generally conserved among many species; however, some differences existed (Fig. 3). Specifically, these genes had a single copy in all tested mammals, including human (*H. sapiens*), mouse (*M. musculus*), cattle (*B. taurus*) and sheep (*O. aries*), while these genes had 0-4 copies in other selected species. Of these species, mammalian *tlr4* homologs were detected only in three reported fish, i.e., zebrafish (*D. rerio*), common carp (*C. carpio*) and channel catfish (*I. punctatus*). The reason for the loss of the *tlr4* gene in some fish species might be the lack of costimulatory molecules essential for LPS activation via *tlr4* [47]. In teleost fish, the *tlr6* and *tlr10* genes were not found in the *tlr1*-subfamily, but some nonmammalian genes, including *tlr14* and *tlr18*, were identified in this subfamily. Therefore, teleost fish *tlr14* and *tlr18* are speculated to be functional substitutes for mammalian *TLR6* and *TLR10* [46,48]. Some other nonmammalian genes, including *tlr19-26,* were classified into the *tlr11*-subfamily according to the results of the phylogenetic analyses, which indicated that these genes appear on the same branch as murine *Tlr11*, *Tlr12* and *Tlr13* (Fig. 1). For example, in Tibet fish (*G. przewalskii*), *tlr19-22* were grouped with their corresponding homologs to form the *tlr11*-subfamily [49]. In channel catfish (*I. punctatus*), the *tlr11*-subfamily comprises *tlr19-21* and *tlr26* [8]. In Atlantic salmon (*S. salar*), the *tlr19-22* molecules constitute the *tlr11*-subfamily [50]. In this study, we identified *tlr13, tlr21, tlr22* and *tlr23* in spotted sea bass as belonging to the *tlr11*-subfamily, which is consistent with miiuy croaker (*M. miiuy*) and puffer fish (*T. rubripes*) (Fig. 1).

3.4. Intron-exon organization and chromosomal location analysis of tlr genes in spotted sea bass

The intron-exon organizations, lengths and numbers have profound consequences on the origin and evolution of the genes in eukaryotic organisms [51–53]. To further investigate the structural diversity of the *tlr* genes, the intron-exon organizations were compared. The exon-intron structure analysis showed that the number of exons varied from 1 (*tlr1-1*, *tlr1-2*, *tlr7*, *tlr8*, *tlr9*, and *tlr21*) to 11 (*tlr2-1*), while 6 genes (*tlr1-1*, *tlr1-2*, *tlr7*, *tlr8*, *tlr9* and *tlr21*) were intronless (Fig. 4). In the *tlr1*-subfamily, both copies of the *tlr1* gene were composed of a single exon and had no intron, and the same situation was found in torafugu (*T. rubripes*) and spotted green pufferfish (*Tetraodon nigroviridis*) [54]. The *Tlr2-1* gene had the largest number of introns/exons in *tlr*-family genes with 11 exons and 10 introns. This exon/intron organization of *tlr2* has also been reported in amazon molly (*Poecilia formosa*), stickleback (*G. aculeatus*), miiuy croaker (*M. miiuy*) and Japanese flounder (*Paralichthys olivaceus*) [55]. Previous reports have hypothesized that a large number of introns were acquired in fish *tlr2* genes after the divergence of vertebrates [45]. In contrast, another gene copy of *tlr2*, i.e., *tlr2-2,* only had 2 exons and 1 intron in spotted sea bass, which was identical to the findings observed in gibel carp (*Carassius auratus gibelio*), while there was only one exon in *tlr2* genes in channel catfish (*I. punctatus*) [19] and zebrafish (*D. rerio*) [13]. It has recently been speculated that an intron

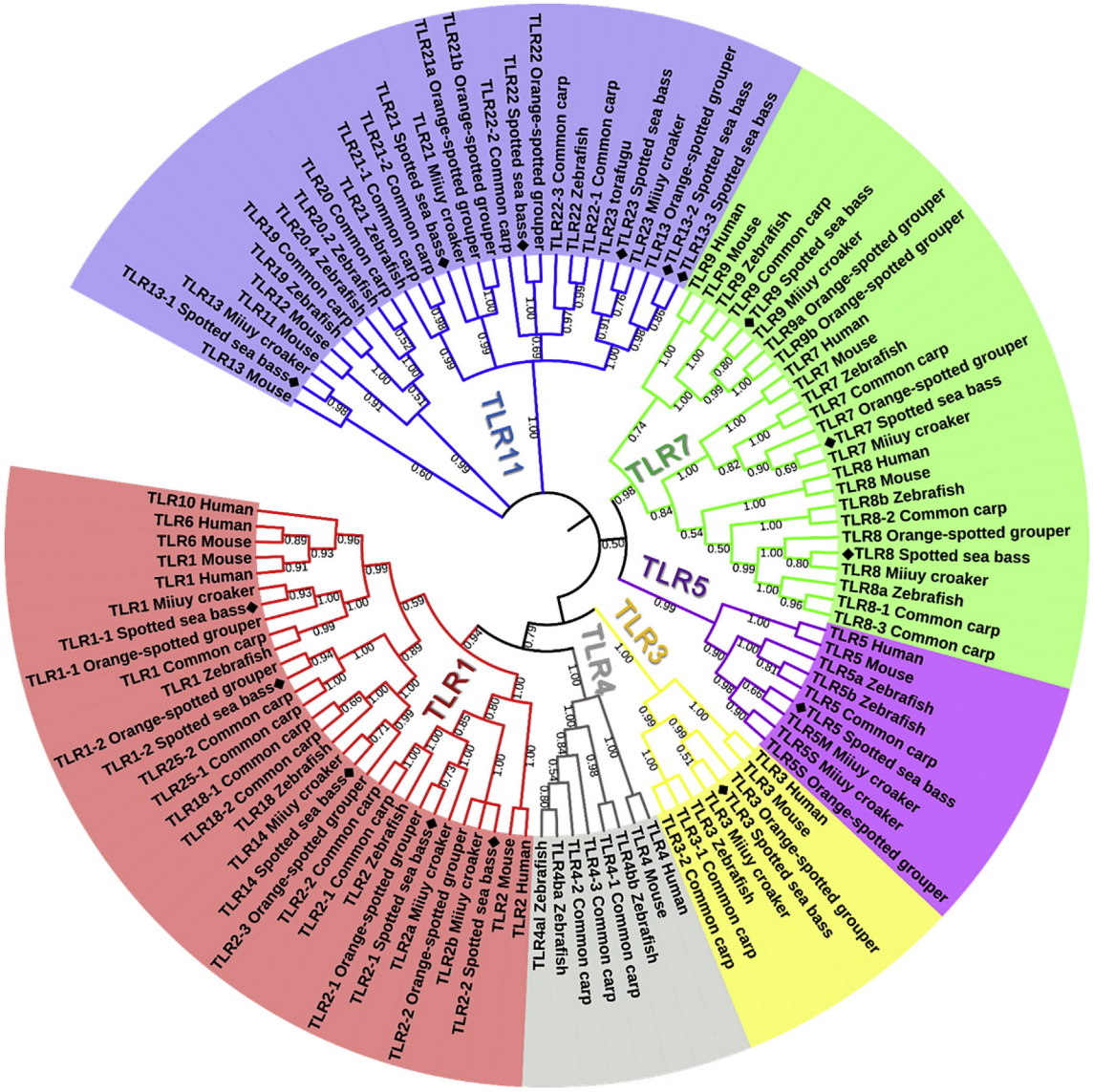

Fig. 1. Phylogenetic tree of the *tlr* gene family. *TLR* gene family genes are divided into the following five subfamilies: *TLR1*-subfamily, *TLR3*-subfamily, *TLR5*-subfamily, *TLR7*-subfamily and *TLR11*-subfamily. The different subfamily genes are denoted by various colors, and the names are annotated in the clade. The black rhombus indicates spotted sea bass genes. The GenBank accession numbers of the sequences are available in Supplemental Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

insertion event likely occurred in exon 1 of miiuy croaker (*M. miiuy*) *tlr2* after its divergence from the ancestor of zebrafish (*D. rerio*) [56]. The *Tlr14* gene in the *tlr1*-subfamily is a nonmammalian gene that might be a functional substitute for mammalian *TLR6* and *TLR10* to generate an immune response against a wide variety of pathogens in the water [57]. The *Tlr14* gene in spotted sea bass comprised 4 exons and 3 introns, which is consistent with the gene structure of Atlantic salmon (*Salmo salar*) [50]. The single-member *tlr-3* gene in the *tlr3*-subfamily contained 5 exons and 4 introns, which is consistent with previous results suggesting that the grass carp *tlr3* gene also has 5 exons and 4 introns [23]. The *Tlr5* gene had 4 exons and 3 introns. The *Tlr7*-subfamily members (*tlr7, tlr8* and *tlr9*) shared a similar exon-intron pattern containing only 1 exon, which is identical to channel catfish (*I. punctatus*) in which *tlr7*-subfamily genes were all intronless [8]. The intron-exon structures of the *Tlr11*-subfamily genes in spotted sea bass were diverse, and no fixed rule was observed; for example, there are 4 exons in *tlr13-1* and *tlr13-2*; 2 exons in *tlr13-3*, *tlr22* and *tlr23;* and 1 exon in *tlr21*. In addition, some genes, including *tlr21, tlr22* and *tlr23,* are teleost-specific genes that are poorly characterized to date.

Sixteen *tlr* genes were distributed among 11 of 24 chromosomes in the spotted sea bass genome (Fig. 5). *Tlr7* and *tlr8* were tandem duplicates located on the same chromosome. A similar genomic organization was also observed in humans (*H. sapiens*), zebrafish (*D. rerio*), rainbow trout (*O. mykiss*) and Japanese flounder (*P. olivaceus*) in which *tlr7* and *tlr8* were located adjacent to each other on the chromosome by tandem duplication [58–60].

3.5. Protein domain analysis of tlr genes in spotted sea bass

Several conserved functional domains were detected in the *tlrs,* including LRR domains, the TIR domain, and the transmembrane region (TM). The *TLR* genes were type I integral membrane glycoproteins characterized by extracellular domains containing varying numbers of LRR domains, a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor, which is known as the TIR domain, and a short trans-membrane region [61]. The LRR domain is involved in pathogen recognition, and the sequence variation determines the specificity of the *TLR* genes [62]. The functional domain analysis of the *tlr*

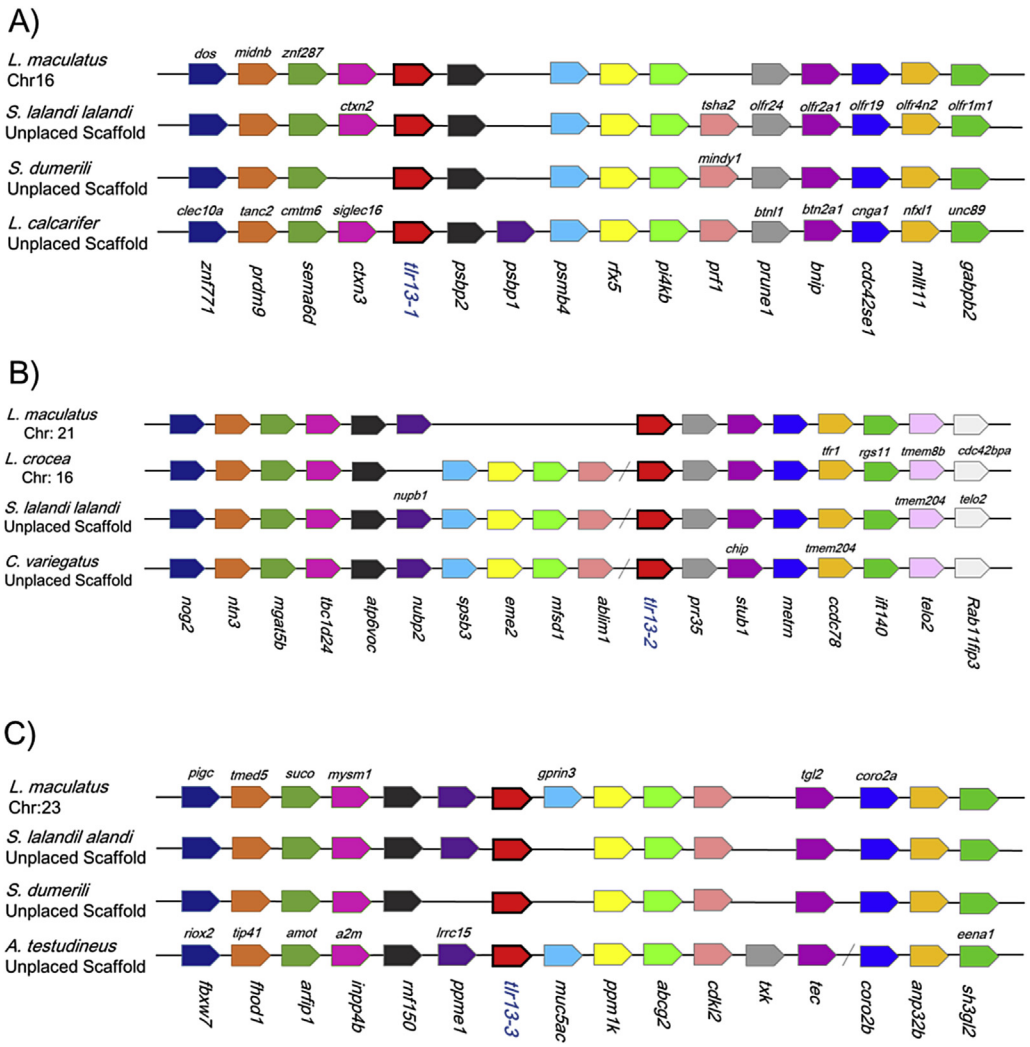

Fig. 2. Syntenic analysis of *tlr* genes in selected vertebrates. These syntenies were generated based on information obtained from the NCBI genome database. The same gene names in each selected species are displayed at the bottom of the picture, and the different gene names are marked above the pentagon. (A) *tlr13-1*, (B) *tlr13-2*, (C) *tlr13-3*. The full gene names and gene IDs are provided in Supplemental Table S2.

	Mammals				Birds		Reptiles		Amphibians	Teleosts								
	H.sapiens	M.musculus	B.taurus	O.aries	G.gallus	M.gallopavo	C.Picta	P.sinensis	X.tropicalis	D.rerio	O.latipes	C.carpio	I.punctatus	T.rubripes	L.crocea	L.calcarifer	M.miluy	L.maculatus
tlr1	1	1	1	1	2	2	1	2	2	1	2	1	1	1	1	1	1	2
tlr2	1	1	1	1	2	2	2	2	3	1	2	2	1	1	3	2	2	2
tlr3	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
tlr4	1	1	1	1	1	1	1	1	1	3	0	4	2	0	0	0	0	0
tlr5	1	1	1	1	1	1	2	2	2	2	2	1	3	2	2	2	2	1
tlr6	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0
tlr7	1	1	1	1	1	1	2	1	1	1	2	2	1	1	2	1	1	1
tlr8	1	1	1	1	0	0	2	3	2	2	1	3	2	1	1	1	1	1
tlr9	1	1	1	1	0	0	2	1	2	1	1	1	1	1	0	0	1	1
tlr10	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
tlr11	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tlr12	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
tlr13	0	1	0	0	0	1	2	3	1	0	0	0	0	0	5	9	1	3
tlr14	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	1
tlr15	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
tlr18	0	0	0	0	0	0	0	0	0	1	1	2	1	1	0	0	0	0
tlr19	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
tlr20	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	0	0
tlr21	0	0	0	0	1	0	0	0	2	1	1	2	1	1	0	0	1	1
tlr22	0	0	0	0	0	0	0	0	1	1	0	3	1	1	0	0	1	1
tlr23	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1
tlr25	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0
tlr26	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Total	10	12	10	10	11	10	16	17	22	18	13	27	19	12	15	17	14	16

Fig. 3. Copy numbers of *TLR* genes in spotted sea bass and several representative vertebrates. The total gene numbers are marked in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

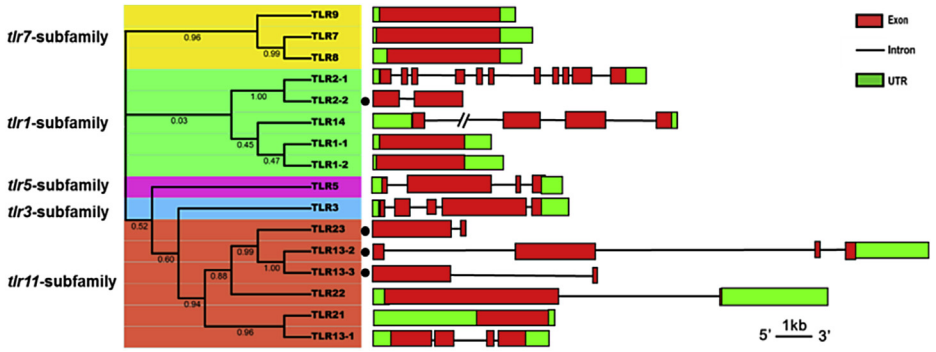

Fig. 4. Exon-intron patterns in *tlr* genes in spotted sea bass. An unrooted tree was constructed based on the amino acid sequences of the *tlr* genes in spotted sea bass using the method of maximum likelihood under the LG + G + F model with 1,000 bootstraps by MEGA7 software. The five subfamilies are indicated by different colors. The black spot represents the partial sequence of *tlr* genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

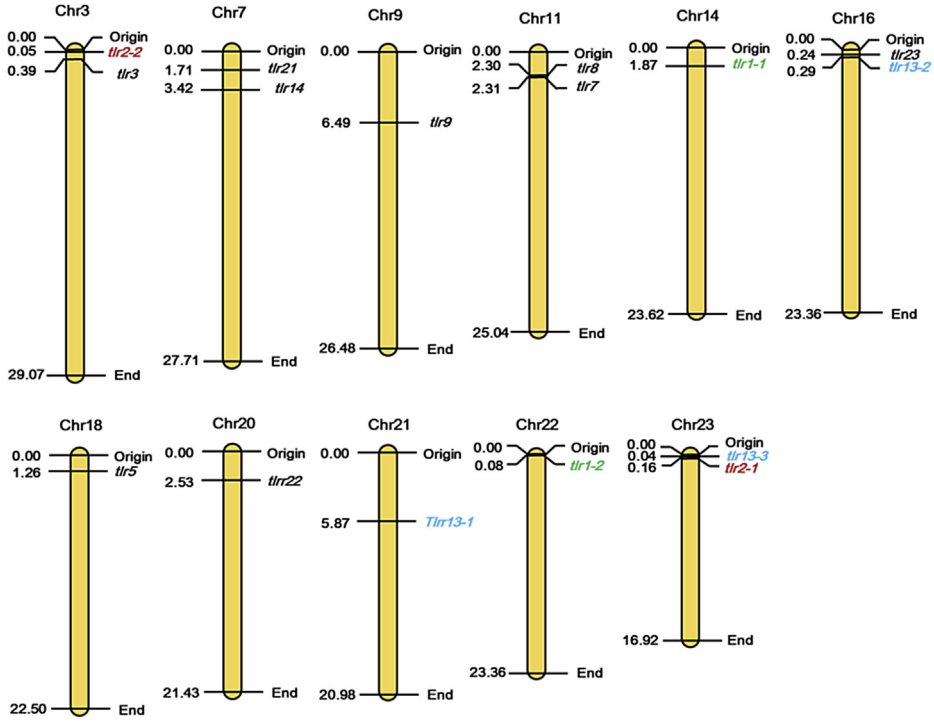

Fig. 5. Chromosomal locations of the *tlr* genes in spotted sea bass. The size of a chromosome is indicated by its relative length. Gene names with the same color indicate paralogs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes in spotted sea bass was predicted by SMART based on their deduced protein sequences. As shown in Fig. 6, the *tlr* genes in spotted sea bass contain various numbers of LRR domains in different genes, ranging from 6 (*tlr13-1*) to 20 LRRs (*tlr21*). Most genes harbor one LRR C-terminal domain (LRR_CT in Fig. 6), except for *tlr13-2*, *tlr13-3* and *tlr23,* which lack this domain. Only a few genes have the LRR N-terminal domain (LRR_NT in Fig. 6), including *tlr3*, *tlr7*, *tlr13-2* and *tlr13-3*. The number of LRR domains in the *TLR* genes differed between teleosts and mammals; for example, in our study, the *tlr* genes in spotted sea bass possessed 6–20 LRRs (with LRR_CT and LRR_NT), which is far less than human (*H. sapiens*) *tlr* genes, which contain 19-25 LRRs [63], but similar to yellow catfish (*Pelteobagrus fulvidraco*) *tlr* genes, which comprise 7–19 LRRs [7]. Except for two copies of *tlr13* (*tlr13-2* and *tlr13-3*), most *tlr* genes in spotted sea bass contained one TIR domain, followed by a single TM region (Fig. 6). A similar gene structure was found in several teleost species, such as zebrafish (*D. rerio*) [64], orange-spotted grouper [11], and common carp (*C. carpio*) [12].

3.6. Expression analysis of spotted sea bass tlr genes after bacterial infection

The head kidney is one of the most important organs in the innate immune system of fish [65–67]. In addition, it has been reported that the expression of *tlrs* have been significantly induced or repressed in head kidney after bacterial infection in several bony fishes [14,15,68,69]. To investigate the potential involvement of *tlr* genes in spotted sea bass in response to bacterial infection, the mRNA expression levels of these *tlr* genes were quantified in the head kidney after a challenge with *V. harveyi*. The expression of all 16 *tlr* genes in spotted sea bass appeared to be affected by the *V. harveyi* infection, while most genes revealed a significant variation immediately at 12 h, whereas *tlr9* significantly changed until 24 h (Fig. 7). Most genes, including *tlr2-1, tlr3, tlr8, tlr9, tlr13-1, tlr13-2, tlr13-3, tlr21, tlr22* and *tlr23*, were downregulated following the *V. harveyi* infection, while only 5 *tlr* genes, including *tlr1-1, tlr1-2, tlr2-2, tlr5*, and *tlr7,* were upregulated (Fig. 7). These *tlr* genes that were upregulated after the bacterial infection were considered important for the recognition of bacterial ligands. A similar expression pattern was found in *tlr1-1, tlr5* and *tlr7* in which the mRNA levels were significantly upregulated (log2FC 1.02-, 0.71- and 3.21-fold increments, respectively) at 12 h ($P < 0.05$) and then returned to the normal level at 24 h postinfection. Among these genes, *tlr7* was the most highly induced gene, and its expression was upregulated by log2FC 3.21-fold. Similarly, in the kidney of tongue sole (*Cynoglossus semilaevis*), the *tlr7* gene was significantly upregulated at 6 h, 12 h, and 24 h with the highest level of induction (180-fold) occurring at 6 h after

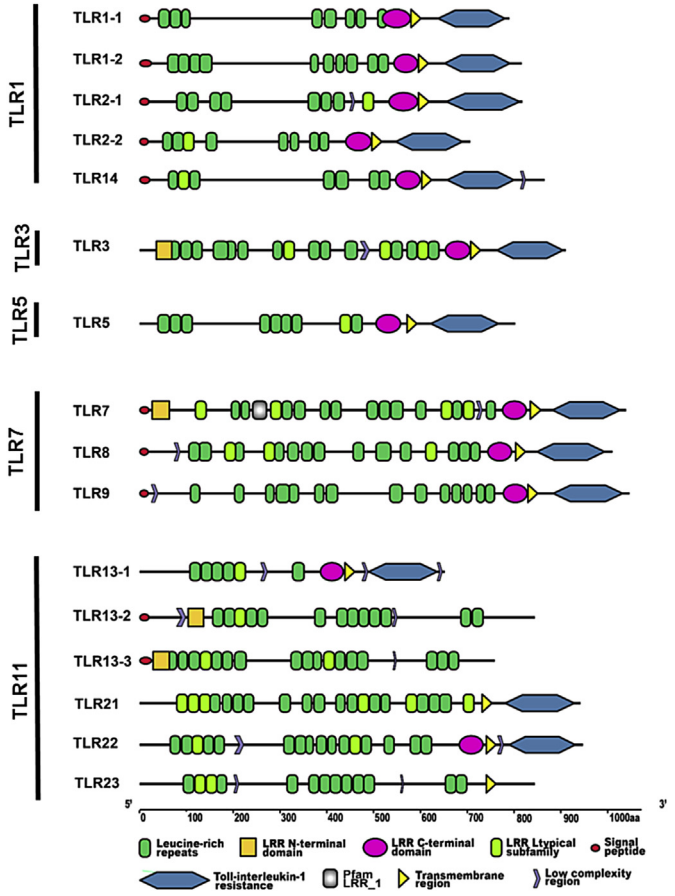

Fig. 6. Schematic representation of the domain architecture of *tlr*s in spotted sea bass. Different colors and shapes indicate the different domains, and the details are shown at the bottom of the chart. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Pseudomonas fluorescence infection [70]. The highly induced expression of *tlr7* in response to bacterial infection indicates that it may play an important immune function in fish. According to our results, the expression of *tlr1-1* was upregulated initially at 12 h (log2FC 1.02-fold. $P < 0.05$) and then dramatically decreased after 24 h (log2FC −1.1-fold, $P < 0.05$), and its expression level at 48–72 h was significantly lower than that at 0 h (log2FC −2.85-fold, −2.00-fold $P < 0.05$, respectively) (Fig. 7). Previous studies have shown that *tlr1* in fish is involved in the inflammatory response to Gram-negative bacteria infection [7]. For example, in orange spotted grouper (*E. coioides*) infected with *Vibrio alginolyticus*, the mRNA expression of *tlr1* was upregulated from 3 days to 7 days, and its level increased from 1.59 to 2.57-folds in the head kidney [14]. In large yellow croaker (*P. crocea*), the mRNA expression of *tlr1* obviously increased at 24 and 48 h (1.68- and 2.13-fold increases, respectively) after LPS induction in head kidney cells [15]. This finding may partially explain the similar immune mechanisms of the *tlr1* gene among different teleost fish. Notably, *tlr2-2* was always drastically induced throughout the entire infection challenge and was upregulated by log2FC 2.87- to 3.06-fold after infection. The *tlr2-2* gene expression patterns in spotted sea bass were consistent with those in large yellow croaker (*L. crocea*), showing that the expression level of *TLR2b* is significantly upregulated in head kidneys infected with *Vibrio parahaemolyticus*, LPS and polyI:C [71,72]. However, *tlr2-1* in spotted sea bass exhibited a distinct expression pattern that was dramatically downregulated at 12 h (log2FC −2.00-fold), and no expression was detected from 48 h to 72 h. These results suggest that two gene copies of *tlr2* might play distinct functions in antibacterial immunity in spotted sea bass. In the present study, 11 *tlr* genes were downregulated at 12 h ranging from log2FC −0.03-fold to −9.55-fold; among these genes, *tlr8, tlr13-2, tlr13-3* and *tlr23* were observed as the most downregulated genes, and these genes were rapidly downregulated with log2FC −0.44-fold, −3.63-fold, and −2.41-fold at 12 h after infection, reaching very low expression values (log2FC −6.12-fold, −9.55-fold, −9.31-fold and −8.25-fold, respectively) at 72 h after infection (Fig. 7). Consistent with this result, in channel catfish (*I. punctatus*), nine *tlr* genes (*tlr3, tlr4, tlr18, tlr19, tlr20-1, tlr21, tlr22, tlr25,* and *tlr26*) were observed to be significantly downregulated in the head kidney within 6 days after *Edwardsiella ictaluri* infection. The two *tlr* genes *tlr20-1* and *tlr21* were the most drastically downregulated genes, indicating that these genes might be the most responsive to bacterial infection as the subpopulation of phagocytes expressing these genes could rapidly migrate out of the head kidney to the infection sites [24]. The downregulation of the *tlr* genes in the head kidney after bacterial infection has also been reported in blue catfish (*Ictalurus furcatus*) [73]

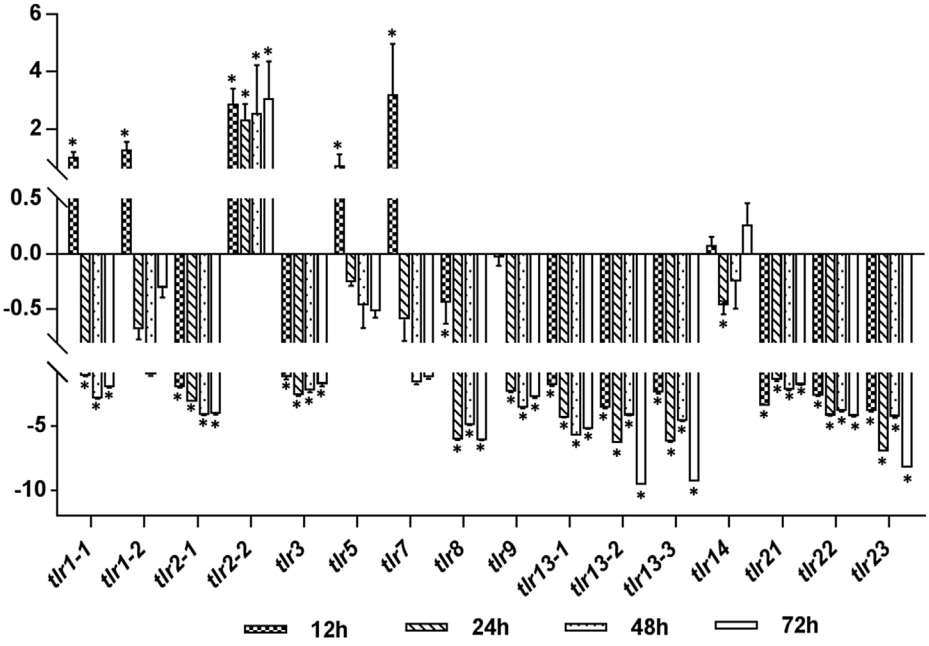

Fig. 7. Expression of *tlr* genes in spotted sea bass in the head kidney following *V. harveyi* infection. Gene expression patterns are presented as fold change relative to the control samples (0 h). Y-axis indicates the mRNA relative expression after logarithm 2 based transformation. Significant differences in the mRNA expression of each gene among the controls and various treatment time points are indicated with an * ($P < 0.05$).

and Indian major carp (*Cirrhinus mrigala*). However, the mechanisms of this downregulation of *tlr* genes in fish are still unclear [74]. While it is difficult to speculate regarding the functional details based on expression levels, it is apparent that these *tlr* genes are involved in immune responses to varying degrees after bacterial infection.

In summary, this study provides comprehensive information about the sequence characteristics, phylogenetic relationships, and chromosome distribution of *tlr* genes in spotted sea bass. The mRNA expression levels of 16 *tlr* genes were quantified in the head kidney in response to *V. harveyi* infection. The present study showed that the expression levels of five *tlr* genes, including *tlr1-1*, *tlr1-2*, *tlr2-2*, *tlr5*, and *tlr7,* were highly upregulated by infection with *V. harvey*, while most of the rest *tlr* genes were significantly downregulated. These findings suggest that these *tlr*s may play crucial roles in the immune response against *V. harveyi* infection in spotted sea bass.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.010.

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