Research Article

Molecular Characterization and Evolution Analysis of Two Forms of TLR5 and TLR13 Genes Base on Larimichthys crocea Genome Data

Lihua Jiang,[1,2] Liyi Pei,[1,2] Ping Wang,[1,2] Liqin Liu,[1,2] Gong Li,[1,2] Binjian Liu,[1,2] Zhenming Li,[1,2] Tabata Hiromasa,[1,2] Hao Pan,[1,2] and Atsushi Ogura[1,2]

[1]National Engineering Research Center for Facilitated Marine Aquaculture, Zhejiang Ocean University, No. 1, South Haida Road, Dinghai District, Zhoushan, China
[2]Nagahama Institute of Bioscience and Technology, Tamura, Nagahama, Shiga, Japan

Correspondence should be addressed to Atsushi Ogura; aogu@whelix.info

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TLRs (Toll-like receptors) are essential in host defense against pathogens. There are two types of TLR5, namely, membrane form of TLR5 (TLR5M) and soluble form of TLR5 (TLR5S), both of which perform a crucial role in flagellin response. TLR13 is a TLR that localizes to endosomes and recognizes nucleic acids released by internal microorganisms, including viruses, bacteria, and fungi. Here, the full-length coding sequence (CDS), protein structure, and immune response and subcellular localization of TLR5 (TLR5S) and TLR13 were characterized in large yellow croaker (Larimichthys crocea). These TLRs share high sequence homology with other ichthyic TLRs, while also having their own characters; qPCR was determined and the results found that the three genes were constitutively expressed in all examined tissues: TLR5M was highly expressed in the spleen and liver; TLR13 expression was high in the kidney, liver, and spleen. And TLRs were upregulated following stimulation with Vibrio parahaemolyticus in the liver, spleen, and kidney. Immuno fluorescence staining revealed that TLR5M were localized in the cytoplasm, while TLR5S and TLR13 were in the endosome. The evolutionary analysis has shown that TLR13 was clustered with TLR11, 19, 20, 21, and 22, while TLR5 and TLR3 were classified into a group; these results suggest that TLRs are vital in the defense of L. crocea against bacterial infection and further increase our understanding of TLR function in innate immunity in teleosts.

1. Introduction

TLRs (Toll-like receptors) were first discovered in Drosophila embryos and control the formation of the dorsal-ventral polarity of early embryos [1]. Further studies have shown that TLRs are involved in the synthesis of antimicrobial peptides and are capable of antifungal infections [2]. In 1997, the Toll ortholog of Drosophila melanogaster was identified in humans and proved to be essential for the activation of the innate or adaptive immunity [3, 4].

TLRs are type I transmembrane proteins composed of extracellular, transmembrane, and intracellular signal domains: the extracellular domain has a repetitive leucine-rich repeat (LRR); the ligand-induced dimerization of TLR triggers the recruitment of adaptor proteins to the intracellular Toll/IL-1 receptor homology domain (TIR), thereby initiating signal transduction [5]. The extracellular domain of the TLR protein usually contains 16-28 LRRs, which are responsible for binding PAMPs and involve a variety of physiological functions including immune response, signal transduction, cell cycle regulation, and enzyme regulation [6]. A single LRR module typically has 20-30 amino acids in length and consists of a variable portion and a conserved “LxxLxLxxN” motif that allows the extracellular domain to form a special horseshoe [5, 7, 8]. The LRRNT and LRRCT modules in the N-terminus and C-terminus of the LRR domain do not function as LRR, but their cysteines are aggregated to form disulfide bonds and protect the hydrophobic
core of the protein from exposure to the solvent, stabilizing the protein structure [7].

The first fish TLR gene was discovered in goldfish in 2002, and several TLRs were retrieved from the genomic data of pufferfish and zebrafish, respectively, in 2004 [9]. So far, more than 20 TLRs have been identified from more than ten species of teleost fish, namely, TLR1, 2, 3, 4, 5M, 5S, 6, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27 [10, 11]. Generally, there are no TLR6 and TLR10 in 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27 which are mutual between humans and ten species of teleost fish. More than 20 TLRs have been identified from more than 2002, and several TLRs were retrieved from the genomic data of human PAMPs; not only respond to virus invasion but also recognize bacterial ligands and functions in fish and mammals. For example, human TLR3 can only recognize viruses, while fish TLR3 can not only respond to virus invasion but also recognize bacterial PAMPs; fish TLR4 may be an inhibitor of the MYD88-dependent signaling pathway [13, 14]. Human TLRs can be broadly divided into two major subgroups: TLRs 1, 2, 4, 5, 6, and 10 located on the cell surface identify lipoproteins, lipopolysaccharides, or flagellin on the surface of microorganisms, while TLR3, 7, 8, and 9 locate on the endosomes or lysosomes and recognize the nucleic acid sequence of the microorganism [15]. In addition to the cross-model TLR5, fish TLR5 also has soluble TLR5 (TLR5S), both of which recognize flagellin on the surface of pathogenic bacteria [16].

TLR5-ligand interaction in mammals, TLR5, is primarily responsible for the detection of a kind of flagellin in bacterial flagella and the specific identification of constant functional domains that are relatively conserved between different bacteria [17]. To fish, they have two forms of TLR5, TLR5M (membrane form) and TLR5S (soluble form), which are responsible for identifying bacterial flagellin [18]. Fish TLR5M is homologous to mammalian TLR5 and contains the LRR domain, transmembrane region, and TIR domain typical of TLR. However, TLR5 is unique to fish and lacks transmembrane and TIR regions. Studies have shown that the recombinant TLR5S of Sparus aurata can bind to flagellin of Vibrio anguillarum, and then, TLR5M activates the inflammatory response. But it is still controversial whether the exact function of TLR5S is to enhance the innate immune response or to avoid the excessive inflammatory response of TLR5M [19]. Therefore, the molecular mechanism of fish TLR5 in the regulation of flagellar-mediated immune responses requires further investigation.

As per previous studies, TLR13 is a receptor for bacterial RNA; small-interfering RNA against TLR13 reduced cyto-kine induction by bRNA in DCs [20]; additionally, TLR13-ligand interaction TLR3, TLR7-9, TLR11, and TLR13 are TLRs localized to endosomes, which basically recognize nucleic acids released by intracellular microorganisms, including viruses, bacteria, and fungi. It has been demonstrated that mouse TLR13 can recognize the highly conserved 5′-CGGAAAGACC-3′ motif of bacterial 23S rRNA [6, 21]. The ssRAN oligomer mutation assay demonstrated that most of the nucleotides in the conserved sequence of the 23S rRNA of bacteria, especially the last eight nucleotides, are essential for triggering TLR13-mediated signaling [21]. In addition, vesicular stomatitis virus (VSV) has also been shown to induce a TLR13-mediated immune response [22]. However, the chemical properties that lead to the activation of TLR13 have been difficult to determine.

Larimichthys crocea is one of the most economically important fish endemic in the eastern and southern coastal waters of China. It has a well-developed innate immune system [23]. However, the wild stocks of L. crocea are seriously damaged by overfishing, while the cultured L. crocea is vulnerable to various pathogens [24, 25]. At present, there are more than 20 kinds of TLRs found in fish, and only 6 L. crocea TLRs are published, while TLR5, which has two forms, namely, membrane form (TLR5M) and soluble form (TLR5S), was poorly understood; meantime, TLR13, one of the important receptor, and the mechanism of innate immunity of large yellow croakers need further study. Therefore, this study was focused on three genes, and the results will contribute to our understanding of the immune system in fish.

2. Materials and Methods

2.1. Fish and Tissue Sampling. The examined fishes (weight 800 ± 15 g), obtained from Zhejiang Dahaiyang Technology Co. Ltd. (Zhoushan, Zhejiang Province, China), were clinically healthy. They were maintained at 25°C in an aerated seawater tank and fed a commercial diet for two weeks prior to the start of the experiments. The water in the tank was changed daily.

For the basal tissue expression analysis, tissues (muscle, spleen, liver, kidney, brain, heart, intestine, gill, swimming bladder, skin, fin, and eye) were isolated from six unchallenged L. crocea after being anaesthetized by immersion in MS222.

To investigate the transcriptional modulations of lctLRs, two independent groups of 100 individuals were infected with V. parahaemolyticus (1 × 10^8 CFU/mL, resuspended in PBS, pH 7.4) and PBS (as control) at a dose of 300 μL/200 g, respectively. Then, the liver, kidney, and spleen tissues were collected from three fishes per group, at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h postinjection. All procedures were performed under the guidelines of the Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People’s Republic of China, November 14, 1988) and were approved by the Animal Ethics Committee of Zhejiang Ocean University (Zhoushan, China).

2.2. RNA Isolation and Molecular Cloning. Total RNA was isolated from examined tissues using the Trizol Total RNA Kit (Invitrogen, USA) following the manufacturer’s instructions. Then, the concentration was measured by a UV-spectrophotometer (Eppendorf, Germany), and the quality of RNA was analyzed by 1% agarose gel by visualizing the intensity of 18 and 28s ribosomal RNA. After that, cDNA was synthesized using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan) following the guidelines of its manufacturer’s instructions. Finally, using the cDNAs as templates, PCR amplification was conducted using a thermal cycler (Bio-Rad, USA) under the following conditions: 50°C for 2 min, 95°C for 30 s, and then 40 cycles of 95°C for 15 s,
| Species | Protein | Accession no. | Species | Protein | Accession no. |
|---------|---------|---------------|---------|---------|---------------|
| L. crocea | TLR1 | AHB51065.1 | Miichthys miiuy | TLR5M | ALJ55566.1 |
| TLR2 | AHB51066.1 | TLR8 | XP_010741343.3 | TLR3 | ALJ55565.1 |
| TLR3 | KKF15845.1 | TLR5S | ALJ55567.1 |
| TLR7 | AGO28200.1 | TLR5S | ALJ55567.1 |
| TLR8 | XP_010741343.3 | Miichthys miiuy | TLR5M | ALJ55566.1 |
| TLR9 | AC66066.1 | TLR8 | ALJ55569.1 |
| TLR21 | A0Z21302.1 | TLR9 | ALJ55570.1 |
| TLR22 | ADK77870.1 | TLR21 | ALJ55573.1 |
| Lates calcarifer | TLR22 | AOV82293.1 | TLR22 | ALJ55574.1 |
| Acanthochromis polyacanthus | TLR1 | XP_022069587.1 | TLR13 | XP_006015139.1 |
| Cathartes aura | TLR15 | KFP51840.1 | TLR8 | XP_023148269.1 |
| TLR1 | ANS71060.1 | TLR1 | XP_006029305.1 |
| TLR2 | XP_023251851.1 | TLR2 | XP_022608643.1 |
| TLR21 | A0Z21302.1 | TLR9 | ALJ55570.1 |
| Scophthalmus maximus | TLR22 | AI7C13095.1 | TLR21 | ALJ55573.1 |
| TLR2 | ANV20861.1 | Andrias davidianus | TLR2 | AHB18364.1 |
| TLR5M | AMQ35501.1 | Anabas testudineus | TLR13 | XP_026256989.1 |
| TLR1 | XP_023251851.1 | TLR2 | XP_022608643.1 |
| TLR2 | XP_022608643.1 | TLR8 | ALJ55569.1 |
| TLR9 | ALI16362.2 | TLR8 | XP_022597011.1 |
| TLR13 | XP_023266978.1 | TLR13 | XP_022598134.1 |
| Seriola lalandi dorsalis | TLR2 | XP_023279904.1 | TLR2 | XP_022625698.1 |
| TLR3 | XP_003449976.2 | TLR1 | XP_006125821.1 |
| Xenopus laevis | TLR3 | XP_013126527.2 | TLR1 | AIZ71264.1 |
| TLR12 | XP_018082190.1 | TLR21 | AIZ72110.1 |
| Oreoichromis niloticus | TLR2 | XP_023279904.1 | TLR2 | XP_022625698.1 |
| TLR5S | XP_019201018.2 | TLR3 | XP_006125821.1 |
| TLR7 | XP_019208739.1 | Pelodiscus sinensis | TLR4 | NP_001273862.1 |
| TLR7 | XP_019208739.1 | TLR7 | XP_014428945.1 |
| Corvus cornix cornix | TLR6 | XP_010400098.3 | TLR3 | XP_009914242.1 |
| Phoenicopterus ruber ruber | TLR5 | XP_026150467.1 | TLR4 | NP_001267776.1 |
| Ctenopharyngodon idella | TLR7 | XP_018082190.1 | TLR4 | NP_001093239.2 |
| Epinephelus lanceolatus | TLR7 | XP_026150467.1 | TLR4 | NP_001267776.1 |
| Epinephelus coioides | TLR18 | AIB55030.1 | Struthio camelus | TLR4 | NP_001267776.1 |
| Oryzias melastigma | TLR20 | AHN49762.1 | Equus caballus | TLR4 | NP_001093239.2 |
| Haplochromis burtoni | TLR21 | AJW66342.1 | TLR11 | XP_001502488.1 |
| Epinephelus coioides | TLR2 | XP_026178049.1 | TLR2 | AVY54488.1 |
| TLR5M | XP_020470010.1 | Halaeacetus leucocephalus | TLR5M | XP_010575998.1 |
| TLR7 | XP_026150467.1 | TLR6 | XP_026267776.1 |
| Oryzias melastigma | TLR8 | XP_02141916.1 | TLR3 | ACR26292.1 |
| TLR5S | XP_005931636.1 | TLR21 | NP_001205729.1 |
| TLR2 | XP_026150467.1 | TLR4 | NP_001267776.1 |
| Epinephelus coioides | TLR21 | ADN34974.2 | Gallus gallus | TLR15 | NP_001032924.1 |
| TLR21 | XP_021334735.1 | Halaeacetus leucocephalus | TLR5M | XP_010575998.1 |
| Alligator mississippiensis | TLR5M | XP_019386797.1 | TLR21 | NP_001205729.1 |
| TLR8 | XP_014463251.1 | Urocitellus parryi | TLR4 | AEC32857.1 |
| TLR6 | XP_005518931.1 | Anser anser | TLR15 | AEC32857.1 |
| TLR7 | XP_021334735.1 | Oryzias latipes | TLR5S | XP_004083935.2 |
| TLR9 | XP_021322433.1 | Dentex tumifrons | TLR9 | AAB79218.1 |
| Danio rerio | TLR19 | NP_001352353.1 | Zonotrichia albicollis | TLR6 | XP_005490558.1 |
| TLR20 | NP_001170914.2 | Ornus orca | TLR6 | XP_012387211.1 |
| TLR21 | NP_001186264.1 | Sparus aurata | TLR9 | AAW81697.1 |
| TLR22 | NP_001122147.2 | Tursiops truncatus | TLR8 | XP_019784091.1 |
| Acanthopagrus schlegelii | TLR9 | ABY79216.1 | Delphinapterus leucas | TLR10 | XP_022421795.1 |
| Rachycentron canadum | TLR9 | AGD79973.2 | Sinocylocheilus grahami | TLR12 | XP_016140610.1 |
58°C for 45 s, and 95°C for 15 s, followed by a final extension at 72°C for 5 min.

2.3. Quantitative Real-Time PCR (qRT-PCR) and Statistical Analysis. Tissue-specific distribution and temporal mRNA expression of TLR5S, TLR5M, and TLR13 upon immune challenge were determined by qRT-PCR. A sample of 2 g of total RNA was reverse transcribed in a reaction mixture using the Primer5.064 software using the CDS of lcTLRs and TLR13. Primers for qRT-PCR (Table 1) were designed was used to normalize the expressions of TLR5M, TLR5S, and TLR13. The mRNA expression in muscle was treated as a control for comparisons with various tissues, and the 0 h time point comprised the control for the expressions post V. parahaemolyticus infection. Two-tailed Student $t$-test was performed to determine the statistical significance of differences observed between the experimental and control groups using SPSS Statistics 19 (IBM). *$p < 0.05$ and **$p < 0.01$ were considered statistically significant and indicated in Results.

2.4. Protein Localization. We used pcDNA3.1-C-eGFP plasmids (Genscript, Nanjing, China) to transform the overexpressed protein TLR5S, TLR5M, and TLR13 with green fluorescence. Transfection was using FTX and PLUS reagents (InvitrogenTM, Calif., USA). Cells were seeded in 24-well plates and cultured. DAPI was used to locate the nucleus, and Dil was used to locate the cytomembrane. The localization of protein TLR5S, TLR5M, and TLR13 was observed under a Nikon TE2000 microscope.

2.5. Sequence Analysis. The gene sequences of TLR5M, TLR5S, and TLR13 were obtained from the L. crocea whole-genome data [24]. Their coding sequences and the amino acid sequences were predicted using GeneScan (http://genes.mit.edu/GENSCAN.html) and ORFinder (https://www.ncbi.nlm.nih.gov/orffinder/). The theoretical molecular weight (MW) and isoelectric point (pI) were determined by Expsy-ProtParam (http://web.expasy.org/protparam/). The domains of the proteins were predicted using the online tool SMART (http://smart.embl-heidelberg.de) and pictured by IBS (http://ibs.biocuckoo.org). The secondary structure and tertiary structure were predicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) [26]. The homology genes were determined with BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and in the Gene Database (https://www.ncbi.nlm.nih.gov/gene/). Multiple sequence alignments were performed with MEGA7 using ClustalW and ESPript3 (http://esprit.ibr.fr/ESPript/ESPript/index.php). The phylogenetic tree was constructed by MEGA7, using the neighbor-joining method.

3. Results

3.1. Sequence Characteristics and Protein Structure Prediction. The coding region (CDS) of the TLR5M gene was deduced to be 2658 bp in length and encodes a protein of 885 aa with a predicted MW of 101.369 kDa and a pI of 5.69.

The 1926 bp length CDS of the TLR5S gene encoded a protein of 641 aa with a theoretical MW of 71.612 kDa and a pI of 8.94. The domains of TLR5M and TLR5S and their homologous genes in other species are shown in Figure 1. TLR5S lacks TIR and transmembrane domains compare to TLR5M, with an additional LRR_NT and 3 LRRs.

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Figure 1: Continued.
had 19 β folds, and the convex surface has 7 α-helices. TLR13 was composed of an intracellular region, a transmembrane domain, and an intracellular region. The extracellular region had 19 β-sheets and 7 α-helices, and the intracellular region was also surrounded by 6 α-helices with 4 β-sheets4 (Figure 2(c)).

The multisequence alignment of TLR5M and other fish TLRs is shown in Figure 2. The LRR_CT (586-638 aa) of TLR5M is capable of stabilizing the extracellular horseshoe structure, containing a cysteine cluster of CxCx(24)Cx(20)C. Among them, C590 and C616, C592 and C637 could form disulfide bonds, respectively, which is the key to the stability of LRR_CT to maintain the extracellular domain of TLR5M, and these four amino acid residues are conserved in TLR5M of all fish (Figure 3).

### 3.2 Expression of TLR Genes after V. parahaemolyticus Challenge (Figure 4).

In this study, in order to detect the expression of TLR5M, qPCR was used to determine the expression in 12 tissues from healthy *L. crocea*. As shown in Table 2, TLR5M was detectable in all tissues. A relatively high expression level of lc TLR5M (89.3-fold) was observed in the heart, followed by the brain (~36.0-fold) and liver (~8.0-fold). In addition to muscle, the expression of lc...
Figure 2: Continued.
Figure 2: Continued.
TLR5M detected in the spleen was pretty low (1.3-fold); unlike TLR5M, TLR13 had the highest expression in the spleen (182.6-fold), followed by the liver and heart, with the lowest expression level in the brain. Except for the spleen, the expression level of TLR13 in all other tissues was lower than that in muscle. Expression profiles of TLR5M and TLR13 after challenging *V. parahaemolyticus* caused upregulation of the expression level of TLR5M in the spleen, liver, and kidney of *L. crocea*. The expression level of TLR5M in the spleen increased during 0-12 h and reached the highest value at 12 h and then decreased.

However, its peak expression in the liver and kidney appeared at 6 h. In addition, the expression level of TLR5M in the liver increased sharply at 6 h and suddenly decreased after 6 h; in the *L. crocea* treated with *V. parahaemolyticus*, the expression levels of TLR13 in the three immune tissues were upregulated, while comparing with the expression pattern of TLR5M, the fluctuation of TLR13 express was lagging behind, the peaks in the liver and kidney appeared at 12 h, and the peak in the spleen appeared at 24 h; it was apparent that the expression level of TLR13 in the kidney was greatly modulated by *V. parahaemolyticus*.

### 3.3. Phylogenetic Analysis

Phylogenetic analysis to elucidate the evolutionary development history of TLR5M, TLR5S, and TLR13, phylogenetic tree analysis on TLRs of *L. crocea*, and other species was performed (Figure 5).

All TLRs were clustered into 4 groups, in which TLR15 and TLR4 were grouped separately (TLR15 group and TLR4 group), TLR1, 2, 6, 8, and 10 were clustered together.
Figure 3: Continued.
Figure 3: Continued.
(TLR1 group), and the remaining TLRs formed a large group (TLR3, TLR5/M/S, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13, TLR19, TLR20, TLR21, and TLR22). The large groups of 13 TLRs could be subdivided into 3 groups: TLR7, 8, and 9 clustered into one group (TLR7 subgroup); TLR3 and TLR5 clustered together (TLR3 subgroup); TLR11, 13, 19, 20, 21, and 22 clustered into a group (TLR11 subgroup). Previously, vertebrate TLRs were divided...
3.4. Subcellular Localization of TLR5S, TLR5M, and TLR13. The correct localization of a protein in a cell is crucial to understand the function of the protein. Hence, we examined the subcellular localization of TLR5M, TLR5S, and TLR13 (Figure 6). As the results show, TLR5M appeared in the nucleus while TLR5S were cytoplasmic in distribution. TLR13 protein was distributed in the endosome; its proteins were localized in the cytoplasm in a punctuate manner.

4. Discussion

4.1. Sequence Characteristics and Protein Structure Prediction. The functional domains of TLRs were highly conserved, and the differences were mainly expressed in the N-terminal LRR domain. The LRR domain is the extracellular domain of the TLR and is involved in the recognition and binding of ligands, so its N-terminal differences should have an effect on TLR recognition and binding ligands.
form a CxCx(24)Cx(16)C motif (Figure 1). Although TLR13 did not have LRR_NT, it had a conserved Cx(11)C cysteine motif. Hwang et al. experimentally obtained the sequence of the human TLR5M-binding region of flagellin (KLQTLDLRDNLTTIHFPSI) [32]. The corresponding sequence in TLR5M was NLRLFLTGNSLRLDLGF PASLPNL, in which fish TLR5M was conserved with motif xLxxLxLTGNSL/IRxLGx(n)AxLPNL/I, while the fish TLR5S...
also contained similar conserved motifs (blue square markers).

TLRs have a high conservation in the functional domain, and the difference is mainly reflected in the LRR domain at the N-terminal; the difference is mainly reflected in the LRR domain at the N-terminal. LRR domain is the extracellular domain of TLR, which is involved in the recognition and binding of ligands, so the difference of the N-terminal of the LRR domain should influence the recognition and binding of TLR. Multiple sequence alignments revealed that the TLR5M/S and TLR13 sequences contained multiple conserved Cys residues that were likely to form disulfide bonds to help maintain the stability of the extracellular structure of TLRs (Figure 2(c)). Previous studies on two types of TLR5 in Ctenopharyngodon idellus yielded similar results of TLR5M/S, and TLR13 contain multiple conserved Cys residues, which are likely to form disulfide bonds to help maintain the stability of the extracellular structure of TLRs (Figure 2). Similar results were obtained from previous studies on two forms of TLR5 in grass carp.

The length and functional domain structure of TLR13 is similar to that of Seriola lalandi dorsalis TLR13 (sldTLR13), but TLR13 has one LRR less than sldTLR13, while sldTLR13 had one N-terminal LRR-TYP more than TLR13 (Figure 2(c)). LRR number of mice TLR13 was much more than fish TLR13, with 11 LRR and 7 LRR_TYP, but LRR_CT is missing. Comparing all of the various types of TLRs, TLR5Ss had only the LRR domain and TLR13s lacked the transmembrane domain, while other TLRs had TIR, transmembrane domain, and LRRs. TLR5Ss lacked the C-terminal TIR and transmembrane domains, so they were the shortest (around 600 bp). Fish TLR13 has been well studied. For example, Liang et al. studied TLR13 (gTLR13) of the grouper (E. coioides), the cDNA of gTLR13 is 3559 bp in length, and the ORF is 2844 bp in length, encoding a protein of 947 amino acid residues (aa). Its domain includes a signal peptide, 13 LRR, a C-terminal LRR, a transmembrane domain, and a TLR structure [33].

Analysis of TLR sequences revealed that all three functional domains (LRRs, transmembrane, and TIR domains) of TLRs, except TLR5S (without transmembrane and intracellular TIR domains) and TLR21 (transmembrane domain deletion), are available (Figure 2). The difference between TLR5M/S and TLR13 of L. crocea and other species was in the number, type, and distribution of N-terminal LRRs [34].

4.2. Expression of TLR Genes after V. parahaemolyticus Challenge. Transcriptional expression of lcTLR5/TLR13 and genes in the TLR signal pathway TLR5 is located on the cell membrane, and it is capable of detecting flagellin and specifically recognizing a relatively conserved constant domain among different bacteria. TLR13 is a TLR that localizes to endosomes and recognizes nucleic acids released by internal microorganisms, including viruses, bacteria, and fungi [35].

Studies have shown that stimulation of C. irritans can cause an increase in TLR5M and TLR5S transcription levels.
in the fins and spleen of *E. colioides* [29]. The expression of *M. miyui* TLR5 in the liver and kidney increased significantly after infection by *Vibrio harveyi* [28]. Jiang et al. used flagellin and LPS to infect *S. maximus* and found that TLR5M was upregulated in fin, head, kidney, and spleen [35]. In addition, TLR13 in the grouper spleen can be stimulated by the 19-mer S. aureus 23S ribosomal RNA-derived oligoribonucleotide (ORN Sa19) [29]. It has been reported that human TLR5 was mainly expressed in the ovary and was expressed in monocytes, immature dendritic cells, and epithelial cells simultaneously [36]. However, mouse TLR5 was mainly expressed in the liver and lungs [37]. In this study, in order to detect the expression of TLR5M, qRT-PCR was used to determine the expression in 12 tissues from healthy *L. crocea*. As shown in Figure 3, TLR5M was detectable in all tissues. A relatively high expression level of TLR5M was observed in the heart, followed by the brain and liver. In addition to muscle, the expression of TLR5M detected in the spleen was pretty low. Unlike lcTLR5, TLR13 had the highest expression in the spleen, followed by the liver and heart, with the lowest expression level in the brain (Figure 6). Except for the spleen, the expression level of TLR13 in all other tissues was lower than that in muscle.

### 4.3. Phylogenetic Analysis

From the phylogenetic tree, we found that all TLRs were clustered into 4 groups, and the TLR5M/S and TLR13 were clustered in one group, although the genetic distance between them is not so close as that between TLR5M/S and TLR13, which may be the reason for the similar 3D model of TLR5M. All TLRs were clustered into 4 groups, in which TLR15 and TLR4 were grouped separately (TLR15 group and TLR4 group), TLR1, 2, 6, 8, and 10 were clustered together (TLR1 group), and the remaining TLRs form a large group (TLR3, TLR5M/S, TLR7, TLR8, TLR9, TLR11, TLR12, TLR12, TLR20, TLR21, and TLR22); the large groups of 13 TLRs could be subdivided into 3 groups: TLR7, 8, and 9 clustered into one group (TLR7 subgroup), TLR3 and TLR5 clustered together (TLR3 subgroup), and TLR11, 13, 19, 20, 21, and 22 clustered into a group (TLR11 subgroup); previously, vertebrate TLRs were divided into six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11) according to evolution. Our evolutionary analysis results were similar to their conclusions, but TLR3 and TLR 5 were classified into one family, and the TLR 11 family was complemented by TLR19 and TLR20.

### 4.4. Subcellular Localization of TLR5S, TLR5M, and TLR13

As per the previous studies, there were two types of TLR5 of fish, namely, membrane form of TLR5 (TLR5M) and soluble form of TLR5 (TLR5S) [38]. Interestingly, TLR5M appeared in the nucleus while TLR5S were cytoplasmic in distribution in *L. crocea*, which was different from other fish; TLR13 localized in endosomes, which basically recognize nucleic acids released by intracellular microorganisms, including viruses, bacteria, and fungi. In this study, TLR13 of *L. crocea* protein was distributed in endosomes; its proteins were localized in the cytoplasm in a punctuate manner.

### Data Availability

The data used to support the findings of this study are included within the article: Wu, C., Zhang, D., Kan, M. et al. The draft genome of the large yellow croaker reveals well-developed innate immunity: Nat Commun 5, 5227 (2014). 10.1038/ncomms6227, and the BioProject accession number is PRJNA237858.

### Conflicts of Interest

The authors declare no conflict of interest.

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### References

[1] K. V. Anderson, L. Bokla, and C. Nüsslein-Volhard, “Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product,” Cell, vol. 42, no. 3, pp. 791–798, 1985.

[2] B. Leemaitre, E. Nicolas, L. Michaut, J.-M. Reichhart, and J. A. Hoffmann, “The dorsoventral regulatory gene cassette spätzle/toll/cactus controls the potent antifungal response in Drosophila adults,” Cell, vol. 86, no. 6, pp. 973–983, 1996.

[3] R. Medzhitov, P. Preston-Hurlbut, and C. A. Janeway Jr., “A human homologue of the Drosophila toll protein signals activation of adaptive immunity,” Nature, vol. 388, no. 6640, pp. 394–397, 1997.

[4] F. L. Rock, G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan, “A family of human receptors structurally related to Drosophila toll,” Proceedings of the National Academy of Sciences of the United States of America, vol. 95, no. 2, pp. 588–593, 1998.

[5] D. Gao and W. Li, “Structures and recognition modes of toll-like receptors,” Proteins: Structure, Function, and Bioinformatics, vol. 85, no. 1, pp. 3–9, 2017.

[6] M. Oldenburg, A. Kruger, R. Ferstl et al., “TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification,” Science, vol. 337, no. 6098, pp. 1111–1115, 2012.

[7] M. S. Jin and J.-O. Lee, “Structures of the toll-like receptor family and its ligand complexes,” Immunity, vol. 29, no. 2, pp. 182–191, 2008.

[8] A. V. Kajava, “Structural diversity of leucine-rich repeat proteins,” Journal of Molecular Biology, vol. 277, no. 3, pp. 519–527, 1998.

[9] Y. Palti, “Toll-like receptors in bony fish: from genomics to function,” Developmental & Comparative Immunology, vol. 35, no. 12, pp. 1263–1272, 2011.

[10] P. R. Rauta, M. Samanta, H. R. Dash, B. Nayak, and S. Das, “Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses,” Immunology Letters, vol. 158, no. 1-2, pp. 14–24, 2014.

[11] P. Boudinot, J. Zou, T. Ota et al., “A tetrapod-like repertoire of innate immune receptors and effectors for coelacanths,” Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, vol. 322, no. 6, pp. 415–437, 2014.
A. Hidmark, A. von Saint Paul, and A. H. Dalpke, “Lamprey TLRs with properties distinct from those of the variable lymphocyte receptors,” The Journal of Immunology, vol. 178, no. 1, pp. 397–406, 2006.

A. Rebl, T. Goldammer, and H. M. Seyfert, “Toll-like receptor signaling in bony fish,” Veterinary Immunology and Immunopathology, vol. 134, no. 3–4, pp. 139–150, 2010.

Y. Lu, C. Li, P. Zhang et al., “Two adaptor molecules of MyD88 and TRAF6 in Apostichopus japonicus toll signaling cascade: molecular cloning and expression analysis,” Developmental & Comparative Immunology, vol. 41, no. 4, pp. 498–504, 2013.

H. Kumar, T. Kawai, and S. Akira, “Pathogen recognition by the innate immune system,” International Reviews of Immunology, vol. 30, no. 1, pp. 16–34, 2011.

T. Tsujita, H. Tsukada, M. Nakao, H. Oshiumi, M. Matsumoto, and T. Seya, “Sensing bacterial flagellin by membrane and soluble orthologs of toll-like receptor 5 in rainbow trout (Onchorhynchus mykiss),” Journal of Biological Chemistry, vol. 279, no. 47, pp. 48588–48597, 2004.

Z. Liang, Y. Kong, C. Zhou, L. Li, G. Nie, and X. Li, “Toll-like receptor recognition of bacteria in fish: ligand specificity and signal pathways,” Fish & Shellfish Immunology, vol. 41, no. 2, pp. 380–388, 2014.

D. Pietretti and G. F. Wiegertjes, “Ligand specificities of toll-like receptors in fish: indications from infection studies,” Developmental & Comparative Immunology, vol. 43, no. 2, pp. 205–222, 2014.

Z. Liang, Y. Kong, C. Luo, Y. Shen, and S. Zhang, “Molecular cloning, functional characterization and phylogenetic analysis of B-cell activating factor in zebrafish (Danio rerio),” Fish & Shellfish Immunology, vol. 29, no. 2, pp. 233–240, 2010.

A. Hidmark, A. von Saint Paul, and A. H. Dalpke, “Cutting edge: TLR13 is a receptor for bacterial RNA,” Journal of Immunology, vol. 189, no. 6, pp. 2717–2721, 2012.

X. D. Li and Z. J. Chen, “Sequence specific detection of bacterial 23S ribosomal RNA by TLR13,” eLife, vol. 1, pp. 101-102, 2012.

Z. Shi, Z. Cai, A. Sanchez et al., “A novel toll-like receptor that recognizes vesicular stomatitis virus,” Journal of Biological Chemistry, vol. 286, no. 6, pp. 4517–4524, 2011.

M. Liu and Y. S. de Mitcheson, “Profile of a fishery collapse: why mariculture failed to save the large yellow croaker,” Fish and Fisheries, vol. 9, no. 3, pp. 219–242, 2008.

C. Wu, D. Zhang, M. Kan et al., “The draft genome of the large yellow croaker reveals well-developed innate immunity,” Nature Communications, vol. 5, no. 1, pp. 5227–5236, 2014.

L. Wang, L. Jiang, C. Wu, and B. Lou, “Molecular characterization and expression analysis of large yellow croaker (Larimichthys crocea) interleukin-12A, 16 and 34 after poly IC and Vibrio anguillarum challenge,” Fish & Shellfish Immunology, vol. 74, pp. 84–93, 2018.

K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using realtime quantitative PCR and the 2−ΔΔCT method,” Methods, vol. 25, pp. 402–408, 2001.

L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, and M. J. E. Sternberg, “The Phyre2 web portal for protein modeling, prediction and analysis,” Nature Protocols, vol. 10, no. 6, pp. 845–858, 2015.

C. Qin, Q. Gong, Z. Wen, D. Yuan, T. Shao, and H. Li, “Molecular characterization and expression of toll-like receptor 5 genes from Pelteobagrus vachelli,” Fish & Shellfish Immunology, vol. 75, pp. 198–207, 2018.

R. Huo, X. Zhao, J. Han, and T. Xu, “Genomic organization, evolution and functional characterization of soluble toll-like receptor 5 (TLRSS) in miyu croaker (Miichthys miyui),” Fish & Shellfish Immunology, vol. 80, pp. 109–114, 2018.

J.-S. Bai, Y.-W. Li, Y. Deng et al., “Molecular identification and expression analysis of TLR5M and TLRSS from orange-spotted grouper (Epinephelus coioides),” Fish & Shellfish Immunology, vol. 63, pp. 97–102, 2017.

P. K. Jayaramu, G. Tripathi, A. Pavan Kumar, J. Keezhedath, M. K. Pathan, and P. P. Kurcheti, “Studies on expression pattern of toll-like receptor 5 (TLR5) in Edwardsiella tarda infected Pangasianodon hypophthalmus,” Fish & Shellfish Immunology, vol. 63, pp. 68–73, 2017.

S. D. Hwang, T. Asahi, H. Kondo, I. Hirono, and T. Aoki, “Molecular cloning and expression study on toll-like receptor 5 paralogs in Japanese flounder, Paralichthys olivaceus,” Fish & Shellfish Immunology, vol. 29, no. 4, pp. 630–638, 2010.

S. B. Mizel, A. P. West, and R. R. Hantgan, “Identification of a sequence in human toll-like receptor 5 required for the binding of gram-negative flagellin,” The Journal of Biological Chemistry, vol. 278, no. 26, pp. 23624–23629, 2003.

Y. Liang, X. Ding, X. Yu et al., “Identification and functional characterization of Toll-like receptor 13 from orange-spotted grouper (Epinephelus coioides),” Fish & Shellfish Immunology, vol. 74, pp. 309–317, 2018.

Y. Jiang, L. He, C. Ju et al., “Isolation and expression of grass carp toll-like receptor 5a (CiTLR5a) and 5b (CiTLR5b) gene involved in the response to flagellin stimulation and grass carp reovirus infection,” Fish & Shellfish Immunology, vol. 44, no. 1, pp. 88–99, 2015.

D. Liu, J. Chen, S. Li, and G. Hu, “Molecular cloning and expression study on toll-like receptor 5M in turbot, Scophthalmus maximus,” Developmental & Comparative Immunology, vol. 85, pp. 44–50, 2018.

K. D. Smith and A. Ozinsky, “Toll-like receptor-5 and the innate immune response to bacterial flagellin,” in Toll-Like Receptor Family Members and Their Ligands, vol. 270 of Current Topics in Microbiology and Immunology, pp. 93–108, Springer, 2002.

G. Sebastiani, G. Levesque, L. Lariviére et al., “Cloning and characterization of the murine toll-like receptor 5 (Tlr5) gene: sequence and mRNA expression studies in Salmonella-susceptible MOLF/Ei mice?,” Genomics, vol. 64, no. 3, pp. 230–240, 2000.